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NEALKOHOLOVÁ TUKOVÁ CHOROBA JATER: MECHANISMY VZNIKU A MOŽNOSTI TERAPIE

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PROHLÁŠENÍ

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V Praze, říjen 2021

.....
podpis

PODĚKOVÁNÍ

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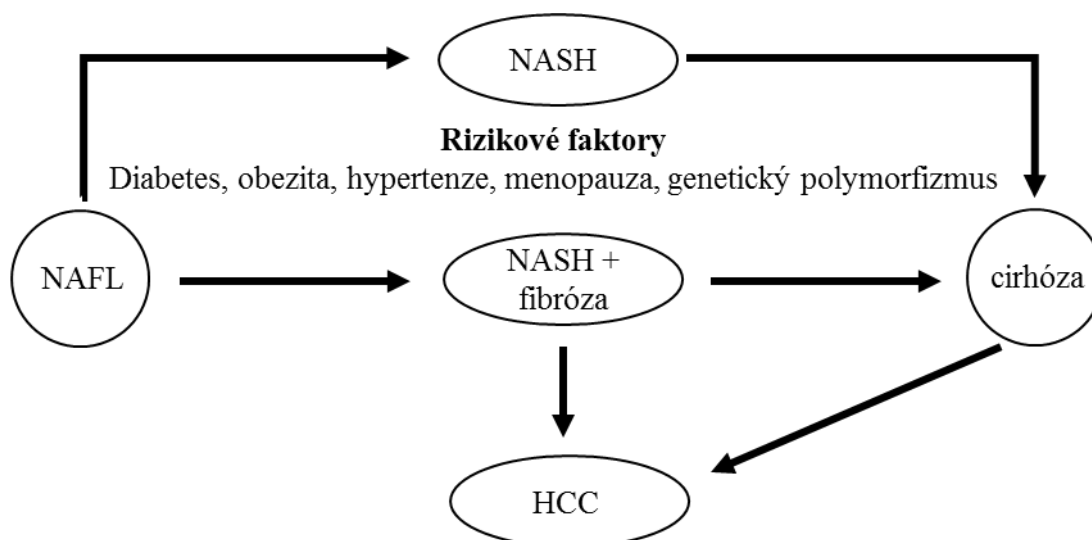
1 ÚVOD

1.1 EPIDEMIOLOGIE NAFLD

Nealkoholová tuková choroba jater (NAFLD) představuje celosvětově nejčastější jaterní onemocnění, odhaduje se, že v současnosti postihuje až 1 miliardu lidí (1). V tzv. rozvinutých (západních) státech se týká až jedné čtvrtiny populace (2). Podle současných modelů se počet pacientů s NAFLD v USA odhaduje na 85,3 mil., v reprezentativním vzorku evropských států (Francie, Německo, Itálie, Španělsko, Velká Británie) na 72,2 mil. a v Číně na 211 mil. Znepokojující je vzrůstající trend incidence NAFLD u dětí a dospívajících (3). Výskyt NAFLD je úzce asociován s dalšími metabolickými dysfunkcemi sdruženými do společného označení metabolický syndrom. V literatuře se objevuje i označení MAFLD (metabolic-associated fatty liver disease), což odráží tuto skutečnost (2). Pacienti s NAFLD vykazují zvýšenou incidenci kardiovaskulárních a onkologických onemocnění, což z tohoto onemocnění činí závažný socioekonomický problém (4). Roční náklady na léčbu NAFLD v roce 2016 byly odhadnuty na 100 mld. dolarů v USA a 35 mld. euro ve čtyřech evropských státech (Velká Británie, Francie, Německo, Itálie) (5). V USA se mezi lety 2004–2013 počet čekatelů na transplantaci jater z důvodů pokročilé NAFLD zvýšil o 170 % (z 804 na 2174), zatímco z důvodů alkoholové jaterní choroby se zvýšil pouze o 45 % (z 1400 na 2024) a z důvodů infekce virem hepatitidy C (HCV) o 14 % (z 2887 na 3291). NAFLD se tak stala druhou nejčastější příčinou transplantace jater. Kromě toho, NAFLD pacienti na čekací listině měli menší pravděpodobnost, že podstoupí transplantaci a menší pravděpodobnost 90denního přežití než pacienti s alkoholovou jaterní chorobou nebo HCV (6). Nealkoholová cirhóza je čtvrtou nejčastější příčinou hepatocelulárního karcinomu po alkoholové cirhóze, virové hepatitidě B a virové hepatitidě C. Vzhledem k výrazným úspěchům v prevenci hepatitidy B (očkování) a léčbě hepatitidy C (účinná farmakoterapie) v posledních letech význam NAFLD jako rizikového faktoru rozvoje hepatocelulárního karcinomu nabývá na důležitosti (7, 8).

1.2 PŘÍČINY VZNIKU A ROZVOJE NAFLD

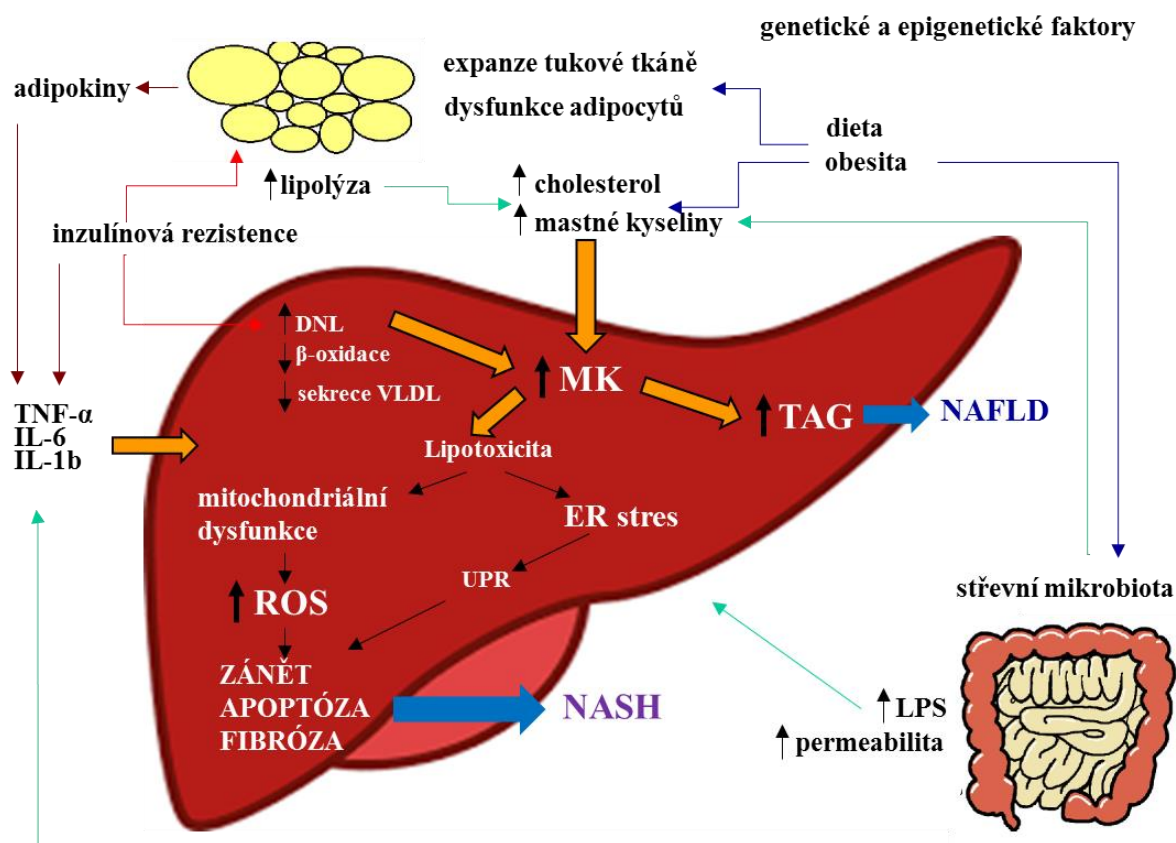
Z klinického hlediska představuje NAFLD kontinuum, které začíná prostou jaterní steatózou (non-alcohol fatty liver, NAFL), která může přecházet u 20–30 % pacientů v jaterní steatohepatitidu (non-alcohol steatohepatitis, NASH) a fibrózu. Zhruba u 10–20 % pacientů s diagnózou NASH onemocnění přechází do stadia jaterní cirhózy (7). V nejvíce závažných případech nealkoholová cirhóza přechází do hepatocelulárního karcinomu nebo jaterního selhání (**obr. 1.1**). Prostá jaterní steatóza je onemocnění zcela benigní, a pokud jsou aplikována adekvátní opatření spočívající zejména ve změně diety a životního stylu, může být zcela reverzibilní. NASH je již charakterizována poškozením hepatocytů, balónováním hepatocytů, přítomností markerů zánětu a různým stupněm fibrózy, která je důsledkem hojení zánětlivých ložisek. Při závažném průběhu může NASH přecházet až v jaterní cirhózu a jaterní selhání.



Obrázek 1.1 *Progrese NAFLD*. Adaptováno dle Calzadilla Bertot a Adams (9). HCC, hepatocelulární karcinom; NAFL, non-alcohol fatty liver (prostá jaterní steatóza); NASH, non-alcohol steatohepatitis (nealkoholová steatohepatitida).

1.2.1 Mechanismy vzniku a rozvoje NAFLD: „Multiple-hits theory“

Faktory zodpovědné za postupnou progresi prosté steatózy v závažnější formy NAFLD nejsou dosud přesně objasněny. Původní „two-hits“ model vycházel z představy, že akumulace triglyceridů v játrech („first-hit“) navozuje situaci zvýšené vnímavosti jaterní tkáně k dalším poškozujícím faktorům („second-hit“) (10). Tato zjednodušená představa byla nahrazena komplexnějším paradigmatickým, které vychází ze synergického působení více faktorů současně (11) (**obr. 1.2**).



Obrázek 1.2 Patogeneze NAFLD. Souhra metabolických, genetických a environmentálních faktorů vede k nerovnováze v sekreci a ukládání lipidů v hepatocytech. Zvýšená tvorba triacylglycerolů v játrech je důsledkem (i) zvýšeného vstupu mastných kyselin do hepatocytů, a to buď z diety, nebo v důsledku zvýšeného uvolňování z tukové tkáně a (ii) zvýšené syntézy mastných kyselin *de novo*. Zvýšená lipolýza v tukové tkáni i stimulace *de novo* lipogeneze je důsledkem inzulínové rezistence. Ta se v tukové tkáni manifestuje mimo jiné jako porucha antilipolytického působení inzulínu. V játrech zůstává zachovaná citlivost transkripčního faktoru SREBP-1c (sterol regulatory element binding protein) k inzulínu i v situaci periferní inzulínové rezistence. Hyperinzulinémie asociovaná s inzulínovou rezistencí pak vede k nadměrné stimulaci SREBP-dependentní dráhy a zvýšené produkci mastných kyselin *de novo*. Deregulace metabolismu lipidů v hepatocytu je spojena s akumulací meziproduktů metabolismu triglyceridů, které mohou způsobit poškození mitochondrií a následnou zvýšenou produkci reaktivních sloučenin kyslíku, stejně tak jako sníženou schopnost oxidace mastných kyselin. Dalším důsledkem intracelulární akumulace meziproduktů lipidového metabolismu je stres endoplazmatického retikula, který též může vyústit v poškození hepatocytu. Nárůst objemu tukové tkáně vede k vyšší sekreci některých adipokinů, zejména leptinu, které mají prozánětlivý účinek. Všechny příčiny poškození hepatocytů vyvolávají prozánětlivou odpověď prostřednictvím aktivace terminálních kináz (JNK) a NF-κB (nukleární faktor kappa B) dráhy. Výzkumy posledních let ukazují, že k rozvoji NAFLD přispívá i složení střevní mikrobioty. DNL, *de novo* lipogeneze; MK, mastné kyseliny; UPR, unfolded protein response; ROS reaktivní sloučeniny kyslíku; TAG, triacylglyceroly; LPS, bakteriální lipopolysacharid. Adaptováno dle Buzzetti et al. (11)

1.2.2 Poruchy metabolismu lipidů v hepatocytech

Hlavním znakem NAFLD je akumulace triacylglycerolů (TAG) v játrech v podobě lipidových kapének. V současnosti převládá názor, že toxickým faktorem nejsou samotné molekuly TAG, které jsou ve své podstatě inertní, ale spíše meziprodukty metabolismu TAG, které se hromadí

v buňce v důsledku narušených regulačních mechanismů (12, 13). Kromě samotných mastných kyselin a jejich derivátů jsou to například ceramidy, složené ze sfingosinu a mastné kyseliny, které mají přímý lipotoxický účinek (14). Samotná steatóza je tedy spíše projev deregulovaného metabolismu mastných kyselin v hepatocytu a může představovat časnou adaptaci na nadbytek mastných kyselin. Některé mastné kyseliny, např. kyselina palmitová, navozují stav stresu endoplazmatického retikula. Součástí adaptivní odpovědi na stres endoplazmatického retikula je i „unfolded protein response“, jejíž součástí je i aktivace terminálních kináz (JNK) a NF- κ B (nukleární faktor kappa B) drah. Pokud se adaptačním mechanismům, které jsou součástí UPR, nepodaří stres endoplazmatického retikula eliminovat, je aktivována regulovaná buněčná smrt (apoptóza) (15).

Dysregulace metabolismu lipidů se projevuje i zhoršenou inkorporací TAG do VLDL (very low density lipoprotein) partikulí, což dále přispívá k akumulaci TAG v hepatocytech (16). Významným zdrojem mastných kyselin a TAG v hepatocytech je lipogeneze *de novo*. Hlavním transkripčním faktorem regulujícím syntézu mastných kyselin je SREBP-1c (17). Tento transkripční faktor je na několika úrovních pozitivně regulován inzulinem a na rozdíl od inzulinem regulovaných metabolických drah v periferních tkáních (sval, tuková tkáň), SREBP-1c dráha zůstává senzitivní k inzulinu i v situaci inzulinové rezistence periferních tkání. Hyperinzulinémie, která je průvodním znakem inzulinové rezistence, za těchto okolností způsobuje hyperstimulaci SREBP-1c dráhy a zvýšenou produkci mastných kyselin *de novo* (18).

1.2.3 Chronický prozánětlivý stav

Obezita je stav charakterizovaný chronickým zánětem. Tuková tkáň se na tomto stavu podílí mimo jiné sekrecí prozánětlivých adipokinů, např. leptinu, resistinu apod. Leptin se kromě stimulace NF- κ B dráhy podílí na aktivaci jaterních hvězdčkových (stelátních) buněk a má tak přímý fibrogenní efekt. Při obezitě je naopak snižená produkce adiponektinu, který inhibuje sekreci prozánětlivých cytokinů (19–21). Charakteristickým rysem hepatocytů s vysokým obsahem TAG je mitochondriální dysfunkce, která se projevuje sníženou schopností oxidovat mastné kyseliny, a zvýšenou produkcí reaktivních sloučenin kyslíku. Důsledkem této dysfunkce je i nižší produkce ATP (22). Produkce reaktivních sloučenin kyslíku v prostředí s vysokým obsahem molekul lipidového charakteru vede k tvorbě lipoperoxidů, což vede mimo jiné i k poruchám transportu elektronů elektron-transportním řetězcem a další tvorbě reaktivních sloučenin kyslíku (23). Vzniká tak pozitivní zpětnovazebná smyčka, která udržuje a potencuje produkci radikálových i neradikálových sloučenin. Vysoká produkce reaktivních sloučenin kyslíku je spojena s produkcí prozánětlivých cytokinů tumor necrosis factor alfa (TNF α) a transforming growth factor beta (TGF β) a udržováním přetrvávajícího zánětu (22).

1.2.4 Genetická predispozice

Charakteristickým rysem NAFLD je značná variabilita v projevech a progresi choroby. Jedním z možných faktorů vysvětlujících tuto variabilitu může být genetická predispozice pacientů. Doposud bylo identifikováno pět variant, které jsou asociovány s rizikem NAFLD, konkrétně Patatin-like phospholipase domain-containing protein 3 (PNPLA3, rs 738409 C>G), transmembrane 6 superfamily member 2 (TM6SF2, rs 58542926 C>T), glucokinase regulator (GCKR, rs 780094 A>G), membrane-bound O-acyltransferase domain-containing protein 7 (MBOAT7, rs2587366) a hydroxysteroid 17-beta dehydrogenase-13 (HSD17B13, rs3816873) (24). Polymorfismy v prvních čtyřech uvedených genech jsou asociovány se zvýšeným rizikem

NAFLD, naopak varianta rs3816873 genu *HSD17B13* má vzhledem k NAFLD protektivní charakter.

Mnohé studie identifikovaly řadu dalších polymorfismů, které mohou být zodpovědné za variabilitu různých aspektů NAFLD. Pozornost se soustředila zejména na geny kódující proteiny účastníci se přenosu inzulínového signálu, metabolismu glukózy a lipidů, regulace imunitní odpovědi, fibrogenese nebo geny, které jsou asociovány s oxidačním stresem (25). Vzdor rozsáhlému výzkumu však genetické varianty vysvětlují jen malou část rizika NAFLD (26). Ukazuje se, že významnou roli mohou hrát i interakce mezi jednotlivými genetickými variantami, mezi geny a prostředím, epigenetické vlivy a podobně. Dosud byla prokázána interakce mezi variantami genu *PNPLA3* a druhem diety (27), příjmem jednoduchých cukrů (28), příjmem ω -6 nenasycených mastných kyselin (29), obezitou a inzulínovou rezistencí (30).

1.2.5 Epigenetické vlivy

Reverzibilní epigenetické změny mohou představovat „most“ mezi geny a prostředím. miRNAs jsou malé nekódující RNA molekuly, které se váží ke komplementárním úsekům v sekvenci mRNA a regulují jejich transkripci. Různé studie ukazují asociaci řady těchto molekul s NAFLD. Nedávná metaanalýza identifikovala tři nejčastěji zmiňované miRNA, konkrétně miRNA-122, miRNA-34a a miRNA-192 (31).

Další směr výzkumu se zaměřuje hlavně na metylační vzorec DNA jaterních buněk u pacientů s diagnózou NAFLD. Dosud byl prokázán vztah mezi metylačním vzorcem jaterní DNA a reverzí steatózy po bariatrické operaci (32) nebo stupněm fibrózy u NAFLD (33). Epigenetické mechanismy hrají rovněž klíčovou úlohu v procesu fetálního metabolického programování náchylnosti k NAFLD (34, 35). Objevují se důkazy pro tvrzení, že zvýšené riziko rozvoje NAFLD v pozdějším životě se zakládá už v průběhu nitroděložního vývoje. Experimenty na zvířatech prokázaly, že pokud matka dostává dietu s vysokým obsahem tuků, dochází k rozsáhlým epigenetickým modifikacím jaterní DNA plodu, které byly asociovány s pozdějším zvýšeným rizikem rozvoje NAFLD u potomstva (36, 37). Je zajímavé, že tyto změny se přenášejí i na další generace, zároveň ale mohou být eliminovány fyzickou aktivitou nebo dietou (38–40). Údaje ze studií na lidech jsou dosud pouze anekdotické, nicméně se ukazuje, že obezita matky a způsob výživy v raném dětství zvyšuje riziko rozvoje NAFLD v období dospívání a v dospělosti (41).

1.2.6 Střevní mikrobiota a rozvoj NAFLD

Výzkum posledních dvaceti let ukázal významnou úlohu mikrobiomu vzhledem k hostitelskému organismu – složení a funkce mikrobioty osídlující konkrétní niky mohou zásadně přispívat jak k udržování zdravého stavu, tak k rozvoji mnoha patologií. Terminologicky je třeba rozlišovat pojem „mikrobiom“, tj. souborný genom všech přítomných mikroorganismů, a „mikrobiota“, tj. soubor všech přítomných mikroorganismů. Ukázalo se, že patologické nastavení vnitřního prostředí ve střevě (složení mikrobioty, jejích produktů a nastavení imunitního systému) je úzce spojeno se vznikem a progresí NAFLD. Za určitých podmínek dochází k významné změně ve funkci střevní bariéry a zvýšení propustnosti pro bakteriální metabolity, mikrobiální antigeny a někdy i celé mikroorganismy. Tyto komponenty se prostřednictvím portálního oběhu dostávají do jater a prozánětlivě stimulují jaterní makrofágy, což přispívá k progresi NAFLD. Osa „střevo-játra“ se tak dostává do centra pozornosti jak z hlediska vysvětlení mechanismu vzniku a rozvoje onemocnění, tak i vzhledem k možným terapiím.

1.2.6.1 Složení střevní mikrobioty a NAFLD

Mikrobi osídlující trávicí trakt ovlivňují svého hostitele buď přímým kontaktem se slizničními buňkami a buňkami imunitního systému, nebo prostřednictvím produktů svého metabolismu. Počáteční optimismus, daný otvírajícími se možnostmi next-generation sequencing a předpokládající identifikaci konkrétních „viníků“ zodpovědných za NAFLD, doznal během doby značné úhony. Ukázalo se, že lidská mikrobiota je extrémně variabilní (42) a shoda mezi konkrétními výstupy různých studií je spíše vzácností než pravidlem. Přesto se ale podařilo shromáždit důkazy svědčící pro konkrétní úlohu střevní mikrobioty v rozvoji obezity, která je jedním z faktorů podmiňujících NAFLD. Klíčová studie Backhed et al. ukázala, že při stejném kalorickém příjmu bezmikrobní myši dosáhly menší váhy a adiposity ve srovnání se zvířaty stejného kmene, ale s běžnou střevní mikroflórou (43). Dále bylo zjištěno, že střevní mikrobiom geneticky shodných ob/ob obézních a štíhlých myší, které se liší pouze v genu pro leptin, je odlišný a charakterizuje jej poměr četnosti zástupců dvou hlavních kmenů, Firmicutes a Bacteroidetes (poměr F:B je vyšší u obézního kmene). Obdobné výsledky byly pozorovány i u lidí, kdy hubnutí bylo provázeno právě změnou poměru Firmicutes a Bacteroidetes (44). Ridaura et al. prokázala, že obézní nebo štíhlý fenotyp lze indukovat u bezmikrobních myší přenosem stolice od štíhlých a obézních lidských dárců, přičemž hlavní rozdíl spočívá právě v poměru zástupců Firmicutes a Bacteroidetes (45). Méně úspěšné jsou však snahy asociovat obezitu s konkrétními bakteriálními druhy a rody. V případě NAFLD se ukazuje, že rozvoj onemocnění je asociován s přerůstáním bakterií v tenkém střevě (46). Několik studií prokázalo rozdíly ve složení mikrobioty mezi NAFLD pacienty a zdravými kontrolami, ale nepodařilo se najít jednotící charakteristiky (47-49).

1.2.6.2 Střevní metabolom a NAFLD

Příčiny rozporupných výsledků studií zaměřených na hledání souvislostí mezi konkrétními mikrobiálními druhy a konkrétními patologiemi lze spatřovat ve značné funkční redundanci druhů osídlujících lidské střevo. Příbuzné druhy bakterií často sdílejí stejné metabolické dráhy a mnohdy lze najít funkční podobnost i mezi fylogeneticky značně vzdálenými druhy, buď v důsledku koevoluce v určitém prostředí, nebo díky horizontálnímu přenosu genů. V řadě studií se podařilo najít příčinné souvislosti právě mezi studovaným jevem a složením střevního metabolomu. V případě NAFLD jsou nejvíce diskutovanými produkty bakteriálního metabolismu sekundární žlučové kyseliny, mastné kyseliny s krátkým řetězcem a etanol.

Žlučové kyseliny

Žlučové kyseliny jsou v podobě primárních konjugovaných žlučových kyselin vylučovány do dvanácterníku a veškerý jejich další metabolismus (dekonjugace, transformace na sekundární žlučové kyseliny) je výlučně důsledkem činnosti mikroorganismů (50). Dekonjugace žlučových kyselin, tj. odštěpení glycinového nebo taurinového zbytku, je důsledkem aktivity enzymu BSH (hydroláza žlučových kyselin), který je přítomen hlavně v metagenomu bakterií patřících k rodům *Lactobacillus* a *Bifidobacterium*. Transformace primárních žlučových kyselin na sekundární, tj. 7 α -dehydroxylace, je katalyzována enzymy BAI operonu (bile acid inducible operon), který se nachází v genomu bakterií rodu *Clostridium*. Žlučové kyseliny mají nejen zásadní úlohu v trávení tuků, ale jejich deriváty současně hrají i důležitou roli v regulaci glukózové, lipidové a energetické homeostázy (51, 52) a pro- nebo protizánětlivého nastavení imunitního systému (53). Žlučové kyseliny mají i přímé antimikrobiální účinky, protože díky svým detergentním vlastnostem narušují mikrobiální membrány. Toxicita žlučových kyselin

závisí na míře hydrofobicity a rovněž odolnost specifických bakterií k jednotlivým žlučovým kyselinám je různá. Žlučové kyseliny tak ovlivňují složení střevní mikrobioty a současně složení střevní mikrobioty ovlivňuje spektrum žlučových kyselin (54). Množství a složení spektra žlučových kyselin je tedy výsledkem složité rovnováhy závisující jak na metabolismu hostitele, tak na složení a funkční kapacitě střevní mikrobioty.

Změny ve složení spektra žlučových kyselin, jejich množství a na nich závislých signálních drahách jsou dávány do souvislosti s řadou metabolických chorob včetně NAFLD (55). U pacientů trpících steatohepatitidou se střevní dysbióza projevuje i zvýšením obsahu primárních žlučových kyselin, vyšším poměrem primárních a sekundárních žlučových kyselin a vyšší celkovou koncentrací žlučových kyselin v séru i ve stolici (55, 56). U pokročilého poškození jater lze pozorovat vyšší zastoupení rodu *Clostridium* a naopak pokles zastoupení jiných zástupců kmene Firmicutes, např. Lachnospiraceae, *Roseburia*, Ruminococcaceae a *Blautia*, kteří jsou producenti spíše prospěšných metabolitů jako jsou mastné kyseliny s krátkým řetězcem (56).

Mastné kyseliny s krátkým řetězcem

Mastné kyseliny s krátkým řetězcem, zejména acetát, propionát a butyrát, jsou produkty bakteriální fermentace polysacharidů. V prostředí lidského střeva jde zejména o polysacharidy obsahující vazby, které lidská enzymatická výbava neumí štěpit (57). Štěpení těchto polysacharidů v tlustém střevě umožňuje dodatečné získání energie, množství závisí na jejich obsahu v dietě (58). Acetát je považován za obezitogenní substrát, který stimuluje lipogenezi a syntézu cholesterolu v tukové tkáni a v játrech. V krvi je ze všech mastných kyselin s krátkým řetězcem přítomen v největším množství (59). Propionát a butyrát jsou naopak považovány za antiobezitogenní látky. Acetát a propionát je produkován hlavně zástupci kmene Bacteroidetes, butyrát je syntetizován bakteriemi patřícími ke kmeni Firmicutes (60). Butyrát slouží jako hlavní zdroj energie pro buňky slizniční výstelky tlustého střeva, kde pomáhá udržovat správnou funkci střevní bariéry. Tato funkce souvisí i s jeho protektivním účinkem vzhledem k metabolické endotoxemii (59). Butyrát stimuluje sekreci GLP-1 (glucagon-like peptide-1) a GIP (gastric inhibitory polypeptide), což pozitivně ovlivňuje senzitivitu tkání k inzulínu (61) a stimuluje proliferaci protizánětlivě působících T regulačních lymfocytů (62). Butyrát i propionát zvyšují expresi anorexigenního adipokinu a leptinu (63). Otázka, zda nedostatečná produkce mastných kyselin s krátkým řetězcem, zejména butyrátu, může přispívat k rozvoji NAFLD není dosud vyřešena, i když některé studie poukazují na nižší zastoupení jeho producentů u NAFLD pacientů (56).

Etanol

Etanol je běžným produktem činnosti střevních bakterií (64). U NAFLD pacientů, kteří nepřijímali alkohol perorálně, byly v krvi a vydechovaném vzduchu prokázány vyšší koncentrace etanolu a acetaldehydu ve srovnání se zdravými kontrolami (65). Otázkou, zda lze prokázat souvislost mezi střevní dysbiózou a endogenní produkcí etanolu, se zabýval Zhu et al ve studii, která srovnávala střevní mikrobiom pacientů se steatohepatitidou a obézních jedinců bez diagnózy NAFLD (66). Rozdíly mezi oběma skupinami se týkaly zástupců kmene Proteobacteria, čeledi Enterobacteriaceae a druhu *E. coli*, přičemž některé z druhů více zastoupených u NAFLD pacientů patřily zároveň k identifikovaným producentům etanolu. Obsah etanolu v krvi koreloval s mírou závažnosti steatohepatitidy.

1.2.6.3 *Střevní mikrobiota a permeabilita střevní stěny*

Vzhledem ke své anatomické poloze jsou játra orgánem, který je jako první vystaven všem komponentám, které přecházejí přes střevní bariéru do krve, konkrétně do portálního oběhu. Kromě živin to mohou být i prospěšné nebo škodlivé produkty bakteriálního metabolismu, části mikroorganismů nebo dokonce celé virové partikule či bakterie (67), a jednou z hlavních funkcí jater je tyto potenciálně škodlivé látky eliminovat z oběhu. Množství potenciálně toxických komponent, které je uvolněno ze střeva do cirkulace, závisí na permeabilitě střevní stěny. Ve zdravém střevě k sobě buňky slizničního epitelu těsně adherují pomocí „tight-junction“ (těsně spojujících) proteinů (68). Složení střevní mikrobioty a jí produkovaných metabolitů může výrazně ovlivňovat expresi těchto proteinů, propustnost střevní stěny a přispívat nebo bránit rozvoji jaterní steatózy, steatohepatitidy a fibrózy (69). Na myším modelu i u NAFLD pacientů byla prokázána snížená exprese „tight junction“ proteinů a vyšší translokace bakteriálních antigenů do oběhu (70, 71). Kauzální souvislost zvýšené permeability střevní stěny a míry poškození jater byla prokázána i experimentálně na myším modelu kolitidy indukované dextran sulfátem. Míra steatózy a stupeň poškození jater (zánět, fibróza) byl závažnější u zvířat krmených vysokotukovou dietou a vystavených působení dextran sulfátu než u zvířat pouze krmených touto dietou (72). Vztah mezi zvýšenou střevní permeabilitou, endotoxemií a rozvojem steatohepatitidy potvrzují i další studie u NAFLD pacientů (73, 74).

1.3 MODEL Y NAFLD

Experimentální studium mechanismů podmiňujících konkrétní patologii a možnosti terapie zásadním způsobem závisí na dostupnosti vhodných zvířecích modelů, které by co nejvěrněji rekapitulovaly obdobné děje probíhající u lidí. V případě NAFLD ani jeden z dostupných modelů tuto podmínku nesplňuje dokonale. Podle způsobu intervence můžeme dostupné modely rozdělit do čtyř kategorií: (i) modely založené na podávání obezitogenních diet; (ii) modely využívající deficienci konkrétních nutrientů; (iii) genetické modely a (iv) modely využívající hepatotoxické látky. Žádný z dostupných zvířecích modelů nezahrnuje komplexní patofyziologii NAFLD, ale s vědomím jejich omezení je lze úspěšně využít pro studium konkrétních jednotlivých aspektů tohoto onemocnění.

1.3.1 *Modely využívající obezitogenní diety*

Model fyziologicky nejbližší NAFLD u lidské populace je indukce poškození jater pomocí podávání obezitogenních diet. Podávání obezitogenní diety myším nebo potkanům vede k manifestaci průvodních známek metabolického syndromu. Limitujícím faktorem, který znesnadňuje interpretaci výsledků získaných na zvířecích modelech založených na podávání obezitogenních diet, je velká heterogenita v přesném složení použitých diet. Používané diety mohou být založené buď pouze na vysokém obsahu tuku (až 60 % energie ve formě tuků), nebo na kombinaci tuků a jednoduchých cukrů. Dalšími důležitými faktory jsou druh a forma jednoduchých cukrů (glukóza vs. fruktóza, v tuhém skupenství nebo jako kapalina), skutečnost, zda do diety byl nebo nebyl přidán cholesterol (75) a druh, popř. kmen použitých experimentálních zvířat.

1.3.1.1 *Vysokotuková dieta*

Vysokotuková dieta je často využívána k navození NAFLD jak u potkanů, tak u myší. U této diety je 45–75 % energie dodáváno v podobě tuku s převahou nasycených mastných kyselin

(76). U těchto modelů lze prokázat společné rysy s NASH, i když s méně závažnými projevy (77). Existují výrazné mezidruhové rozdíly v efektu vysokotukové diety na rozvoj NAFLD. Potkani jsou k vysokotukové dietě citlivější než myši, což se projevuje kratší dobou, která je nutná k vyvolání patologických příznaků (78). Kromě toho je nutné brát v úvahu i rozdílnou citlivost různých kmenů. U potkanů bylo prokázáno, že podávání vysokotukové diety indukovalo stetohepatitidu u kmene Sprague–Dawley (79), zatímco u potkanů kmene Wistar se projevy zánětu vyvolat nepodařilo (80). U myši byla závažnější steatóza vyvolána u kmene BALB/c ve srovnání s kmenem C57Bl/6J (81). Nevýhodou modelů založených na vysokotukové dietě je nepřítomnost některých složek metabolického syndromu jako je např. hyperinzulinemie. Pokud obsah tuku v dietě představuje 55 % nebo více, tak již dochází ke stimulaci ketogeneze, což je stav zcela netypický pro metabolický syndrom.

1.3.1.2 Western-type dieta

Charakteristickými rysy diety vedoucí k NAFLD u lidí je vysoký obsah tuků, zejména těch bohatých na nasycené a *trans*-nenasycené mastné kyseliny, a jednoduchých cukrů, zejména fruktózy. Tato dieta se často označuje jako „western-type“. Charakteristická dieta NAFLD pacientů obsahuje 36 % tuků, 46 % sacharidů s vysokým podílem jednoduchých cukrů a 18 % proteinů (82). Western-type dieta používaná k indukci NAFLD u zvířat obsahuje obdobné složení základních živin a její výhodou je, že rekapituluje klíčové aspekty metabolického syndromu, jako jsou hyperinzulinemie a periferní i jaterní inzulínová rezistence. U C57Bl/6J myši podávání western-type diety po dobu 16 týdnů vedlo k obezitě, inzulínové rezistenci, steatóze a v játrech byl prokázán oxidační stres, infiltrace makrofágů a známky fibrózy (83). Při podávání diety s obdobným složením obohacené navíc o 0,2% cholesterol bylo dosaženo podobných výsledků, přičemž bylo navíc prokázáno balónovutí hepatocytů a progresivní fibróza (84). Všechny typy western-type diety vedou k akumulaci triacylglycerolů v játrech v podobě makrovezikulární steatózy, ale pouze při podávání této diety obohacené cholesterolem lze prokázat i mikrovezikulární steatózu (85).

1.3.2 Modely využívající diety deficitní v určitých nutrientech

1.3.2.1 Metionin-cholin deficitní dieta

Nejčastěji využívanou nutričně deficitní dietou při indukci NAFLD je metionin-cholin deficitní dieta (MCD). Metionin je jedním z klíčových prekurzorů syntézy glutathionu, důležité složky antioxidační ochrany buňky. Jeho nedostatek se projevuje rozvojem jaterního poškození zahrnujícím zánět a fibrózu. Cholin je nezbytný pro sekreci VLDL částic a jeho nedostatek se manifestuje rozvojem makrovezikulární steatózy. Podávání MCD vede u myši k rozvoji výrazné steatózy již v horizontu 4–6 týdnů (68, 86). Nicméně mechanismus vzniku steatózy je odlišný než v případě jaterní steatózy u lidí, kde se jedná spíše o nadměrně stimulovanou lipogenezi. MCD dieta vede k rychlému rozvoji zánětu v játrech (87), který se manifestuje balónovutím hepatocytů a zvýšenou hladinou prozánětlivých cytokinů v cirkulaci (68, 87). Významná je i skutečnost, že zánět, tj. steatohepatitida, indukovaný MCD dietou je reverzibilní při návratu ke standardní dietě. Tento fakt je v souladu s pozorovanou reverzí steatohepatitidy u lidí při úpravě životosprávy (88, 89). Vliv MCD diety do značné míry závisí na druhu, kmeni a pohlaví zvířat. Kirsch et al. (90) srovnávali citlivost tří kmenů laboratorního potkana (Wistar, Sprague-Dawley a Long-Evans) a jednoho myšního kmene (C57Bl/6J) k MCD dietě. Samci reagovali na dietu výrazněji než samice. Všechny kmeny laboratorního potkana vyvinuly po podávání MCD diety jaterní steatózu, nejvýrazněji se vliv diety projevil u samců kmene Wistar.

Nicméně markery zánětu nebyly výrazné a fibróza se nevyvinula. Naopak, u samců myši kmene C57Bl/6J byla po podávání MCD diety prokázán kromě steatózy i výrazné známky zánětu, nekrózy, oxidačního stresu a změny v histologické stavbě jater, což z tohoto modelu činí výborný nástroj ke studiu jaterních projevů NASH. Nedostatkem MCD modelu je skutečnost, že poškození jater není spojeno s dalšími charakteristickými znaky metabolické nerovnováhy, která je typická u lidí trpících NAFLD, proto bývá MCD dieta kombinovaná s genetickými modely NAFLD (db/db nebo ob/ob myši).

1.3.2.2 CDAA dieta

I tato dieta je, stejně jako MCD dieta, deficitní v obsahu cholinu. Na rozdíl od MCD diety neobsahuje komplexní proteiny, ale odpovídající směs L-aminokyselin (91). Podobně jako u MCD modelů, podávání CDAA (choline-deficient amino acid) diety stimuluje syntézu lipidů, zvyšuje oxidační stres a zánět v játrech a vede k rozsáhlé fibróze (36). Manifestace jaterního poškození vyžaduje delší čas než v případě MCD diety, ale výsledná intenzita, měřeno podle vzestupu koncentrace ALT (alaninaminotransferázy) v séru, je závažnější (92). Ani CDAA dieta neindukuje metabolické změny charakteristické pro NAFLD u lidí, tj. periferní a hepatickou inzulinovou rezistenci, hyperinzulinemii a obezitu.

1.3.3 Genetické modely

Existuje řada modelů, většinou myších, u nichž dochází k manifestaci některých aspektů NAFLD v důsledku spontánních mutací nebo cílených zásahů do dědičné informace. Jejich společnou nevýhodou je skutečnost, že tyto mutace nebyly prokázány u lidí trpících NAFLD. U většiny genetických modelů dochází spontánně ke zvýšené akumulaci triacylglycerolů v játrech, ale ne už progresi směrem ke steatohepatitidě a fibróze. Řada studií je proto postavena na kombinaci genetického modelu a některé z výše uvedených diet. Používané modely cílí na jednu ze tří oblastí metabolismu lipidů, a to (i) syntéza a ukládání lipidů (ob/ob, db/db, PTEN KO, SREBP-1c overexpression); (ii) transport mastných kyselin (CD36 KO, ApoE KO) nebo (iii) oxidace mastných kyselin (PPAR α KO, AOX KO) (77).

1.3.4 Modely využívající hepatotoxické látky

Chemické, resp. farmakologické, modely NAFLD využívají obvykle jednu z těchto tří látek – streptozotocin, chlorid uhličitý (tetrachlormetan, CCl₄) nebo tetracyklin. Streptozotocin aplikovaný novorozeným zvířatům ničí inzulin produkující beta buňky pankreatu a navozuje diabetes (93, 94). V kombinaci s vysokotukovou dietou může představovat model NAFLD zahrnující steatózu, steatohepatitidu, fibrózu a ojediněle i hepatocelulární karcinom. Nevýhodou tohoto modelu je, že destrukce beta buněk pankreatu implikuje spíše situaci diabetu 1. typu než inzulinovou rezistenci a chronický zánět charakteristický pro lidskou NAFLD.

CCl₄ je hepatotoxická látka navozující akutní poškození jater provázené nekrózou. Aplikace CCl₄ vede k stimulaci buněk imunitního systému a rychlému rozvoji zánětu. U tohoto modelu je vysoká míra úmrtnosti v krátkém časovém horizontu. Patogeneze, progresi onemocnění a histomorfologické změny jsou výrazně odlišné od klinického obrazu NAFLD u lidí (95, 96).

Tetracyklin je látka, která se akumuluje v mitochondriích a blokuje replikaci a transkripci mitochondriální DNA. Důsledkem je blokáda sekrece VLDL a rychlé hromadění lipidů v hepatocytech (97). Aplikace tetracyklinu v kombinaci s vysokotukovou dietou vedla k rychlému rozvoji steatohepatitidy u myšího modelu (98). Společnou nevýhodou všech

modelů založených na hepatotoxických látkách je rozdílná etiopatogeneze onemocnění a zásadní odlišnosti v metabolických charakteristikách ve srovnání s NAFLD u lidí.

1.4 TVORBA LIPIDOVÝCH KAPÉNEK

Mastné kyseliny, které slouží jako substrát pro syntézu TAG v játrech, se do jater dostávají ze tří zdrojů, (i) z oběhu, (ii) procesem *de novo* lipogeneze přímo v játrech a (iii) recyklací lipoproteinových partikulí pohlcovaných hepatocyty v procesu endocytózy. Hlavním zdrojem mastných kyselin za většiny podmínek jsou mastné kyseliny přiváděné do jater krevním oběhem (99). Jejich podíl se dále zvyšuje za hladovění a při inzulínové rezistenci v důsledku zvýšené lipolýzy v tukové tkáni. V postprandiálním stavu se zvyšuje podíl mastných kyselin pocházejících z úniku (spillover) při štěpení lipoproteinových partikulí lipoproteinovou lipázou ve tkáních, z vychytávaných remnantních chylomikrů a *de novo* lipogeneze. Při NAFLD se podíl *de novo* lipogeneze významně zvyšuje a může dosáhnout až 30–40 % všech jaterních mastných kyselin (18, 100).

Pro další osud mastných kyselin v hepatocytu je klíčové, zda budou nasměrovány k oxidaci nebo k esterifikaci. V hepatocytech se nacházejí četné ACSLs (long-chain acyl-CoA synthetases), které katalyzují vazbu určitých mastných kyselin na koenzym A a určují tak jejich další metabolické využití. Rozdíly v subcelulární lokalizaci, preferenci k určitým druhům substrátu a enzymová kinetika podmiňují intracelulární osud mastných kyselin. ACSL5 směřuje mastné kyseliny především k syntéze TAG, zatímco ACSL4 reguluje metabolismus kyseliny arachidonové, což dále ovlivňuje syntézu fosfolipidů a sekreci VLDL (101). Dosud není plně objasněno, která nebo které ACSL směřují mastné kyseliny v játrech k oxidaci. Dalšími klíčovými enzymy, stojícími na křižovatkách metabolismu TAG/mastných kyselin v játrech, jsou karnitin palmitoyl acyltransferáza a glycerol-3-fosfát acyltransferáza, které řídí utilizaci mastných kyselin pro oxidaci a esterifikaci. V hepatocytech existuje několik izoform glycerol-3-fosfát acyltransferázy a na ně navazujících diacylglycerol acyltransferáz, které mají různou afinitu k různým mastným kyselinám (102–105). Jejich relativní zastoupení a četnost tedy ovlivňují zastoupení jednotlivých mastných kyselin v TAG uložených v lipidových kapénkách.

1.5 STRUKTURA LIPIDOVÝCH KAPÉNEK

Lipidové kapénky jsou specializované orgány, které slouží k uchovávání neutrálních lipidů ve tkáních (106). Stavba lipidových kapének je shodná napříč tkáněmi. Vnitřek kapénky tvoří hydrofobní jádro, které obsahuje zejména TAG a estery cholesterolu. Na povrchu lipidové kapénky se nachází jednoduchá vrstva fosfolipidů, která obsahuje integrované i periferně asociované proteiny, např. perilipin 2 (PLIN2), adipofilin, tail-interacting protein 47 kDa (TIP47), S3-12 (adipocyte specific protein) (107). Lipidové kapénky byly původně považovány za zcela inertní zásobárny TAG, ale pozdější výzkumy ukázaly, že se prostřednictvím povrchových proteinů aktivně podílí na regulaci mnoha vnitrobuněčných procesů lipidového metabolismu, energetické homeostázy a signálních funkcích (108). Tyto děje zahrnují přímou interakci lipidových kapének s dalšími organelami jako je endoplazmatické retikulum, mitochondrie, peroxisomy a autofagolysosomy (109, 110). Narušení správné interakce lipidových kapének s dalšími organelami je jednou z příčin podmiňující rozvoj patofyziologických stavů jater včetně NAFLD. Progrese prosté steatózy směrem k NASH bývá spojena se stresem endoplazmatického retikula, mitochondriální a lysozomální dysfunkcí a se zvýšenou expresí povrchových proteinů lipidových kapének, což vede k jejich stabilizaci (107).

1.6 KATABOLISMUS LIPIDOVÝCH KAPÉNEK

Degradace lipidových kapének je komplexní proces, který zahrnuje koordinovaný katabolismus povrchových proteinů, fosfolipidové membrány a neutrálních lipidů (111). Hlavním proteinem

na povrchu lipidových kapének v játrech je PLIN2 (112), který zamezuje lipázám v přístupu k uskladněným TAG. Za podmínek stimulujících lipolýzu je PLIN2 degradován prostřednictvím chaperon-mediated autofagické dráhy (113). Tohoto procesu se pravděpodobně účastní i AMP-aktivovaná kináza (AMPK), která PLIN2 fosforyluje a usnadňuje jeho autofagickou degradaci (114). Na vlastní degradaci TAG se v hepatocytech podílí několik lipáz s rozdílnou specifikou a podřízených rozdílným regulačním mechanismům.

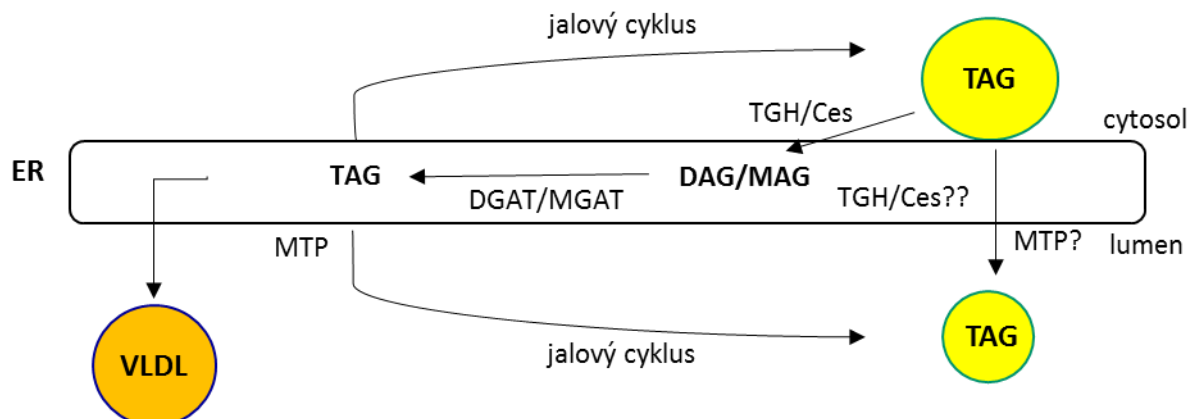
1.6.1 Enzymy podílející se na katabolismu lipidů v játrech

V hepatocytech byla prokázána přítomnost několika lipáz. ATGL katalyzuje odštěpení prvního acylového zbytku z molekuly TAG (115). Je exprimována především v bílé tukové tkáni, a dále pak v srdci (116) a kosterním svalu (117). Význam ATGL v játrech není dosud plně objasněn. Za fyziologických podmínek je exprese ATGL v játrech nízká (118). U myši, u kterých byl gen pro ATGL cíleně vyřazen, bylo dosaženo rozdílných výsledků. Někteří autoři pozorovali masivní steatózu (119), a obdobné výsledky byly získány i na modelu primárních hepatocytů (120). Jiní však zaznamenali pouze mírné zvýšení obsahu TAG v játrech nebo dokonce nepozorovali žádný efekt (121). Genetickou manipulací indukovaná zvýšená tvorba ATGL (adipose tissue glycerol lipase) v játrech vedla ke zvýšení oxidace mastných kyselin, přímému exportu mastných kyselin z hepatocytů a snížení míry steatózy (118). Jedna z hypotéz vysvětlujících tyto rozpory navrhuje, že ATGL cíleně směřuje mastné kyseliny pouze do některých metabolických drah, konkrétně k oxidaci. Opírá se o pozorování, že u ATGL-KO (knock-out) modelu nebyly prokázány žádné změny v koncentraci sérových TAG nebo volných mastných kyselin a rovněž nebyla ovlivněna rychlost sekrece TAG nebo apolipoproteinu B (118). Druhá teorie předpokládá, že ATGL se v podstatné míře účastní lipolýzy v játrech pouze za určitých podmínek, např. při metabolickém stresu. Fuchs demonstroval, že u ATGL deficitních zvířat nedošlo k akumulaci TAG v játrech za fyziologických podmínek, ale po indukci stresu endoplazmatického retikula tunicamycinem se rozvinula masivní steatóza během 48 hodin (118).

Monoacylglycerol lipáza katalyzuje finální krok hydrolýzy triacylglycerolů, tj. hydrolýzu monoglyceridů. Významně se podílí na metabolismu endokanabinoidů, protože štěpí 2-arachidonoylglycerol, který je významný ligand endokanabinoidových receptorů, na arachidonovou kyselinu (AA), která je prekursorem syntézy prostaglandinů. Monoacylglycerol lipáza tak představuje významný regulační prvek, který ovlivňuje tvorbu biologicky aktivních lipidů zasahujících do četných metabolických drah jako je metabolismus mastných kyselin, zánět, kontrola příjmu potravy a bolesti (122).

Další skupinou enzymů zasahujících do metabolismu lipidových kapének jsou karboxylesterázy (TGH/Ces). Tyto enzymy mají vysokou afinitu k TAG obsahujícím mastné kyseliny s krátkým řetězcem a výrazně nižší k TAG obsahujícím mastné kyseliny s dlouhým řetězcem. U lidí jsou exprimovány zejména v játrech, tukové tkáni a tenkém střevě. V buňce jsou lokalizovány na cytosolické straně endoplazmatického retikula a na povrchu lipidových kapének. V játrech se vyskytují pouze v hepatocytech těsně přiléhajících ke kapilárám. Exprese TGH/Ces je závislá na dietních faktorech, byl prokázán pozitivní regulační vliv cholesterolu. Zvýšený přísun mastných kyselin v dietě expresi TGH/Ces neovlivňuje (123). Výsledky některých studií naznačují, že mastné kyseliny uvolňované prostřednictvím TGH/Ces jsou využity pro tvorbu VLDL. McArdle-RH7777 buňky (potkaní buněčná hepatocytární linie) neexprimují TGH/Ces a nejsou schopny sekretovat VLDL bez dodání exogenních mastných kyselin (124). Stabilní transfekce TGH/Ces cDNA do těchto buněk vedla k depleci intracelulárních TAG zásob a obnovila jejich schopnost sekretovat lipoproteiny typu VLDL. Efekt transfekce byl anulován použitím inhibitoru serinesteráz (E600) nebo specifických inhibitorů TGH/Ces. Na základě těchto výsledků formulovali autoři hypotetický model, podle něž TGH/Ces štěpí TAG v lipidových kapénkách asociovaných s endoplazmatickým

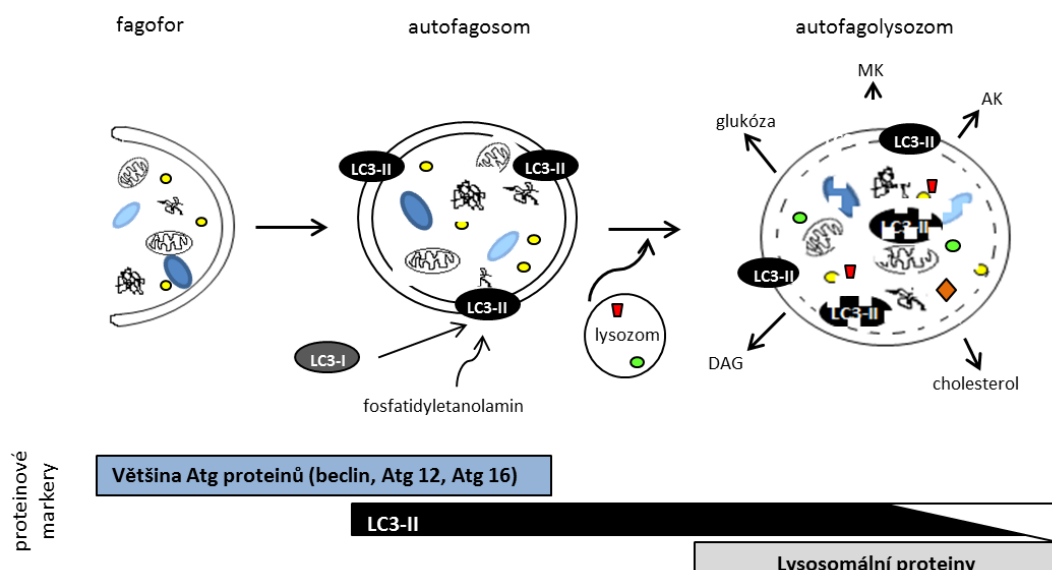
retikulem. Uvolněné mastné kyseliny jsou potom přenášeny do lumen endoplazmatického retikula, kde jsou využity k resyntéze TAG zabudovávaných do vznikající lipoproteinové partikule (123) (**obr. 1.3**).



Obrázek 1.3 Úloha TGH/Ces v tvorbě sekrečních lipoproteinových partikulí. DAG, diacylglycerol; MAG, monoacylglycerol; ER, endoplazmatické retikulum; MGAT/DGAT, monoacyl-/diacylglycerol transferáza; MPT, microsomal triacylglycerol transfer protein. Adaptováno dle Dolinsky et al. (125).

1.6.2 Lipoautofagie

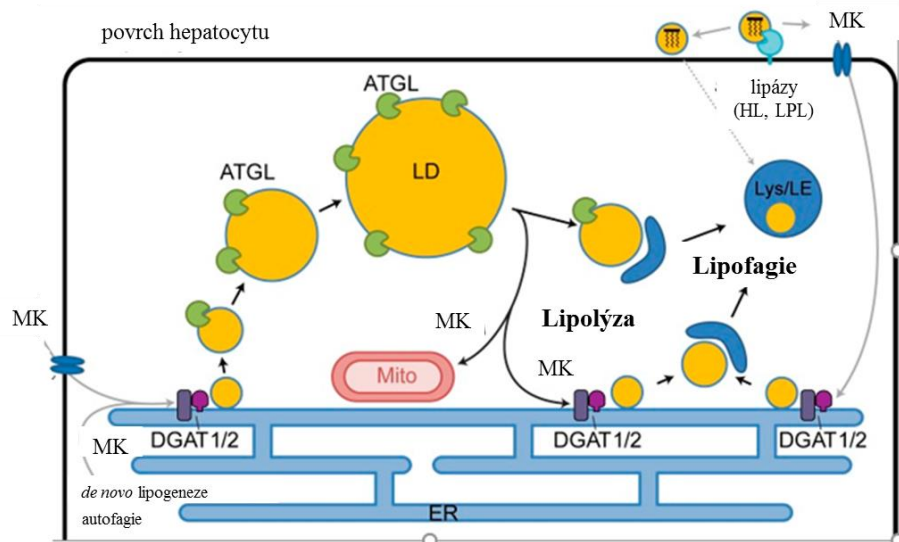
Zcela specifické vlastnosti má lysozomální lipáza (LAL), která se podílí na degradaci lipidů autofagicko-lysozomální dráhou. V buňce se vyskytuje výlučně v lysozomech, čemuž odpovídá i pH optimum tohoto enzymu, 4,5–5,0. Lysozomální lipáza v játrech byla poprvé popsána českými autory (126). Zásadní role LAL v degradaci intracelulárních TAG byla potvrzena na LAL-KO modelu, u kterého byla pozorována masivní steatóza jater (127). Jako všechny lysozomální enzymy, LAL může degradovat svůj substrát (TAG) pouze v případě, že je dopraven do lysozomu. Existují dva možné mechanismy – endocytóza a autofagie. Endocytózou je do lysozomu dopravován materiál z extracelulárního prostředí (např. LDL partikule, patogeny apod.), zatímco autofagická dráha je vyhrazena pro transport intracelulárních komponent. Autofagicko lysozomální dráha byla dlouho považována výlučně za způsob, jak do lysozomu dopravit buď poškozené orgány (např. mitochondrie) nebo proteiny. Nicméně, autofagie a lipolýza sdílí řadu společných znaků. Oba děje jsou aktivovány za podmínek hladovění a deprivace základních živin a odpovídají shodně na hormonální regulaci, tj. jsou aktivovány glukagonem a inhibovány inzulínem (128). Debeer (129) na izolovaných hepatocytech demonstroval, že látky inhibující činnost lysosomů (lysozomotropní činidla) snižují jednak produkci ketolátů z endogenních lipidů, jednak degradaci proteinů; a navrhl autofagii jako mechanismus, kterým jsou lipidové kapénky dopravovány do lysozomu. Dalším nepřímým důkazem pro účast lysozomální lipázy na metabolismu TAG v játrech je skutečnost, že inhibice lysozomální aktivity významně snižuje produkci VLDL (130). Singh (131) formuloval hypotézu, podle níž jsou lipidové kapénky inkorporovány do autofago(lipo)zomu stejně jako orgány nebo proteiny a v této podobě dopravovány do lysozomu (**obr. 1.4**). Tato hypotéza byla založena na těchto skutečnostech: (i) inhibitory autofagie zvyšují obsah intracelulárních TAG *in vivo* i *in vitro*; (ii) lipidové kapénky byly pozorovány uvnitř autofagozomů; (iii) u myši s vyřazeným genem *atg7* (klíčový protein autofagického procesu) v játrech byla pozorovaná masivní steatóza doprovázená akumulací TAG a cholesterolu v játrech a sníženou sekrecí VLDL. Poruchy autofagie tak mohou představovat další mechanismus přispívající k rozvoji jaterní steatózy.



Obrázek 1.4 Schéma tvorby autofagolysosomu. AK, aminokyseliny; DAG, diacylglycerol; LC3, mikrotubule-associated protein 1A/1B-light chain 3; MK, mastné kyseliny.

1.6.3 Koordinace lipolýzy v játrech

Nedávné studie ukazují, že jednotlivé lipolytické dráhy jsou navzájem vysoce propojené a koordinované. První krok představuje degradace PLIN2 v procesu chaperon-mediated autofagie, což umožní přístup lipáz a proteinů účastnících se tvorby autofagozomů k povrchu lipidové kapénky. ATGL zprostředkovaná lipolýza a lysosomální degradace pravděpodobně představují dva na sebe navazující kroky. ATGL nejprve hydrolyzuje velké lipidové kapénky a redukuje jejich velikost. Degradace menších lipidových kapének je dokončena v autofagolysosomech (132) (**obr. 1.5**). ATGL zprostředkovaná lipolýza rovněž aktivuje SIRT1 signalizaci a následnou deacetylaci/aktivaci četných proteinů přímo se účastnících lipoautofagie (133) a přímo interaguje s proteinem LC3, klíčovou komponentou tvorby autofagosomu. Zvláštní větev pravděpodobně představuje činnost TGH/Ces, která se podílí na degradaci intracelulárních TAG, které jsou směřovány k sekreci z buňky ve formě VLDL.



Obrázek 1.5 Degradace lipidových kapének v hepatocytu. ATGL, adipose tissue glycerol lipase; HL, jaterní lipáza; LD, lipid droplets; LPL, lipoproteinová lipáza; Lys/LE, lysosom; MK, mastné kyseliny. Převzato z Schott et al. (132).

1.7 MASTNÉ KYSELINY JAKO SIGNÁLNÍ MOLEKULY

Mastné kyseliny nepředstavují pouze významný zdroj energie, ale i důležité signální molekuly, které regulují četné metabolické procesy, mimo jiné zajišťující precizní koordinaci uvolňování mastných kyselin dle aktuálních potřeb buňky a organismu. Mastné kyseliny a jejich deriváty regulují buněčné procesy na mnoha úrovních – genové transkripce, post-transkripční modifikace (acylace apod.) a přímé modulace aktivity enzymů v podobě koaktivátorů.

1.7.1 Procesy nezávislé na modulaci transkripce

Tato skupina zahrnuje řadu velmi různorodých mechanismů. Druh mastných kyselin, zejména stupeň saturace acylových zbytků ve fosfolipidech, ovlivňuje membránovou fluiditu, strukturu lipidových raftů a v důsledku toho i přenos signálu (134, 135). Membránová fluidita stoupá se stoupajícím zastoupením nenasycených mastných kyselin v pořadí nasycené mastné kyseliny (SFA) < mononenasycené mastné kyseliny (MUFA) < *n*-6 polynenasycené mastné kyseliny (PUFA) < *n*-3 PUFA. SFA působí jako agonisté Toll-like receptorů (TLR2 a TLR4) a aktivují Src-kinázy, zatímco ω -3 PUFA působí jako antagonisté těchto dějů, a to jak modifikací struktury membránových mikrodomén, kde jsou receptory lokalizovány, tak ovlivněním jejich funkce (134, 136). Acylové zbytky, které jsou prostřednictvím fosfolipáz odštěpovány z membránových fosfolipidů, slouží jako substráty pro syntézu četných pro- a protizánětlivých mediátorů, např. eikosanoidů, resolvinů a protektinů (137). Omega-6 PUFA jsou převážně prekurzory pro-zánětlivých cytokinů, zatímco od ω -3 PUFA se odvozují spíše protizánětlivé mediátory (138). Omega-3 mastné kyseliny kromě toho přímo modulují aktivitu klíčového prozánětlivého faktoru NF- κ B tím, že interferují s I- κ B fosforylací a znemožňují tak aktivaci NF- κ B (139). SFA, zejména kyselina palmitová a stearová, indukují stres endoplazmatického retikula (140) a fungují jako spouštěč NLRP3 inflamasomu (141) v odpověď na infekci nebo buněčný stres. Omega-3, ale nikoli ω -6 nebo ω -9 PUFA, aktivaci NLRP3 inflamasomu inhibují.

1.7.2 Mastné kyseliny jako ligandy transkripčních faktorů

Mastné kyseliny jsou agonisté mnoha nukleárních transkripčních faktorů. V aktivovaném stavu se tyto faktory váží k promotorovým oblastem specifických genů a řídí jejich transkripci. Obecně lze říci, že SFA a PUFA aktivují opačné děje. SFA jsou asociovány s ději, které podporují vznik jaterní steatózy. Zvyšují expresi genů spadajících do SREBP dráhy stimulující lipogenezi (SREBP1) a sterol-O-acyltransferázu, klíčový enzym homeostázy cholesterolu (142). Naproti tomu ω -3 PUFA stimulují dráhy vedoucí k oxidaci mastných kyselin a inhibují *de novo* syntézu mastných kyselin (143). Je logické, že výrazný účinek jako signální molekuly mají velmi málo abundantní mastné kyseliny, např. EPA (kyselina eikosapentaenová) nebo DHA (kyselina dokosaheptaenová), zatímco v dietě hojně zastoupené mastné kyseliny, např. kyselina olejová, mají mnohem slabší regulační účinky.

Nukleární transkripční faktory závislé na mastných kyselinách jako ligandech patří do několika rodin – PPAR α (Peroxisome Proliferator-Activated Receptor alpha), SREBP, LXR (liver X Receptor), FXR (Farnesoid X Receptor) a Nrf2 (Nuclear factor erythroid 2-related factor 2). PPAR skupina transkripčních faktorů je tvořena čtyřmi rodinami (α , β , γ , δ) s rozdílnou tkáňově specifickou expresí a funkcí. V játrech je dominantní rodina PPAR α , která je klíčovým regulátorem metabolismu lipidů. Jejich nejúčinnějšími ligandy jsou ω -3 mastné kyseliny, EPA a DHA. PPAR α signalizace představuje jednu z hlavních strategií buňky v adaptaci na hladovění, neboť stimuluje utilizaci mastných kyselin jako hlavního zdroje energie a vede k minimalizaci utilizace glukózy (144).

SREBP1 aktivuje především geny, které kódují enzymy účastníce se *de novo* lipogeneze, zatímco SREBP-2 aktivuje geny pro proteiny účastníce se syntézy a vychytávání cholesterolu (145). Mastné kyseliny regulují aktivitu SREBP na transkripční a post-transkripční úrovni.

PUFA, ale nikoli MUFA nebo SFA, účinně inaktivují SREBP1c na všech úrovních a potlačují tak expresi genů účastnících se *de novo* lipogeneze (146), desaturace mastných kyselin (147) a jejich elongace (148). Vzhledem k tomu, že se desaturázy a elongázy účastní i syntézy PUFA, fungují tyto mastné kyseliny i jako zpětnovazebný regulátor vlastní syntézy.

Nrf2 je hlavním transkripčním faktorem řídícím expresi genů kódujících enzymy účastnící se obrany proti oxidačnímu stresu způsobenému vzestupem obsahu reaktivních sloučenin kyslíku (149). K tomu dochází mimo jiné i při zvýšeném příjmu PUFA a následné aktivaci jejich mitochondriální a peroxisomální oxidace. Stimulační efekt PUFA na Nrf2 je zprostředkován pravděpodobně oxidovanými formami těchto mastných kyselin (150), které stabilizují Nrf2 mRNA.

Vzhledem k velmi nízké rozpustnosti mastných kyselin v cytosolu je vhodné zařadit mezi intracelulární receptory mastných kyselin i FABPs (fatty acid-binding proteins), které zajišťují jejich transport od buněčné membrány přes cytoplasmu do jádra. Dosud bylo popsáno devět tříd FABP s výrazně tkáňově specifickou expresí (151). Existují doklady, že jednotlivé typy FABP selektivně sekvestrují a distribuují různé typy mastných kyselin a přispívají tak k regulaci signalizace a aktivity jednotlivých enzymů (152). Jaterní forma FABP (L-FABP) interaguje s PPAR α a PPAR γ , ale nikoli s PPAR δ nebo RXR α . Na modelu izolovaných myších hepatocytů bylo prokázáno, že L-FABP funguje jako pozitivní regulátor PPAR α aktivity, přičemž aktivita nukleárního receptoru je striktně závislá na dostupnosti L-FABP (153).

1.7.3 Mastné kyseliny a signalizace v játrech

Játra představují orgán s druhou největší kapacitou pro ukládání lipidů v těle (po tukové tkáni). Mimo to jsou játra jediným orgánem, který je schopen transformovat mastné kyseliny na ketolátky, které jsou zdrojem energie pro mozek v době hladovění. Centrální role jater v lipidovém a energetickém metabolismu vyžaduje, aby regulace všech účastnících se metabolických procesů byla velmi dobře koordinovaná. V játrech je regulace metabolické odpovědi, tj. preference oxidace nebo ukládání mastných kyselin, silně závislá na stupni saturace mastných kyselin, které fungují jako ligandy klíčových transkripčních faktorů. Vysoké zastoupení ω -3 a ω -6 PUFA v dietě (tj. pokud je 2-5 % energetického přísunu pokryto PUFA) vede k potlačení lipolytických genů. Hlavním mechanismem je inhibice SREBP1c transkripční aktivity a indukce genů účastnících se oxidace mastných kyselin prostřednictvím stimulace aktivity PPAR α (154).

Ne zcela vyjasněná zůstává otázka, zda signální funkce mastných kyselin závisí na jejich původu, tj. zda exogenní mastné kyseliny (vychytané z cirkulace) a endogenní mastné kyseliny (vzniklé štěpením intracelulárních lipidů, popř. syntetizované *de novo*) jsou zastupitelné. Clarke (154) demonstroval, že pouze v dietě přijaté ω -3 a ω -6 PUFA a jejich produkty vzniklé Δ -6 desaturázovou dráhou mohou modulovat jaterní lipidový metabolismus, zatímco produkty vzniklé elongací a desaturací SFA a MUFA nemají žádný efekt. Naproti tomu Chakravarthy (155) přinesl důkazy o tom, že i produkty syntézy mastných kyselin, tj. kyselina palmitová (C 16:0) a její deriváty, mohou efektivně modulovat expresi některých PPAR α cílových genů. Ong (120) na ATGL „gain-and-loss-function model“ ukázal, že mastné kyseliny uvolněné pomocí ATGL ovlivňují oxidaci mastných kyselin, ale nemají žádný vliv na sekreci VLDL. Snížená exprese ATGL v jaterní tkáni u ATGL-KO modelu korelovala se sníženou expresí PPAR α a jeho cílových genů (120). Další úhel pohledu je založen na teorii, že jak mastné kyseliny syntetizované *de novo*, tak mastné kyseliny uvolněné lipolýzou mohou ovlivňovat genovou expresi, ale tyto zdroje nejsou vzájemně zastupitelné (155).

2 HLAVNÍ CÍLE A ČÁSTI HABILITAČNÍ PRÁCE

Předkládaná habilitační práce je založena na výsledcích experimentální práce, kterých žadatelka se svými spolupracovníci a spolupracovníky dosáhla v oblasti výzkumu mechanismů rozvoje a terapeutických možností ovlivnění nealkoholové jaterní steatózy. Experimentální část je rozdělena do tří celků.

První část habilitační práce (*Kapitola 3: Mechanismy podílející se na rozvoji dietně indukované NAFLD*) popisuje na experimentálním zvířecím modelu (potkan) vliv cílených dietních manipulací (podávání diety s vysokým obsahem sacharózy nebo vysokým obsahem tuků) na jednotlivé metabolické dráhy zapojené do metabolismu triacylglycerolů a mastných kyselin v játrech. Diskutován je vliv těchto manipulací na lipolýzu, oxidaci mastných kyselin a sekreci VLDL. Zvláštní pozornost je věnována degradaci intracelulárních lipidů v lysozomech a významu lipoautofagie v metabolismu lipidů v játrech.

Druhá část habilitační práce (*Kapitola 4: Farmakologické nástroje ovlivnění NAFLD*) se zaměřuje na možnosti farmakologické terapie v léčbě jaterní steatózy. Jsou zde uvedeny výsledky týkající se vlivu perorálního antidiabetického léčiva metforminu a potravního doplňku karnitinu. Jsou zde diskutovány základní mechanismy působení obou látek, zejména jejich vliv na mitochondriální respiraci a oxidační stres. Tato část se opírá zejména o experimenty *in vitro* provedené na submitochondriálních strukturách, izolovaných mitochondriích nebo permeabilizovaných buňkách. Kromě toho je prezentován i efekt podávání těchto látek *in vivo* na zvířecích modelech dietně (vysokotuková dieta) a geneticky (kmen hereditárně hypertriglyceridemických potkanů) navozené jaterní steatózy.

Třetí část habilitační práce (*Kapitola 5: specifické případy jaterní steatózy*) se věnuje jaterní steatóze spojené se zvláštními fyziologickými situacemi, a to steatóze provázející parenterální aplikaci výživy (parenteral nutrition-associated liver disease, PNALD) a steatóze jaterního štěpu po transplantaci jater. Mechanismus rozvoje PNALD a možný terapeutický vliv ω -3 mastných kyselin byl studován na zvířecím modelu (potkan), markery rozvoje steatózy štěpu byly sledovány díky možnosti analyzovat jaterní biopsie pacientů, kteří podstoupili transplantaci jater v Institutu klinické a experimentální medicíny (IKEM).

Detailnější informace o zaměření a dílčích cílech provedeného výzkumu je uvedena v následujících odstavcích, odkazujících na jednotlivé přílohy s původními pracemi.

Mechanismy podílející se na rozvoji dietně indukované NAFLD

Význam lipoautofagie v metabolismu triacylglycerolů v játrech (Přílohy I–V).

Vliv diet na oxidaci mastných kyselin a sekreci VLDL v játrech (Příloha VI).

Farmakologické nástroje ovlivnění NAFLD

Mechanismus působení biguanidů na mitochondriální respirační řetězec (Přílohy VII a VIII).

Vliv metforminu na oxidační stres při dietně indukované steatóze (Příloha IX).

Vliv karnitinu na oxidační stres a poruchy metabolismu lipidů v játrech u genetického modelu steatózy a hypertriglyceridemie (Příloha X).

Specifické případy jaterní steatózy

Omega-3 polynenasycené mastné kyseliny jako možný terapeutický nástroj při PNALD (Příloha XI).

Využití miRNA jako potenciálních markerů rozvoje poškození jater při PNALD (Příloha XII).

Transkriptomický profil odlišující jaterní štěp s rozvinutou steatózou a bez steatózy, průřezová studie u pacientů po transplantaci jater (Příloha XIII).

Sérové hladiny miR-33a jako marker steatózy a zánětu jaterního štěpu u transplantovaných pacientů (Příloha XIV).

3 METABOLISMUS LIPIDŮ PŘI DIETNĚ INDUKOVANÉ NAFLD

Diety s vysokým obsahem jednoduchých sacharidů nebo tuků vedou k nadměrnému ukládání TAG v játrech a rychlému rozvoji steatózy. Lipidové kapénky v cytosolu hepatocytů nepředstavují pouze inertní zásobárnu energeticky bohatého substrátu, ale podstupují kontinuální cyklus lipolýzy a reesterifikace, tzv. jalový cyklus (156, 157). Toto zdánlivě paradoxní uspořádání umožňuje pružně reagovat na měnící se metabolické požadavky buňky i organismu.

Položili jsme si otázku, jak jednotlivé metabolické dráhy utilizace lipidů přispívají k rozvoji steatózy a zda jsou odlišně regulovány při různých dietních manipulacích. Soustředili jsme se na degradaci TAG lipoautofagickou dráhou, oxidaci mastných kyselin a sekreci VLDL. Výsledky uvedené v této kapitole byly získány na zvířecích modelech, konkrétně na potkaních kmene Wistar a na kmeni hereditárně hypertriglyceridemických potkanů (HHTg), který je od kmene Wistar odvozen na základě vysoké citlivosti k vysokosacharóзовé dietě (HSD). Potkani kmene HHTg manifestují většinu znaků metabolického syndromu, tj. zhoršenou glukózovou toleranci, hyperinzulinemii, hypertriglyceridémii, zvýšený krevní tlak a inzulínovou rezistenci. Použili jsme dvě experimentální diety, dietu s vysokým obsahem tuků (vysokotukovou, HFD) a dietu s vysokým obsahem sacharózy (HSD), které jsme potkanům podávali po dobu 2 (HF-2, HS-2), 4 (HF-4, HS-4) nebo 10 týdnů (HF-10, HS-10). Kontroly (SD) byly krmeny standardní laboratorní dietou pro hlodavce. Jednotlivé metody jsou detailně popsány v přílohách. Dietní intervence vedla dle očekávání k zvýšení obsahu TAG v játrech ($SD < HS-2 < HS-4 < HF-2 < HF-10$). Obsah β -hydroxybutyrátu v séru lačných zvířat se zvýšil po podávání HSD i HFD, v sytém stavu pouze u zvířat krmených HFD (**tab. 3.1**).

Tabulka 3.1 Obsah TAG v játrech a β -hydroxybutyrátu v séru. β -OH, β -hydroxybutyrát. Obsah TAG v játrech je uveden v $\mu\text{mol g}^{-1}$ v.v., obsah β -hydroxybutyrátu v $\mu\text{mol l}^{-1}$. * $p < 0.05$, *** $p < 0.001$ vs SD; ### $p < 0.001$ vs HS.

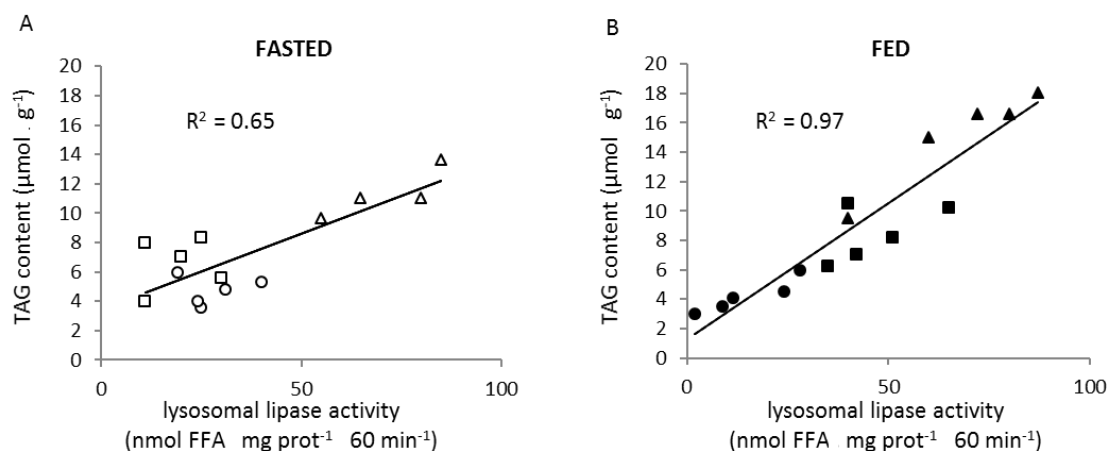
	SD	HS-2	HF-2	HS-4	HF-4	HF-10
TAG v játrech	4,1 $\pm$ 0,8	6,6 $\pm$ 0,6*	14,7 $\pm$ 1,1***	7,6 $\pm$ 0,6***	20,8 $\pm$ 1,5***	50,6 $\pm$ 9,6***
β -OH v séru lačné	1,2 $\pm$ 0,04	2,5 $\pm$ 0,1***	3,2 $\pm$ 0,25***	2,2 $\pm$ 0,14***	3,8 $\pm$ 0,2***	2,4 $\pm$ 0,2***
β -OH v séru syté	0,02 $\pm$ 0,01	0,07 $\pm$ 0,01	0,28 $\pm$ 0,05***,###	0,03 $\pm$ 0,02	0,45 $\pm$ 0,05***,###	0,50 $\pm$ 0,09***

3.1 ÚLOHA LIPOAUTOFAGIE V ODBOURÁVÁNÍ INTRACELULÁRNÍCH TAG V JÁTRECH

3.1.1 Lysozomální lipáza se v játrech podílí na odbourávání intracelulárních TAG

Otázka, zda kyselá jaterní (lysozomální) lipáza přispívá nebo je dokonce dominantní při odbourávání intrahepatocytárních lipidů byla donedávna sporná. V našich pokusech jsme proto v první řadě testovali tuto hypotézu. Vycházeli jsme z předpokladu, že pH optimum LAL leží ve výrazně kyselé oblasti. Navrhli jsme pokus, kde jediným zdrojem substrátu byly intracelulární lipidy přítomné v jaterním homogenátu a měřili jsme nárůst obsahu produktů degradace TAG, tj. mastných kyselin a diacylglycerolu (DAG), při pH=4,5 (optimum LAL), a při pH=8,0 (optimum TGH/CeS3 nebo HL). Za těchto podmínek jsme nezjistili přítomnost žádných degradačních produktů lipidů při pH 8,0. Naopak, při pH=4,5 jsme prokázali přítomnost mastných kyselin a DAG což prokazuje lipolytickou aktivitu. Pokud jsme použili

homogenáty z jater zvířat krmných SD nebo HSD, viděli jsme i závislost aktivity LAL na prandiálním stavu (útlum v sytém stavu, vzestup při lačnění), která nebyla patrná u zvířat krmných HFD. Aktivita LAL vzhledem k endogennímu substrátu pozitivně korelovala s obsahem TAG v játrech (**obr. 3.1**).



Obrázek 3.1 Korelace mezi aktivitou lysozomální lipázy v játrech a obsahem TAG v játrech. Aktivita lysozomální lipázy byla stanovena jako uvolňování mastných kyselin z intracelulárních TAG. Prázdné symboly: lačná zvířata; plné symboly: sytá zvířata. ○ SD, □ HSD, △ HFD. Převzato z Cahova M. et al. doi: 10.1155/2012/757205.

Toto pozorování je v přímém rozporu s předpokladem, že steatóza je důsledkem snížené aktivity lipolytických enzymů. Pokus jsme zopakovali s použitím exogenního radioaktivně značeného substrátu (³H-triolein). Za těchto podmínek jsme detekovali lipolytickou aktivitu při pH=8,0 i pH=4,5, přičemž závislost na prandiálním stavu jsme opět viděli pouze u LAL v jaterním homogenátu zvířat krmných SD a HSD. Naše výsledky prokazují, že LAL se účastní štěpení endogenních TAG v játrech a její aktivita je regulována v závislosti na fyziologickém stavu organismu a v důsledku dietních intervencí.

3.1.2 Intracelulární TAG jsou v játrech odbourávány autofagicko-lysozomální dráhou

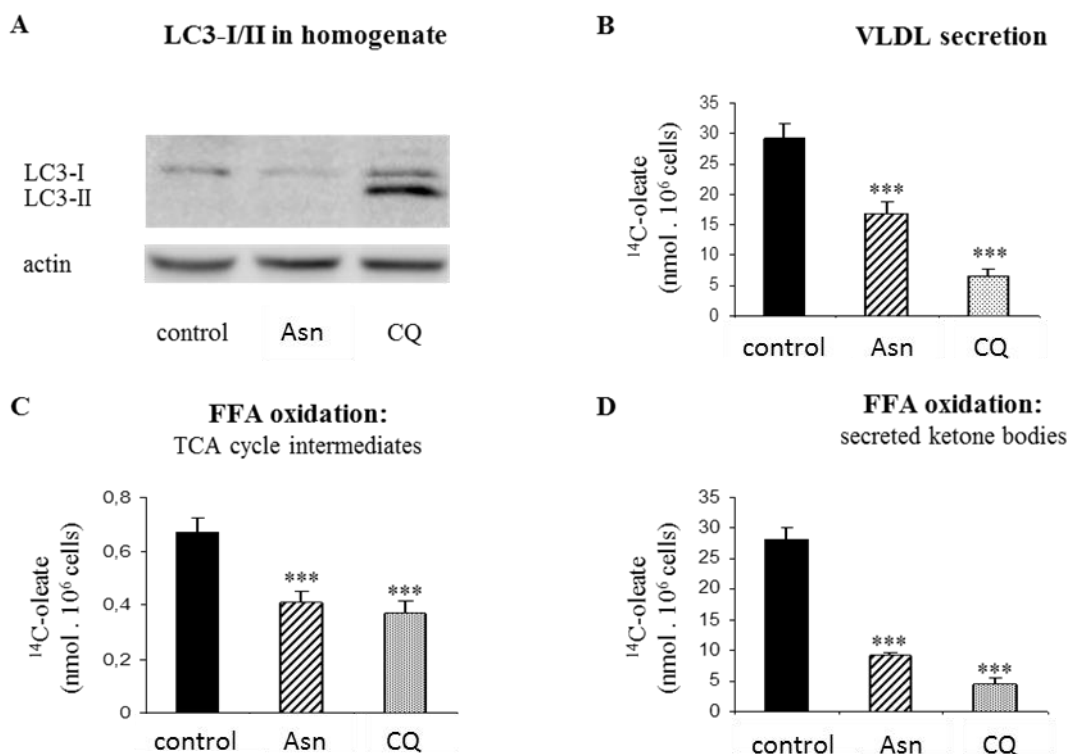
Dále jsme testovali hypotézu, že odbourávání intracelulárních lipidů souvisí s procesem autofagie a LAL může degradovat TAG pouze v případě, že lipidové kapénky se nacházejí v aktivovaných autofagolysosomech. Využili jsme skutečnost, že neaktivované lysozomy a aktivní autofagolysosomy obsahující lipidové kapénky mají rozdílnou hustotu a je možné je separovat pomocí centrifugace v hustotním gradientu. Oddělili jsme proto obě frakce a lipolytickou aktivitu v jednotlivých frakcích jsme stanovili s použitím exogenního značeného substrátu při kyselém pH. Lipolytická aktivita ve frakci těžkých lysozomů byla konstantní a neměnila se ani v závislosti na prandiálním stavu, ani na dietě zvířete. Ve frakci aktivovaných autofagolysosomů jsme naopak pozorovali oba typy závislostí, což podporuje hypotézu o degradaci lipidů v játrech autofagickou dráhou.

3.1.3 Manipulace intenzity autofagie ovlivňuje utilizaci mastných kyselin v primárních hepatocytech

Pokud je autofagická dráha cestou, kterou jsou intracelulární lipidy transportovány do lysozomů k degradaci, lze prokázat, že ovlivnění intenzity autofagie ovlivní i degradaci intracelulárních TAG a intenzitu využití uvolněných mastných kyselin. Sledovali jsme proto vliv zásahů do procesu autofagie na metabolický osud intracelulárních TAG v primárních

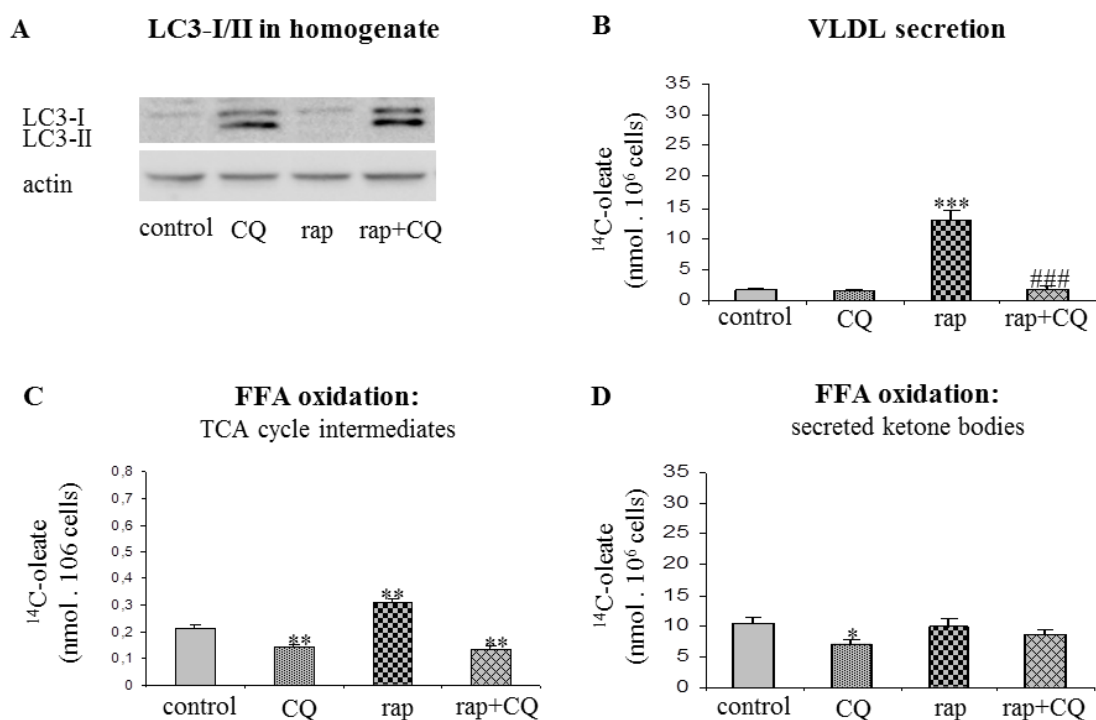
hepatocytech. Izolované hepatocyty, získané od potkana krmeného standardní dietou, jsme kultivovali 18 hod v přítomnosti buď ^{14}C -glycerolu nebo ^{14}C -olejové kyseliny konjugované s hovězím sérovým albuminem (BSA) („pulse“). Za těchto podmínek byly všechny nově syntetizované TAG radioaktivně značené buď na glycerolové kostře nebo acylovém zbytku molekuly TAG. Poté bylo médium odstraněno a následovala „chase“ perioda (6 hod), kdy jsme simulovali (i) lačnění, tj. kultivace ve Williamsově mediu pouze s přidavkem pyruvátu nebo (ii) sytý stav, kdy byly hepatocyty inkubovány ve Williamsově mediu obohaceném o 10% FCS (fetal calf serum), glutamin, laktát a inzulín. Jako inhibitor autofagie jsme použili asparagin (20 mmol l^{-1}), jako stimulator rapamycin (100 nmol l^{-1}) a jako inhibitor lysozomální aktivity chloroquin ($500 \text{ } \mu\text{mol l}^{-1}$) (158). Intenzitu autofagie jsme hodnotili podle akumulace LC3-II (Microtubule-associated protein 1A/1B-light chain 3) v přítomnosti inhibitoru lysozomální degradace chloroquinu. LC3 je syntetizován jako neaktivní preprotein, který je posléze enzymaticky konvertován na formu LC3-I. V procesu tvorby autofagozomů je na LC3-I navázán fosfatidylethanolamin (PE) a vzniká LC3-II. LC3-II je jediný proteinový marker fagoforu, autofagozomů a plně maturovaného autofagolysosomu (159). Nicméně, LC3-II je rovněž kontinuálně uvnitř lysosomu degradován a jednorázové stanovení obsahu LC3-II o intenzitě autofagie nevypovídá dostatečně. Měřili jsme proto velikost přírůstku obsahu LC3-II za podmínek inhibice lysozomální degradace (160). Intenzitu jednotlivých metabolických drah utilizace mastných kyselin jsme stanovili podle radioaktivity inkorporované do meziproductů cyklu trikarboxylových kyselin (TCA) a ketolátek (oxidace) a sekretované do media (tvorba VLDL).

Výsledky experimentu v podmínkách lačnění ukazuje **obr. 3.2**. Akumulace LC3-II potvrdila probíhající autofagický proces (**obr. 3.2A**). ^{14}C -olejová kyselina byla stejnoměrně využívána k tvorbě ketolátek a sekreci VLDL, v meziproductech TCA cyklu jsme zachytili pouze asi 1 % z celkové radioaktivity (**obr. 3.2B-D**). Jak inhibice autofagie asparaginem, tak i inhibice aktivity lysozomů chloroquinem významně snížila dostupnost ^{14}C -olejové kyseliny pro všechny metabolické dráhy.



Obrázek 3.2 Vliv inhibice autofagie a lysozomální aktivity na utilizaci mastných kyselin z intracelulárních TAG v lačných hepatocytech. **A:** LC3-I/II v homogenátu; **B:** inkorporace ^{14}C -olejové kyseliny do sekretovaných TAG; **C:** inkorporace ^{14}C -olejové kyseliny do meziproductů TCA cyklu; **D:** inkorporace ^{14}C -olejové kyseliny do ketolátek. Asn = asparagin (inhibitor autofagie); CQ = chloroquin (inhibitor lysozomální aktivity). Hodnoty představují aritmetický průměr $\pm$ S.E.M ze tří nezávislých měření. *** $p < 0.001$ vs control. Převzato z Škop V. et al. doi: 10.33549/physiolres.932285

V další sérii experimentů jsme se soustředili na otázku, zda experimentálně navozená stimulace autofagie povede ke zvýšené rychlosti degradace intracelulárních TAG a zvýšené utilizaci mastných kyselin a zda inhibice lysozomální aktivity bude působit opačně. Hepatocyty s radioaktivně značenými intracelulárními TAG byly inkubovány v podmínkách simulujících sytý stav s cílem snížit bazální intenzitu autofagie. Část hepatocytů byla inkubována se stimulem autofagie rapamycinem a část s rapamycinem a inhibitorem lysozomální aktivity chloroquinem (**obr. 3.3**). Rapamycin významně zvýšil inkorporaci ^{14}C -olejové kyseliny do sekretovaných TAG a tento efekt byl eliminován přítomností chloroquinu (**obr. 3.3B**). Rapamycin měl však jen velmi slabý stimulační vliv na inkorporaci ^{14}C -olejové kyseliny do meziproductů TCA cyklu a neovlivnil využití intracelulárních TAG pro tvorbu ketolátek (**obr. 3.3C,D**). V procesu ketogeneze jsou tři vysoce regulované kroky: (i) dostupnost mastných kyselin (z potravy, z lipolýzy v tukové tkáni, z intracelulárních TAG); (ii) vstup mastných kyselin do mitochondrií a (iii) mitochondriální enzym HMG-CoA syntáza (hydroxymethylglutaryl-CoA syntáza). Druhý a třetí krok jsou v sytém stavu potlačeny a samotná zvýšená dostupnost mastných kyselin je nemůže kompenzovat (161). V důsledku toho může být nadbytek mastných kyselin směřován pouze do tvorby VLDL, což ukázaly i výsledky našich experimentů.



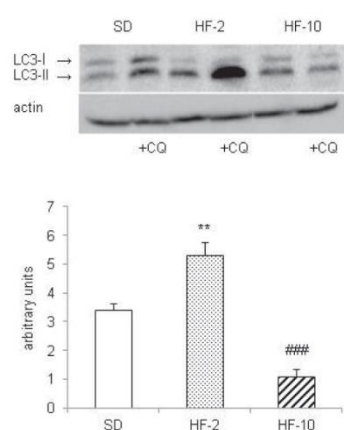
Obrázek 3.3 Vliv inhibice autofagie a lysozomální aktivity na utilizaci mastných kyselin z intracelulárních TAG v sytých hepatocytech. **A:** LC3-I/II v homogenátu; **B:** inkorporace ^{14}C -olejové kyseliny do sekretovaných TAG; **C:** inkorporace ^{14}C -olejové kyseliny do meziproductů TCA cyklu; **D:** inkorporace ^{14}C -olejové kyseliny do ketolátek. rap = rapamycin (stimulátor autofagie); CQ = chloroquin (inhibitor lysozomální aktivity). Hodnoty představují aritmetický průměr $\pm$ S.E.M ze tří nezávislých měření. ** $p < 0.01$ *** $p < 0.001$ vs control; ### $p < 0.001$ CQ+rap vs rap. Převzato z Škop V. et al. doi: 10.33549/physiolres.932285

V poslední sérii experimentů jsme se zaměřili na osud glycerolové kostry intracelulárních TAG. Významná část celkové radioaktivity inkorporované do glycerolové kostry intracelulárních TAG byla na konci chase periody detekována v kultivačním médiu což značí, že byla sekretována z hepatocytů. Zásahy, které směřovaly k oslabení autofagicko-lysozomální dráhy (simulovaný sytý stav, asparagin, chloroquin), vedly ke snížení sekrece TAG obsahujících ^{14}C -glycerol, zatímco zásahy vedoucí ke stimulaci této dráhy měly opačný efekt. Potvrdili jsme tak účast autofagicko-lysozomální dráhy v procesu sekrece VLDL. Kromě toho jsme však i nepřímo potvrdili zapojení LAL. Přítomnost ^{14}C -glycerolu v sekretovaných TAG implikuje, že produkty částečné degradace TAG (mono- a diacylglyceroly) byly využity pro syntézu VLDL partikulí. LAL preferenčně odštěpuje acylové zbytky z TAG za vzniku DAG, zatímco její afinita k DAG jako substrátu je řádově nižší (162). Kromě toho jsme nezaznamenali významné naředění ^{14}C -glycerolu v sekretovaných TAG ve srovnání s intracelulárními lipidy, k čemuž by došlo, kdyby byla molekula TAG v lysozomu degradována úplně a značený glycerol byl při resyntéze TAG pro tvorbu VLDL nahrazen neznačeným. Výsledky pokusů na izolovaných primárních hepatocytech potvrdily, že zásahy ovlivňující intenzitu autofagicko-lysozomální dráhy ovlivňují rovněž rychlost degradace intracelulárních TAG a využití produktů lipolýzy k oxidaci nebo VLDL sekreci. Z toho vyvozujeme, že intracelulární TAG jsou alespoň částečně degradovány v lysozomech a funkční autofagický proces je nezbytnou podmínkou tohoto děje.

3.1.4 Dlouhodobá expozice vysokotukové dietě zvyšuje aktivitu lysozomálních enzymů

V následující studii jsme se věnovali studiu faktorů, které mohou ovlivňovat intenzitu lipoautofagie *in vivo*. Zajímal nás zejména vliv dietně indukované steatózy na aktivitu lysozomálních enzymů. Stanovili jsme proto *in vitro* aktivitu LAL, tj. enzymu zodpovědného za degradaci TAG, a dvou dalších lysozomálních enzymů, glukuronidázy a kyselé fosfatázy. Aktivita LAL a kyselé fosfatázy byla stejná ve skupinách SD a HF-2 a zvýšená u skupiny HF-10. Aktivita glukuronidázy byla stejná ve skupinách SD a HF-10, zatímco ve skupině HF-2 byla mírně snížena. Celkové množství LAL proteinu v jaterním homogenátu bylo po 10 týdnech podávání HFD významně vyšší. mRNA exprese genů pro všechny tři enzymy nebyla podáváním HFD ovlivněna.

3.1.5 Dlouhodobá a krátkodobá expozice vysokotukové dietě má opačný efekt na intenzitu autofagie

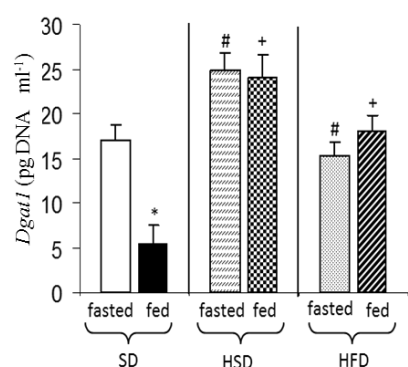


Intenzita autofagie, měřená jako rychlost nárůstu obsahu LC3-II v lysozomální frakci v přítomnosti chloroquinu, a exprese mRNA pro dva klíčové markery (beclin 1 a ATG16), se v závislosti na době podávání HFD měnila. Zatímco po dvou týdnech podávání HFD (HF-2) došlo k významnému nárůstu ve srovnání s kontrolní skupinou, po deseti týdnech (HF-10) intenzita autofagie klesla pod úroveň kontrolní skupiny (**obr. 3.4**).

Obrázek 3.4 Vliv HFD na intenzitu autofagie. Lačná zvířata byla v celkové narkóze podrobena částečné hepatektomii a vzorek jaterní tkáně (A) byl okamžitě zamražen v tekutém dusíku. Následně byl

aplikován inhibitor aktivity LAL chloroquin (120 mg/kg tělesné hmotnosti, i.p.). Pokus byl ukončen po čtyřech hodinách, kdy jsme odebrali vzorek jaterní tkáně B. Intenzita autofagie byla stanovena jako nárůst obsahu LC3-II ve vzorcích s chloroquinem (CQ). Výsledky jsou vyjádřeny jako intenzity bandů B (post-CQ) ku A (pre-CQ), aritmetický průměr $\pm$ S.E.M. ** $p < 0.01$ HF-2 vs SD, ### $p < 0.001$ HF-10 vs SD. Převzato z Papáčeková Z. et al. doi: 10.33549/physiolres.932394.

3.1.6 Vyšší lipolytická aktivita v játrech je spojena s vyšší esterifikační kapacitou



Volné mastné kyseliny jsou potenciálně vysoce toxické látky a jako takové jsou v buňkách buď bezprostředně využity (oxidace, ketogeneze) nebo uloženy zpět do TAG (re-esterifikace). Dgat1 (diacylglycerol O-acyltransferáza 1) je enzym zodpovědný za finální krok syntézy TAG, kdy katalyzuje přenos acylové skupiny na diacylglycerol-fosfát. V našich pokusech exprese *Dgat1* mRNA částečně kopírovala aktivitu LAL, zejména se zvýšila exprese *Dgat1* sytých zvířat krměných HFD (**obr. 3.5**).

Obrázek 3.5 Expresa *Dgat1* mRNA v jaterním homogenátu. Sloupce představují aritmetický průměr $\pm$ S.E.M., $n=7$. * $p < 0.05$ fed vs fasted; # $p < 0.05$ vs SD fasted; + $p < 0.05$ vs SD fed. Převzato z Cahová M. et al. PMID 20974050.

3.2 VYUŽITÍ MASTNÝCH KYSELIN UVOLNĚNÝCH LIPOLÝZOU INTRACELULÁRNÍCH TAG

Mastné kyseliny uvolněné lipolýzou uvnitř hepatocytů mohou být využity buď k resyntéze TAG, oxidaci nebo tvorbě ketolátek. Preference jednotlivých drah úzce souvisí s prandiálním stavem, kdy ketogeneze je výrazně preferována ve stavu lačnění. Dle očekávání jsme u potkanů krmených SD našli vyšší koncentraci β -hydroxybutyrátu v séru v lačném než v sytém stavu. Krátkodobé podávání HSD i HFD signifikantně zvýšilo obsah ketolátek v séru nalačno, v případě HFD i v sytém stavu. Aktivita LAL pozitivně korelovala s obsahem β -hydroxybutyrátu v séru v lačném ($r=0,81$) i v sytém stavu ($r=0,62$).

Využití mastných kyselin uvolněných z intracelulárních lipidů jsme studovali pomocí *in vivo* radioaktivně značených TAG. Potkanům byla aplikována ^{14}C -palmitová kyselina ve 4% albuminu *i.v.* Dle Francone je za 90 min 90 % radioaktivity inkorporováno do cytosolických TAG v játrech a v tukové tkáni (130). Následně jsme inkubovali jaterní řízky obsahující radioaktivně značené TAG *ex vivo* a stanovili jsme radioaktivitu inkorporovanou v meziproduktech TCA cyklu, v β -hydroxybutyrátu a v CO_2 , tj. podíl mastných kyselin využitý pro oxidaci, a radioaktivitu inkorporovanou v TAG sekretovaných do média (VLDL sekrece) (**tab. 3.2**).

Tabulka 3.2 Inkorporace ^{14}C kyseliny palmitové do CO_2 , meziproduktů TCA cyklu, sekretovaných TAG a β -hydroxybutyrátu v jaterních řízcích *ex vivo*. Hodnoty udávají nmol kyseliny palmitové na g tkáně (nmol g^{-1} v.v.) a jsou vyjádřeny jako aritmetický průměr $\pm$ S.E.M. $n = 6$. β -OH, β -hydroxybutyrát.

* $p < 0.05$ ** $p < 0.01$ HS-4 lačné vs SD lačné; † $p < 0.05$, †† $p < 0.001$ HS-4 syté vs SD syté; $^{\#\#\#}$ $p < 0.001$ HF-4 lačné vs SD lačné; $^{\text{xx}}$ $p < 0.01$, $^{\text{xxx}}$ $p < 0.001$ HF-4 syté vs SD syté.

	SD		HS-4		HF-4	
	lačné	syté	lačné	syté	lačné	syté
CO_2	16 $\pm$ 1,2	11,9 $\pm$ 1	11 $\pm$ 1,5*	8,3 $\pm$ 0,3 †	15 $\pm$ 0,7	9,8 $\pm$ 0,8
meziprodukty TCA cyklu	55,3 $\pm$ 1,6	41,1 $\pm$ 3,4	46,6 $\pm$ 1,8	25,7 $\pm$ 1,5 ††	54,2 $\pm$ 4,2	40,3 $\pm$ 1
β -OH	269 $\pm$ 9	204 $\pm$ 8	380 $\pm$ 18**	178 $\pm$ 15	452 $\pm$ 22 $^{\#\#\#}$	480 $\pm$ 11 $^{\text{xxx}}$
TAG	581 $\pm$ 35	401 $\pm$ 44	713 $\pm$ 29**	825 $\pm$ 29 ††	314 $\pm$ 16 $^{\#\#\#}$	286 $\pm$ 35 $^{\text{xx}}$

Ve srovnání se zvířaty krmenými SD, podávání HSD vedlo ke snížení oxidace mastných kyselin v TCA cyklu, zvýšení lačné ketogeneze (o 40 %) a zvýšení sekrece VLDL (lačná +23 %, sytá +100 %). Podávání HFD neovlivnilo oxidaci mastných kyselin v TCA cyklu, významně zvýšilo ketogenezi (lačná +68 %, sytá +135 %) a výrazně snížilo sekreci VLDL (lačná -46 %, sytá -29 %). I v systému *ex vivo* byly zachovány regulační mechanismy fungující *in vivo*, tj. jaterní řízky získané od lačných zvířat produkovaly významně více β -hydroxybutyrátu než jaterní řízky připravené z jater sytých zvířat. Lačná ketogeneze byla potencována podáváním HSD i HFD, sytá ketogeneze byla zvýšená pouze u zvířat krmených HFD. Naše výsledky ukazují souhlasné změny v ketogenezi i v aktivitě LAL za fyziologických (lačnění/sytost) i patofyziologických (dietní intervence) stavů.

Sekreci VLDL jsme měřili rovněž *in vivo* s využitím Tritonu WR-1339, který blokuje vychytávání lipoproteinů z krevního řečiště. Výsledky, uvedené v **tab. 3.3**, potvrdily data získaná *in vitro*, tj. snížení sekrece VLDL po podávání HFD a významné zvýšení po podávání HSD.

Tabulka 3.3 Vliv HSD a HFD na produkci VLDL in vivo. TAG₀ koncentrace TAG v séru před podáním WR 1339; TAG₉₀ koncentrace TAG v séru 90 min po podání WR 1339. Rychlost produkce VLDL (objevování TAG v séru) podle vzorce: TG entry rate ($\mu\text{mol } 100 \text{ g b.wt.}^{-1} \text{ hr}^{-1}$) = $((T_{90} - T_0)/1.5) \times V \times (W/100)$. Hodnoty jsou uvedeny jako aritmetický průměr $\pm$ S.E.M. n = 6. ⁺⁺⁺ p < 0.001 HS-4 vs SD; ^{**} p < 0.01 HF-4 vs SD; ^{***} p < 0.001 HF-4 vs HS-4.

	SD	HS-4	HF-4
TAG ₀ ($\mu\text{mol ml}^{-1}$)	3,9 $\pm$ 0,2	6,4 $\pm$ 0,5 ⁺⁺⁺	3,0 $\pm$ 0,1 ^{***}
TAG ₉₀ ($\mu\text{mol ml}^{-1}$)	7,4 $\pm$ 0,4	11,7 $\pm$ 0,75 ⁺⁺⁺	5,2 $\pm$ 0,44 ^{***}
TAG entry rate ($\mu\text{mol hr}^{-1} \text{ kg}^{-1}$)	11,2 $\pm$ 0,7	45,7 $\pm$ 6,1 ⁺⁺⁺	8,2 $\pm$ 0,5 ^{***}

Naše výsledky vyvracejí hypotézu, že steatóza vzniká v důsledku snížené aktivity lipolytických enzymů, konkrétně LAL. Naopak poukazují na možnou úlohu kompenzačních mechanismů, které mají za úkol omezit zátěž potenciálně toxickými volnými mastnými kyselinami. Dále jsme ukázali, že převažující tuky nebo sacharidy v dietě ovlivňují různé metabolické dráhy. Dieta bohatá na jednoduché sacharidy stimuluje především VLDL sekreci a omezuje oxidaci mastných kyselin, zatímco vysokotuková dieta vede k omezení sekrece VLDL a stimulaci ketogeneze. V souladu s tímto je i pozorování, že podávání HSD je asociováno s výraznou hypetriglyceridemií v séru, zatímco u HFD je akcentována jaterní steatóza.

SHRNUTÍ HLAVNÍCH POZNATKŮ

1. Lysozomální lipáza se účastní degradace intracelulárních lipidů v játrech.
2. Lipidy jsou do lysozomů transportovány prostřednictvím autofagozomů.
3. Intenzita autofagicko-lysozomální dráhy je závislá na prandiálním stavu, zvyšuje se při lačnění a je potlačena v sytém stavu.
4. Aktivita lysozomální lipázy se zvyšuje úměrně s mírou steatózy.
5. V játrech existují kompenzační mechanismy eliminující možnou lipotoxicitu uvolněných mastných kyselin:
 - v krátkodobé perspektivě zvýšení (re)esterifikační kapacity;
 - v dlouhodobé perspektivě snížení aktivity autofagické dráhy a tím i omezení přísunu substrátu (lipidové kapénky) do místa degradace (lysozomy).
6. Tyto kompenzační mechanismy přispívají k rozvoji a udržení steatózy.
7. Mastné kyseliny uvolněné z intracelulárních TAG jsou dostupné pro oxidaci v TCA cyklu, ketogenezi a sekreci VLDL. Diety s převažujícím obsahem tuků nebo jednoduchých cukrů různě ovlivňují jednotlivé metabolické dráhy.

4 FARMAKOLOGICKÉ NÁSTROJE OVLIVNĚNÍ NAFLD

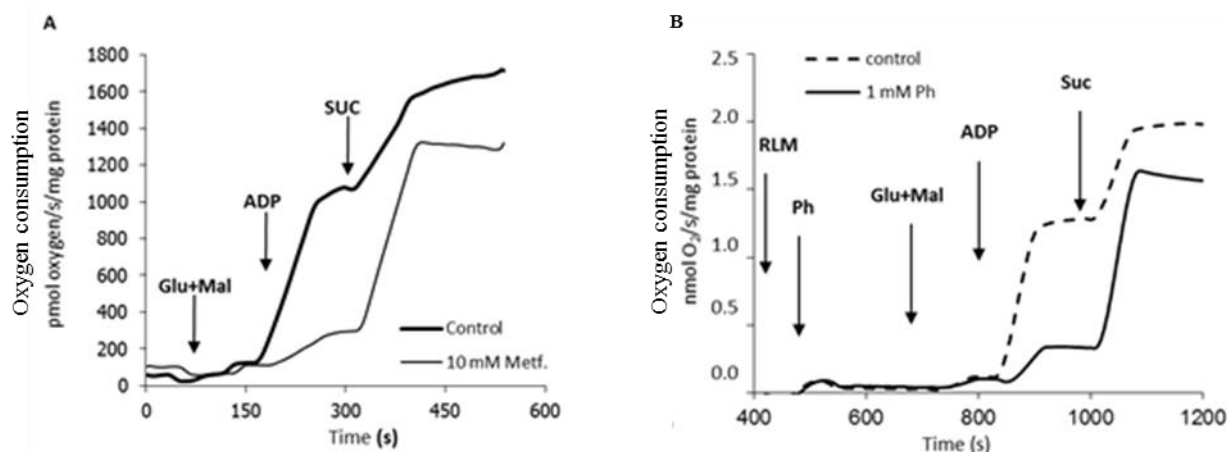
4.1 METFORMIN

Metformin patří mezi biguanidy a v léčbě diabetu 2. typu (T2D) je využíván od 50. let 20. století (163). Metformin je lékem první volby především díky své schopnosti snížit glykémii (164). Rovněž se používá při léčbě NAFLD, která téměř vždy T2D provází, protože pozitivně ovlivňuje body mass index (BMI), množství celkové i viscerální tukové tkáně a stupeň poškození jater měřený dle sérové hladiny transamináz (zejména aspartátaminotransferázy, AST) (165). Existují četné doklady (166, 167), že podávání metforminu snižuje obsah ATP v játrech a poměr ADP/ATP v jaterních buňkách. Za hlavní mediátor vlivu metforminu byla tedy logicky považována AMP-aktivovaná protein kináza (AMPK), enzym citlivě reagující na změny energetické bilance buňky (168, 169). Překvapivé bylo proto zjištění, že hypoglykemický efekt metforminu byl plně zachován u myši s nefunkčním genem pro AMPK (170). Z uvedených údajů vyplývá, že vzdor tomu, že metformin je nejdéle a nejhojněji používaným lékem v léčbě T2D, mechanismy jeho účinku dosud zdaleka nejsou objasněny. V naší práci jsme se soustředili na dvě oblasti: (i) studium mechanismu účinků biguanidů (metformin a fenformin) na jednotlivé komponenty oxidoredukčního řetězce *in vitro* na modelu izolovaných jaterních mitochondrií; (ii) vliv metforminu na oxidační stres na modelu dietně indukované NAFLD (potkan).

4.1.1 Mechanismus účinku biguanidů na mitochondriální respirační řetězec

4.1.1.1 Primárním cílem biguanidů je komplex I mitochondriálního respiračního řetězce

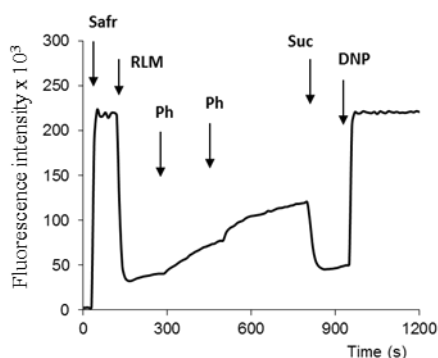
Pokusy jsme prováděli na jaterním homogenátu a na izolovaných jaterních mitochondriích potkana. Pracovali jsme se dvěma zástupci biguanidů, metforminem (N,N-dimethylimidodicarbonimidic diamine) a fenforminem (N-2-phenyletylimidodicarbonimidic diamine). Metformin i fenformin byly používány v humánní medicíně, ale fenformin byl v 70. letech stažen vzhledem k vysokému výskytu komplikací v podobě laktátové acidózy. Obě látky existují za fyziologických podmínek pouze jako pozitivně nabitě protonované formy, což umožňuje jejich akumulaci uvnitř mitochondrií. Právě postupným zadržováním v mitochondriích, a tím i zvýšením efektivní koncentrace, se vysvětluje dlouhodobě pozorovaný rozdíl v koncentracích efektivních *in vitro* a *in vivo*. Ve srovnání s metforminem má fenformin více lipofilní charakter a fenylová a etylová skupina mu dovolují snadnější průchod lipidovou dvojvrstvou. Z tohoto důvodu jsme část našich experimentů realizovali právě s použitím fenforminu. Ukázali jsme, že oba biguanidy v koncentraci 10 mmol l⁻¹ (metformin) a 1 mmol l⁻¹ (fenformin) inhibují mitochondriální respirační řetězec pouze na úrovni komplexu I (**obr. 4.1**).



Obrázek 4.1 Vliv metforminu (A) a fenforminu (B) na spotřebu kyslíku izolovanými jaterními mitochondriemi. Šipky vyznačují přidání jednotlivých chemikálií: 1. Glutamát (10 mmol l⁻¹) + malát (2,5 mmol l⁻¹); 2. ADP (1,5 mmol l⁻¹); 3. sukcinát (10 mmol l⁻¹). RLM, rat liver mitochondria (mitochondrie z jater potkana); Ph, fenformin. Záznam oxygrafu představuje reprezentativní vzorek tří nezávislých měření. Převzato z Páleníčková E. et al. doi: 10.33549/physiolres.932193.

Inhibiční vliv biguanidů na oxidaci glutamátu a malátu, tj. substrátů spojených s produkcí NADH (nikotinamidadenindinukleotid) může být způsoben buď inhibicí NADH dehydrogenáz produkujících NADH nebo přímo inhibicí NADH-oxidoreduktázy v komplexu I. Tuto otázku jsme řešili s využitím dezintegrovaných mitochondrií, jejichž celistvost byla porušena opakovaným zamražením a rozmražením a NADH substrát měl tak přímý přístup ke komplexu I. V přítomnosti 1 mM fenforminu je rotenon-senzitivní oxidace NADH (tj. oxidace na komplexu I) v dezintegrovaných mitochondriích snížena obdobně jako oxidace glutamátu + malátu + ADP na intaktních mitochondriích. Rovněž závislost spotřeby O₂ na koncentraci fenforminu byla u obou modelů stejná. Tento výsledek tedy vylučuje vliv biguanidů na NADH produkující dehydrogenázy a identifikuje NADH-oxidoreduktázu v komplexu I jako cílovou molekulu.

V dalších pokusech jsme vyloučili hypotézu, že biguanidy fungují jako odpřahovače. Na **obr. 4.2** jsou znázorněny změny mitochondriálního membránového potenciálu měřené pomocí fluorescentní sondy safranin O, která je aktivně vychytávána a zadržována uvnitř mitochondrií s nábojem na membráně. Vysoká hodnota fluorescence znamená uvolnění safraninu O z mitochondrií a pokles membránového potenciálu. Výchozí médium obsahovalo glutamát + malát a safranin O. Po přidání suspenze mitochondrií došlo k prudkému poklesu fluorescence, tj. vytvoření membránového potenciálu, který se postupně snižoval po přidání fenforminu. Přidání sukcinátu však vedlo k prudkému obnovení membránového potenciálu i v přítomnosti fenforminu, což vylučuje jeho působení v roli odpřahovače.



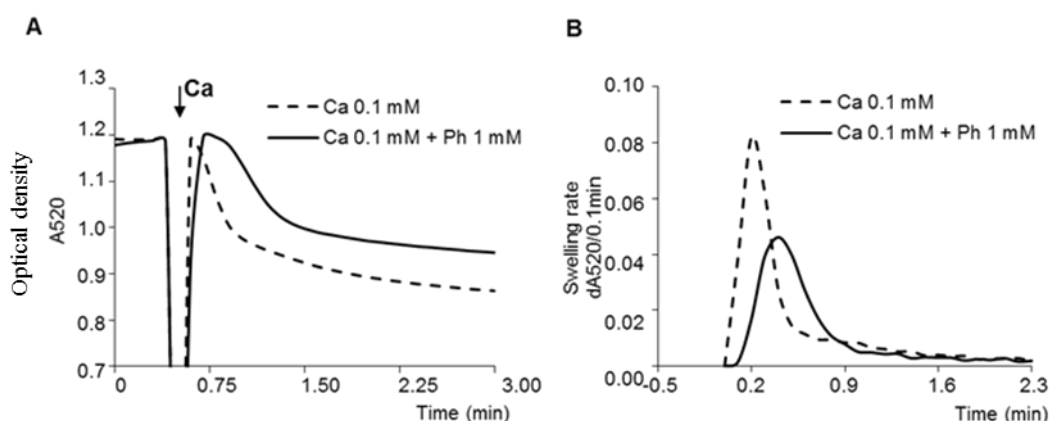
Obrázek 4.2 Vliv 1 mM fenforminu na membránový potenciál jaterních mitochondrií. Šipky indikují přidání jednotlivých chemikálií; 1. Safranin O (Safr); 2. rat liver mitochondria (RLM); 3. fenformin (Ph, 1 mmol l⁻¹); 4. sukcinát (Suc, 10 mmol l⁻¹); 5. 2,4-dinitrofenol (DNP, 0,05 mmol l⁻¹). Excitační vlnová délka 495 nm, emisní vlnová délka 586 nm. Reprezentativní výsledek tří nezávislých měření. Převzato z Páleníčková E. et al. doi: 10.33549/physiolres.932600.

4.1.1.2 Vysoké koncentrace biguanidů inhibují i komplex II a komplex IV mitochondriálního respiračního řetězce

V případě metforminu se nám v podmínkách *in vitro* nepodařilo prokázat inhibiční vliv na další komponenty dýchacího řetězce kromě komplexu I. Naproti tomu fenformin v koncentraci 6 mmol l⁻¹ vykazoval významný inhibiční účinek i na komplex II a IV. Tato koncentrace však násobně přesahuje koncentrace pozorované v krvi při podávání *in vivo*. Nelze však vyloučit, že srovnatelné koncentrace lze dosáhnout uvnitř mitochondrií díky retenci biguanidů v důsledku jejich pozitivního náboje.

4.1.1.3 Fenformin zvyšuje odolnost přechodového póru mitochondriální permeability k Ca²⁺ iontům

Přechodový pór mitochondriální permeability (MTPT) je protein, který se tvoří za podmínek buněčného stresu a je lokalizován ve vnitřní mitochondriální membráně. Reaguje na signály stresu buňky, např. zvýšení intracelulární hladiny Ca²⁺ iontů. Při otevření MTPT dochází ke zvýšení propustnosti vnitřní mitochondriální membrány, bobtnání mitochondrií a jejich zániku. Na izolovaných jaterních mitochondriích jsme prokázali, že v přítomnosti 1 mM fenforminu se snížila míra a rychlost bobtnání mitochondrií po přidání Ca²⁺ iontů o 20 % a 44 %, resp. (**obr. 4.3**). Tento výsledek ukazuje další mechanismus, nezávislý na transformaci energie, kterým biguanidy ovlivňují procesy v mitochondriích.

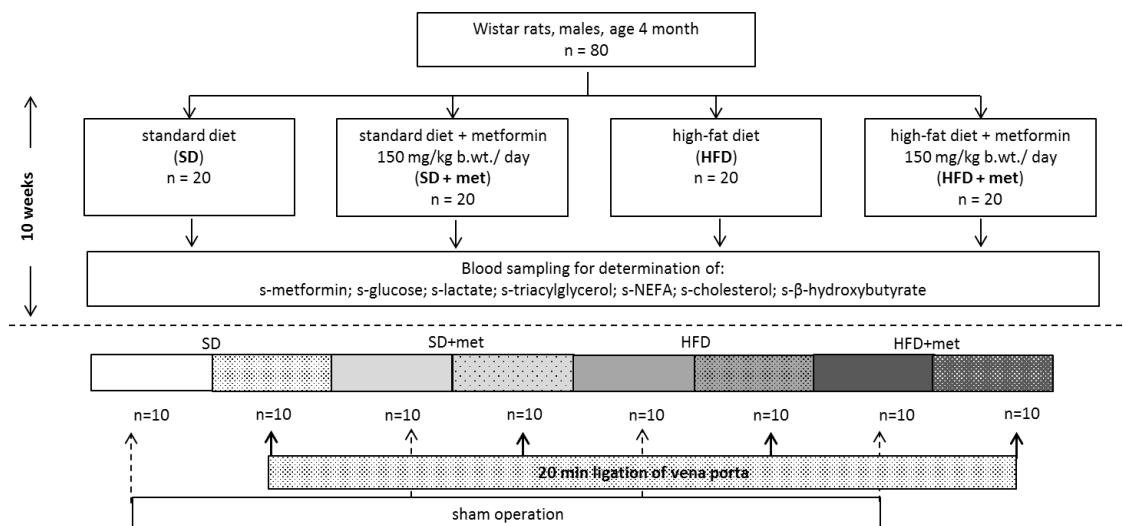


Obrázek 4.3 Vliv fenforminu na bobtnání mitochondrií indukované Ca²⁺ ionty. A: míra bobtnání určená podle změny optické denzity. B: rychlost bobtnání určená jako první derivace originálních dat. Záznam představuje reprezentativní výsledek tří nezávislých měření. Převzato z Páleníčková E. et al. doi: 10.33549/physiolres.932600.

4.1.2 Vliv metforminu na oxidační stres při NAFLD

Poškození navozené reaktivními sloučeninami kyslíku a dusíku, tj. oxidační stres, se považuje za jednu z příčin vedoucí k rozvoji závažného poškození jater, steatohepatitidě. Existují doklady o protektivním účinku metforminu v různých tkáních, avšak mechanismus tohoto působení nebyl dosud plně objasněn. Jedno z možných vysvětlení vychází z hypoglykemického účinku metforminu. Zvýšená dostupnost glukózy v některých tkáních může vést k intenzivní glykolýze a vyšší tvorbě redukováných ekvivalentů vstupujících do elektron transportního řetězce. Zvýšená tvorba superoxidu a dalších ROS je pak důsledkem zvýšeného průchodu elektronů. Proti této hypotéze mluví skutečnost, že metformin působil protektivně i v několika modelech oxidačního stresu, které nejsou spojeny s hyperglykemií. Formulovali jsme hypotézu, že protektivní vliv metforminu je zprostředkován, alespoň částečně, přímou interakcí

s mitochondriálním respiračním řetězcem. Pokusy jsme prováděli na modelu potkana, u kterého byla NAFLD indukována podáváním HFD (10 týdnů). Akutní oxidační stres byl navozen krátkodobou parciální ischemií a následnou reoxygenací (ischemie/ reperfúze, I/R). Části potkanů byl po celou dobu podáván metformin v dávce 150 mg kg⁻¹ tělesné hmotnosti. Výsledná koncentrace metforminu v séru se pohybovala v rozmezí 1–10 μmol l⁻¹, což odpovídá hodnotám stanoveným u lidí. Schéma pokusu viz **obr. 4.4**.



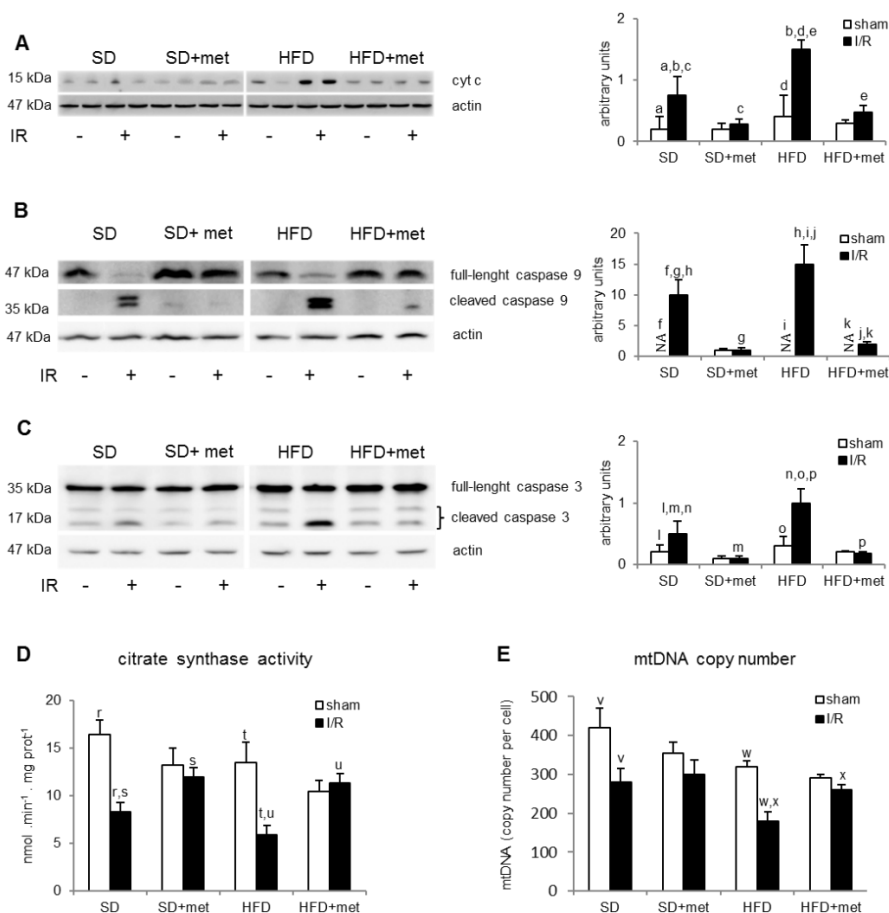
Obrázek 4.4 *Uspořádání pokusu.* Převzato z Cahova M. et al. doi: 10.1152/ajpgi.00329.2014.

4.1.2.1 Metformin snižuje histologické poškození jater

Ischemie a následná reperfúze vedla k rozvoji srovnatelné centrilobulární nekrózy u zvířat krmených SD i HFD. U zvířat, kterým byl před zásahem dlouhodobě podáván metformin, se podobné poškození nerozvinulo. Toto pozorování potvrdila i kvantitativní analýza obsahu produktů lipoperoxidace, tj. TBARS (látky reaktivní s kyselinou thiobarbiturovou) a 4-HNE (4-hydroxy nonenal), v jaterní tkáni.

4.1.2.2 Metformin omezuje poškození mitochondrií a míru apoptózy vyvolané akutním oxidačním stresem

Poškození mitochondrií je silným pro-apoptotickým signálem. Dle očekávání jsme zjistili, že akutní oxidační stres vedl k poškození vnější mitochondriální membrány (měřeno dle uvolňování cytochromu c do cytosolu), vyšší intenzitě apoptózy (odhadnuto na základě štěpení kaspázy 3 a kaspázy 9) a snížení počtu mitochondrií (měřeno podle aktivity výlučně mitochondriálního enzymu citrát syntázy a počtu kopií mitochondriální DNA po normalizaci na ekvivalent jaderné DNA). Míra poškození byla vyšší u mitochondrií izolovaných ze steatózních jater. Dlouhodobé podávání metforminu vedlo k normalizaci všech sledovaných parametrů (**obr. 4.5**). U mitochondrií izolovaných ze steatózních jater jsme měřili i funkční parametry *in vivo*. Dále jsme ukázali, že jak samotný akutní oxidační stres, tak i podávání metforminu snižuje maximální respirační kapacitu, ale tento efekt není aditivní. Z toho vyplývá, že ačkoli samotné podávání metforminu snižuje respirační kapacitu mitochondrií, nejsou tyto mitochondrie dále poškozeny expozicí k akutnímu oxidačnímu stresu.

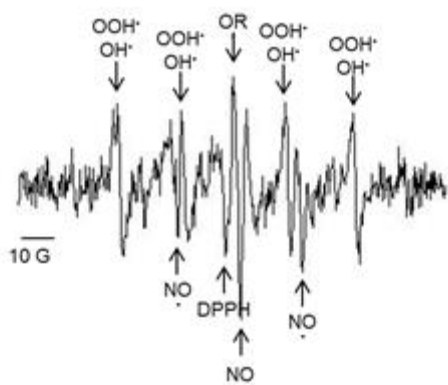


Obrázek 4.5 Vliv metforminu na jaterní mitochondrie a intenzitu apoptózy v játrech. A: obsah cytochromu c v cytosolu, abundance cyt c je vyjádřena jako poměr intenzity specifického signálu cyt c k signálu pro aktin; B: a C: aktivace kaspázy 9 a kaspázy 3, resp. Grafy ukazují poměr intenzit signálů produktů štěpení a neštěpeného enzymu; D: aktivita citrát syntázy v jaterním homogenátu; E: počet kopií mitochondriální DNA na buňku. mtDNA, mitochondriální DNA; Cyt C, cytochrom c. Sloupčky označené stejným písmenem jsou vzájemně významně odlišné. a,b,c,d,h,l,n,o,s,u,v,x $p < 0.05$; e,m,p,r,t,w $p < 0.01$; f,g,i,j,k $p < 0.001$. Převzato z Cahova M. et al. doi: 10.1152/ajpgi.00329.2014.

4.1.2.3 Metformin snižuje tvorbu ROS z NADH a sukcinátu

Transport elektronů mitochondriálním respiračním řetězcem je spojen s možným vznikem ROS a toto riziko je významně zvýšeno během reoxygenace po ischemii. Ve studiích *in vitro* byly identifikovány tři možné děje vedoucí ke vzniku ROS, (i) „forward“ tok elektronů přes komplex I, (ii) „reverse“ tok elektronů z komplexu II na komplex I a (iii) přenos elektronů zprostředkovaný mitochondriální glycerol-3-fosfát dehydrogenázou (mGPDH). Vliv dlouhodobého podávání metforminu na vznik ROS na jednotlivých komponentách mitochondriálního respiračního řetězce jsme studovali *in vitro* na modelu submitochondriálních partikul (SMP) s využitím fluorescenční sondy dichlorofluorescein diacetate (DCFDA). NADH jsme použili jako zdroj elektronů pro komplex I, sukcinát pro komplex II a glycerol-3-fosfát pro mGPDH. Na tomto modelu jsme prokázali, že I/R zátěž vede ke zvýšené produkci ROS jak na komplexu I, tak v důsledku reverzního toku elektronů z komplexu II na komplex I. Metformin vliv I/R eliminoval. Neprokázali jsme významný vliv I/R ani metforminu na mGPDH.

Metoda elektronové paramagnetické rezonance je jediný způsob, kterým lze přímo měřit vznik radikálových sloučenin. Tuto metodu jsme použili k analýze vzniku ROS v situaci, kdy NADH fungoval jako donor elektronů na modelu SMP. Po přidání NADH k SMP jsme byli schopni identifikovat čtyři typy radikálů, (i) OR $\cdot$ radikál; (ii) nitroxidový radikál (NO $\cdot$); (iii) superoxidový radikál (OOH $\cdot$) a (iv) hydroxylový radikál (OH $\cdot$) (**obr. 4.6**).



Obrázek 4.6 Reprezentační záznam EPR spektra. Radikálové sloučeniny byly stabilizovány pomocí DMPO (5,5-Dimethyl-1-Pyrroline-N-Oxide) a identifikovány na základě g faktoru. DPPH (2,2-Diphenyl-1-picrylhydrazyl) jsme použili jako interní standard ($g=2,0036$). Převzato z Cahova M. et al. doi: 10.1152/ajpgi.00329.2014.

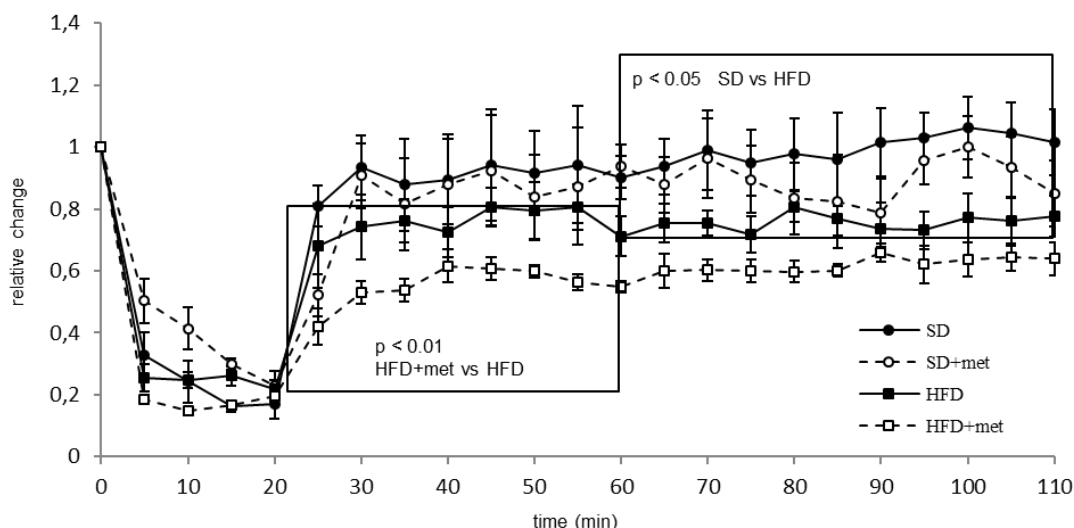
Prokázali jsme významné zvýšení produkce OH $\cdot$ radikálu a trend ke zvýšení produkce superoxidového radikálu OOH $\cdot$ ($p = 0,061$) u zvířat vystavených I/R. Dlouhodobé podávání metforminu nemělo žádný efekt na kontrolní zvířata, ale zcela eliminovalo vliv I/R zátěže na tvorbu OH $\cdot$ a OOH $\cdot$ (**tab. 4.1**). Na základě těchto pozorování dovozujeme, že dlouhodobé podávání metforminu vede ke snížení tvorby ROS v mitochondriálním respiračním řetězci na úrovni komplexu I při akutním oxidačním stresu.

Tabulka 4.1 Produkce radikálových sloučenin *in vitro*. Údaje představují aritmetický průměr z pěti nezávislých měření $\pm$ S.E.M. Hodnoty označené stejným písmenem jsou statisticky významně odlišné, ^{a,b,c,d} $p < 0.05$.

	HFD	HFD+met	HFD+I/R	HFD+met+I/R
radikál OR $\cdot$	7,9 $\pm$ 1,1	7,9 $\pm$ 0,3	7,6 $\pm$ 0,8	6,9 $\pm$ 0,3
nitroxidový radikál NO $\cdot$	3,8 $\pm$ 0,8	4,1 $\pm$ 0,2	4,3 $\pm$ 0,5	4,2 $\pm$ 0,6
superoxidový radikál OOH $\cdot$	4,1 $\pm$ 0,2 ^a	4,0 $\pm$ 0,25	5,1 $\pm$ 0,3 ^{a,b}	3,3 $\pm$ 0,4 ^b
hydroxylový radikál OH $\cdot$	1,2 $\pm$ 0,03 ^c	1,1 $\pm$ 0,1	1,7 $\pm$ 0,2 ^{c,d}	1,2 $\pm$ 0,05 ^d

4.1.2.4 Metformin zpomaluje obnovení zásob ATP v játrech *in vivo*

Dosud shromážděné výsledky indikují, že metformin snižuje mitochondriální respiraci. Za tohoto předpokladu by mělo platit, že metformin rovněž snižuje tvorbu ATP (adenosintrifosfát). Tuto hypotézu jsme testovali měřením rychlosti obnovy zásob ATP během reperfúze po částečné ischemii jater pomocí ³¹P magnetické rezonanční spektroskopie. V SD skupině se obsah ATP plně obnovil během prvních 15 minut reperfúze. V HFD skupině byla rychlost resyntézy ATP stejná jako v SD, ale výsledná hladina ATP dosáhla pouze 80 % hodnot před ischemií. Ve skupině HFD+metformin byla rychlost resyntézy ATP pomalejší než u ostatních zvířat a maximální hodnota ATP po I/R dosáhla pouze 60 % před ischemií. Ve skupině SD se vliv metforminu neprojevil (**obr. 4.7**). Naše výsledky potvrzují, že metformin snižuje tvorbu ATP v steatózních játrech po akutní ischemicko/reperfúzní zátěži.



Obrázek 4.7 Vliv metforminu na replaci ATP během reoxygenace po ischemii. Částečná ischemie byla navozena ligací vena porta (čas 0) a uvolněna po 20 min (čas 20). Obsah ATP byl stanoven pomocí ^{31}P magnetické rezonanční spektroskopie, měření byla prováděna v 5 min intervalech. Hodnoty jsou vyjádřeny jako relativní změna ve srovnání s časem 0. Hodnoty představují aritmetický průměr z pěti nezávislých měření $\pm$ S.E.M. Převzato z Cahova M. et al. doi: 10.1152/ajpgi.00329.2014.

SHRNUTÍ HLAVNÍCH POZNATKŮ

1. *In vitro*, biguanidy (metformin a fenformin) inhibují mitochondriální respiraci, a to v závislosti na použité dávce.
2. Inhibiční vliv biguanidů se soustřeďuje zejména na komplex I, ve vyšších dávkách lze pozorovat i vliv na komplex II a komplex IV.
3. Biguanidy snižují citlivost mitochondriálního MPTP ke kalciovým iontům a redukují rychlost a rozsah bobtnání mitochondrií.
4. *In vivo*, metformin chrání jak steatózní, tak zdravá játra před důsledky akutního oxidačního stresu navozeného ischemicko-reperfúzním poškozením.
5. Mechanismus ochranného působení metforminu je založen na snížení tvorby ROS v mitochondriích (především na úrovni komplexu I), což vede ke zmenšení poškození mitochondrií a omezení apoptotické a nekrotické buněčné smrti.
6. Důsledkem snížení mitochondriální respirace je nižší schopnost tvorby ATP ve steatózních játrech.

4.2 KARNITIN

Karnitin zprostředkovává transport acylových zbytků přes vnitřní mitochondriální membránu do matrix mitochondrií a je zcela nezbytný pro normální funkci mitochondrií. Karnitin a jeho analogy byly intenzivně studovány v souvislosti s možným pozitivním účinkem na mitochondriální dysfunkci (171), zejména v souvislosti s T2D a dalšími metabolickými chorobami. Klinické studie přinesly rozporné výsledky (172, 173). Zdá se, že účinek karnitinu jako monoterapie je sporný u obezity (nezávisle na přítomnosti T2D) (174–176) a u prediabetu (177). Podávání karnitinu mělo pozitivní účinky u T2D pacientů jako doplňková terapie k léčbě antidiabetiky (178), hypokalorické dietě (179) nebo přiměřené pohybové aktivitě (180, 181). Karnitin se rovněž ukázal jako účinný u nově diagnostikovaných pacientů s T2D (182).

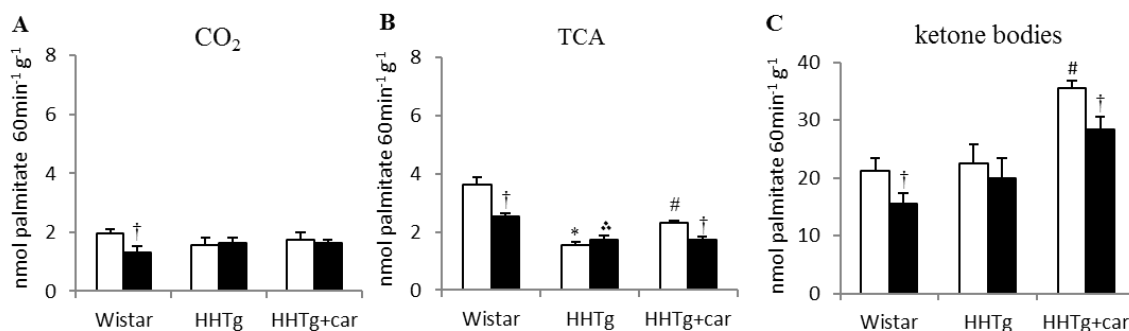
V této části práce předkládám výsledky naší studie zaměřené na vliv dlouhodobého podávání karnitinu na jaterní steatózu. Využili jsme HHTg potkany odvozené od kmene Wistar. U tohoto kmene se manifestují charakteristické rysy metabolického syndromu, tj. hypetriglyceriemie, hyperinzulinemie, inzulinová rezistence, narušená glukózová homeostáza a ektopické ukládání tuku včetně jaterní steatózy (183, 184). Zaměřili jsme se na vliv karnitinu na ektopické ukládání tuku v játrech, na funkci jaterních mitochondrií, oxidační stres a metabolickou flexibilitu. HHTg potkani byli rozděleni náhodně do dvou skupin. Skupina označená HHTg+car dostávala po dobu 8 týdnů 1% karnitin v pitné vodě, druhá skupina (HHTg) nikoli. Skupinu Wistar jsme zařadili jako referenční standard, bez prokázané patologie glukózového nebo lipidového metabolismu.

4.2.1 Karnitin zlepšuje některé parametry lipidového metabolismu

HHTg i Wistar potkani vykazovali po celou dobu experimentu stabilní váhový přírůstek (Wistar > HHTg) zatímco ve skupině HHTg+car zůstala váha zvířat po celou dobu konstantní. Suplementace karnitinem rovněž významně snížila obsah TAG v játrech HHTg potkanů ve srovnání s nesuplementovanou skupinou (Wistar: $4,6 \pm 0,7$; HHTg: $10,5 \pm 0,6$; HHTg+car: $7,9 \pm 0,7$ mmol TAG g⁻¹). Neprokázáli jsme vliv karnitinu na obsah TAG v kosterním svalu, v srdci a v séru, ani snížení sérové koncentrace nenasycených mastných kyselin.

4.2.2 Karnitin zvyšuje oxidaci mastných kyselin v játrech

Jedním z možných vysvětlení pozitivního vlivu karnitinu na jaterní steatózu může být zvýšená utilizace mastných kyselin. Na jaterních řízciích jsme proto *ex vivo* měřili oxidaci radioaktivně značeného palmitátu do CO₂, inkorporaci do meziproductů cyklu trikarboxylových kyselin (TCA) a produkci ketolátů (**obr. 4.8**). HHTg se od Wistar potkanů lišili sníženou inkorporací radioaktivity do meziproductů TCA cyklu, zatímco produkce CO₂ a ketogeneze byla u obou skupin srovnatelná. Suplementace karnitinem vedla k částečné normalizaci inkorporace do meziproductů TCA cyklu a k výrazně zvýšené produkci ketolátů.



Obrázek 4.8 Utilizace ^{14}C -palmitátu v jaterních řízcích *ex vivo*. A: inkorporace ^{14}C -palmitátu do CO_2 ; B: inkorporace ^{14}C -palmitátu do meziproductů TCA cyklu; C: inkorporace ^{14}C -palmitátu do ketolátek. Otevřené sloupce: KRB médium; uzavřené sloupce: KRB médium + 5 mmol l⁻¹ L-pyruvát. Hodnoty představují aritmetický průměr ± S.E.M., n=8-10. * p<0.05 HHTg vs Wistar (basal); ‡ p<0.05 HHTg vs Wistar (+ pyruvát); # p<0.05 HHTg+car vs HHTg (basal); † p<0.05 KRB vs KRB+pyruvát. Převzato z Cahova M. et al. doi: 10.1139/apnm-2014-0163.

Významným aspektem metabolické flexibility je schopnost vybírat nejvhodnější substrát k produkci energie vzhledem k aktuální dostupnosti a metabolickým potřebám organismu. V metabolicky flexibilní tkáni dochází v přítomnosti sacharidů k utlumení oxidace mastných kyselin, zatímco pro diabetickou tkáň je charakteristické, že na změnu dostupnosti substrátů nereaguje. V našich *ex vivo* pokusech jsme tuto situaci modelovali přítomností neznačeného pyruvátu, který je konečným produktem glykolýzy a prostřednictvím pyruvát dehydrogenázy (PDH) vstupuje do TCA cyklu. Ve skupině Wistar se potvrdil předpoklad, že v přítomnosti pyruvátu se sníží utilizace palmitátu jak pro přímou oxidaci (produkce CO_2 a meziproductů TCA cyklu), tak pro tvorbu ketolátek. Naopak ve skupině HHTg přídavek pyruvátu utilizaci palmitátu vůbec neovlivnil. Suplementace karnitinem vedla k částečnému obnovení metabolické flexibility v játrech HHTg potkanů.

Klíčovým enzymem umožňujícím metabolický přesmyk mezi mastnými kyselinami a sacharidy je PDH. Stanovili jsme aktivitu tohoto enzymu v jaterním homogenátu *in vitro* a prokázali jsme, že aktivita pyruvátdehydrogenázy (PDH) je v játrech HHTg potkanů suplementovaných karnitinem skutečně signifikantně vyšší než u potkanů Wistar nebo HHTg, kterým karnitin nebyl podáván.

4.2.3 Karnitin stimuluje biogenezi mitochondrií

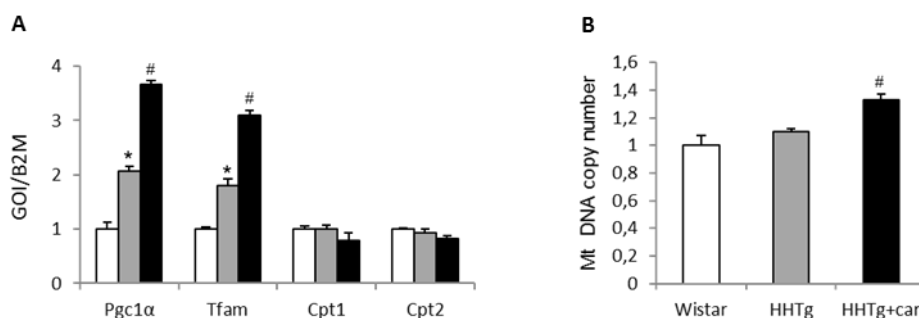
Na izolovaných jaterních mitochondriích jsme měřili respirační kapacitu vzhledem k různým typům substrátů (**tab. 4.2**). Mitochondriální respirační kapacita byla u všech skupin srovnatelná v případě, kdy byl jako substrát použit malát+glutamát nebo sukcinát. Pokud jsme však použili palmitoylkarnitin, byla mitochondriální respirace ve skupině HHTg významně nižší než u kontrol a tento defekt vymizel po dlouhodobém podávání karnitinu.

Tabulka 4.2 Respirační kapacita jaterních mitochondrií *in vivo*.

Hodnoty jsou uvedeny v pmol O_2 min⁻¹ mg prot⁻¹ a vyjádřeny jako aritmetický průměr ± S.E.M. M+G, malát + glutamát; RC, respirační kontrola. Všechna měření byla prováděna v přítomnosti ADP (stav 3). *p<0.05 HHTg vs Wistar; #p<0.05 HHTg+car vs HHTg.

	M+G	Palmitoylkarnitin	Sukcinát	RC
Wistar	363 ± 32	261 ± 19	473 ± 41	3,4 ± 0,2
HHTg	347 ± 48	214 ± 14 [*]	446 ± 68	2,8 ± 0,3
HHTg+car	421 ± 28	266 ± 13 [#]	555 ± 35	3,5 ± 0,1

Vzhledem k tomu, že jsme jako substrát při měření mitochondriální respirace *in vitro* použili palmitoylkarnitin a nikoli samotnou kyselinu palmitovou, nelze stimulační vliv karnitinu na respiraci palmitátu vysvětlit pouze zvýšením transportní kapacity pro mastné kyseliny. V dalším kroku jsme proto testovali hypotézu, že podávání karnitinu ovlivňuje biogenezi mitochondrií. Zjistili jsme, že exprese klíčových genů kontrolujících tento proces, *Pgc1α* (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) a *Tfam* (mitochondrial transcription factor A), je skutečně ve skupině HHTg+car signifikantně zvýšená a tomu odpovídá i vyšší počet kopií mitochondriální DNA vztažený na genomovou DNA (**obr. 4.9**). Naše výsledky tedy ukazují, že dlouhodobá suplementace karnitinem vede ke zvýšení počtu mitochondrií v játrech.



Obrázek 4.9 Expresa některých mitochondriálních genů (A) a počet kopií mitochondriální DNA (B). Expresa GOI byla kvantifikována metodou $\Delta\Delta C_t$ po normalizaci na housekeeper gen (B2M) při použití průměrné exprese ve skupině Wistar jako kalibrátoru. Počet kopií mitochondriální DNA byl normalizován na počet kopií jaderné DNA. Hodnoty představují aritmetický průměr $\pm$ S.E.M., $n=8-10$. GOI, gene of interest; B2M, beta-2 mikroglobulin; MtDNA, mitochondriální DNA. * $p<0.05$ HHTg vs Wistar; # $p<0.05$ HHTg+car vs HHTg. Převzato z Cahova M. et al. doi: 10.1139/apnm-2014-0163.

4.2.4 Karnitin snižuje oxidační stres v játrech

Zvýšená oxidace mastných kyselin v respiračním řetězci může být doprovázena vyšší tvorbou ROS a větším oxidačním stresem v játrech. Stanovili jsme proto kvantitativní markery oxidačního stresu, obsah oxidovaného (GSSG) a redukovaného (GSH) glutathionu a obsah lipoperoxidů (TBARS). V rozporu s výchozí hypotézou jsme zjistili, že oxidační stres charakterizovaný poměrem GSSG/GSH a obsahem TBARS byl sice významně vyšší u skupiny HHTg ve srovnání se skupinou Wistar, ale podávání karnitinu míru oxidačního stresu snížilo až na úroveň kontrolní skupiny (**tab. 4.3**).

Tabulka 4.3 Antioxidační markery v játrech. Hodnoty představují aritmetický průměr $\pm$ S.E.M. $n=8-10$. * $p<0.05$ HHTg vs Wistar; # $p<0.05$ HHTg+car vs HHTg.

	Wistar	HHTg	HHTg+car
GSH ($\mu\text{mol g}^{-1}$ v.v.)	4,01 $\pm$ 0,40	3,32 $\pm$ 0,01*	3,10 $\pm$ 0,25
GSSG ($\mu\text{mol g}^{-1}$ v.v.)	0,13 $\pm$ 0,04	0,21 $\pm$ 0,02*	0,11 $\pm$ 0,01#
GSH/GSSG	28,9 $\pm$ 4,3	14,9 $\pm$ 0,5*	28,3 $\pm$ 0,9#
Konjugované dieny (nmol mg^{-1} prot)	25,1 $\pm$ 0,9	28,0 $\pm$ 3,0	26,0 $\pm$ 3,0
TBARS (nmol mg^{-1} prot)	0,77 $\pm$ 0,02	0,97 $\pm$ 0,03*	0,84 $\pm$ 0,04#

Zaměřili jsme se proto na stanovení kapacity antioxidačních systémů. Zjistili jsme, že aktivita tří klíčových enzymů antioxidační ochrany, superoxid dismutázy, glutathion peroxidázy a

katalázy, je v játrech u skupiny HHTg významně nižší než u skupiny Wistar, a dlouhodobé podávání karnitinu vede k normalizaci na úroveň kontrolní skupiny (**tab. 4.4**).

Tabulka 4.4 *Aktivity antioxidantních enzymů.* Hodnoty představují aritmetický průměr $\pm$ S.E.M., n=8-10. GR, glutathion reduktáza; GSH-Px, glutathion peroxidáza; SOD, superoxid dismutáza.

* p<0.05 HHTg vs Wistar; # p<0.05 HHTg+car vs HHTg

	Wistar	HHTg	HHTg+car
SOD ($\mu\text{kat mg prot}^{-1}$)	2,80 $\pm$ 0,05	2,28 $\pm$ 0,18*	2,78 $\pm$ 0,15#
GSH-Px ($\mu\text{kat mg prot}^{-1}$)	4,35 $\pm$ 0,25	3,97 $\pm$ 0,17*	4,63 $\pm$ 0,20#
GR ($\mu\text{kat mg prot}^{-1}$)	2,7 $\pm$ 0,2	2,9 $\pm$ 0,3	2,6 $\pm$ 0,3
Kataláza ($\mu\text{kat mg prot}^{-1}$)	21,0 $\pm$ 0,9	15,1 $\pm$ 0,4*	19,3 $\pm$ 0,8#

Naše výsledky poukazují na určitý paradox, na jedné straně podávání karnitinu vede k vyšší oxidaci mastných kyselin, což je děj spojovaný s tvorbou ROS, a přitom nacházíme méně známek působení ROS na biologické struktury (TBARS, GSSG). Tento rozpor je možné vysvětlit antioxidantními vlastnostmi samotného karnitinu, který je schopen přímo reagovat se superoxidovými a peroxidovými radikály (185, 186). Karnitin by pak fungoval nejen jako látka stimulující oxidaci mastných kyselin, ale zároveň i jako antioxidant eliminující nežádoucí vedlejší účinky tohoto děje.

4.2.5 Suplementace karnitinem normalizuje obsah karnitinu ve tkáních a zvyšuje vylučování (acyl)karnitinů v moči

V krvi, játrech a kosterních svaích potkanů HHTg jsme prokázali snížený obsah volného karnitinu a různých acylkarnitinů, zejména konjugátů karnitinu a acylových zbytků s krátkým řetězcem (C2–C5). Dlouhodobé podávání karnitinu vedlo k normalizaci koncentrace karnitinu v krvi a v játrech na úroveň zjištěnou u kmene Wistar. Zároveň se také vysoce významně zvýšilo vylučování karnitinu a acylkarnitinů v moči. Tento efekt byl nejvýznamnější u acetyl karnitinu (300násobně), C3–C5 acylkarnitinů (200násobně) a volného karnitinu (95násobně). Neprokázali jsme akumulaci acylkarnitinů ve tkáních (játra, kosterní sval).

Patofyziologické stavy jako obezita, inzulinová rezistence a T2D jsou často provázeny mitochondriální dysfunkcí a vysokou mírou neúplné oxidace mastných kyselin (187, 188). K tomu dochází, když přísun acylových zbytků do TCA cyklu přesahuje jeho kapacitu. Hladký chod TCA cyklu a elektron-transportního řetězce je mimo jiné závislý na dostatečném množství volného koenzymu A. V případě nadbytku mastných kyselin se jich mitochondrie mohou zbavit prostřednictvím karnitinu. Tato molekula nejenom umožňuje vstup acylových zbytků do mitochondrií, ale také zprostředkovává transport acylových zbytků opačným směrem, z mitochondrie ven. Klíčovým prvkem tohoto procesu je mitochondriální enzym karnitin acyltransferáza, která katalyzuje přesun acylových zbytků na volný karnitin za vzniku acylkarnitinu a volného koenzymu A (CoA) (189). Acylkarnitiny s krátkým řetězcem jsou pak vyloučeny z organismu v moči. Naše výsledky ukazují, že obnovení hladin karnitinu u HHTg potkanů přispělo k obnovení funkce mitochondrií mimo jiné i zvýšením kapacity pro odsun přebytečných acylových zbytků.

SHRNUTÍ HLAVNÍCH POZNATKŮ

U zvířecího modelu metabolického syndromu jsme prokázali:

1. Nedostatek karnitinu v krvi, játrech a kosterním svalu.
2. Zvýšené vylučování volného karnitinu a snížené vylučování C3- a C5-acylkarnitinů v moči.
3. Dlouhodobé podávání karnitinu bylo asociováno se stagnací nárůstu tělesné hmotnosti a snížením jaterní steatózy.
4. Karnitin pozitivně ovlivnil mitochondriální dysfunkci (zvýšení kapacity oxidace mastných kyselin a stimulace biogeneze mitochondrií).
5. Zvýšení oxidace mastných kyselin nebylo provázeno zvýšením oxidačního stresu v játrech, důvodem mohou být antioxidační vlastnosti karnitinu jako lapače radikálových sloučenin.
6. Jedním z mechanismů účinku karnitinu může regenerace volného CoA v důsledku zvýšeného odsunu nadbytečného substrátu (acylů mastných kyselin) prostřednictvím vylučování acylkarnitinů s krátkým řetězcem v moči.

5 SPECIÁLNÍ PŘÍPADY NAFLD

5.1 STEATÓZA JATERNÍHO ŠTĚPU

Běžná prevalence NAFLD v populaci vyspělých zemí dosahuje 19–31 % (190), avšak u pacientů po transplantaci jater se steatóza štěpu vyskytuje až v 40 % případů (191–193) a u pacientů transplantovaných z důvodů cirhózy prevalence dosahuje 39–70 % (194, 195). Studie provedená na pacientech Transplantačního centra IKEM prokázala v dlouhodobé perspektivě steatózu jaterního štěpu u 56 % transplantovaných, což vysoce přesahuje průměr v ČR (196). V této studii byla steatóza asociována s kratší dobou dožití, především kvůli onkologickým a kardiovaskulárním onemocněním, což je v souladu s poznatky z jiných center (197).

V klinické praxi lze NAFLD odhalit pomocí ultrazvukového vyšetření, počítačové tomografie nebo magnetické rezonance. Tato vyšetření však spolehlivě prokazují NAFLD až v rozvinutém stádiu. Možnost identifikovat markery indikující „pro-steatotické“ podmínky v jaterním štěpu před vlastní manifestací choroby by umožnila začít s léčbou v časných stádiích a zvrátit negativní trend vývoje. V této kapitole předkládám výsledky studie zaměřené na analýzu genových expresních profilů v jaterních biopsiích odebraných pacientům různě dlouhou dobu po transplantaci jater a identifikaci markerů charakteristických pro steatózní a zdravou jaterní tkáň.

5.1.1 Pacienti s histologicky prokázanou NAFLD vykazují zhoršené funkční jaterní testy a známky poruch glukózového metabolismu

V celé sledované kohortě (n=91) bylo 37 pacientů se steatózou 1. stupně, 11 pacientů se steatózou 2. nebo 3. stupně a 43 pacientů bez známek steatózy. Pro pacienty s NAFLD všech stupňů byly charakteristické vyšší hodnoty BMI a větší obvod pasu. V ostatních sledovaných parametrech se subjekty s mírnou steatózou (stupeň 1) nelišily od zdravých účastníků studie. Pacienti se závažnější steatózou (stupeň 2 a 3) měli ve srovnání s kontrolami vyšší hladiny ALT, triacylglycerolů a cholesterolu v séru, i když jejich hodnoty zůstávaly v stále v normálním rozmezí. Parametry asociované s glukózovou homeostázou, tj. lačná glykemie, glykovaný hemoglobin, lačná inzulinemie, C-peptid a HOMA index (homeostatic model assessment) byly vyšší ve srovnání se subjekty bez steatózy, přičemž inzulinemie, C-peptid a HOMA index byly vyšší i ve srovnání se skupinou se steatózou 1. stupně.

5.1.2 Rozdíly v transkriptomickém profilu steatózního a zdravého jaterního štěpu

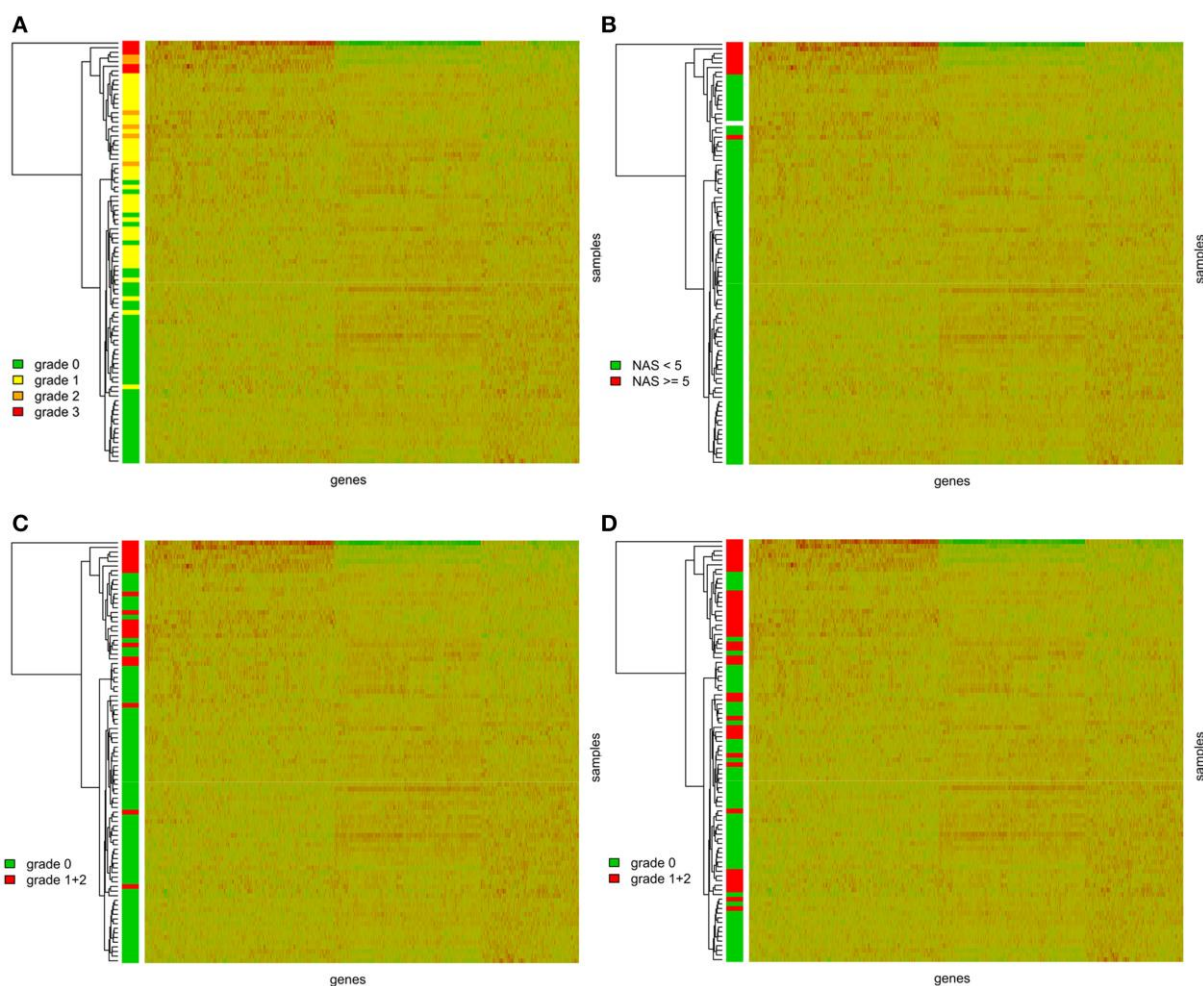
Část jaterní tkáň odebraná při pravidelné protokolární biopsii byla využita ke stanovení transkriptomického profilu vzorku z jaterní biopsie metodou microarray. Současně byla druhá část biopsované tkáň vyšetřena histologicky a bylo možné tak přesně určit stupeň steatózy. Pro účely analýzy jsme všechny pacienty se steatózou sloučili do jedné skupiny nezávisle na jejím stupni. Po provedení statistické korekce na mnohonásobné srovnávání jsme identifikovali 747 transkriptů odlišně exprimovaných ve steatózním a zdravém štěpu, z toho bylo 326 upregulovaných a 421 downregulovaných u pacientů s NAFLD. Představuje to 1,4 % z celkového počtu 53 617 identifikovaných transkriptů. Expres šestnácti genů se lišila více než 1,5x, jejich seznam je v **tab. 5.1**.

Tabulka 5.1 Nejvýznamnější transkripty odlišně exprimované ve steatózním štěpu. FC, poměr mediánů exprese ve steatózním a zdravém jaterním štěpu. Jména genů jsou přiřazena dle HGNC guidelines.

Symbol	Název genu	FC	p-hodnota
P4HA1	Prolyl 4-hydroxylase subunit alpha 1	-2,26	1,03E-07
CYP1A1	Cytochrome P450 family 1 subfamily A member 1	-1,98	7,75E-05
IGF1	Insulin-like growth factor 1	-1,73	3,27E-06
SHBG	Sex hormone binding globulin	-1,72	3,10E-07
SLC2A12	Solute carrier family 2 member 12	-1,66	2,71E-06
ENST00000560967	Predicted non-coding transcript	-1,59	3,46E-07
IGFBP2	Insulin-like growth factor binding protein 2	-1,58	7,75E-08
PROZ	Protein Z, vitamin K dependent plasma glycoprotein	-1,50	6,29E-04
APOA4	Apolipoprotein A4	1,55	1,95E-04
ME1	Malic enzyme 1	1,55	4,30E-05
SERPINE1	Serpin family E member 1	1,56	5,73E-04
PLIN1	Perilipin 1	1,65	5,33E-05
SCUBE1	Signal peptide, CUB domain and EGF like domain containing 1	1,66	3,42E-07
MAMDC4	Mam domain containing 4	1,67	2,31E-07
DOPEY2	Dopey family member 2	1,69	3,43E-04
LOC101928635	Uncharacterized LOC101928635	1,70	2,73E-04

Dříve publikovaná systematická meta-analýza transkriptomických dat nashromážděných při studiu NAFLD identifikovala 280 genů, jejichž exprese je deregulovaná při NAFL/NASH (198). Námi identifikované geny se s tímto seznamem překrývají ve třiceti genech. Tyto shodné geny kódují proteiny účastníci se odpovědi na buněčný stres (*annexiny*, *DNAJC12*), mitochondriálního metabolismu (*PPARGC1*, *ATP1A1*), metabolismu lipidů a cholesterolu (*PPARA*, *LPIN1*, *ABC transporters*, *APOF*), regulace genové exprese (*H2AFY*) nebo metabolismu extracelulární matrix (*LAMA2,3/LAMA 5*, *PCOLCE2*).

Dále nás zajímalo, zda změněný transkriptomický profil souvisí s některými dalšími rysy charakteristickými pro histologický obraz NAFLD/NASH. Neprokázali jsme souvislost se stupněm fibrózy nebo s intervalem od transplantace. Našli jsme významnou asociaci s NAS skóre ($p < 0,0001$), balónováním hepatocytů ($p < 0,0001$) a zánětem ($p < 0,0001$) (**obr. 5.1**). Balónováním hepatocytů je známkou mitochondriální dysfunkce, zánět je typický znak pokročilé NAFLD. Asociace mezi pozorovaným transkriptomickým profilem a výše zmíněnými charakteristikami podporuje předpoklad, že námi identifikované geny jsou významné pro predikci negativní progrese steatózy.



Obrázek 5.1 Hierarchické shlukování dle transkriptomických profilů. Vzorky jsou barevně kódovány dle (A) stupně steatózy klasifikované dle Kleinerova skóre; (B) NAS skóre; (C) balónovutění; (D) stupně zánětu.

5.1.3 Procesy odlišně regulované ve steatózních játrech

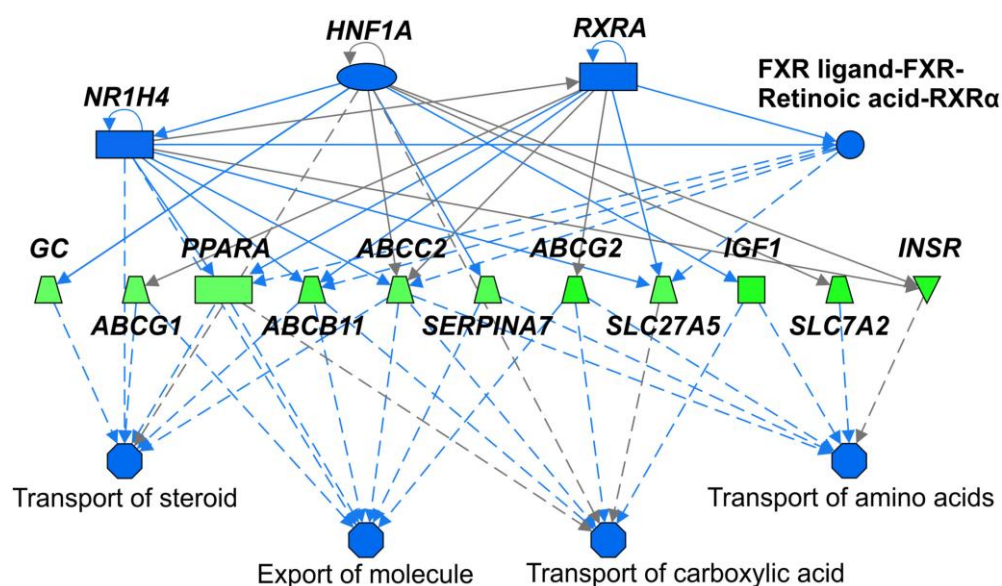
Pochopení dějů probíhajících ve steatózních játrech může napomoci identifikace klíčových deregulovaných metabolických procesů a funkcí. Porovnali jsme soubor odlišně exprimovaných genů s hlavními dostupnými databázemi, tj. IPA (Ingenuity pathways), KEGG (Kyoto Encyclopedia for Genes and Genomes) and DAVID (Database for Annotation, Visualization and Integrated Discovery). Identifikovali jsme tyto odlišně regulované procesy: krevní srážlivost, syntézu a transport žlučových kyselin, redoxní homeostázu, metabolismus lipidů, cholesterolu a aminokyselin, signální dráhy AMPK a glukagonu, transmetylační reakce a zánětlivé procesy.

Nedávné studie ukazují, že progresse NAFLD směrem k závažnějším stádiím jaterního onemocnění není spojena se samotnou akumulací lipidů, ale spíše s poruchami metabolismu cholesterolu (199). I v našem souboru jsme zaznamenali downregulaci drah účastnících se cholesterolového metabolismu, např. FXR/RXR a LXR/RXR signalizaci, syntézy a vylučování žlučových kyselin, ABC transportní systém. Toto pozorování naznačuje, že konverze cholesterolu na žlučové kyseliny, exkrece žlučových kyselin z hepatocytů a transport cholesterolu do apoA1/HDL partikul jsou u pacientů se steatózou štěpu narušeny. V důsledku toho může docházet k akumulaci cholesterolu v hepatocytech a dalšímu rozvoji jejich poškození.

Oxidační stres (200) a zánět (201) jsou typické znaky patofyziologie NAFLD. V naší kohortě pacientů s NAFLD jaterního štěpu jsme pozorovali downregulaci 15 genů důležitých

pro udržení redox homeostázy, což naznačuje přinejmenším zvýšenou citlivost k oxidačnímu poškození. Dále jsme ve steatózním štěpu pozorovali snížení exprese genů biosyntetické dráhy S-adenosylmetioninu, *MAT1A* a *MAT1B*. S-adenosylmetionin je kofaktorem metyltransferáz, které jsou klíčovými enzymy mnoha důležitých biologických procesů. Patří k nim metylace fosfolipidů buněčných membrán (202), což ovlivňuje jejich fluiditu, nebo epigenetická modifikace DNA (203). Jejich role v rozvoji NAFLD byla prokázána na zvířecích modelech (204) i v humánních studiích (205). Míra exprese *MAT1A* odlišuje pacienty s mírnou a pokročilou NAFLD.

S využitím databáze IPA jsme predikovali potenciální upstream regulační faktory, které mohou modulovat genovou expresi ve steatózních játrech včetně FXR-RXR α , NR1H4, RXR α , HNF1 α , a INSR (**obr. 5.2**). S výjimkou INSR patří tyto transkripční faktory do rodiny „liver-enriched transcription factors“, které řídí expresi typických jaterních enzymů (206). Ty kontrolují procesy jako metabolismus glukózy a mastných kyselin, syntézu koagulačních faktorů, detoxifikaci (CYP450) a metabolismus žlučových kyselin.



Obrázek 5.2 Upstream regulátory a cílové geny a procesy. ABCG1, ATP-binding cassette sub-family G member 1; ABCG2, ATP-binding cassette super-family G member 2; ABCB11, ATP-binding cassette, sub-family B member 11; ABCC2, ATP-binding cassette sub-family C member 2; FXR, farnesoid X receptor; GC, vitamin D-binding protein; IGF-1, insulin like growth factor 1; INSR, insulin receptor; HNF1A, hepatocyte nuclear factor 1 homeobox A; NR1H4, Nuclear Receptor Subfamily 1 Group H Member 4; PPARA, Peroxisome proliferator-activated receptor alpha; RXRA, retinoid X receptor alpha; SERPINA7, thyroxine-binding globulin; SLC7A2, solute carrier family 7 member 2; SLC27A5, solute carrier family 27 member 5.

5.1.4 Cirkulující miR-33a je nezávislým prediktorem steatózy štěpu

miRNA jsou malé nekódující molekuly RNA, které se účastní post-transkripční regulace genové exprese (207). Specifická interakce konkrétní miRNA a odpovídající mRNA vede většinou k negativní regulaci translace a syntézy proteinu kódovaného příslušnou mRNA (208). Výskyt těchto miRNA není omezen pouze na vnitřek buňky, ale jsou nacházeny i v extracelulárních tělních tekutinách včetně krve (209). Mnoho miRNA je tkáňově specifických a ukazuje se, že mohou sloužit jako neinvazivní biomarkery různých chorob včetně NAFL (210) a NASH (211).

miR-33a se účastní regulace lipidového metabolismu v játrech (212, 213) a byla publikována zpráva demonstrující asociaci zvýšených sérových hladin miR-33a s NASH u obézních lidí

(214). Testovali jsme proto hypotézu, zda existuje vazba mezi sérovou koncentrací miR-33a a steatózou štěpu u pacientů po transplantaci jater. Prokázali jsme, že obsah miR-33a v séru je skutečně o 17 % vyšší u pacientů s prokázanou steatózou štěpu ($p=0,034$) a o 25 % vyšší u pacientů s prokázaným zánětem štěpu ($p=0,003$) než u pacientů bez steatózy a bez zánětu, resp. S použitím multivariantní regresní analýzy jsme zjistili, že sérové hladiny miR-33a jsou nezávislým prediktorem jaterní steatózy (odds ratio = 2,9) a zánětu (odds ratio = 4).

SHRNUTÍ HLAVNÍCH POZNATKŮ

1. Transkriptomický profil steatózního a zdravého štěpu se liší.
2. Odlišně regulované procesy zahrnují:
 - krevní srážlivost;
 - syntézu a transport žlučových kyselin;
 - redoxní homeostázu;
 - metabolismus lipidů, cholesterolu a aminokyselin;
 - signální dráhy AMPK a glukagonu;
 - transmetylační reakce;
 - zánětlivé procesy.
3. Obsah miRNA33a v séru je nezávislý prediktor steatózy a zánětu jaterního štěpu.

5.2 PARENTERAL NUTRITION-ASSOCIATED LIVER DISEASE (PNALD)

Parenterální výživa (PN) představuje život zachraňující nutriční podporu v situacích, kdy výživa enterální cestou není možná nebo nepokrývá kalorickou potřebu. Bohužel, PN je provázána řadou negativních vedlejších dopadů, k nimž patří i závažné poškození jater, PNALD. Etiologie rozvoje PNALD není dosud plně známa, ale nesporně se na ní podílí i druh, množství a frekvence aplikace lipidové emulze. První lipidová emulze používaná v PN (Intralipid) byla založena na ω -6 PUFA. Vedle nesporných výhod (vysoká energetická hodnota, zdroj esenciálních mastných kyselin, minimalizace metabolického stresu) však ω -6 PUFA slouží jako prekurzory pro syntézu prozánětlivých prostaglandinů a používání Intralipidu bylo spojeno se zvýšenými projevy zánětu a oxidačního stresu v játrech. Další mezník představovalo zavedení ω -3 PUFA do parenterálních směsí, které se na klinické úrovni projevilo často zlepšením stavu pacientů. Mechanismus pozitivního vlivu ω -3 PUFA při PNALD však není dosud s jistotou objasněn.

V této studii jsme se zaměřili na detailní popis jaterního lipidomu, proteomu a spektra žlučových kyselin v závislosti na typu lipidové emulze (ω -6 vs ω -3 PUFA) a způsobu podání (enterální vs parenterální cesta) na zvířecím modelu (potkan). Cílem bylo identifikovat potenciálně významné markery nebo procesy, které mohou přispívat k rozvoji PNALD. Studie provedená na lidských pacientech byla zacílena na identifikaci cirkulujících miRNA, které by mohly sloužit jako neinvazivní marker stupně poškození jater a rozvoje PNALD.

5.2.1 Charakteristika jednotlivých skupin

Potkani kmene Wistar byli rozděleni do pěti skupin: kontroly (Plasmalyte *i.v.*, granule *per os*); ENIL (Plasmalyte *i.v.*, Intralipid *per os*, granule *per os*); ENILOV (Plasmalyte *i.v.*, Intralipid + Omegaven *per os*, granule *per os*); PNIL (nutriční směs + Intralipid *i.v.*); PNILOV (nutriční směs + Intralipid + Omegaven *i.v.*). Směs Intralipid je založena na sójovém oleji, přípravek Omegaven obsahuje pouze EPA a DHA v poměru 1:1. Všechny nutriční režimy byly kaloricky srovnatelné (251 kJ/den), experiment trval dva týdny. V žádné experimentální skupině jsme nepozorovali významné poškození jater (normální hladiny ALT a AST). V obou skupinách na parenterální výživě jsme zjistili zvýšení sérového bilirubinu, v případě PNIL statisticky významné ($p < 0,05$), v případě skupiny PNILOV se jednalo pouze o trend ($p = 0,056$). Parenterální podávání obou lipidových směsí bylo spojeno s jaterní steatózou, která byla významně vyšší ve skupině PNIL ve srovnání s PNILOV (**tab. 5.2**). Histologické hodnocení toto pozorování potvrdilo.

Tabulka 5.2 Charakteristika experimentálních skupin. prot, protein. * $p < 0,05$ vs kontroly; # $p < 0,05$ vs ENIL; † $p < 0,05$ vs PNIL; ‡ $p < 0,05$ vs ENILOV.

		Kontroly	ENIL	ENILOV	PNIL	PNILOV
Sérum	ALT $\mu\text{kat l}^{-1}$	0,7 $\pm$ 0,2	0,7 $\pm$ 0,1	0,9 $\pm$ 0,3	0,4 $\pm$ 0,1	0,5 $\pm$ 0,2
	AST $\mu\text{kat l}^{-1}$	2,5 $\pm$ 0,7	1,8 $\pm$ 0,3	1,9 $\pm$ 0,4	2,3 $\pm$ 0,8	2,1 $\pm$ 0,5
	TAG mmol l^{-1}	0,7 $\pm$ 0,2	0,6 $\pm$ 0,2	0,4 $\pm$ 0,2*	0,6 $\pm$ 0,1	0,5 $\pm$ 0,3*
	celkový bilirubin $\mu\text{mol l}^{-1}$	1,5 $\pm$ 0,3	1,4 $\pm$ 0,6	1,6 $\pm$ 0,7	2,3 $\pm$ 0,4*,#	2,1 $\pm$ 0,6
	přímý bilirubin $\mu\text{mol l}^{-1}$	0,6 $\pm$ 0,2	0,6 $\pm$ 0,3	0,5 $\pm$ 0,1	1,3 $\pm$ 0,2*,#,‡	0,7 $\pm$ 0,2†
Játra	TAG $\mu\text{mol mg prot}^{-1}$	3,3 $\pm$ 1,3	3,0 $\pm$ 2,0	4,1 $\pm$ 2,1	47,8 $\pm$ 21*,#	13,1 $\pm$ 8,5†

5.2.2 Parenterální výživa, oxidační stres a markery zánětu v játrech

Oxidační stres jsme hodnotili na základě obsahu malondialdehydu (MDA) v játrech, antioxidační kapacity a exprese některých genů asociovaných s oxidačním stresem (**tab. 5.3**). U enterálně kmených zvířat jsme neprokázali žádné známky oxidačního stresu v játrech. Parenterální výživa rovněž nevedla k významnému zvýšení obsahu MDA, ale u obou skupin jsme zjistili významné zvýšení exprese některých genů nebo proteinů pozitivně regulovaných oxidačním stresem. Na úrovni exprese mRNA se jednalo o hemoxidázu (Hmox-1), NAD(P)H dehydrogenázu (chinon) 1 (Nqo1) a glutamát-cystein ligázu (Gclc). U dvou posledně jmenovaných enzymů jsme pozorovali i zvýšený obsah odpovídajících proteinů. Expres mRNA prozánětlivých cytokinů *Ccr2* (C-C motif chemokine receptor 2) a *IL-1β* (interleukin 1 beta) byla zvýšená pouze ve skupině PNIL. Expres *IL-8* (interleukin 8) mRNA byla zvýšena ve skupinách PNIL i PNILOV vzhledem ke kontrolám, ale ve skupině PNILOV dosáhla pouze poloviční hodnoty ve srovnání s PNIL.

Tabulka 5.3 Markery oxidačního stresu a zánětu v játrech. Expres cílových genů je kvantifikovaná pomocí $\Delta\Delta C_t$ metody, po normalizaci na referenční gen; průměr C_t kontrolní skupiny slouží jako kalibrátor. Kvantifikace proteinů je odvozena z intenzity signálu. *Ccr2*, C-C motif chemokine receptor 2; *IL-1β*, interleukin 1 beta; *IL-8*, interleukin 8; *Gclc*, glutamát-cystein ligáza, katalytická podjednotka; *Hmox1*, hemoxidáza; MDA, malondialdehyd; *Nqo1*, NAD(P)H dehydrogenáza (chinon) 1; *TNFα*, tumor necrosis factor alpha. * $p < 0,05$ vs kontroly; # $p < 0,05$ vs ENIL; † $p < 0,05$ vs PNIL; ‡ $p < 0,05$ vs ENILOV; & $p < 0,05$ vs PNILOV.

	Kontroly	ENIL	ENILOV	PNIL	PNILOV
MDA μmol g ⁻¹	1,5±0,8	0,5±0,2	1,6±2,0	1,7±1,6	1,8±1,5
antioxidační kapacita	125±13	127±20	119±12	96±12*	113±23
<i>Gclc</i> mRNA	1,0±0,2	1,2±0,1 ^{†,&}	0,9±0,3 [†]	1,6±0,4*	2,3±0,3*
<i>Gclc</i> protein	226±42	253±24 ^{†,&}	234±12 ^{†,&}	415±81*	465±153*
<i>Hmox1</i> mRNA	1,0±0,1	0,7±0,1 ^{*,&}	0,8±0,1 ^{*,&}	2,3±0,6*	2,1±0,7*
<i>Hmox1</i> protein	62±9 ^{&}	63±5 ^{&}	63±7 ^{&}	72±8 ^{&}	106±24*
<i>Nqo1</i> mRNA	1,0±0,5	1,0±0,7 ^{†,&}	1,0±0,4 ^{†,&}	3,2±0,9*	4,3±1,7*
<i>Nqo1</i> protein	128±48	99±54 ^{†,&}	95±55 ^{†,&}	236±91*	199±52*
<i>Ccr2</i> mRNA	1,0±0,2	1,0±0,3 [†]	0,9±0,2 [†]	3,0±1,7*	1,1±0,2 [†]
<i>Ccr2</i> protein	1,0±0,2	0,7±0,1 [†]	0,9±0,3 [†]	1,8±0,2*	0,9±0,1 [†]
<i>IL-8</i> mRNA	1,0±0,6	0,4±0,4 ^{*,†,&}	0,4±0,1 ^{*,†,&}	5,9±2,8*	2,6±0,9 ^{*,†}
<i>TNFα</i> mRNA	1,0±0,4	0,6±0,3	0,7±0,3	1,1±0,5	0,9±0,5

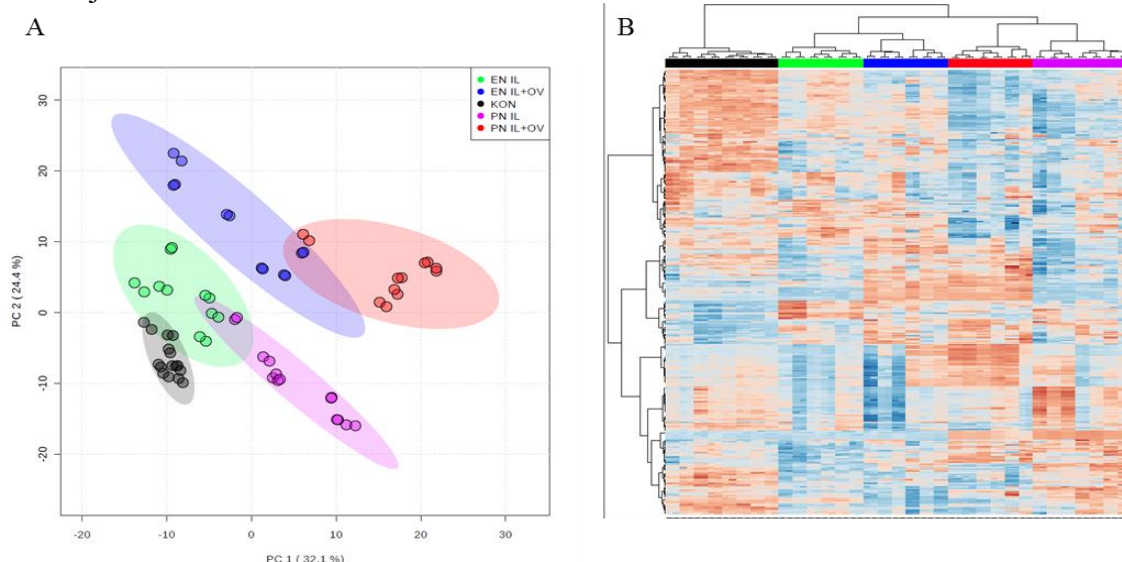
5.2.3 Spektrum žlučových kyselin v játrech

Celkem jsme v játrech identifikovali 15 žlučových kyselin. Deset z nich bylo srovnatelně zastoupeno ve všech skupinách. Obsah kyseliny glykochenodeoxycholové (gCDCA), deoxycholové (DCA), glykodeoxycholové (gDCA) a lithocholové (LCA) byl vyšší, zatímco obsah kyseliny tauroursodeoxycholové (tUDCA) nižší ve skupině PNIL ve srovnání se všemi ostatními skupinami. Skupina PNILOV byla co do spektra žlučových kyselin srovnatelná se skupinami na enterální lipidové výživě a kontrolami.

5.2.4 Složení jaterního lipidomu

V játrech jsme identifikovali 456 lipidových signálů. Pomocí ANOVA testu jsme identifikovali 435 signálů, které se významně ($p < 0,05$; $FDR < 0,1$) lišily alespoň mezi dvěma skupinami. Multivariantní statistická analýza potvrdila separaci vzorků jak podle druhu lipidové emulze, tak podle způsobu aplikace (**obr. 5.3A**). Hierarchické shlukování potvrdilo tento výsledek, přičemž způsob aplikace je nadřazen druhu emulze (**obr. 5.3B**).

Dále nás zajímalo, které konkrétní lipidy jsou charakteristické pro způsob podávání nebo druh lipidové emulze. Nezávisle na složení emulze, parenterální aplikace byla asociována s vysoce zvýšeným (100násobně) obsahem esterů hydroxy-mastných kyselin (FAHFA) a naopak sníženým obsahem fosfatidylcholinu (PC) (20:0/20:4). Charakteristickým rysem enterálně podávaného Intralipidu (ENIL) ve srovnání s parenterálně podávaným Intralipidem (PNIL) a kontrolami je zvýšený obsah oxidovaných mastných kyselin (18:2 + O; 22:4 + O; 20:4 + O; 20:5 + O, 18:3 + O) a fosfatidylglycerolu obsahujícího arachidonovou kyselinu (AA) (20:4). Parenterální aplikaci Intralipidu charakterizuje snížený obsah některých fosfolipidů obsahujících AA většinou v kombinaci s mastnými kyselinami s lichým počtem uhlíků a zvýšený obsah fosfolipidů obsahujících kyselinu linolenovou, dokosapentaenovou (22:5 FA), DHA a kyselinu dihomo- γ -linolenovou (20:3). Parenterální podávání ω -3 PUFA (PNILOV) bylo asociováno se zvýšenou koncentrací DAG obsahujících DHA a sníženým obsahem DAG obsahujícího AA.



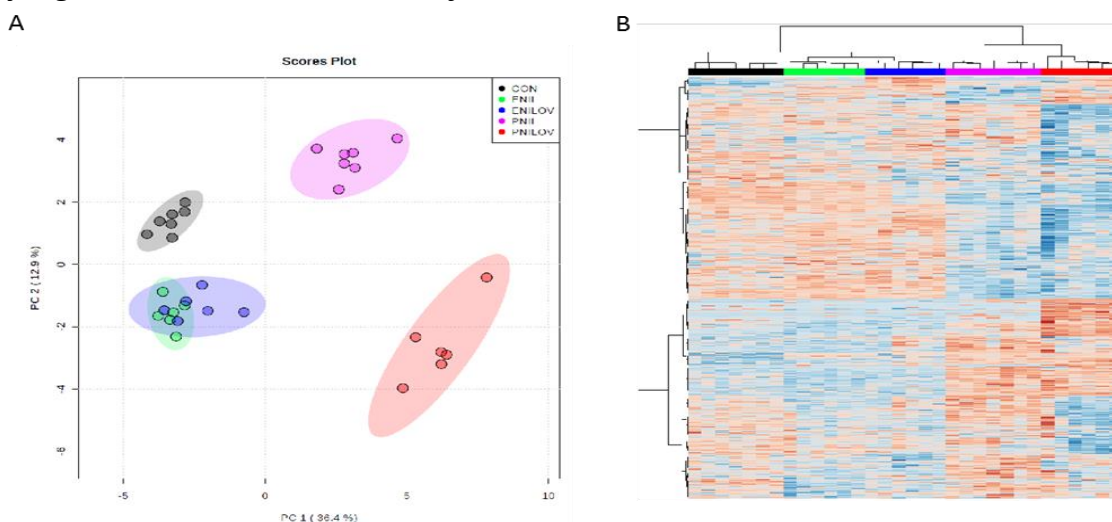
Obrázek 5.3 Složení lipidomu v játrech. A: PCA. Vzorky byly analyzovány v dubletech. B: Lipidomová heatmapa a shlukovací dendrogram. Vzorky jsou barevně kódovány podle příslušnosti ke skupinám. Převzato z Bechynska K. et al. doi: 10.1038/s41598-019-54225-8.

Vliv druhu lipidové emulze, tj. srovnání skupin ENIL vs ENILOV a PNIL vs PNILOV, se dle očekávání projevil zvýšeným obsah různých druhů lipidů obsahujících kyselinu EPA (20:5) a DHA (22:6) v játrech zvířat, kterým byly podávány ω -3 mastné kyseliny, a dále zvýšeným obsahem oxidované formy DHA (22:6+O). Lipidy nejvíce diskriminující mezi ENIL a

ENILOV skupinami byly kyselina dihomog- γ -linolová, EPA a dokosatetraenová kyselina 22:4 (zvýšené v ENIL) a fosfolipidy a plasmenyly obsahující kyseliny AA a dokosatetraenová (zvýšené v ENILOV). Při srovnání parenterálně podávaných emulzí se jako nejvíce diskriminující jeví FAHFA. Ačkoliv byly tyto sloučeniny dramaticky zvýšeny v játrech všech parenterálně krmených zvířat, podávání ω -3 PUFA jejich obsah ve srovnání s emulzí založenou pouze na ω -6 PUFA významně snížilo. Ve srovnání s PNIL jsme ve skupině PNILOV pozorovali významně zvýšený obsah DHA a lipidů obsahujících DHA.

5.2.5 Složení jaterního proteomu

V játrech jsme identifikovali 3439 proteinů. Pomocí ANOVA jsme vybrali 1560 proteinů, jejichž abundance se signifikantně lišila ($p < 0,01$, FDR 0.1) mezi alespoň dvěma skupinami. Multivariantní analýza prokázala jasnou separaci kontrol, PNILOV a PNIL skupin. Skupiny ENIL a ENILOV vytvořily jeden homogenní shluk oddělený od ostatních. Hierarchické shlukování ukázalo jasnou separaci všech skupin, přičemž metoda aplikace lipidové emulze byla nadřazena jejímu složení (**obr. 5.4**). Použití řízené multivariantní analýzy nám umožnilo identifikovat nejvýznamnější proteiny přispívající k separaci jednotlivých experimentálních skupin od kontrol. Pomocí KEGG databáze jsme se tyto proteiny snažili přiřadit konkrétním metabolickým drahám. Ve srovnání s kontrolami, enterální podávání obou lipidových emulzí ovlivnilo jaterní proteom méně než parenterální. Ve skupině ENIL bylo odlišně regulovaných 40 proteinů, 8 z nich přísluší ke čtyřem vzájemně se překrývajícím drahám (terpenoid backbone biosynthesis; synthesis and degradation of ketone bodies; butanoate metabolism; valine, leucine and isoleucine degradation). Čtyři proteiny se účastní syntézy acetoacetyl-CoA. Skupina ENILOV se od kontrol lišila v 33 proteinech, které se ale nepodařilo zařadit do metabolických drah. PNIL skupina se od kontrol odlišovala v abundanci 134 proteinů, které spadají širokého spektra metabolických drah zahrnujících metabolismus sacharidů (propanoate and butanoate metabolism), aminokyselin (tryptophan, valine, leucine, isoleucine biosynthesis and degradation), mastných kyselin, terpenoidů, steroidů a retinolu. Některé z drah se překrývají se skupinou ENIL, ale specifickým rysem skupiny PNIL je deregulace proteinů patřících do rodiny CYP450. Ve skupině PNILOV jsme identifikovali 305 odlišně exprimovaných proteinů. Identifikovali jsme čtyři metabolické dráhy (chemical carcinogenesis, glutathione metabolism, metabolism of xenobiotics by CYP450 a drug metabolism-CYP450). Dominantním proteinem byla glutathion-S-transferáza, která je součástí všech těchto drah.



Obrázek 5.4 Složení proteomu v játrech. A: PCA. Vzorky byly analyzovány v dubletech. B: Proteomová heatmapa a shlukovací dendrogram. Vzorky jsou barevně kódovány podle příslušnosti ke skupinám. Převzato z Bechynska K. et al. doi: 10.1038/s41598-019-54225-8.

Obr. 5.5 znázorňuje kvantitativní změny v zastoupení proteinů, které spadají do odlišně regulovaných metabolických drah. Většina enzymů, které se podílí na degradaci nebo syntéze mastných kyselin, mají nejnížší abundanci ve skupině PNILOV. Proteiny, které se účastní transportu mastných kyselin (CD36) a uchovávání mastných kyselin (PLIN2) byly významně zvýšeny ve všech experimentálních skupinách, zatímco lysozomální fosfolipáza A2 (PLA2G15) byla zvýšena pouze ve skupinách suplementovaných ω -3 PUFA. Klíčové enzymy glukózového metabolismu (glukokináza, hexokináza 3, fosfoenolpyruvát karboxyláza 2) byly zvýšeny pouze u parenterálně krmených zvířat. Všechny enzymy účastnící se metabolismu xenobiotik a metabolismu glutathionu byly významně méně exprimovány ve skupině PNILOV. Deset proteinů spadajících do rodiny CYP450 bylo deregulováno v důsledku intervencí studovaných v tomto pokusu, ale nelze vysledovat jednoznačný vzorec určující tyto změny.

PUFA (AA, EPA a DHA) slouží jako prekurzory pro syntézu bioaktivních lipidů. Prvním stupněm jejich syntézy je uvolnění příslušné mastné kyseliny z pozice *sn*-2 membránově vázaného fosfolipidu nebo diacylglycerolu, druhým jejich konverze katalyzovaná jedním ze tří systémů COX (cyclooxygenáza), LOX (lipooxygenáza) nebo cytochrom P-450 (CYP450) (CYP2C, CYP2J, CYP4A). AA je metabolizována všemi enzymatickými systémy srovnatelně, ale EPA a DHA jsou metabolizovány téměř výlučně prostřednictvím CYP450. EPA a DHA tak soutěží s AA o katalytickou aktivitu CYP450. Kombinace dostupnosti prekurzoru spolu s aktivitou enzymatického systému určuje výsledné složení produkovaných mediátorů a konečný biologický efekt. Funkčně jsou produkované metabolity velmi heterogenní, ale lze s jistým zjednodušením předpokládat, že mediátory odvozené od AA jsou spíše prozánětlivé a metabolity odvozené od EPA a DHA spíše protizánětlivé. Naše výsledky ukazují, že podávání směsi obohacené o ω -3 mastné kyseliny vede k významnému zvýšení obsahu fosfolipidů a DAG obsahujících EPA a DHA, které mohou sloužit jako substrát pro syntézu bioaktivních lipidů. Rovněž jsme prokázali zvýšený obsah oxidované DHA a EPA, které jsou produktem konverze těchto mastných kyselin prostřednictvím CYP450. V souladu s tímto pozorováním jsme ve skupině, které byly ω -3 mastné kyseliny parenterálně podávány, prokázali i zvýšený obsah enzymů CYP4A skupiny, které tuto reakci katalyzují. Na základě těchto zjištění nabízíme hypotézu, že obohacení nutriční směsi o ω -3 mastné kyseliny vede jak ke zvýšené dostupnosti substrátu, tak i stimuluje aktivitu příslušných enzymových systémů, a moduluje výsledný eikosanoidový profil směrem k protizánětlivému nastavení.

Metabolismus lipidů

	ENIL	ENILOV	PNIL	PNILOV
Cyp1a2	1,1	1,1	-2	-1,3
Cyp17a1	11,9	26,1	8,1	18,4
Cyp2b2	9,6	29,8	4,9	20,5
Cyp2c11	-1,2	-1,3	-2,1	-4,3
Cyp2c12	-1,1	-1,2	6,1	3,2
Cyp2d4	-1,2	-1,7	-4,7	-4,4
Cyp3a9	1,9	-1,5	-2,2	-2,9
Cyp4a10	2,1	6,7	2,1	6,5
Cyp4a14	2,3	3,5	3,5	3,7
Cyp51a1	-1,4	-3,8	-23,8	-14,1
SULT1E1	1,3	-1,3	-2,1	-3,3
HSD17B7	-1,4	-2,0	-21,1	-21,1
STE2	-1,0	-2,0	-3,1	-3,9
PPT1	1,2	1,4	3,3	4,5
ACOX1	-2,1	1,8	1,1	-2,3
ACADSB	1,2	1,0	-1,6	-2,0
ACSL1	1,2	1,4	-1,4	-1,2
ACADM	1,1	1,4	-1,2	-1,1
EHHADH	1,3	3,4	-1,1	1,9
FADS1	-1,7	-1,4	-68,4	-10,9
FADS2	-2,8	-1,6	-3,5	-3,7
HADH	1,1	1,4	1,0	-1,4
TECR	-1,1	-1,4	-1,5	-2,2
ECI1	1,8	4,1	1,7	1,9
ACSL1	1,3	1,4	-1,4	-1,2
APOA1	1,5	1,5	-1,2	-1,5
APOA2	1,6	-1,0	-1,5	-2,4
APOC	1,6	1,7	1,0	-1,8
FABP5	-1,6	-2,9	-1,1	-1,0
CD36	8,9	17,2	9,9	15,1
PLA2G15	-1,0	2,2	-1,0	7,1
PLIN2	13,4	10,6	14,2	29,7

Metabolismus sacharidů

	ENIL	ENILOV	PNIL	PNILOV
HSD11B1	-1,4	-1,5	-1,3	-2,7
FMO1	1,8	1,4	-1,4	-1,9
FMO5	1,0	1,2	-1,1	-1,6
MGST3	1,4	1,4	-2,1	-1,8
GSTM1	-1,1	-1,3	-1,4	-2,2
GSTM2	1,1	-1,3	-1,8	-2,4
GSTM3	1,6	1,2	-1,2	-2,5
GSTA4	-1,1	-1,2	-1,4	-1,8
GSTA5	1,1	-1,2	-1,5	-1,7
GSTK1	-1,0	-1,2	-1,5	-1,9

Metabolismus xenobiotik

	ENIL	ENILOV	PNIL	PNILOV
LDHA	1,3	1,4	1,5	1,5
ACACB	-1,2	-1,3	-24,9	-24,9
ACSS2	-1,5	-3,0	-3,7	-2,6
ME1	-1,0	-1,1	2,8	3,6
ALDH1B1	2,1	1,2	-1,3	1,4
PKLR	1,2	-1,3	2,9	2,0
PCK1	1,1	-1,2	-1,9	-1,6
PCK2	-1,2	-1,3	2,1	2,2
GCK	1,1	-1,3	2,4	2,2
HK3	-1,7	-1,7	1,5	1,3

Obrázek 5.5 *Expres vybraných proteinů v játrech.* Hodnoty (x) představují poměr intenzit signálu proteinu (experimentální vs kontrolní skupina), pokud $x < 1$ použili jsme rovnici $-1/x$. Jména genů jsou přiřazena dle HGNC guidelines. Červeně podbarvené hodnoty jsou zvýšené a modře podbarvené hodnoty jsou snižené vzhledem k hodnotám kontrolní skupiny. Intenzita barvy je úměrná velikosti změny. ACACB, acetyl-CoA carboxylase beta; ACADM, acyl-CoA dehydrogenase medium chain; ACADSB, acyl-CoA dehydrogenase short/branched chain; ACOX1, acyl-CoA oxidase 1; ACSL1, acyl-CoA synthetase long chain family member 1; ACSS2, acyl-CoA synthetase short chain family member 2; ALDH1B1, aldehyde dehydrogenase 1 family member B1; APOA, apolipoprotein A; APOC, apolipoprotein C; CD36, CD36 molecule; CYP, cytochrome P450 monooxygenase; ECI1, enoyl-CoA delta isomerase 1; EHHADH, enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase; FABP5, fatty acid binding protein 5; FADS, fatty acid desaturase 1; FMO, flavin containing dimethylaniline monooxygenase; GCK, glucokinase; GSTM, glutathione S-transferase mu; GSTA, glutathione S-transferase A; GSTK1, glutathione S-transferase kappa 1; HADH, hydroxyacyl-CoA dehydrogenase; HK3, hexokinase 3; HSD11B1, hydroxysteroid 11-beta dehydrogenase 1; HSD17B7, hydroxysteroid 17-beta dehydrogenase 7; LDHA, lactate dehydrogenase A; ME1, malic enzyme 1; MGST, microsomal glutathione S-transferase 1; PCK, phosphoenolpyruvate carboxykinase; PKLR, pyruvate kinase L/R.

PLA2G15, phospholipase A2 group XV; PLIN2, perilipin 2; PPT1, palmitoyl-protein thioesterase 1; SULT1E1, sulfotransferase family 1E member 1; TECR, trans-2,3-enoyl-CoA reductase. Převzato z Bechynska K. et al. doi: 10.1038/s41598-019-54225-8.

5.2.6 miRNA jako neinvazivní markery PNALD

Na základě dosud publikovaných údajů jsme vytypovali 52 miRNA, u kterých byla prokázána asociace s některým z těchto typů poškození jater: NAFLD, NASH, cholestáza. Do studie bylo zařazeno 12 pacientů se syndromem krátkého střeva, kteří byli sledováni v intervalu tří měsíců po dobu dvou let. Jedenáct pacientů mělo dlouhodobě zhoršenou funkci jater. Při každé návštěvě bylo provedeno jak rutinní vyšetření funkce jater, tak byla odebrána krev na stanovení panelu miRNA. Šest miRNA bylo odlišně zastoupeno v séru zdravých jedinců a pacientů s PNALD. Tři z nich (miR-122-p, miR-1273g, miR-500a) byly u pacientů zvýšeny vzhledem ke kontrolám, tři (miR-505, miR-199a, miR-139) měly naopak nižší expresi. Abundance miR-122-p v séru pozitivně korelovala s aktivitou ALT a AST, abundance miR-1273g korelovala pozitivně s obsahem C-reaktivního proteinu (CRP) a aktivitou gama-glutamyltransferázy (GGT). Abundance miR-505, miR-199a a miR-139 korelovala negativně s aktivitou GGT. Identifikovali jsme tak panel šesti miRNA, jejichž výskyt v krvi je významně odlišný u pacientů s PNALD a které korelují s markery poškození jater a zánětu. Validace na větší nezávislé kohortě může ověřit jejich klinický význam pro zpřesnění diagnostiky PNALD.

SHRNUTÍ HLAVNÍCH POZNATKŮ

1. Enterální podávání lipidových emulzí má na metabolismus jater menší vliv než parenterální aplikace.
2. Významným znakem asociovaným s parenterálně podávanou výživou je zvýšení obsahu bioaktivních lipidů, FAHFA, v játrech. Jejich funkce není dosud známa.
3. Parenterální podávání ω -3 PUFA vedlo ke snížení jaterní steatózy, normalizaci antioxidační kapacity v játrech a normalizaci, popř. snížení markerů zánětu.
4. Zařazení ω -3 PUFA do parenterálně podávaných nutričních směsí je asociováno s:
 - vyšším zastoupením lipidů obsahujících EPA a DHA;
 - zvýšenou abundancí CYP450 izoform, které se mohou podílet na tvorbě bioaktivních lipidů;
 - zvýšeným zastoupením oxidovaných EPA a DHA, které mohou být jejich produktem a mají potenciálně protizánětlivé účinky;
 - sníženou abundancí CYP450 enzymů, které se účastní metabolismu xenobiotik, což může představovat regulační mechanismus omezující negativní dopady oxidace mastných kyselin, tj. zvýšenou produkci ROS;
 - normalizaci složení spektra žlučových kyselin v játrech.
5. Tyto mechanismy se mohou podílet na klinicky pozorovaném protektivním účinku ω -3 PUFA.
6. Abundance šesti miRNA (miR-122-p, miR-1273g, miR-500a, miR-505, miR-199a, miR-139) v séru koreluje s markery poškození jater a zánětu.

6 ZÁVĚR

Předložená habilitační práce přispívá originálními vědeckými poznatky k problematice rozvoje a terapie NAFLD. Rozšiřuje znalosti v oblasti (dys)regulace metabolismu lipidů v játrech při NAFLD indukované různými dietními intervencemi a přispívá k pochopení mechanismu účinku některých látek používaných ve farmakoterapii. Zaměřuje se i na děje probíhající v játrech za specifických patofyziologických situací (parenterální výživa, transplantace jater) charakterizovaných rozvojem steatózy.

V experimentální části věnované metabolismu lipidů v játrech při dietně indukované steatóze shrnuji zejména poznatky týkající se degradace intracelulárních lipidů autofagicko-lysozomální cestou. V době, kdy se naše laboratoř tomuto tématu věnovala, se jednalo o zcela nový koncept a naše práce pomohly k prokázání této hypotézy. Na různých modelech, např. subcelulární systémy, izolované hepatocyty i pokusy *in vivo*, jsme prokázali, že lysozomální lipáza degraduje intracelulární triglyceridy a že v intaktních buňkách je degradace lipidových kapének v lysozomech závislá na procesu autofagie. Rovněž jsme se zabývali otázkou možné deregulace těchto dějů při dietně indukované steatóze a jako první popsali mechanismus odlišné regulace lysozomálních dějů a autofagie ve steatózních játrech. Ukázali jsme, jak tyto mechanismy přispívají k udržení steatózy. Námi vyvinutá metoda kvantifikace intenzity autofagie („autofagy flux“) v podmínkách *in vivo* byla zahrnuta i do mezinárodních Guidelines for monitoring of autophagy, které již dosáhly několika tisíc citací.

V části zabývající se možnostmi farmakologické terapie NAFLD prezentuji výsledky studia mechanismu působení dvou používaných látek, léčiva metforminu a doplňku stravy karnitinu. V případě metforminu jsme se zaměřili na základní výzkum mechanismu jeho působení na subcelulární úrovni a ukázali jsme způsob, jakým interaguje s jednotlivými komponentami mitochondriálního respiračního řetězce a jak ovlivňuje i děje nezávislé na transformaci energie. Na zvířecím modelu jsme pak rozvedli tyto poznatky a demonstrovali jak metformin, v důsledku interakce s mitochondriálním respiračním řetězcem, chrání jaterní tkáň před oxidačním stresem. V případě karnitinu jsme na zvířecím modelu metabolického syndromu (hereditárně hypetriglyceridemických potkanek) prokázali jeho specifický vliv na snížení obsahu lipidů v játrech. Navrhli jsme i mechanismus podmiňující tento efekt, kterým je regenerace volného koenzymu A v důsledku zvýšeného odsunu nadbytečných mastných kyselin prostřednictvím vylučování acylkarnitinů s krátkým řetězcem v moči.

NAFLD představuje i komplikaci některých patofyziologických stavů. Jedním z nich je steatóza jaterního štěpu u pacientů, kteří podstoupili transplantaci jater. Prospektivní studie ukazují, že prevalence steatózy je u transplantovaných pacientů vyšší než v běžné populaci a zároveň, že steatóza štěpu je asociovaná s kratší dobou dožití. Vypracování postupů umožňujících časnou diagnostiku steatózy tak může přispět k včasnému zahájení léčby a zvrácení nepříznivého vývoje. Využili jsme pravidelných protokolárních biopsií realizovaných u pacientů transplantovaných v IKEM a analyzovali jsme transkriptomický profil steatózního a nesteatózního štěpu. Identifikovali jsme hlavní odlišně regulované geny a procesy související se steatózou. Po validaci na nezávislé kohortě mohou naše poznatky napomoci k definici biomarkerů počínající steatózy štěpu.

Steatóza přecházející v závažnější poškození jater je průvodním znakem dlouhodobé závislosti na parenterální výživě. Etiologie jejího rozvoje je multifaktoriální, ale nesporně se na ní podílí i druh, množství a frekvence aplikace lipidové emulze. V současnosti se používají emulze založené na ω -6 nebo ω -3 mastných kyselinách a jejich směsi. Žádná z používaných

kombinací není bez vedlejších účinků, ω -6 mastné kyseliny slouží jako prekurzory prozánětlivých cytokinů, zatímco ω -3 mastné kyseliny mohou zvyšovat riziko oxidačního stresu vzhledem k vyššímu počtu dvojných vazeb. V Centru experimentální medicíny IKEM jsme zavedli zvířecí model parenterální výživy a využili jsme možnosti komplexní analýzy (omics metody) jaterního lipidomu, proteomu a profilu žlučových kyselin k identifikaci potenciálně významných markerů nebo procesů, které mohou přispívat k rozvoji postižení jater. Prokázali jsme obecně prospěšný vliv zařazení ω -3 mastných kyselin do nutriční směsi, zejména ve smyslu snížení jaterní steatózy, normalizaci antioxidační kapacity a normalizaci, resp. snížení markerů zánětu v játrech. Na základě výsledků analýz jsme navrhli možné mechanismy, které pozorované efekty ω -3 mastných kyselin vysvětlují.

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8 SEZNAM ZKRATEK

AA, arachidonic acid (kyselina arachidonová)
ABC transporters, ATP-binding cassette transporters
ACSLs, long-chain acyl-CoA synthetases
ALT, alanine aminotransferase (alaninaminotransferáza)
AMPK, AMP-activated protein kinase
ANOVA, analysis of variance
AOX, acyl-coenzyme A oxidase (acylkoenzym A oxidáza)
ApoE, apolipoprotein E
ApoF, apolipoprotein F
Asn, asparagine (asparagin)
AST, aspartate aminotransferase (aspartátaminotransferáza)
ATGL, adipose triglyceride lipase
ATP, adenosine triphosphate (adenosintrifosfát)
ATP1A1, sodium/potassium-transporting ATPase subunit alpha-1

BAI operon, bile-acid inducible operon
BMI, body mass index (index tělesné hmotnosti)
BSA, bovine serum albumin (hovězí sérový albumin)
BSH, bile salt hydrolase (hydroláza žlučových kyselin)
B2M, beta-2 microglobulin (beta-2 mikroglobulin)

CCl₄, chlorid uhličitý, tetrachlormetan
Ccr2, C-C motif chemokine receptor 2
CDAA, choline-deficient amino acid (diet) (cholin-deficitní aminokyselinová (dieta))
CoA, coenzyme A (koenzym A)
COX, cyclooxygenase (cyklooxygenáza)
CQ, chloroquine (chloroquin)
CRP, C-reactive protein (C-reaktivní protein)
CVD, cardiovascular disease (kardiovaskulární choroba)
CYP 450, cytochrome P-450 (cytochrom P-450)
Cyt C, cytochrome C (cytochrom C)

DAG, diacylglycerole (diacylglycerol)
DAVID, Database for Annotation, Visualization and Integrated Discovery
DCA, deoxycholic acid (kyselina deoxycholová)
DCFDA, dichlorofluorescein diacetate
Dgat1, diacylglycerol O-acyltransferase 1 (diacylglycerol O-acyltransferáza 1)
DHA, docosahexanoic acid (kyselina dokosaheptaenová)
DNAJC1, DnaJ homolog subfamily C member 1

EPA, eicosapentaenoic acid (kyselina eikosapentaenová)
EPR, elektronová paramagnetická rezonance

FABP, fatty acid binding protein
FAHFA, fatty acid esters of hydroxy fatty acids (větvené estery mastných kyselin)
FCS, fetal calf serum
FFA, free fatty acids (volné mastné kyseliny)
FXR, farnesoid-X-receptor

GCLC, glutamate-cysteine ligase, catalytic subunit (glutamát-cystein ligáza, katalytická podjednotka)

GGT, gamma-glutamyl transferase (gama-glutamyl transferáza)

GIP, gastric inhibitory polypeptide

GLP-1, glucagon-like peptide-1

GOI, gene of interest

GR, glutathione reductase (glutathion reduktáza)

GSH, glutathione (glutathion)

GSH-Px, glutathione peroxidase (glutathion peroxidáza)

GSSG, oxidized state of glutathione (oxidovaný glutathion)

gCDCA, glykochenodeoxycholic acid (kyselina glykochenodeoxycholová)

gDCA, glykodeoxycholic acid (kyselina glykodeoxycholová)

HCC, hepatocellular carcinoma (hepatocelulární karcinom)

HCV, hepatitis C virus (virus hepatitidy C)

HDL, high density lipoprotein (lipoproteiny o vysoké hustotě)

HFD, high-fat diet (vysokotuková dieta)

HHTg, hereditary hypertriglyceridemic (rat) (hereditárně hypetriglyceridemický potkan)

HL, hepatic lipase (jaterní lipáza)

HMG-CoA syntáza, hydroxymethylglutaryl-CoA synthase (hydroxymethylglutaryl-CoA syntáza)

Hmox-1, heme oxygenase 1 (hemoxidáza)

HOMA index, homeostatic model assessment

HSD, high-sucrose diet (vysokosacharózová dieta)

HSD17B13, hydroxysteroid 17-beta dehydrogenase-13

IL-1 β , interleukin 1 beta

IL-8, interleukin 8

I/R, ischemie/reperfúze

IPA, ingenuity pathways

i.v., intravenózní

JNK, c-Jun N-terminal kinase (c-Jun N-terminální kinázy)

KEGG, Kyoto Encyclopedia for Genes and Genomes

KO, knock-out

KRB, Krebs-Ringer bicarbonate

LAL, lysosomal lipase (lysozomální lipáza)

LAMA, laminin subunit alpha

LCA, lithocholic acid (kyselina lithocholová)

LC3, microtubule-associated protein 1A/1B-light chain 3

LD, lipid droplets (lipidové kapénky)

LDL, low-density lipoprotein (lipoproteiny o nízké hustotě)

LOX, lipoxygenase (lipoxygenáza)

LPL, lipoprotein lipase (lipoproteinová lipáza)

LPS, bacterial lipopolysaccharide (bakteriální lipopolysacharid)

LXR, liver X receptor

MAFLD, metabolic-associated fatty liver disease
 MAG, monoacylglycerole (monoacylglycerol)
 MAT1(2), CDK-activating kinase assembly factor MAT1(2)
 MBOAT7, membrane-bound O-acyltransferase domain-containing protein 7
 MCD, methionine/choline deficient (diet) (metionin/cholin deficitní (dieta))
 MDA, malondialdehyde (malondialdehyd)
 miRNA, microRNA (mikroRNA)
 MGAT/DGAT, monoacyl-/diacylglycerol transferase (monoacyl-/diacylglycerol transferáza)
 mGPDH, mitochondrial glycerol-3-phosphate dehydrogenase (mitochondriální glycerol-3-fosfát dehydrogenáza)
 MK, mastné kyseliny
 mtDNA, mitochondrial DNA (mitochondriální DNA)
 MTPT, mitochondrial permeability transition pore (přechodový pór mitochondriální permeability)
 MUFA, monounsaturated fatty acid (mononenasycené mastné kyseliny)

NADH, nicotinamide adenine dinucleotide (nikotinadenindinukleotid)
 NAFLD, non-alcohol fatty liver disease (nealkoholová tuková choroba jater)
 NAFL, non-alcohol fatty liver (prostá jaterní steatóza)
 NASH, steatohepatitis (steatohepatitida)
 NF- κ B, nuclear factor kappa B (nukleární faktor kappa B)
 NQO1, NAD(P)H quinone dehydrogenase 1 (NAD(P)H dehydrogenáza (chinon) 1)
 Nrf2, nuclear factor-erythroid 2-related factor 2

PC, phosphatidylcholine (fosfatidylcholin)
 PCOLCE2, procollagen C-endopeptidase enhancer 2
 PDH, pyruvate dehydrogenase (pyruvát dehydrogenáza)
 PE, phosphatidylethanolamine (fosfatidylethanolamin)
 PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator 1-alpha
 PLIN2, perilipin 2
 PN, parenteral nutrition (parenterální výživa)
 PNALD, parenteral nutrition-associated liver disease
 PNPLA3, patatin-like phospholipase domain-containing protein 3
 PPAR, peroxisome proliferator-activated receptor
 PPARGC1, PPARG coactivator 1 alpha
 PTEN, phosphatase and tensin homolog
 PUFA, polyunsaturated fatty acids (polynenasycené mastné kyseliny)

RC, respiratory control (respirační kontrola)
 ROS, reactive oxygen species (reaktivní sloučeniny kyslíku)
 RXR, retinoid X receptor

SCFA, short-chain fatty acids (mastné kyseliny s krátkým řetězcem)
 SD, standard diet (standardní dieta)
 SFA, saturated fatty acid (nasycené mastné kyseliny)
 SOD, superoxide dismutase (superoxid dismutáza)
 SREBP, sterol-regulatory element binding protein
 S3-12, adipocyte specific protein

TAG, triacylglycerols (triglyceridy, triacylglyceroly)

TBARS, thiobarbituric acid-reacting substance (látky reaktivní s kyselinou thiobarbiturovou)

TCA, tricarboxylic acid cycle (cyklus trikarboxylových kyselin)

Tfam, mitochondrial transcription factor A

TGF β , transforming growth factor beta

TGH/Ces triacylglycerol hydrolase/ carboxylesterase

TIP47, tail-interacting protein 47 kDa

TLR, toll-like receptors

TM6SF2, transmembrane 6 superfamily member 2

TNF α , tumor necrosis factor alpha

tUDCA, tauroursodeoxycholic acid (kyselina tauroursodeoxycholová)

T2D, diabetes 2. typu

UPR, unfolded protein response

VLDL, very low density lipoproteins (lipoproteiny o velmi nízké hustotě)

v.v., vlhká váha

4-HNE, 4-hydroxy nonenal

9 PŘÍLOHY

PŘÍLOHA I

Škop V, Cahová M, Papáček Z, Páleníčková E, Daňková H, Baranowski M, Zabielski P, Zdychová J, Zídková J, Kazdová L. *Autophagy-lysosomal pathway is involved in lipid degradation in rat liver*. *Physiol Res*. 2012;61(3):287-97. doi: 10.33549/physiolres.932285.

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PŘÍLOHA III

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PŘÍLOHA V

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PŘÍLOHA X

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PŘÍLOHA I

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Autophagy-Lysosomal Pathway Is Involved in Lipid Degradation in Rat Liver

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Summary

We present data supporting the hypothesis that the lysosomal-autophagy pathway is involved in the degradation of intracellular triacylglycerols in the liver. In primary hepatocytes cultivated in the absence of exogenous fatty acids (FFA), both inhibition of autophagy flux (asparagine) or lysosomal activity (chloroquine) decreased secretion of VLDL (very low density lipoproteins) and formation of FFA oxidative products while the stimulation of autophagy by rapamycin increased some of these parameters. Effect of rapamycin was completely abolished by inactivation of lysosomes. Similarly, when autophagic activity was influenced by cultivating the hepatocytes in "starving" (amino-acid poor medium) or "fed" (serum-supplemented medium) conditions, VLDL secretion and FFA oxidation mirrored the changes in autophagy being higher in starvation and lower in fed state. Autophagy inhibition as well as lysosomal inactivation depressed FFA and DAG (diacylglycerol) formation in liver slices *in vitro*. *In vivo*, intensity of lysosomal lipid degradation depends on the formation of autophagolysosomes, i.e. structures bringing the substrate for degradation and lysosomal enzymes into contact. We demonstrated that lysosomal lipase (LAL) activity in liver autophagolysosomal fraction was up-regulated in fasting and down-regulated in fed state together with the increased translocation of LAL and LAMP2 proteins from lysosomal pool to this fraction. Changes in autophagy intensity (LC3-II/LC3-I ratio) followed a similar pattern.

Key words

Autophagy • Lysosomes • Lipolysis • Hepatocyte • Lysosomal lipase

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Introduction

The liver has an enormous capacity to store excessive fat in the form of lipid droplets. Growing evidence indicates that lipid droplets in the hepatocytes are not the physiologically inactive particles but that the liver is a site of a continuous lipolysis/reesterification cycle of stored TAG (triacylglycerol). Most of the FFA transported into hepatocytes are rapidly esterified and must be released prior any further utilization, i.e. for oxidation or VLDL particles assembly, by lipolysis (Zechner *et al.* 2005, Lankester *et al.* 1998, Yang *et al.* 1995). Thus, the lipolysis is an important part of hepatocellular TAG metabolism, but little is known about the lipases responsible for controlling hepatic TAG hydrolysis or about how this process is regulated. Several lipases have been identified in rat liver. Triacylglycerol hydrolase (TGH, EC 3.1.1.1) is expressed in the liver as well as in the heart, kidney, small intestine and adipose tissue and localizes to the endoplasmic reticulum and lipid droplets (Dolinsky *et al.* 2004). Adipose triglyceride lipase (ATGL, alias PNPLA2, desnutrin, FP17548, TTS-2.2) is expressed predominantly in adipose tissue but its expression in the liver is relatively low (Haemmerle *et al.* 2006).

Lipolytic activity in a low pH range (4.5-5) in the liver was first demonstrated by Vavrinkova and Mosinger (1965). The lipase responsible for this phenomenon is of lysosomal origin (lysosomal lipase, LAL, EC 3.1.1.13). Its involvement in endogenous TAG degradation was first demonstrated by Debeer *et al.* (1979) who showed on isolated hepatocytes that lysosomotropic agents (chloroquine or NH_4Cl) that inactivate lysosomal enzymes *via* elevation of intralysosomal pH significantly inhibited ketogenesis from endogenous (intracellular TAG) but not exogenous (added oleate) sources. Furthermore, Kalopissis and colleagues demonstrated in the same model that in the absence of exogenous fatty acids chloroquine not only reduced ketogenesis but also attenuated VLDL secretion (Francone *et al.* 1992). The essential role of LAL in TAG hydrolysis in the liver was confirmed in a LAL null mice model. *Lal* $^{-/-}$ mice exhibited massive TAG and cholesterol storage in the adult liver, adrenal glands and small intestine, severe hepatosplenomegaly and shortened life span (Du *et al.* 2001). This phenotype could be reversed by LAL cDNA adenoviral transfection (Du *et al.* 2002).

The main objection made against LAL involvement in the degradation of intracellular lipid droplets was the different intracellular localisation of lipolytic enzyme (LAL sequestered in lysosomes) and substrate (lipid droplets in cytoplasm). Only recently did Singh *et al.* (2009) bring evidence supporting the hypothesis that lipid droplets in hepatocytes can enter the autophagic degradation pathway in the same manner as damaged proteins or organelles *via* formation of autophago(lipo)somes that carry the cargo to the final degradation in lysosomes. The intensity of autophagy is closely linked with the nutrient sensing being up-regulated in starvation and suppressed in nutrient excess. The regulatory and functional similarities between autophagy and lipolysis indicate that autophagy may contribute to the TAG breakdown in the liver.

The aim of our study was to address the issue concerning the contribution of autophagy-lysosomal pathway to intracellular lipid degradation in the liver. We determined the effect of autophagy inhibition/stimulation alone or in combination with lysosomal inactivation in the formation of lipid degradation products in primary hepatocytes and liver slices incubated *in vitro*. We further assessed the correlation between the effect of starvation on both autophagy intensity and lysosomal lipid degradation in the liver *in vivo*.

Material and Methods

Animals and experimental protocol

Male Wistar rats (AnLab, Prague; b.wt. 300 ± 20 g) were kept in temperature-controlled room at 12:12-h light-dark cycle. Animals had free access to drinking water and standard chow-diet if not stated otherwise. All experiments were performed in agreement with the Animal Protection Law of the Czech Republic 311/1997 which is in compliance with Principles of Laboratory Animal Care (NIH Guide to the Care and Use of Laboratory Animals, 8th edition, 2011) and were approved by the ethical committee of the Institute for Clinical and Experimental Medicine. Animals designed as “starved” were deprived of food 24 hrs prior the experiment, “fed” animals had free access to food.

Preparation and cultivation of primary hepatocytes

The cells were prepared as described before (Rousar *et al.* 2009). Briefly, rats were narcotized, the abdomen was opened and heparin was injected into *v. cava inferior*. Cannula was fixed in *v. portae* and the liver was then perfused by solution without Ca^{2+} and collagenase (137 mM NaCl; 5.4 mM KCl; 0.4 mM MgSO_4 ; 0.35 mM Na_2HPO_4 ; 0.45 mM KH_2PO_4 ; 26 mM NaHCO_3 ; 0.5 mM EGTA; pH=7.4). Next, the liver was removed from the rat and perfused by solution with Ca^{2+} and collagenase (Sigma) (137 mM NaCl; 5.4 mM KCl; 0.4 mM MgSO_4 ; 1 mM HEPES; 4 mM CaCl_2 ; 0.25 g/l collagenase; pH 7.4) for 15 min. The liver was placed into Krebs-Henseleit medium and the tissue was disintegrated by slow shaking. Cell suspension was filtered through gauze and washed three times. Isolated hepatocytes were suspended in Williams' medium E supplemented with 10 % fetal bovine serum (FBS) and plated in collagen-coated 25 cm² T-flask, at the density of 4×10^6 cells per flask. The viability of the hepatocytes was >86 % determined according to the Trypan Blue exclusion. After adherence, the medium was changed and the cells were incubated overnight under conditions promoting TAG accumulation in Williams' medium E supplemented with 0.6 mM oleic acid conjugated to bovine serum albumin (Sigma), 10 % FBS, 100 nM dexamethasone, 2 mM glutamine, 1 mM pyruvate, 10 mM lactate, 80 mU/ml insulin (Humulin R, Eli Lilly). During this “pulse” period, the newly synthesized TAG were labeled by ¹⁴C-oleate (S.A.1 $\times 10^6$ dpm/ μmol). After 18 hrs, the medium was removed and replaced with non-radioactive medium (“chase”). During this period the

cells were kept either in starving (amino acid-poor Williams' medium E supplemented only with 1 mM pyruvate) or in fed conditions (the same medium supplemented with 10 % FBS, 20 mM glutamine, 10 mM lactate, 80 mU/ml insulin). The cells were incubated for further 6 hrs in the medium alone or in the presence of autophagy flux inhibitors asparagine 20 mM, autophagy stimulator rapamycin 100 nM or inhibitor of lysosomal activity chloroquine 500 μ M (Berg *et al.* 1998).

Determination of lipid degradation products in primary hepatocytes

At the end of the chase period, the incubation medium was collected; the cells were quickly frozen by immersion of the cultivation flask into liquid nitrogen and then stored in -40°C until the analysis. For the determination of TCA (tricarboxylic acid cycle) intermediates content, the hepatocytes were homogenised by sonication (2 x 30 sec, Hielsler ultrasonic homogeniser UP200S, Teltow, Germany) in 150 mM NaCl, extracted by petroleum ether and the radioactivity remaining in the water fraction was counted. According to Kawamura (Kawamura *et al.* 1981) this fraction represents mostly TCA cycle intermediates (>80 %) and minor part are amino acids derived from FFA via TCA cycle (<20 %). The ^{14}C oleate incorporation into VLDL was determined in chloroform extract of the incubation medium. The conversion of ^{14}C oleate into secreted water-soluble oxidation products (i.e. predominantly ketone bodies) was assessed according to the radioactivity remaining in the aqueous fraction of incubation medium after chloroform extraction.

Determination of FFA and DAG production in liver slices in vitro

Another set of experiments was designed to assess the effect of autophagy inhibition or lysosomal inactivation on FFA and DAG production in liver slices *ex vivo*. Precisely cut liver slices (150 ± 8 mg; approx. 1 mm width) were incubated in Krebs Ringer phosphate medium (2 ml) supplemented with 0.01 M KCN for 2 hours. As KCN prevents oxidation of released FFA their accumulation in the tissue serves as the indicator of lipolysis. At the end of the incubation period, the tissue was frozen in liquid nitrogen and stored in -80°C until the analysis. The linearity of the assay was tested up to 3 hours of incubation. Samples were grinded in aluminum mortar precooled in liquid nitrogen. Lipids were extracted by Folch method (Folch *et al.* 1957). The

fractions of diacylglycerols (DAG) and nonesterified fatty acids (FFA) were separated by thin-layer chromatography (TLC) according to Roemen and van der Vusse (1985). Authentic lipid class standards were spotted on the outside lanes of the TLC plates to enable localization of sample lipid fractions. The gel bands corresponding to the standards were scrapped from the plates, transferred into fresh tubes and then transmethylated in 14 % methanolic boron trifluoride (Sigma) at 100°C for either 2 (FFA) or 10 (DAG) minutes. The content of resulting fatty acid methyl esters was determined by means of gas-liquid chromatography as previously described in detail (Nawrocki *et al.* 2004). Heptadecanoic acid and 1,2-diheptadecanoin (Larodan Fine Chemicals) were used as internal standards.

Preparation of lysosomal and phagolysosomal fractions

Lysosomes and autophagolysosomes were separated according to their density by differential centrifugation (Seglen *et al.* 1985). The liver homogenate (20 % wt/vol) was prepared by homogenization of liver tissue in 0.25 M sucrose; 0.001 M EDTA pH=7.4; heparin 7 IU/ml, 1 mM PMSF, leupeptin 10 $\mu\text{g}/\text{ml}$, aprotinin 10 $\mu\text{g}/\text{ml}$ by teflon pestle homogenizer. The crude impurities were removed by brief centrifugation at 850 g. Fat cake and all traces of fat remaining on the tube walls were carefully removed in order to prevent contamination of the homogenate. An aliquot of the homogenate was kept at 4°C until lipase assay (max. 2 hour), the rest was centrifuged for 10 000 g 20 min 4°C and the resulting pellet and supernatant were separated. The supernatant preferentially contains the less dense lysosomes with higher TAG content ("autophagolysosomes", PhL), the pellet is formed by more dense particles.

Determination of autophagy flux

The most frequently used autophagy marker is the quantification of microtubule-associated protein 1 light chain 3 (LC3). LC3 is initially synthesized in an unprocessed form, proLC3, which is converted into proteolytically processed form lacking amino acids from the C-terminus, LC3-I, and is finally modified into a phosphatidylethanolamine-conjugated form, LC3-II. LC3-II is the only protein marker that is reliably associated with phagophores, sealed autophagosomes and mature autophagosomes/autolysosomes (Rubinsztein 2009). Nevertheless, LC3-II is localized both in the luminal and cytosolic site of the autophagosome and undergoes rapid degradation within the lysosome.

Consequently, a “snapshot” measurement of LC3-II content or LC3-II: LC3-I ratio is not sufficient for the determination autophagy flux as the increased LC3-II may be the consequence of either its increased formation as well as its attenuated degradation. Similarly, the low LC3-II expression may indicate its low formation as well as its increased degradation due to the increased lysosomal activity. In our study, we estimated the autophagy flux by inferring LC3-II turnover by western blot in the presence and absence of lysosomal degradation (Klionsky *et al.* 2007). The hepatocytes were incubated either alone or in the presence of 100 μ M chloroquine, a lysomotropic agent that increases intralysosomal pH and thus completely blocks the activity of lysosomal hydrolases. The autophagy flux is proportional to the rise in LC3-II content in the treated vs. untreated sample.

Separation and immunodetection of proteins

Homogenate, PhL and dense lysosomal fraction were prepared as described above. Proteins in particular fraction were separated by SDS-PAGE and the protein of interest was recognized by specific antibody (LAL: mouse monoclonal to lysosomal acid lipase; LAMP2: rabbit polyclonal to LAMP2, Abcam, Cambridge, UK). LC3-II content was determined in the 20 % liver homogenate lysed by 2 % SDS at 100 °C. LC3 in the lysates was recognized with rabbit polyclonal to LC3A/B (Abcam, Cambridge, UK). LC3-I and LC3-II were distinguished by difference in molecular weight (18 and 16 kDa). Despite the increased molecular weight than LC3-I, LC3-II migrates more rapidly in SDS-PAGE compared to LC3-I, likely due to higher hydrophobicity associated with the phosphatidylethanolamine group (Barth 2010). The loading control was performed using rabbit polyclonal antibody to beta actin (Abcam, Cambridge, UK). The bands were visualized using chemiluminescence and quantified using FUJI LAS-3000 imager (FUJI FILM, Japan) and Quantity One software (BioRad, Hercules, CA).

Assay of lysosomal lipase activity

The optimal conditions for the lipase assay (pH optimum, substrate concentration, reaction temperature and linear range of the assay) were determined as described previously (Cahova 2011). 4 % liver homogenate or lysosomal sub-fractions prepared from the fresh tissue under iso-osmotic conditions (250 mM sucrose) were used for the assay. The reaction medium

(92.5 kBq 3 H triolein, 100 μ M cold triolein, 110 μ M lecithin, 0.15 M NaCl) was emulsified by sonication (Hielsler ultrasonic homogeniser UP200S, Teltow, Germany) in 0.1 M acetate buffer (pH=4.5) containing 6 % FFA-free BSA. The assay itself was performed under hypo-osmotic conditions in order to ensure the release of the enzyme sequestered within the lysosomes. The substrate was incubated with the enzyme (liver homogenate or subcellular fractions) for 60 min at 30 °C. The released fatty acids were extracted according to Belfrage and Vaughan (Belfrage *et al.* 1969) and counted for radioactivity.

Statistical analysis

Data are presented as mean $\pm$ SEM. Statistical analysis was performed using Kruskal-Wallis test with multiple comparisons ($n=5-7$). Differences were considered statistically significant at the level of $p<0.05$. The correlation between FFA and DAG content in *ex vivo* incubated liver slices was evaluated using Spearman's correlation coefficient.

Results

Effect of autophagy and lysosomal inhibition on the utilization of lipids-derived FFA in hepatocytes

The aim of the following experiment was to evaluate the effect of either autophagy inhibition or lysosomal inactivation in the formation of VLDL or FFA oxidation products under the situation when the intracellular TAG's are the only source for FFA-utilizing metabolic pathways. The experiment was carried out on hepatocytes cultivated in “starved” conditions, i.e. in the situation when autophagy is promoted. It was based on the rationale that if autophagy-lysosomal pathway is a significant contributor to intracellular FFA pool than the inhibition of either autophagy flux (by asparagine) or lysosomal activity (by chloroquine) would decrease the formation of lipid degradation-derived products. As shown in Fig. 1A, chloroquine effectively blocked the LC3-II degradation in autophagolysosomes. The accumulation of LC3-II in chloroquine-treated samples confirmed that the autophagy flux was preserved in our experimental setting, but that the final step, i.e. lysosomal degradation, was inhibited.

The FFA released from intracellular TAG that are not re-esterified back can either be utilized for VLDL formation or transported into mitochondria for oxidation in order to produce acetyl-CoA. Acetyl-CoA can enter

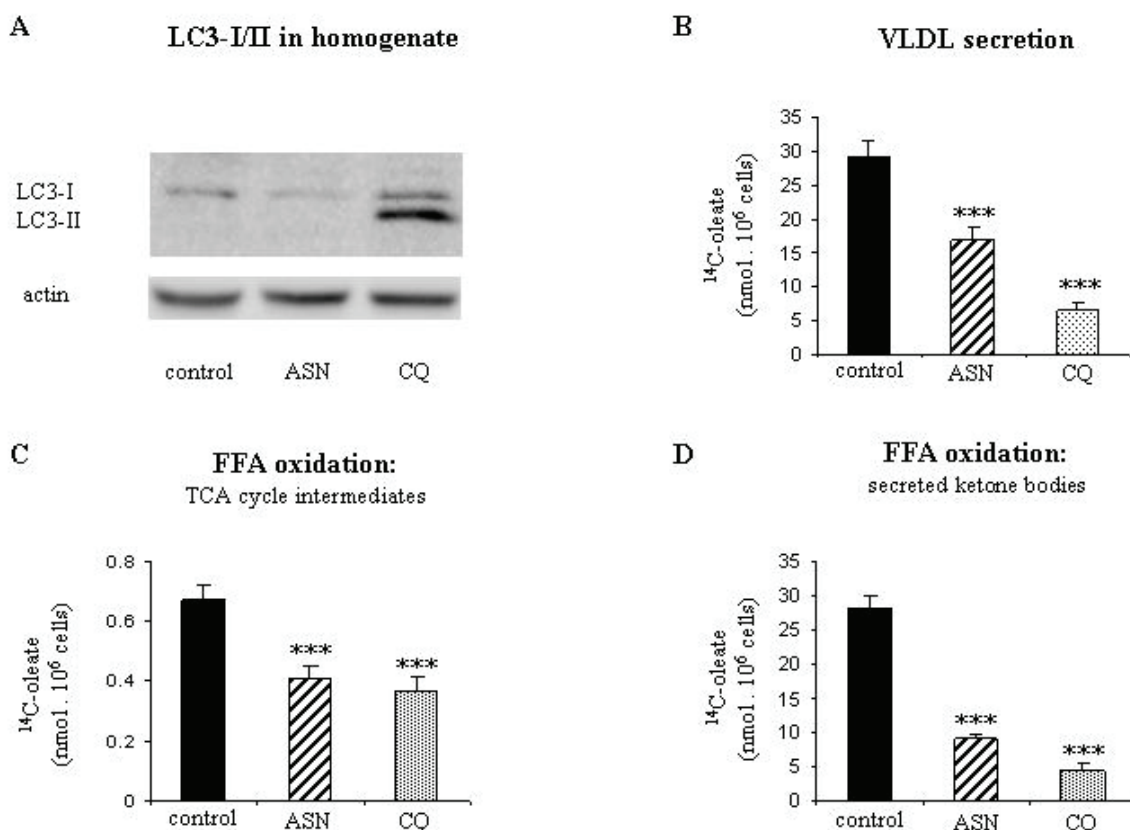


Fig. 1. Effect of autophagy inhibition and lysosomal inactivation on the utilization of lipids-derived FFA in starved hepatocytes. **A:** LC3-I/II content in homogenate; **B:** ^{14}C -oleate incorporation into secreted TAG; **C:** ^{14}C -oleate incorporation into TCA cycle intermediates; **D:** ^{14}C -oleate incorporation into secreted ketone bodies. The hepatocytes were pre-labeled with ^{14}C -oleate overnight and then incubated in serum- and amino acid-free medium for 6 hours without radioactive label. During this period the hepatocytes were left in the medium alone (control) or in the presence of autophagy flux inhibitor asparagine (ASN, 20 mM) or in the presence of lysosomotropic agent chloroquine (CQ, 100 μM) that prevent the degradation of material internalized in lysosomes. ^{14}C -oleate incorporation into secreted TAG was determined as the radioactivity recovered in the chloroform extract of the incubation medium. TCA intermediates formation was assessed according to the ^{14}C -oleate incorporation into intracellular water-soluble fraction. Ketone bodies production was determined as the radioactivity recovered in the aqueous fraction of the incubation medium. Data are given as mean $\pm$ S.E.M from three independent experiments. *** $p < 0.001$ vs. control

several metabolic pathways – it can pass through the TCA cycle and proceed to the oxidation in mitochondrial respiratory chain or, to a lesser extent, be converted into amino acids (Kawamura *et al.* 1981) or may be used for ketone body production. The effect of anti-autophagic and lysosomotropic treatment on FFA availability for further metabolic utilization is demonstrated in Fig. 1B-D. ^{14}C -oleate released from intracellular lipids was nearly equally used for VLDL synthesis and for ketogenesis. TCA intermediates represented only approx 1 % of ^{14}C -oleate derived radioactivity. The inhibition of autophagy flux by asparagine as well as the inactivation of lysosomes similarly decreased the availability of ^{14}C -oleate for all these pathways. Taken together, we showed that both the inhibition of autophagy and the inactivation of lysosomes had significant and comparable effect in the formation of lipid degradation-dependent

products. These data indicate that the autophagy lysosomal pathway is one of the pathways involved in the degradation of intracellular TAG.

Effect of autophagy stimulation in combination with lysosomal inhibition on the utilization of lipids-derived FFA in hepatocytes

A different set of experiments was undertaken in order to answer the question whether the experimentally induced autophagy stimulation may increase the lipid degradation and FFA availability in hepatocytes and whether this effect could be prevented by the inhibition of lysosomal degradation. The hepatocytes pre-labeled with ^{14}C -oleate were incubated in a “fed” condition in order to depress the autophagy intensity. Part of the hepatocytes were treated either with rapamycin, that counteracts the inhibitory effect of amino acids thereby stimulating

autophagy, or by a combination of rapamycin and lysomotrophic agent chloroquine. The results are shown in Fig. 2. The positive effect of rapamycin on autophagy intensity was confirmed by the increase of LC3-II content in the presence of rapamycin and chloroquine compared with chloroquine-only treated cells (Fig. 2A). We found no effect of the rapamycin itself on LC3-II formation and we ascribe this finding to the increased LC3-II turnover due to the stimulated autophagy. Rapamycin treatment led to a significant increase of ^{14}C -oleate content in secreted TAG (Fig. 2B). The addition of chloroquine to rapamycin-treated hepatocytes completely abolished this effect and attenuated VLDL secretion from control hepatocytes as well. We found only subtle effect of rapamycin in the TCA cycle

intermediates formation and no effect on ketogenesis (Fig. 2C,D). The ^{14}C -oleate utilization for VLDL secretion or ketogenesis was significantly decreased in control “fed” hepatocytes (Fig. 2B,D) compared with control “starved” cells (Fig. 1B,D) which suggest the partial inhibition of lipid degradation under fed conditions. The conclusions derived from this experiment show that FFA availability in isolated hepatocytes is lower in the situation when autophagy is depressed (“fed” condition), that FFA availability for VLDL formation (and to a much smaller degree for FFA oxidation) could be increased by autophagy stimulation and that this effect is completely dependent on preserved activity of lysosomes.

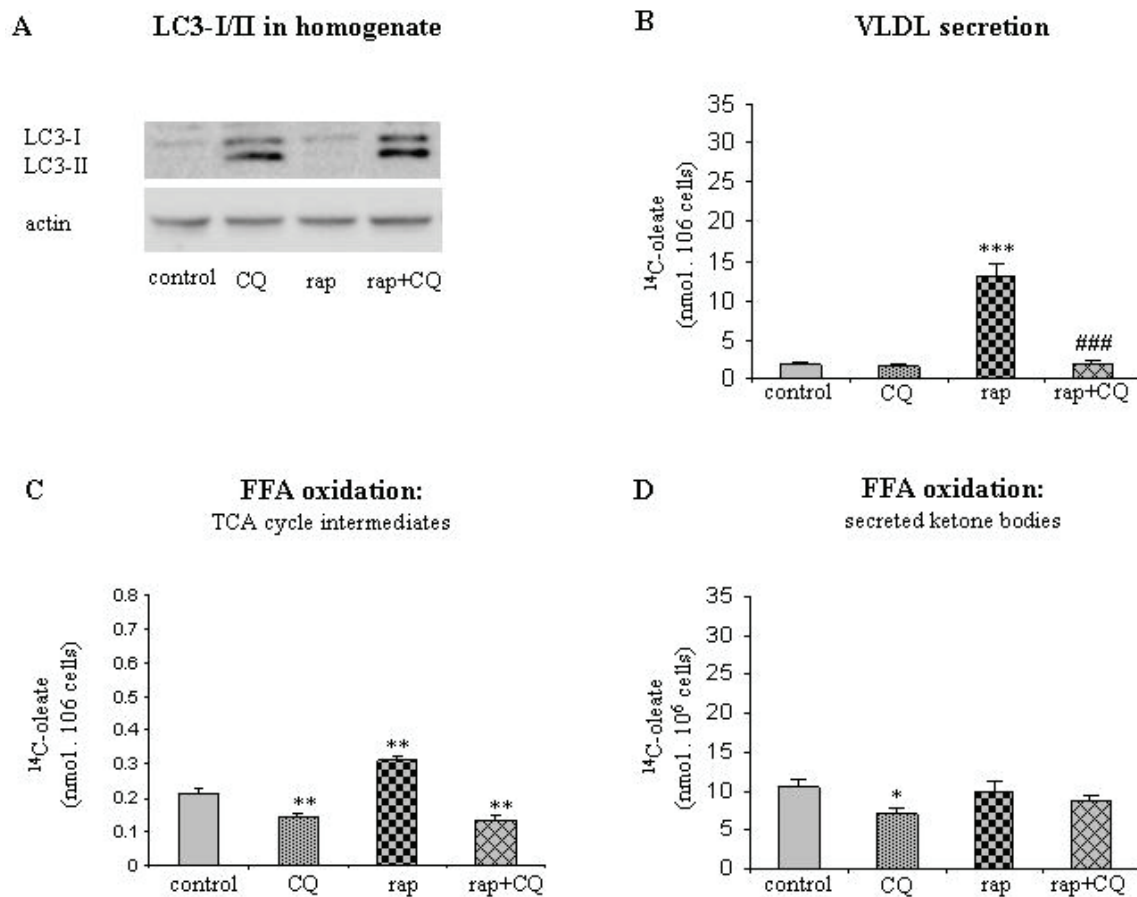


Fig. 2. Effect of autophagy stimulation in combination with lysosomal inactivation on the utilization of lipids-derived FFA in fed hepatocytes. **A:** LC3-I/II content in homogenate; **B:** ^{14}C -oleate incorporation into secreted TAG; **C:** ^{14}C -oleate incorporation into TCA cycle intermediates; **D:** ^{14}C -oleate incorporation into secreted ketone bodies. The hepatocytes were pre-labeled with ^{14}C -oleate overnight and then incubated medium supplemented with 10 % FBS for 6 hours without radioactive label. During this period the hepatocytes were left in the medium alone (control) or in the presence of autophagy flux stimulator (rapamycin 100 nM) or in the presence of lysomotrophic agents (chloroquine 100 μM) that prevents the degradation of material internalized in lysosomes. ^{14}C -oleate incorporation into secreted TAG was determined as the radioactivity recovered in the chlorophorm extract of the incubation medium. TCA intermediates formation was assessed according to the ^{14}C -oleate incorporation into intracellular water-soluble fraction. Ketone bodies production was determined as the radioactivity recovered in the aqueous fraction of the incubation medium. Data are given as mean $\pm$ S.E.M from three independent experiments. ** $p < 0.01$ *** $p < 0.001$ vs. control; ### $p < 0.001$ CQ+ rap vs. rapamycin only treated cells.

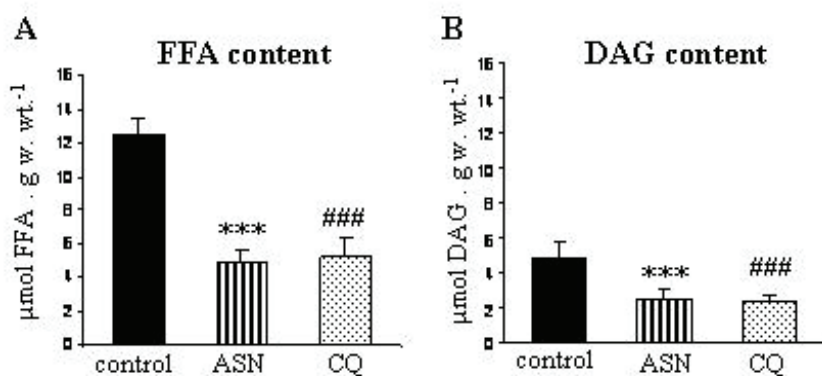


Fig. 3. Effect of autophagy inhibition and lysosomal inactivation on FFA (**A**) and DAG (**B**) production in liver slices *in vitro*. Liver slices from fasted rats administered either SD diet were incubated under non-oxidative conditions (0.01 M KCN) in the KRF medium alone (control) or in the presence of autophagy flux inhibitor asparagine (ASN, 20 mM) or lysosomal activity inhibitor chloroquine (CQ, 100 μM) for 120 min. At the end of the incubation, the FFA and DAG content in the tissue was determined as described in Material and Methods. Data represent mean $\pm$ S.E.M., $n=6$. *** $p<0.001$ asparagine-treated vs. control samples; ### $p<0.001$ CQ-treated vs. control samples.

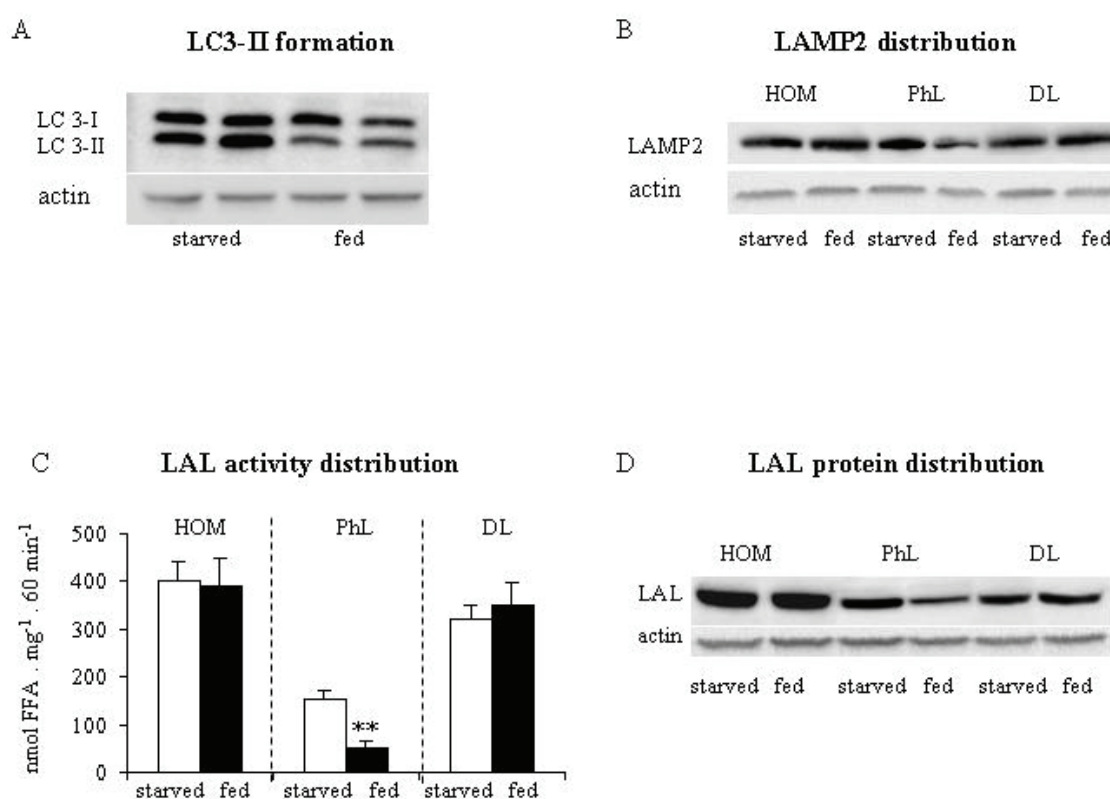


Fig. 4. The effect of starvation on autophagolysosome formation and lipolytic activity *in vivo*. **A:** LC3-I and LC3-II protein expression in homogenate. **B:** LAMP2 expression in subcellular fractions. The PhL and DL fractions were prepared by differential centrifugation (see Material and Methods). **C:** LAL activity in homogenate and subcellular fractions. The activity of LAL was measured as the release of FFA from ^3H -triolein at pH=4.5. **D:** LAL protein expression in homogenate and subcellular fractions. Values represent means $\pm$ S.E.M. of 7 animals. Open bars = starved animals; black bars = fed animals, ** $p<0.01$ fed vs. starved.

Effect of autophagy and lysosomal inhibition on lipid degradation products formation in liver slices *in vitro*

In order to verify the data obtained on primary hepatocytes we determined the effect of autophagy inhibition and lysosomal inactivation on the production of FFA and DAG in liver slices from fasted animals incubated in the presence of 0.01 M KCN. Under such conditions the oxidative utilization of FFA is completely

blocked but lysosomal lipolysis is unaffected and is linear for at least 4 hours (Mosinger *et al.* 1965). Consequently, the released FFA and DAG remain in the tissue. There was a linear correlation between DAG and FFA content ($R^2=0.87$). As shown in Fig. 3 the formation of lipid degradation products, FFA and DAG, was significantly diminished by both autophagy flux inhibitor asparagine and by lysosomotropic agent chloroquine.

Effect of starvation on autophagolysosome formation and lipolytic activity in vivo

The effect of starvation on autophagy, lysosomal translocation into autophagolysosomes and LAL activity and protein intracellular distribution was determined in the liver of rats administered SD who had either free access to food ("fed") or were deprived of food for 24 hours ("starved"). The autophagosome formation was estimated according to the LC3-II/LC3-I ratio. As shown in Fig. 4A, the LC3-II/LC3-I ratio was elevated in the SD group after starvation and low in the fed condition. In accordance with the hypothesis that autophagy pathway involves the conversion of lysosomes into autophagolysosomes and consequently the degradation of their cargo (lipid droplets), we found that starvation led to the increased abundance of lysosomal marker, LAMP2 protein, in autophagolysosomal fraction (Fig. 4B) but it had no effect on total LAMP2 expression. If our presumption that autophagy mediates the formation of activated autophagolysosomes containing LAL is correct, than the corresponding changes in its intracellular distribution should occur in response to starvation. LAL activity in particular subfractions (Fig. 4C) was determined using exogenous substrate (^3H -triolein). Starvation did not affect the total LAL activity in the whole-liver homogenate and in the fraction of dense lysosomes (DL) that represents the reserve pool of the enzyme which is not in contact with the substrate (lipid droplets) and thus probably inactive under physiological condition. In contrast, starvation significantly increased LAL activity associated with PhL fraction being 100 % higher in starved animals compared with the fed ones. These results corresponded well with the effect of starvation on the distribution of LAL protein among subcellular fractions (Fig. 4D). We found no effect of prandial status on LAL protein expression in homogenate and in DL fraction but a significant elevation of LAL protein amount in response to starvation was found in PhL fraction.

Discussion

In this study we present evidence that the autophagy-lysosomal pathway contributes to the degradation of intracellular lipids in the liver. Our conclusions are based on following findings: 1) in primary hepatocytes, inhibition of autophagy as well as inhibition of lysosomal activity, decreased formation of lipid degradation-derived metabolites, i.e. TCA cycle

intermediates and ketone bodies, and VLDL secretion; 2) in the same model, stimulation of autophagy by rapamycin resulted in the increased VLDL production and this effect was completely abolished by the inactivation of lysosomes; 3) in liver slices incubated *in vitro*, both inhibition of autophagy and lysosomal inactivation prevented the FFA and DAG formation from intracellular lipids; 4) the autophagolysosome formation (determined as LC3-II/I ratio and LAMP2 translocation into PhL fraction) and the translocation of lysosomal lipase into autophagolysosomal fraction share the same prandial-dependent pattern of regulation in the liver of rats fed SD, i.e. stimulation in response to starvation and down-regulation in fed state.

The first hints suggesting the importance of autophagy in lipid metabolism came from Debeer *et al.* (1979) who demonstrated on isolated hepatocytes that the inhibition of lysosomal activity decreased not only protein degradation but that it inhibited also ketone body production. He suggested that autophagocytosis may be the likely mechanism of how the intracellular triglycerides may be taken up by the lysosomes. Thirty years later, Singh *et al.* (2009) using an electron microscopy approach, showed that lipid droplets or their parts are engulfed in autophagosome-like structures during starvation and are degraded in lysosomes *via* specific autophagy-related pathway, macrolipophagy. In contrast to their findings, Shibata *et al.* (2009) proposed an alternative role for autophagic machinery in lipid metabolism in hepatocytes. They suggested that LC3 is involved not only in lipid degradation but also in the process of lipid droplet formation in non-adipose tissues. Surprisingly both authors, using the same model, reported different effects of autophagy deficiency on TAG storage. Singh observed an increased TAG accumulation and lipid droplets content while Shibata reported decreased amount of lipid upon fasting in the same model of autophagy-deficient hepatocytes. This apparent contradiction clearly indicates some differences in experimental design – in fact, the experiments of Shibata were carried out on young, one month old mice while the data reported by Singh were obtained on 5-month old animals. Further studies are necessary in order to clarify this issue.

Our data supports the hypothesis of autophagy-mediated lipid degradation in the liver. In our experimental setting, the freshly isolated hepatocytes were pre-incubated with ^{14}C -oleate overnight in order to label intracellular lipids on acyl moiety and the cells were then cultivated in the absence of any exogenous fatty

acids for 6 hrs. Under these conditions, the secreted VLDL and products of fatty acid oxidation had to be derived only from intracellular lipids and quantification of these products may serve as an indicator of lipolysis. As stated in Materials and Methods the lipid fraction either from cells or medium was extracted according to Folch (1957) and the individual lipid classes of intracellular lipids were not separated by thin layer chromatography. Nevertheless we suppose that most of the radioactivity detected in TCA, water-soluble fraction or VLDL are derived from TAG. Our assumption is based on the data published by Woodside *et al.* (1989) who analyzed the deposition of ^{14}C -oleate into lipid fractions in isolated hepatocytes after 90 min incubation and demonstrated that most (88 %) of radioactivity was found in TAG. Francone *et al.* (1992) reported that incorporation of ^{14}C -oleate after *in vivo* application into TAG, PL and cholesteryl esters (CE) is different in microsomes (consisting mostly of endoplasmic reticulum) and floating fat (consisting mostly of cytosolic lipid droplets). While in microsomes nearly half of the ^{14}C -oleate was incorporated into PL, in floating fat 97.6 % of radioactivity was detected in TAG, 2.26 % in phospholipids (PL) fraction and 0.21 % in CE. Autolipophagy is supposed to be involved solely in the degradation of cytosolic lipid droplets in which the PL and CE labeling with ^{14}C -oleate is rather insignificant.

When the hepatocytes were cultivated in amino acid-poor medium (i.e. under the autophagy-promoting conditions), we found that inhibition of autophagy flux by asparagine (Berg *et al.* 1998) decreased the formation of VLDL, TCA cycle intermediates and ketone bodies. A comparable effect was observed also when the autophagy was not influenced but the lysosomal activity was inhibited by chloroquine. Interestingly, Wiggins and Gibbons (1992) found no effect of chloroquine on TAG mobilization for VLDL secretion. To our opinion this discrepancy could be explained by differential experimental setting. In the study of Wiggins and Gibbons the hepatocytes were cultivated in the presence of amino acid mixture during the experimental phase (the composition and the concentrations were not described in detail). Amino acids are widely used inhibitors of autophagy and if the autophagy is down-regulated, the inhibition of lysosomal activity could have no additional effect. In accordance with this we also saw virtually no effect of chloroquine on VLDL secretion in fed hepatocytes as it will be discussed further. Furthermore, the dose of chloroquine used by Wiggins and Gibbons

was quite low (10 μM). In comparable studies focused on the manipulations of lysosomal degradation of either proteins or lipids the chloroquine concentrations administered *in vitro* vary from 0.1 mM (Francone *et al.* 1992) to 1 mM (Furuya *et al.* 2001).

In another experimental setting we tested the possibility of increasing the lipid degradation by stimulation of autophagy. The hepatocytes were incubated in the presence of exogenous proteins and amino acids in order to attenuate autophagy and in some of them autophagy was stimulated by rapamycin that attenuates amino acid-dependent inhibition of autophagy via mTOR/TORC1 signaling. The stimulation of autophagy significantly increased the formation VLDL and this effect was completely prevented by the inhibition of lysosomal activity. The autophagy stimulation by rapamycin did not elevate the ketone body production in this setting, i.e. under “fed” conditions. Ketogenesis has three highly regulated control points: 1. FFA supply (from the diet, from adipose tissue or from intrahepatic TAG), 2. mitochondrial fatty acids entry and 3. mitochondrial HM-CoA synthase. The second and third processes are down-regulated in a fed state and a simple increase of FFA availability cannot overcome this regulation. Consequently, the FFA surpluses are directed only into VLDL secretion (Fukao *et al.* 2004). Furthermore, the formation of lipid degradation-dependent products was significantly lower in hepatocytes cultivated in “fed” conditions (with serum) than in those incubated in “starved” conditions (without serum) which correlated with the autophagy intensity. Nevertheless, the inhibition of autophagy-lysosomal pathway did not abolish the lipid degradation completely which suggests that this pathway is an important but not the only mechanism involved in liver TAG metabolism.

The evidence obtained on primary hepatocytes were further confirmed by the analysis of the effect of autophagy/lysosomal inhibition on DAG and FFA production in liver slices incubated *in vitro* in the presence of KCN. Under these conditions, the lipolysis occurs but the further utilisation of lipid degradation products is completely blocked (Vavrinkova and Mosinger 1971). Similarly to primary hepatocytes, asparagine as well as chloroquine significantly decreased FFA and DAG formation. The FFA : DAG ratio was 2.46 which suggests that approx. one half of DAG undergoes further degradation. The DAG molecule may be further degraded by some other lipase (i.e. by TGH) or remodelled by transacylation (Sugiura *et al.* 1988). Similar

results were reported by Lankester on primary hepatocytes culture (Lankester *et al.* 1998). This data indicate that at least part of the TAG in the liver may be degraded in lysosomes and that the normal autophagy flux is necessary for this process.

In vivo, the regulation of autophagy is determined by its main function which consists in maintaining energy balance during the period of nutrient shortage. It has been shown that autophagy is increased during acute (24 hrs) starvation. The increased autophagy determines the increased delivery of intracellular material into autophagolysosomes and consequently, its decreased degradation and release of degradation products. In our study we proved this regulatory pattern in animals *in vivo*. In this group, fasting was associated with higher autophagy together with the increased translocation of LAL activity, LAL protein and LAMP2 lysosomal marker into autophagolysosomal fraction in fasted compared to fed animals. Nevertheless, the exact data directly showing the contribution of the autophagy-derived FFA to VLDL production or FFA oxidation *in vivo* are still lacking. In this context it is important to mention the finding of Du *et al.* (2001) who showed that LAL KO mice exhibit normal triglyceridemia. This rather surprising observation could be explained as the result of double defect in two metabolic pathways, both of them

associated with LAL – the VLDL production and LDL removal. This assumption is supported by the fact that LDL fraction is highly significantly enriched in agarose electrophoretogram of LAL KO mice serum. Unfortunately, VLDL production was not determined in this paper but we suppose that if the VLDL secretion was normal concomitantly with impaired LDL removal it would have resulted into significant hypertriglyceridemia that was not observed in this model.

In conclusion, we have demonstrated that the manipulation of either autophagy or lysosomal activity independently and comparably affects intracellular lipid degradation and lipid-degradation products formation in primary hepatocytes and liver slices incubated *in vitro*. Furthermore, we demonstrated that autophagy and lysosomal lipolysis are regulated similarly *in vivo*. Based on these findings we propose that at least part of the TAG in the liver is degraded in lysosomes and that the normal autophagy flux is necessary for this process.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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PŘÍLOHA II

Papáčková Z, Cahová M. *Important role of autophagy in regulation of metabolic processes in health, disease and aging*. Physiol Res. 2014;63(4):409-20. doi: 10.33549/physiolres.932684.

REVIEW

Important Role of Autophagy in Regulation of Metabolic Processes in Health, Disease and Aging

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Summary

Autophagy is the basic catabolic mechanism that involves degradation of dysfunctional cellular components through the action of lysosome as well as supplying energy and compounds for the synthesis of essential biomacromolecules. This process enables cells to survive stress from the external environment like nutrient deprivation. Autophagy is important in the breakdown of proteins, carbohydrates and lipids as well. Furthermore, recent studies have shown that autophagy is critical in wide range of normal human physiological processes, and defective autophagy is associated with diverse diseases, including lysosomal storage disease, myopathies, neurodegeneration and various metabolic disorders. This review summarizes the most up-to-date findings on what role autophagy plays in metabolism.

Key words

Autophagy • Lysosome • Metabolism • Autophagosome • Protein • Lipid • Carbohydrate

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Introduction

Autophagy, or the process of degradation of intracellular components in lysosomes, has been traditionally linked to cellular energy balance and nutritional status (Mizushima *et al.* 2004). Lysosomes are

cellular organelles responsible for an important portion of the degradative activity that is necessary for maintaining cellular homeostasis and defense (Dell'Angelica *et al.* 2000). Plasma membrane proteins and their ligands are targeted into lysosomes by the endosomal pathway when destined for degradation. Extracellular pathogens reach lysosomes for destruction as part of cellular defense *via* fusion of phagosomes with lysosomes. Intracellular components that need to be degraded, either because they are dysfunctional or to meet cellular energetic or metabolic demands, reach lysosomes by several pathways collectively termed “autophagy” (Eskelinen and Saftig 2009). The discovery of the autophagic molecular machinery has been rapidly followed by numerous studies supporting the occurrence of autophagic alteration in different common human disorders such as cancer, neurodegenerative and muscular diseases, metabolic syndrome, and infectious disorders, among others. Most of these connections of autophagy with cellular physiology and disease have emphasized the important function of autophagy in quality control and clearance of altered and damaged intracellular proteins and organelles, its contribution to cellular remodeling through degradation of structural components, or its role in cellular defense as part of both innate and acquired immunity (Mizushima *et al.* 2008).

Autophagic pathways

The process of autophagy is conserved from yeast to mammals. During the past decade researchers

have uncovered the existence of yeast autophagy genes and the molecular mechanisms of autophagy have been studied extensively in *Saccharomyces cerevisiae*. These discoveries were followed by the identification of mammalian orthologs with similar roles and provide a series of reagents for characterizing the molecular machinery of the autophagy system (Klionsky 2007). The process of autophagy is similar in yeast and mammalian cells, but there are some distinct differences.

Autophagy, the definition of which is “self-eating”, can be induced by starvation or other forms of nutrient deprivation to supply a variety of substrates for cellular energy generation (Finn and Dice 2006). Three forms of autophagy have been defined: microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy. In microautophagy, organelles or protein are taken up within an invagination of the lysosomal membrane for breakdown (Yorimitsu and Klionsky 2005). CMA is a specific process that removes individual proteins that contain a specific peptide motif recognized by the chaperone protein Hsp70 (70 kDa heat shock cognate protein). The chaperone-protein complex translocates to the lysosome where it binds to lysosome-associated membrane protein 2A (LAMP-2A) for protein internalization and degradation (Orenstein and Cuervo 2010).

Macroautophagy is a non-specific process occurring when a portion of cytosol is engulfed by a double-membrane structure, termed an autophagosome, that fuses with a lysosome whose enzymes degrade the cellular constituents sequestered in the autophagosome (Mehrpour *et al.* 2010). The regulation of this process is

complex and controlled by the coordinated actions of autophagy-regulated genes (Atgs), over 30 of which have been identified both in yeast and humans (Mizushima and Levine 2010). Studies in yeast indicate that an initial structure called an isolation membrane, or phagophore, becomes a nascent autophagosome whose ends elongate until they form the completely enclosed autophagosome. The source of the double membrane is controversial, but it might be derived from the endoplasmic reticulum (ER), mitochondria or plasma membrane (Hamasaki and Yoshimori 2010). The exact mechanism for the formation and elongation of the autophagosomal double membrane is unclear. However, a number of multi-protein complexes are known to be involved in these processes, as discussed below.

There are three major pathways that regulate the process of macroautophagy (Fig. 1). The first one is dependent on the target of rapamycin complex 1 (TORC1) pathway. In direct response to nutrients supply, or nutrient-induced insulin secretion, class I phosphatidylinositol 3-kinase (PI3K) activates protein kinase B (Akt) and TORC1. This signaling pathway blocks macroautophagy through the ability of TORC1 (mammalian homologue of mammalian target of rapamycin mTOR) to inhibit Atg1 (mammalian homologue ULK1/2) from recruiting its partner Atg13 (mammalian homologue mAtg13) and Atg17 (mammalian homologue FIP200) (Neufeld 2010). The Atg1-Atg13-Atg17 (ULK1/2-mAtg13-FIP200) complex recruits and organizes other proteins for the developing autophagosome.

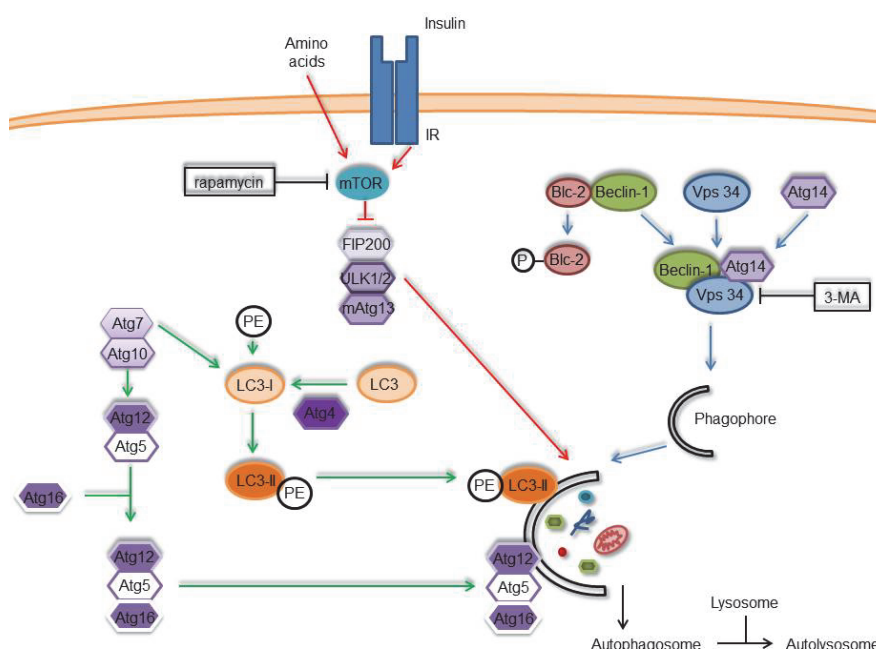


Fig. 1. The three major pathways that regulate process of macroautophagy. The first is an inhibitory pathway in which nutrient or insulin stimulation of the mTOR signaling pathway blocks autophagosome formation (**red line**). Two other pathways are stimulatory. The first, phosphorylation of Bcl-2 dissociates it from beclin-1, which allows beclin-1 to form complex with Vps34 and Atg14, which is required for induction of autophagy (**blue line**). The other pathway involves a series of conjugation steps that generate LC3-II and the Atg5-Atg12-Atg16 protein complex, which are both necessary for autophagosome formation (**green line**). IR: insulin receptor, PE: phosphatidylethanolamine, 3-MA: 3-methyladenine.

The second pathway that regulates autophagy is mediated by Atg6 (mammalian homologue beclin1), which forms a complex with the class III PI3K Vps34. Activation of the Atg1-Atg13-Atg17 (ULK1/2-mAtg13-FIP200) complex leads to organization of the Atg6 (beclin1)-Vps34 complex on the lipid membrane. Vps34 produces phosphatidylinositol 3-phosphate, which is involved in recruiting other proteins to the autophagy complex (Cheong *et al.* 2005). It is important to distinguish this PI3K from the insulin-activated, class I PI3K, which activates mTOR. Vps34 is the target of the widely used pharmacologic inhibitor of autophagy 3-methyladenine (Seglen and Gordon 1982). Beclin 1 is an important interface between the autophagic and cell death pathway because the anti-apoptotic protein Bcl-2 and Bcl-X_L bind beclin 1 to inhibit autophagy (Levine *et al.* 2008).

The third pathway that mediates autophagosome formation and elongation involves 2 ubiquitin-like conjugation processes that generate membrane-bound protein complexes. In the first, Atg7 and Atg10 mediate the conjugation of Atg12 to Atg5, which subsequently interact with Atg16. The Atg12-Atg5 complex associates with the membrane and then dissociates when the autophagosome is fully formed. Another critical conjugation reaction involves Atg8 (mammalian analog microtubule-associated protein 1 light chain 3, LC3). In nutrient-rich condition Atg8 labels the PAS to the vacuole. Moreover, upon starvation, the Atg8 translocate the PAS to autophagosomes (Cheong and Klionsky 2008). LC3 is constitutively cleaved by Atg4 to produce LC3-I. When autophagic signal is induced, Atg7 and Atg3 mediate the conjugation of LC3-I to the membrane lipid phosphatidylethanolamine, to form LC3-II (Ichimura *et al.* 2000). LC3-II associates with the autophagosomal membrane, where the lipidated protein can mediate membrane elongation and closure. LC3-II is degraded late in the autophagic pathway after autophagosome fusion with the lysosome (Tanida *et al.* 2005).

Taken together, the maturation and degradation of autophagosome and autolysosome is a complicated process regulated by many autophagy-associated proteins. Physiologically, autophagy occurs at a low basal level, which is enhanced during starvation as well as in the response to the accumulation of non-required cellular components in order to accomplish its homeostatic mission. Pathologically, however, dysfunctions of autophagy are the cause of several diseases.

Autophagy in carbohydrate homeostasis

The regulatory function of autophagy has a role in glucose metabolism, particularly in glycogenolysis. Glycogen degradation by phosphorylase and debranching enzymes occurs primarily in cytosol, but lysosomal acid glycosidases also contribute to glycogen breakdown. There is an important role for autophagy in regulating cellular glycogen stores, and indeed, glucagon, one of the most important hormones controlling glycogen metabolism, was the first hormone known to activate autophagy (Schworer *et al.* 1979). Autophagy is acutely required during childbirth in which the neonatal liver induces autophagy to mobilize glycogen stores to increase availability of glucose (Kalamidas and Kotoulas 2000). At birth the cardiac muscle of mice has a very high glycogen content, but glycogen levels fall coinciding with the appearance of glycogen-containing autophagic vacuoles. Similarly, glucagon and adrenalin enhance the breakdown of cardiac muscle glycogen by increasing the number, size, and total volume of glycogen-containing autophagic vacuoles, and the activity of acid glucosidase (lysosomal glycogen-hydrolyzing enzyme) (Kotoulas *et al.* 2004). Lysosomal mannose 6- and glucose 6-phosphatase modulate the phosphorylation of glucose favoring its exit from the lysosome.

Autophagy can indirectly impact glucose metabolism by modulating pancreatic β -cell mass and function. Mice with β -cell-specific inhibition of macroautophagy reveal progressive β -cell degradation and decreased insulin secretion. These models demonstrated that the absence of autophagy within β -cells resulted in the impaired insulin secretion, as well as overall impaired glucose homeostasis in the animal (Ebato *et al.* 2008). β -cells are characterized by active mitochondrial respiration. Several independent lines of evidence indicate that autophagy, particularly mitophagy (autophagy of mitochondria), is of essential importance for β -cell homeostasis as it removes damaged mitochondria. Accordingly, any disruption of this quality control mechanism is expected to result in accumulation of damaged mitochondria and increases in the accompanying negative consequences, such as, elevated ROS production, severe oxidative stress and β -cell apoptosis. Another essential role of autophagy lies in the regulation of intracellular insulin stores and, more generally, in whole protein turnover (Marsh *et al.* 2007). Rab3A knockout mouse exhibits a dysfunction in insulin secretion due to a defect in β -cell granule transport

concomitantly with a normal rate of proinsulin synthesis and processing. Despite this disconnection between insulin production and secretion, insulin content in isolated islets from Rab3A knockout mice is normal due to a marked up-regulation of autophagy that keeps the granule number constant.

Studies in *db/db* mice with diet-induced obesity and insulin resistance have revealed increased autophagosome formation and β -cell expansion. It is possible that autophagy protects against chronic lipid stress in the pancreas in these settings, particularly since β -cell autophagy deficient rodents failed to display similar increases in β -cells mass (Jung *et al.* 2008). Closer examination of the β -cells revealed these cells had accumulated aggregates of polyubiquitinated proteins and structurally abnormal mitochondria. These results are consistent with the housekeeping and known recycling function of autophagy. As such, these models suggest that a functional autophagic system is necessary to maintain β -cell health and that disruption of autophagy can lead to profound metabolic impairment.

Autophagy in protein catabolism

Although lysosomes contain a broad array of hydrolases (lipases, proteases, glycosidases and nucleotidases) that allows them to degrade many different molecules, most of the functional studies on autophagy have focused on protein breakdown. In fact, for a long time, changes in the rate of degradation of long-lived proteins were used to monitor autophagy. In the liver, autophagy was estimated to degrade from 1.5 to 5 % of total proteome per hour under fed or starved conditions (Deter *et al.* 1967). Autophagy was thus responsible for up to 70 % of intracellular protein breakdown in this organ during prolonged (24 h) fasting, which was later confirmed in mice with hepatic knockouts of essential autophagy genes (Komatsu *et al.* 2005). There are two purposes for protein breakdown: to utilize amino acids for cellular fueling and to replenish the intracellular pool of amino acids required to maintain protein synthesis. Amino acids can be utilized in fasting to provide substrates for gluconeogenesis and ketogenesis (in liver) and to replenish the intracellular pool of amino acids. The contribution of different proteolytic systems to these processes seems to be timed with the duration of starvation. Studies in cultured cells have shown that the proteasome system contributes most of the amino acids to this pool during first hours of starvation (Vabulas and

Hartl 2005), whereas macroautophagy starts soon after and reaches a peak at about 6-8 h later (Deter *et al.* 1967). In fact, although autophagosomes are still visible up to 24 h of starvation, the maximal rate of autophagosome formation is reached approximately at the sixth hour of starvation and decline progressively after that. The exact mechanism by which amino acids signal through mTOR to downregulate macroautophagy is still unclear, but the contribution of Vps34, Ras-related small GTPases (that relocate mTOR to the lysosomal compartment) and a bidirectional transporter that exchanges L-glutamine for essential amino acids have all been involved in this signaling process (Nicklin *et al.* 2009).

Autophagy in lipid metabolism

Lipid droplets (LDs) are intracellular deposits of lipid esters surrounded by a monolayer of phospholipids and separated from hydrophilic cytosolic environment by a coat of structural proteins, known as perilipins (Fujimoto and Parton 2011). As with many other organelles, LDs have been shown to adapt to changes in the cellular environment and to interact with other intracellular compartments in a regulated manner, with different outcomes. First, the interaction between LDs and other organelles may be a source of membrane lipids. Second, LD interaction with mitochondria or peroxisomes may enhance the provision of lipid for β -oxidation. Finally, lipid droplets originating from the ER and maintaining a close connection with this organelle may facilitate exchange of lipids and proteins between both compartments to meet metabolic requirements of the cell (Ohsaki *et al.* 2009). Mobilization of the lipids inside the LD occurs through lipolysis that is activated in response to the increased energy demand but also in response to a large affluence of lipids to prevent stores from becoming compromisingly enlarged for cells (Lass *et al.* 2011). The rate of lipolysis is modulated by the interaction of lipases present at the surface of the LD with the structural proteins that surround LD and with inhibitory proteins in the cytosol.

Although mobilization of LD by lipolysis has been attributed to the LD-associated cytosolic lipases like adipose triacylglycerol lipase (ATGL) or hormone sensitive lipase (HSL), recent studies have revealed a role for autophagy in LD breakdown. Lipolysis and autophagy share striking similarities. Both are essential catabolic pathways activated in response to nutrient deprivation.

They are under identical hormonal control, being inhibited by insulin or activated by glucagon (Finn and Dice 2006). Intracellular lipids were not previously considered autophagic substrates, but the similarities between lipolysis and autophagy, together with the existence of lysosomal lipases, suggested a possible link between these two pathways. An interrelationship between the two processes has been demonstrated by the finding that autophagy mobilizes lipids from lipid

droplets for metabolism, through a process termed, “lipophagy” (Singh *et al.* 2009) (Fig. 2). We have previously shown that manipulation of either autophagy or lysosomal activity independently and comparably affects intracellular lipid degradation and lipid-degradation product formation in primary hepatocytes and liver slices incubated *in vitro*. At least part of TAG in the liver is degraded in lysosomes and normal autophagic flux is necessary for this process (Skop *et al.* 2012).

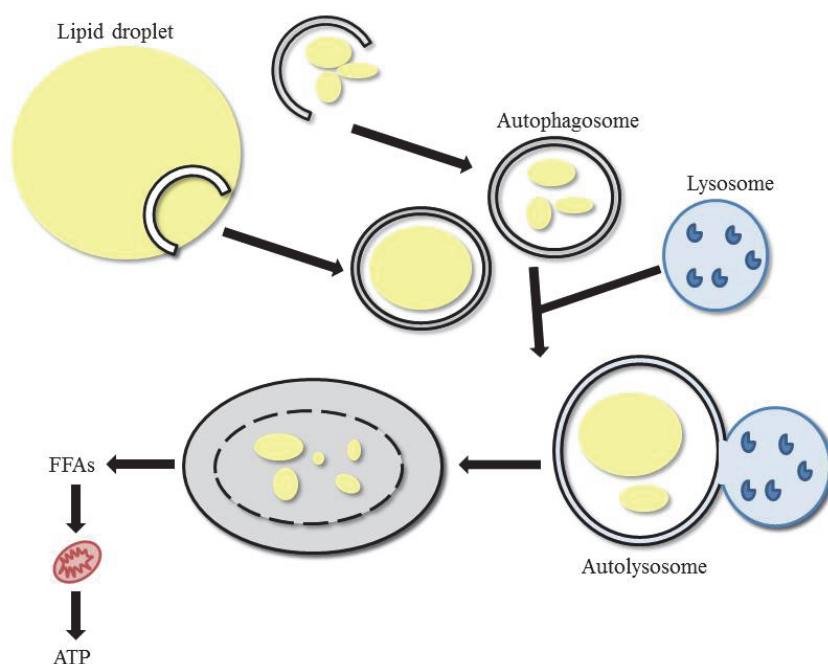


Fig. 2. Process of LD breakdown by lipophagy. Portion of large LD or small LD are enclosed with an autophagosomal double-membrane. Autophagosomes fuse with lysosomes to form autolysosomes and lysosomal enzymes are mixed with the autophagosomal cargo. It leads to degradation of lipids and releases FFAs into the cytoplasm. FFAs are important for stable mitochondrial β oxidation for generation of ATP to maintain cellular energy homeostasis.

The presence of lipases in the lysosomal lumen, along with a large variety of hydrolases such as proteases, glycoses and nucleases, has been acknowledged since the discovery of this organelle. Lysosomal lipase was thought to serve mainly for the degradation of lipids contributed by the diet through endocytosis or those present in the membranes of the organelles digested during the autophagic process. The elevation of LD to the category of cytosolic organelles was in part a motivation to address their turnover by autophagy. The first hint that LDs could become substrates of the autophagic process originated from studies in cultured hepatocytes knocked-down for Atg5, one of the genes essential for the formation of autophagosomes. In addition, hepatocytes respond to an acute oleate challenge by increasing lipolysis, which would prevent massive enlargement of the LD compartment. An oleate challenge resulted in a marked increase in the number and size of LD in cells with compromised macroautophagy. The same was true *in vivo* when the knockout of another essential autophagy

gene (Atg7) in the liver led to an accelerated development of liver steatosis (fatty liver) when compared to control animals (Singh *et al.* 2009).

Lipophagy is not limited to hepatocytes but occurs in almost every cell type investigated to date. In addition to the initial observation obtained in cultured hepatocytes and embryonic fibroblasts, it has been confirmed that disruption of macroautophagy leads to intracellular accumulation of lipids in endothelial cells, lymphoblasts, dendritic cells, glial cells, and even in neurons (Koga *et al.* 2010), suggesting a generalized function of macroautophagy in cellular lipid mobilization. The accumulation of LD upon blocking macroautophagy even in the absence of any nutritional challenge, supports the hypothesis that lipophagy is a constitutive process in many cells (Singh *et al.* 2009).

The determinants for autophagic initiation on the surface of LD remain unknown. Polyubiquitination has been detected in polarized areas of LD in part resulting from the accumulation of clusters of polyubiquitinated

apolipoprotein B (ApoB) on their surface (Ohsaki *et al.* 2006). It seems that ApoB can undergo both proteasomal and lysosomal degradation as proteasome inhibition caused an increase of autophagic vacuoles abundance and ApoB content in lysosomes. Whether or not the lysosomal degradation of ApoB occurs as a result of the activation of lipophagy and how the accumulation of this protein contributes to the initiation of the process require future investigation. Of particular interest is the fact that LDs have been shown to dynamically interact with two of the organelles – the ER and mitochondria – that have been proposed as sites of formation of the limiting membrane of the autophagosomes. Interaction with the ER may be related to LD biogenesis, as this is the compartment from where these organelles originate, but may also favor the distribution of lipids from the LD towards other organelles through the endosecretory pathway. In the case of mitochondria, the proximity of LDs to the outer-membrane of this organelle could facilitate delivery of the FFA for mitochondrial β -oxidation. However, considering the described association of the autophagic initiation complex to precise area in the membrane of ER and the mitochondria, and the formation of cup-like precursors of the limiting membrane of the autophagosome from these regions, it is tempting to at least propose that the previously described interactions of LD with these organelles could contribute to the initiation of their autophagic degradation.

Mobilization of LD by autophagy was first observed both in cultured hepatocytes in response to fatty acid exposure and in the liver of mice maintained on a diet enriched in fat for prolonged period of time (Singh *et al.* 2009). The liver responds to the massive influx of lipids from the blood by up-regulating LD biogenesis as a defense mechanism against the toxicity of FFAs, which upon esterification get converted into TAG that are stored in LDs (Lass *et al.* 2011). However, in order to prevent uncontrolled expansion of LD, activation of lipolysis also occurs under these conditions and contributes to maintaining of LD size. Failure to regulate lipid accumulation in hepatocytes may be the basis of pathologic conditions such as liver steatosis and steatohepatitis (Christian *et al.* 2013). Autophagy has now been added to the mechanism that controls the growth of the hepatic LD under these conditions (Greenberg *et al.* 2011).

After the first observations demonstrating the existence of lipophagy and the upregulation of this

process in response to lipid challenge, numerous studies have confirmed the stimulatory effect of dietary lipids on the autophagic process. In contrast, an equal number of studies have reported inhibition of autophagy in response to high concentrations of particular lipids. In animals exposed to a high-fat diet for prolonged periods of time, it is possible to detect an increase in autophagic activity during the first weeks of treatment that is followed by a gradual decrease in autophagy. This decrease in autophagy further contributes to the expansion of the LD compartment, eventually leading to hepatotoxicity and steatosis (Singh *et al.* 2009). Other results showed that in the liver, the autophagic response to the increased fat supply in the diet is biphasic. At the beginning of high-fat diet feeding autophagy flux is stimulated but over time autophagy flux is nearly completely impaired (Papackova *et al.* 2012).

Recently, a significant role for autophagy in lipid metabolism has been revealed in human enterocytes (Khalidoun *et al.* 2014). Enterocytes have to deal with massive alimentary lipids upon food consummation. They orchestrate complex lipid trafficking events that lead to secretion of TAG-rich lipoproteins and the transient storage of lipids in LDs. The authors showed that delivering alimentary lipid micelles to polarized human enterocytes induced an immediate autophagic response. This was accompanied by rapid capture of newly synthesized LDs by nascent autophagosomal structures at the ER membrane and hence targeting them to the lysosomes. They proposed an interesting hypothesis according to which the autophagosomes, despite their primary lysosomal-delivery function, could also be used as a “hiding” compartment in the cell in order to avoid excessive TAG accumulation from the membranes where lipid biosynthesis occurs. Such a local program could act as a global protection and adaptation response to the arrival of neutral lipids, as is the situation for enterocytes during the postprandial phase (Khalidoun *et al.* 2014).

Autophagy in other disease

A basal and constitutive level of autophagy is indispensable for intracellular homeostasis and quality control for healthy individuals. The molecular dissection of autophagy and the growing number of physiological function attributed to this process are leading to a better understanding of the role of autophagy in disease. Mounting evidence has demonstrated that many of its

physiological function is strongly related to human diseases such as cancer, myopathies, bacterial and viral infections and neurodegenerative, liver and heart diseases (Fig. 3).

Direct evidence for impaired autophagy in a myopathy was first obtained when the gene encoding a lysosomal membrane protein (*lamp2*) was knocked out in mice (Saftig *et al.* 2001). The predominant phenotype of these mice is a massive accumulation of autophagic vacuoles in cells of the liver, muscle and heart. Despite the increased number of autophagic vesicles, the rate of lysosomal degradation of proteins is reduced because the clearance of autophagosomes through lysosomal fusion is impaired. Autophagy of glycogen is not limited to the

liver. In fact, altered autophagic degradation of glycogen stores may underlie the basis of some muscle disorders that are now classified as autophagic vacuolar myopathies such as Danon disease, X-linked vacuolar myopathy with excessive autophagy and Pompe disease (Fukuda *et al.* 2007). Glycogen granules accumulate in muscles of Danon disease patients resulting in cardiomyopathy, proximal muscle weakness and mental retardation (Nishino *et al.* 2000). The histological resemblance of the skeletal and heart muscles from the *lamp-2* knockout mice to those from patients with Danon disease led to the identification of mutations in *LAMP2* as the primary defect in this lysosomal storage disease (Yamamoto *et al.* 2001).

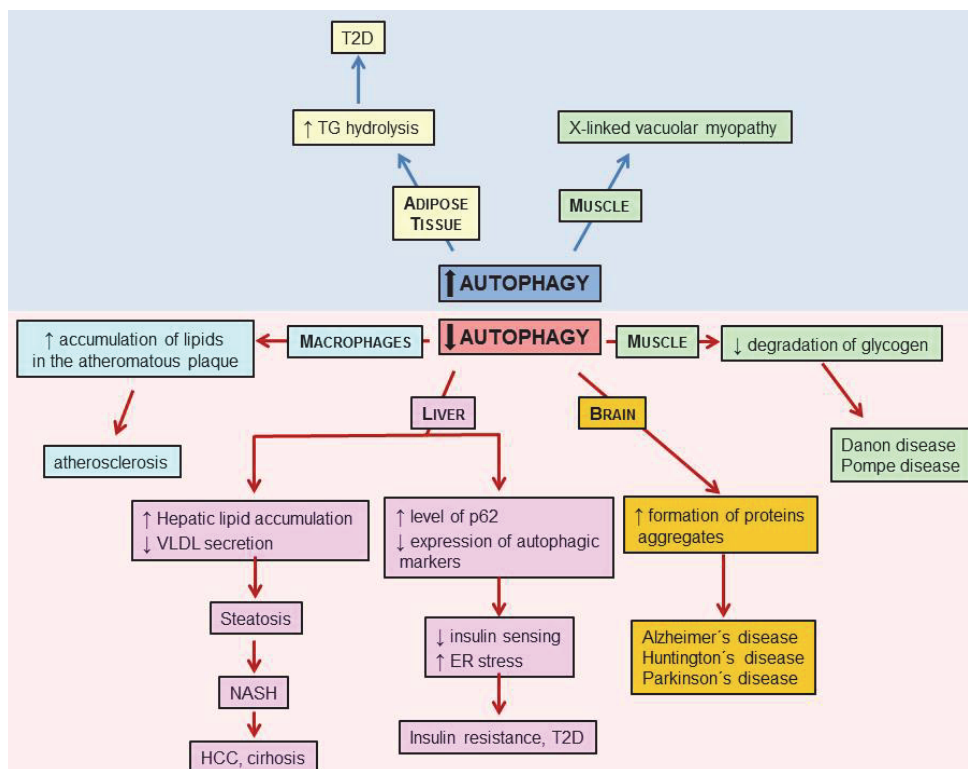


Fig. 3. Autophagy in disease. Intensity of autophagy is important for the homeostasis in all organisms. Decreased or excessively increased autophagic activity could progress various disorders.

Degradation of proteins by autophagy contributes to quality control and prevents proteotoxicity associated with accumulation of abnormal proteins. In fact, defective autophagy often associates with formation of proteins aggregates and it is likely the basis for protein conformational disorders such as Alzheimer's, Huntington's and Parkinson's disease (Komatsu *et al.* 2006). Although the mutated protein is different in each of these disorders, the sequence of events leading to protein aggregation is apparently exactly the same and proceeds as follows: 1) the abnormal conformation of affected protein exposes normally hidden hydrophobic

residues; 2) the cell responds to these abnormal proteins by activating chaperone system and cytosolic proteases; and 3) in the initial stages of the disorder, chaperones and proteases can sometimes revert, or at least slow down, protein aggregation. However, as the levels of the pathogenic protein increase, the process becomes irreversible and even the "helpful" proteins become trapped in the aggregates. Although this might have originally been a defensive mechanism against hydrophobic patches, the hydrophobic nature of these proteins makes them resistant to attack by cytosolic proteases, leaving removal by macroautophagy as the

only viable possibility (Michalik and Van Broeckhoven 2003). Recent studies show that, at least in experimental systems, the activation of macroautophagy facilitates the removal of newly formed aggregates (Webb *et al.* 2003).

Insulin signaling is critical for the regulation of glucose homeostasis in the adipose and muscle. Eating an unhealthy diet and obesity contribute to the development of insulin resistance where hepatic, adipose and muscle tissues no longer respond to insulin signaling, thus resulting in hyperglycemia (Martyn *et al.* 2008). Recently, a role for autophagy in insulin signaling has been discovered. Intriguingly, it seems that the role of autophagy in the regulation of insulin signaling in different tissues may be opposite. Hyperinsulinemic high-fat diet fed mice and *ob/ob* mice both display impaired hepatic autophagy activity indicated by decreased expression of autophagy markers and increased levels of p62, a protein that is normally degraded by autophagy. Decreased autophagy in *ob/ob* mice led to a decreased insulin signaling and induction of ER stress, both of which were rescued by atg7 overexpression (Yang *et al.* 2010). This suggests that insulin signaling down-regulates autophagy activity in the liver during a hyperinsulinemic state. In contrast, autophagy was shown to be up-regulated in adipose tissue of obese and T2D patients. Kovsan *et al.* (2011), in a human study, reveals direct correlation between different types and degrees of obesity and autophagic activity and fat deposits size. Surprisingly, autophagy was extreme high in omental fat tissue extract from obese individuals and was also increased in insulin-resistant obese subjects (Kovsan *et al.* 2011). It has been suggested that diabetic state is associated with pseudo starved state, leading to increased adipocyte autophagy, elevated TAG hydrolysis and enhanced plasma fatty acid concentration (Ost *et al.* 2010).

One of the prominent features of T2D clinical manifestation is the defective angiogenesis and consequent microvascular complications. Some conditions that are tightly associated with T2D, like inflammation or chronic hyperglycemia, are connected also with alterations in autophagy regulation, but the relationship between altered autophagy and the development of these complications is still not fully understood. It has been reported that inflammatory molecules as MCP-1, TNF- α , IL-1 β and IL-8 are known to promote angiogenesis. Roy *et al.* (2012) demonstrated that all these pathways converge at the MCP-induced protein (MCPIP), that is able to switch on the cascade of

oxidative stress, ER stress, autophagy and angiogenic differentiation in HUVEC cells. Interestingly, inhibition of each particular step caused inhibition of each subsequent step that was postulated. These data suggest that cellular stress evoked by the diabetic milieu may be translated into *de novo* angiogenesis (Roy and Kolattukudy 2012). Somewhat contradictory results were reported by Liu *et al.* (2012), who showed that methylglyoxal (MGO) (a small carbohydrate compound that is elevated in T2D) stimulated autophagic degradation of VEGFR2 in endothelial cells. Suppression of autophagy either by inhibitors or siRNA, but not of the proteasome and caspase, normalized both the VEGFR2 protein levels and angiogenesis. Conversely, induction of autophagy either by rapamycin or overexpression of LC3 and Beclin-1 reduced VEGFR2 and angiogenesis (Liu *et al.* 2012).

Retinopathy belongs to the serious pathologies associated with T2D progression. Most retinal cells are fully or terminally differentiated cells; therefore, a steady supply of nutrients, cell growth and cell cycle control are important for long-term survival (Dyer and Cepko 2000, Lee *et al.* 2006) and make these cells extremely sensitive to metabolic disturbance. Recently, it has been published that the crucial role in the process of retinopathy plays a pro-oxidant and pro-apoptotic thioredoxin interacting protein (TXNIP) (Singh, 2013). TXNIP contributes to the oxidative and nitrosative stress by significant attenuation of antioxidant cell capacity, induction of mitochondrial damage and consequent stimulation of mitophagy. In the end, this cascade of events may program autophagic cell death involving caspase-3 or cellular energy collapse. Nonetheless, in the broader context the autophagy could serve as an adaptive and even protective mechanism preventing excessive bursts of reactive oxygen species production and necrotic cell death.

Aging

Morphological alterations in the lysosomal system and changes in its enzymatic content are common in almost all tissues from older mammals (Ward 2002). Considering the physiological functions of autophagy, the cellular consequences of diminished autophagy flux are easily inferred and include inefficient removal of damaged intracellular structures, alterations in cellular homeostasis, inability to adapt to extracellular changes and poor defensive response against damaging agents. Decreased protein degradation plays an important role in

aging with regard to prolonging protein lifespan, which increases the probability of their undesired alteration. The defects in the autophagic/lysosomal proteolytic system may be the main cause of reduced protein degradation, since the proteasomal system cannot digest larger proteins or impaired organelles. Indeed, there is evidence of gradual reduction in autophagy with age (Cuervo *et al.* 2005). Autophagy activation may protect an organism from aging due to the increased ability to get rid of damaged proteins and organelles. Caloric restriction, the only intervention that delays aging and increases lifespan, reverses the decline in autophagy that occurs with age and may come about through reduced insulin/IGF-1 signaling (Kenyon 2005). Fasting can promote longevity, but it may cause potentially adverse effects of caloric restriction on human health and hence alternative approaches are currently being studied that mimic the beneficial effects of caloric restriction. The application of antilipolytic drugs that increase autophagy and extend longevity is a good example (Bergamini 2005).

Conclusion

Autophagy has been shown to complement “classical pathways” in the catabolism of carbohydrates, proteins and mitochondria in fasting and nutrient

deficiency. It is now proposed that autophagy also participates in the regulation of lipid metabolism. This finding necessitates a re-evaluation of much of the knowledge and assumptions about LD metabolism in light of this new alternative pathway of lipolysis. Lipophagy is likely to be an important metabolic pathway in the supply of energy for specific cellular function. The findings to date indicate that further investigations of lipophagy might increase our understanding of the role of LD breakdown in cell physiology and provide new avenues to treat diseases resulting from defects in lipid metabolism or storage. Autophagy is necessary for regulation of metabolic processes. In addition, malfunctions of autophagy have been implicated in the development of several diseases and so manipulation of this critical cellular process might be a promising therapeutic target.

Conflict of Interest

There is no conflict of interest.

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PŘÍLOHA III

Papáčková Z, Daňková H, Páleníčková E, Kazdová L, Cahová M. *Effect of short- and long-term high-fat feeding on autophagy flux and lysosomal activity in rat liver*. *Physiol Res*. 2012;61(Suppl 2):S67-76. doi: 10.33549/physiolres.932394.

Effect of Short- and Long-Term High-Fat Feeding on Autophagy Flux and Lysosomal Activity in Rat Liver

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Summary

Autophagy-lysosomal pathway is a cellular mechanism ensuring degradation of various macromolecules like proteins or triacylglycerols (TAG). Its disruption is related to many pathological states, including liver steatosis. We compared the effect of short- and long-established steatosis on the intensity of autophagy-lysosomal pathway in rat liver. The experiments were carried out on 3-month old Wistar rats fed standard (SD) or high-fat diet for 2 (HF-2) or 10 (HF-10) weeks. HF diet administered animals accumulated an increased amount of TAG in the liver (HF-2→HF-10). Autophagy flux was up-regulated in HF-2 group but nearly inhibited after 10 weeks of HF administration. The expression of autophagy related genes was up-regulated in HF-2 but normal in HF-10. In contrast, total activities of two lysosomal enzymes, lysosomal lipase (LAL) and acid phosphatase, were unaffected in HF-2 but significantly increased in HF-10 groups. mRNA expression of lysosomal enzymes was not affected by the diet. We conclude that in a state of metabolic unbalance (steatosis), autophagy machinery and lysosomal enzymes expression are regulated independently. The accumulation of TAG in the liver is associated with the increase of total LAL activity and protein expression. In contrast, the autophagy response is bi-phasic and after rapid increase it is significantly diminished. This may represent an adaptive mechanism that counteracts the excessive degradation of substrate, i.e. TAG, and eliminate over-production of potentially hazardous lipid-degradation intermediates.

Key words

Autophagy • Lysosomal • Liver • Lysosomal lipase • Biolysis

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Introduction

Autophagy-lysosomal pathway is essential for the maintenance of cellular and metabolic homeostasis. Following nutrient deprivation, the primary cellular response is the induction of autophagy that breaks down redundant cellular components and provides amino acids and additional precursor molecules for processes critical for cellular survival (Singh 2010). For a long time, autophagy was mostly associated with degradation of proteins. Only recently, Singh *et al.* (2009) showed that lipid droplets in hepatocytes can enter the autophagic degradation pathway in the same manner as damaged proteins or organelles *via* formation of autophago(lipo)somes that carry the cargo to the final degradation in lysosomes.

The autophagy-lysosomal pathway consists of two different, but functionally interconnected, parts. Autophagy (autophagocytosis) starts with the engulfing or sequestration of a part of the cytoplasm by a specialised membranous organelle called a phagophore (Seglen 1987). The resulting autophagosome matures into or delivers the sequestered cytoplasm to an amphisome, a collecting vacuole that can also receive material from endocytosis (Gordon *et al.* 1992). Amphisome in turn delivers its content to a lysosome, resulting in exposure of the autophagocytosed cytoplasm to the hydrolytic enzymes of the lysosomes. Lysosomes are permanently present in the cell and formed in the Golgi complex. They contain various hydrolytic enzymes capable of digesting an array of biological polymers, including proteins, nucleic acids,

carbohydrates and lipids (De Duve and Wattiaux 1966).

Autophagy is a complex process that involves the participation of at least 30 proteins (Suzuki *et al.* 2007). These proteins can be divided into 3 functional groups (Levine and Kroemer 2008). The first group are serin/threonine kinases like ATG1 and mTOR that sense the external nutrient environment and initiate the autophagy process (Jung *et al.* 2009). Next, parts of cytoplasm containing material for degradation are engulfed by simple membrane forming phagophore containing a protein complex carrying Beclin 1 and PI3K activity. This complex modifies the nascent autophagosomal membrane (Cao and Klionsky 2007) and the cargo is sequestered in a double membrane structure called autophagosome. During this step a protein called ATG8/LC3-II is inserted into both sides of the autophagosomal membrane and serves as a convenient marker for this structure (Klionsky *et al.* 2008). After formation of autophagosome, the outer membrane fuses with lysosomes while the inner membranes and sequestered contents are subsequently digested by lysosomal hydrolases within the lysosomal lumen (Jager *et al.* 2004).

Mounting evidence has demonstrated that disruption of the physiological function of autophagy is strongly related to human diseases, including neurodegeneration, cancer, liver and heart diseases, ageing, myopathies, and infection (Mizushima *et al.* 2008). The newly described interrelationship between autophagy and lipid metabolism suggests that alteration in autophagic function may play a role in the pathophysiology of human disorders that result from excessive cellular lipid accumulation, such as liver steatosis. Autophagy and lipolysis share striking similarities. Both are essential catabolic pathways activated in response to nutrient deprivation. They are under identical hormonal control, being activated by glucagon and inhibited by insulin (Finn and Dice 2006). The main objection given against autophagy involvement in the degradation of intracellular lipid droplets was different intracellular localisation of lipolytic enzyme (lysosomal lipase sequestered in lysosomes) and substrate (lipid droplets in cytoplasm). Only recently, Singh *et al.* (2009) brought evidence supporting the hypothesis that lipid droplets in hepatocytes can enter the autophagic degradation pathway in the same manner as damaged proteins or organelles *via* formation of autophago(lipo)somes that carry the cargo to the final degradation in lysosomes. Further evidence for the role of autophagy in hepatocyte lipid metabolism came from experiments carried out on mice with a hepatocyte-specific

knockout of the autophagy gene *atg7* that developed a massively enlarged liver. The enlargement was partly caused by markedly increased in triacylglycerol and cholesterol content and decreased VLDL secretion. These findings suggest that a general impairment in autophagy or a specific defect in lipophagy might in part underlie the development of steatotic liver diseases such as non-alcoholic fatty liver disease but the causal relationship remains to be verified.

The aim of our study was to address the issue concerning the effect of the duration steatosis-promoting high-fat diet on the intensity of autophagy-lysosomal pathway in the rat liver. We particularly focused on the regulation of autophagy flux and on the lysosomal activity at the functional (autophagy flux measurements *in vivo*, determination of lysosomal enzymes activities) and transcriptional (determination of mRNA expression) levels.

Material and Methods

Animal and experimental protocol

Male Wistar rats were kept in temperature-controlled room at 12:12-h light-dark cycle. The animals had free access to drinking water and standard chow-diet if not stated otherwise. Starting at the age of 3 months, the rats were fed with standard (SD, 25 w.w. % protein, 4 w.w. % fat, 68 w.w. % starch) or high-fat (HF, 14 w.w. %, protein, 33 w.w. % fat, 20 w.w. % starch) diet for 2 or 10 weeks (Table 1). The main source of fat was lard supplemented with soybean oil (1 %) in order to provide essential fatty acids (Cahova *et al.* 2012). This diet contains approx. 40 % of saturated fatty acid, 50 % of monounsaturated fatty acid (mainly oleic acid) and up to 10 % polyunsaturated fatty acids (mostly linoleic acid). A balanced composition of all necessary nutrients was ensured by the use of dried milk and yeast together with casein as the source of protein. The animals designed as “fasted” were deprived of food for 16 hrs prior the experiment. All experiments were performed in agreement with the Animal Protection Law of the Czech Republic 311/1997 and were approved by the ethical committee of Institute for Clinical and Experimental Medicine.

Determination of autophagy flux

Our assay is based on the method described by Haspel *et al.* (2011) with significant modification. Fasted rats underwent a partial hepatectomy. Through an

Table 1. Composition of the diets. The content of the macronutrients is given in weight to weight percent.

MACRONUTRIENTS			
	w.w.	SD	HF
<i>Crude protein</i>	%	22	14
<i>Fat</i>	%	3	33
<i>Fiber</i>	%	3	3
<i>Ash</i>	%	5.5	–
<i>Starch</i>	%	68	17
MINERALS & VITAMINS			
<i>Cu</i>	mg/kg	25	25
<i>Vitamin A</i>	IU/kg	30	30
<i>Vitamin D3</i>	µg/kg	3	3
<i>Vitamin E</i>	mg/kg	120	200
<i>Vitamin C</i>	mg/kg	120	120
<i>Vitamin PP</i>	mg/kg	36	36
<i>Vitamin B1</i>	mg/kg	2.8	2.8
<i>Vitamin B2</i>	mg/kg	3.2	3.2
<i>Vitamin B6</i>	mg/kg	4	4
<i>Calcium pantothenate</i>	mg/kg	13.2	13.2

incision of mid-abdomen wall under general isoflurane anaesthesia, the median and left lateral lobes of the liver were ligated and then resected (Higgins and Anderson 1931). This piece of liver was marked as sample A and was immediately frozen in liquid nitrogen. The abdominal cavity was closed and chloroquine (120 mg/kg b.wt.) was administered intraperitoneally in order to inhibit lysosomal activity in the liver. Rats were terminated by exsanguination from abdominal aorta 4 hrs later. We visually checked the abdominal cavity and we did not observe any significant bleeding. We removed the rest of liver that was marked as sample B and was dissected and immediately frozen in liquid nitrogen. Samples of tissue were stored in –40 °C until processing. Autophagy intensity was assessed as the accumulation of autophagy marker LC3-II in chloroquine-treated samples.

The isoflurane anaesthesia was employed as it is well tolerated by the animals fed a 10 week high fat diet exhibiting severe steatosis. This type of anaesthesia is associated with a significant decrease by serum FFA concentration which is the consequence of the inhibitory effect of isoflurane on isoproterenol-stimulated lipolysis in adipose tissue (Prokocimer *et al.* 1987). We found no information indicating any effect of isoflurane on liver

lysosomal lipase.

Separation and immunodetection of proteins

The liver homogenate (20 % wt/vol) was prepared by homogenisation of fresh liver tissue (part of sample A) in 0.25 M sucrose; 0.001 M EDTA pH=7.4; heparin 7 IU/m, 1 mM PMSF, leupeptin 10 µg/ml, aprotinin 10 µg/ml by Teflon pestle homogeniser. The crude impurities were removed by brief centrifugation at 850 g. Fat cake and all traces of the fat remaining on the tube wall were carefully removed in order to prevent contamination of the homogenate. Proteins in fraction were separated by SDS-PAGE and the protein of interest was recognised by specific antibody. LAL content was determined in 20 % homogenate and recognised with mouse monoclonal [9G7F12] to lysosomal acid lipase, Abcam, Cambridge, UK. LC3-II content was determined in 20 % liver homogenate lysates by 2 % SDS at 100 °C. LC3 in the lysates was recognised with rabbit polyclonal to LC3A/B (Abcam, Cambridge, UK). LC3-I and LC3-II were distinguished by difference and molecular weight (18 and 16 kDa). The bands were visualised using ECL and quantified using FUJI LAS-3000 imager (FUJI FILM, Japan).

Assay of lysosomal lipase activity

4 % homogenate prepared from fresh tissue under isosmotic conditions (250 mM sucrose) were used for the assay. The reaction medium (92.5 kBq ³H triolein, 100 µM cold triolein, 110 µM lecithin, 0.15 M NaCl) was emulsified by sonification (Hielsler ultrasonic homogeniser UP200S, Teltow, Germany) in 0.1 M acetate buffer (pH=4.5) containing 6 % BSA free fatty acids. The assay itself was performed under hypoosmotic conditions in order to ensure the release of the enzyme sequestered within the lysosomes. The substrate was incubated with the enzyme for 60 min at 30 °C. The released fatty acids were extracted according to Belfrage and Vaughan (1969) and counted for radioactivity.

Determination of glucuronidase and acid phosphatase activities

Enzyme activities were determined in 20 % homogenate diluted in saline and 0.1 M acetate buffer (pH=4.5). Glucuronidase (GU) (EC 3.2.1.31) activity was assayed according to Koldovský and Palmieri (1971). The activity of acid phosphatase (AP) (EC 3.1.3.2) was determined employing Acid Phosphatase Assay Kit (Sigma Aldrich, Saint Louis, USA).

Triglyceride content in tissues

Lipids were extracted from 1 g of fresh tissue homogenised in 4 ml of H₂O. 1 ml of the homogenate was extracted in 15 ml of 2:1 chloroform : methanol for 24 hours. The organic and aqueous phases were removed by adding of 6 ml KH₂PO₄ and centrifugation at 3000 rpm for 20 min. 1 ml of the organic phase was completely dried, resuspended in 100 µl of isopropylalcohol and 10 µl were used for the analysis. The triglyceride concentration in this aliquot was determined using commercially available kit (Pliva-Lachema Diagnostics, Czech Republic).

Real-time RT-PCR

The samples of liver tissue (sample A) were dissected immediately after decapitation and frozen in liquid nitrogen. Total-RNA was extracted from tissue samples using Trizol reagent (Invitrogen) according to standard protocol as described previously (Brinkhof *et al.* 2006). A DNAase step was included to avoid possible DNA contamination. The standard amount of total RNA

(500 ng) was used to synthesise the first-strand cDNA (High Capacity RNA-to-cDNA kit, Applied Biosystems, Foster City, CA). No template control was included in the test for contamination. RT-PCR amplification mixtures (25 µl) contained 1 µl template cDNA, SYBER Green master mix buffer (Quanti-Tect, Qiagen, Hilden) and 400 nM (10 pmol/reaction) forward and reverse primer (Table 2). Reactions were run on Applera 7300H Fast Real-Time PCR detector (Applied Biosystems). The results were analysed by SDS software vs. 2.3 (Applied Biosystems). All primers were designed using Primer3 software. The expression of genes of interest was normalised to the housekeeper gene beta-2 microtubulin (B2M) and calculated using $\Delta\Delta C_t$ method.

Statistic analysis

Data are presented as mean $\pm$ SEM. Statistical analysis was performed using Kruskal-Wallis test with multiple comparison ($n=5-7$). Differences were considered statistically significant at the level of $p<0.05$.

Table 2. Primer characteristics.

Gen	NCBI Ref. Sequence	Left primer	Right primer
<i>Glucuronidase</i>	NM_017015.2	CGAACCAGTCACCACTGAGAG	ATTGTGAACGGTCTGCTTCC
<i>Acid phosphatase</i>	NM_016988.1	CTGCAGACACAGAGGTGATTG	TTTGGGCGTAGGTAATCAGG
<i>Lysosomal lipase</i>	NM_012823.1	CAATCAGAGTTATCCACCCTTG	CCACAAGAACATTCCCGAGT
<i>Beta-2 microglobulin</i>	NC_005102.2	ATTCAGAAAACCTCCCAAATTC	AAAGACCAGTCCTTGCTGAAG

Table 3. Characteristics of experimental groups.

		SD	HF-2	HF-10
<i>Body weight (g)-14 wks</i>		305 $\pm$ 5	309 $\pm$ 5	299 $\pm$ 4
<i>Body weight (g)-16 wks</i>		343 $\pm$ 6	359 $\pm$ 7	327 $\pm$ 4
<i>Body weight (g)-24 wks</i>		415 $\pm$ 9	—	402 $\pm$ 8
<i>Adiposity (g)</i>	<i>fasting</i>	1.2 $\pm$ 0.11	1.4 $\pm$ 0.04	2 $\pm$ 0.08 ^{###}
<i>s-glucose (mmol/l)</i>	<i>fasting</i>	6.3 $\pm$ 0.2	7.4 $\pm$ 0.2 ^{**}	7.4 $\pm$ 0.1 ^{##}
<i>s-insulin (ng/l)</i>	<i>fasting</i>	112 $\pm$ 9	121 $\pm$ 6	165 $\pm$ 10 ^{##}
<i>s-TAG (mmol/l)</i>	<i>fasting</i>	2.3 $\pm$ 0.7	3.1 $\pm$ 0.4	3 $\pm$ 0.4
<i>β-hydroxybutyrate (μmol/l)</i>	<i>fasting</i>	1.8 $\pm$ 0.1	3.6 $\pm$ 0.14 ^{***}	2.4 $\pm$ 0.2 [#]
	<i>fed</i>	0.1 $\pm$ 0.04	0.48 $\pm$ 0.03 ^{***}	0.5 $\pm$ 0.09 ^{###}
<i>Liver TAG (μmol/g w. wt.)</i>	<i>fasting</i>	10.8 $\pm$ 2.0	39.6 $\pm$ 6.0 ^{***}	50.6 $\pm$ 9.6 ^{##}

Adiposity was expressed as weight of epididymal fat pad per 100 grams body weight. Results are shown as mean $\pm$ SEM, $n=6-10$. ^{**} $p<0.01$, ^{***} $p<0.001$ HF-2 vs. SD, [#] $p<0.05$, ^{##} $p<0.01$, ^{###} $p<0.001$ HF-10 vs. SD

Results

Characteristics of experimental groups

We found no difference in body weight among experimental groups (Table 3). The adiposity (expressed as weight of epididymal fat pad per 100 grams body weight) was similar in SD and HF-2 but significantly higher in HF-10. Fasting glycaemia was elevated in both HF-2 and HF-10 groups but hyperinsulinemia was proved only in HF-10. The alterations in glucose metabolism were not accompanied by fasting hypertriglyceridemia. The increased fat utilisation for energy production was documented by the elevation of β -hydroxybutyrate content in serum in HF-2 and HF-10 animals. Interestingly, ketogenesis in fasting is lower in HF-10 compared with HF-2 group ($P=0.035$) which suggests a tendency to the attenuation of TAG utilisation in the liver. As expected, compared with the SD group, the HF diet administered animals accumulated an increased amount of TAG in the liver depending on the length of diet administration.

Effect of high fat diet on autophagy flux

The aim of our experiment was to evaluate the effect of a high fat diet on the autophagy flux, which we determined as the change of the amount of the autophago(lyso)somal membrane marker LC3-II before and after chloroquine treatment *in vivo*. The difference in LC3-II content before and after chloroquine treatment could be interpreted as the rate of LC3-II formation and may thus serve as the indicator of autophagy intensity. The results are shown in Figure 1. The autophagy flux was significantly elevated in HF-2 compared with SD group. In contrast, no autophagy flux was detected in HF-10 group. Taken together, these results indicate that

short- and long-term lasting steatosis has opposite effects on autophagy in the liver, the latter leading to its profound impairment.

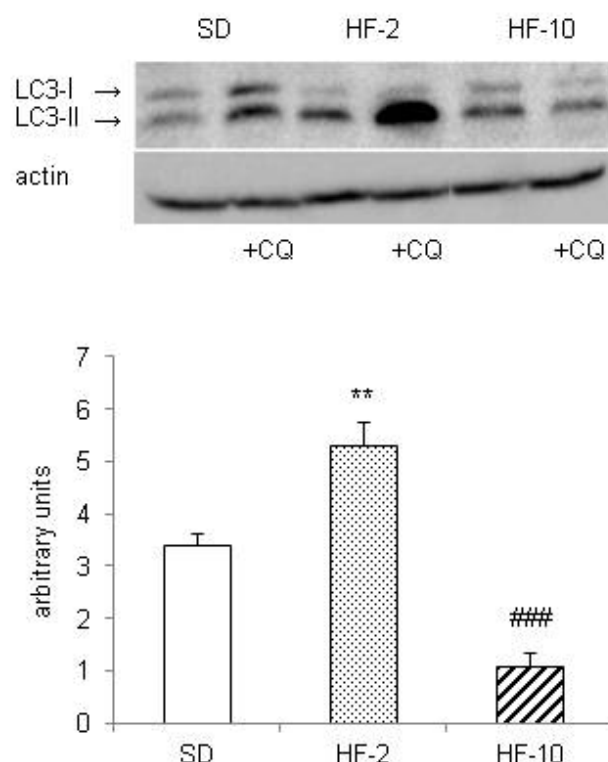


Fig. 1. Effect of HF diet on autophagy intensity. Fasted animals in isoflurane narcosis were subjected to the partial hepatectomy and sample of liver tissue (A) was immediately frozen in liquid nitrogen. Lysosomal activity was inhibited by chloroquine (120 mg/kg b.wt., i.p.). The experiment was terminated 4 hrs later and the liver tissue sample B was dissected and frozen. Autophagy flux was assessed as the accumulation of autophagy marker LC3-II in chloroquine-treated samples. Results are expressed as the ratio of LC3-II post- (B) and pre- (A) chloroquine treatment; given as mean $\pm$ S.E.M., $n=6$. ** $p<0.01$ HF-2 vs. SD; ### $p<0.005$ HF-10 vs. SD

Table 4. mRNA expression of selected autophagy markers and lysosomal enzymes.

		SD	HF-2	HF-10
Autophagy markers	<i>Beclin</i>	1	$1.26 \pm 0.06^*$	1 ± 0.03
	<i>Atg 16</i>	1	$1.45 \pm 0.04^{**}$	$0.63 \pm 0.15^{\#}$
Lysosomal enzymes	<i>Lysosomal lipase</i>	1	0.9 ± 0.04	1 ± 0.06
	<i>Glucuronidase</i>	1	1.02 ± 0.08	0.76 ± 0.06
	<i>Acid phosphatase</i>	1	1.16 ± 0.1	0.89 ± 0.06

mRNA was quantified by RT-PCR, with expression in standard diet (SD) arbitrary set at 1. * $p<0.05$, ** $p<0.01$ HF-2 vs. SD; # $p<0.05$ HF-10 vs. SD

Effect of high fat diet on the expression of autophagic genes

As described above, we found that a high fat diet had serious impact on autophagy flux and we were further interested if this effect is regulated at the transcriptional level. We tested the mRNA expression of two autophagy markers, Beclin 1 and Atg 16 (Table 4). The former is implicated in the proximal part of autophagy pathway and initiates the formation of phagophore, the latter is important for modification the nascent autophagosomal membrane and it is involved in the lipidation of LC3 protein and in the translocation of LC3-II into the autophagosomal membrane. We found small but significant increase in the expression of beclin ($P=0.048$) and more pronounced increase in the expression of Atg 16 ($P=0.015$) in HF-2 group which indicates that the rise in autophagy is reflected also at the transcriptional level. In rats fed HF diet for 10 weeks (HF-10), the expression of beclin returned to the normal level and the Atg16 expression was significantly down-regulated. This data indicates that HF diet induced changes in autophagy flux are, at least partly, mediated also at the mRNA transcription level.

Effect of high fat diet on the activity and expression of lysosomal enzymes

The availability of lysosomal enzymes represents the necessary condition of successful degradation of material *via* autophagy-lysosomal pathway. At first, we focused on the effect of high fat diet on the activity and expression of an enzyme responsible for TAG degradation in lysosomes, lysosomal lipase. We found that the total activity of lysosomal lipase is similar in SD and HF-2 groups, but that it is significantly elevated in HF-10 (Figure 2A). The expression of LAL protein followed a partly different pattern. After 2 weeks of HF diet feeding, LAL protein content was significantly diminished compared with the SD group (Figure 3). In contrast, long-term HF diet administration resulted in the significant increase of LAL protein detected in liver homogenate.

We further determined the activities of two other enzymes generally accepted as lysosomal markers (glucuronidase, acid phosphatase). We observed no uniform trend in the influence of the HF diet on their activities due to short- or long-term diet administration. The activity of glucuronidase was attenuated in HF-2 compared with HF-10 and SD groups (Figure 2B) while the activity of acid phosphatase was unaffected in HF-2

and elevated in HF-10 group (Figure 2C).

The mRNA expression of any of the three lysosomal enzymes tested (LAL, GU, AP) was not affected by the length of HF diet administration and was not different from the SD group (Table 4).

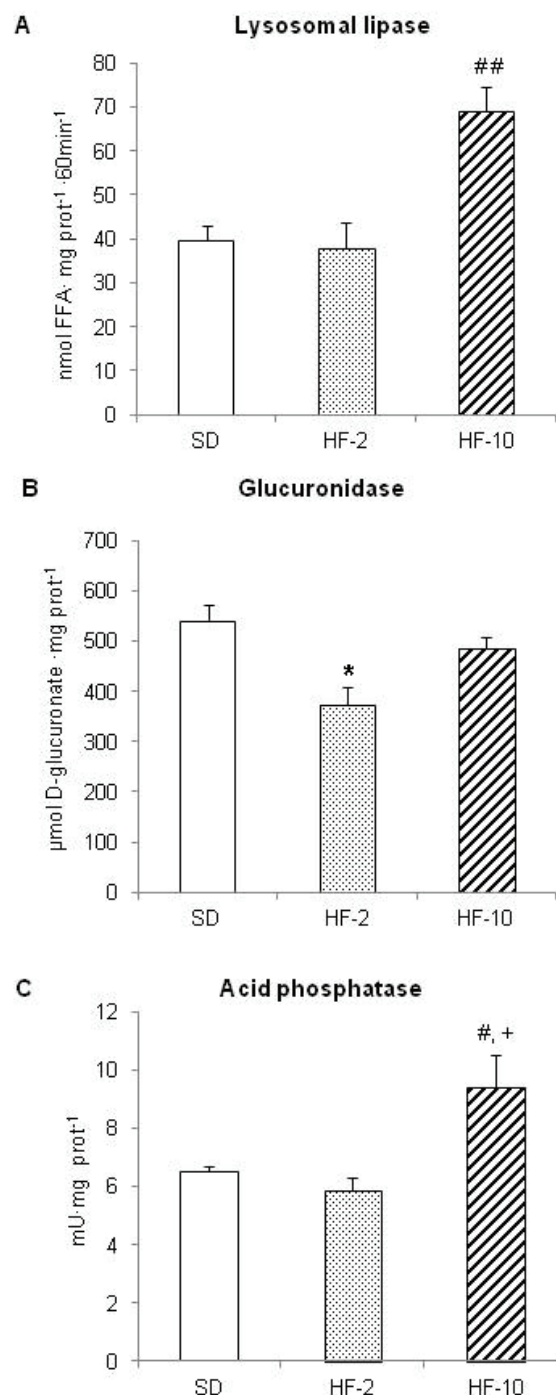


Fig. 2. Effect of HF diet on lysosomal enzymes activities. **A:** lysosomal lipase, **B:** glucuronidase, **C:** acid phosphatase. The enzyme activities were determined in liver homogenates prepared from sample A (i.e. before chloroquine treatment). Results are shown as arithmetic mean $\pm$ SEM, $n=6$. * $p<0.05$ HF-2 vs. SD, # $p<0.05$ ## $p<0.01$ HF-10 vs. SD, + $p<0.05$ HF-10 vs. HF-2

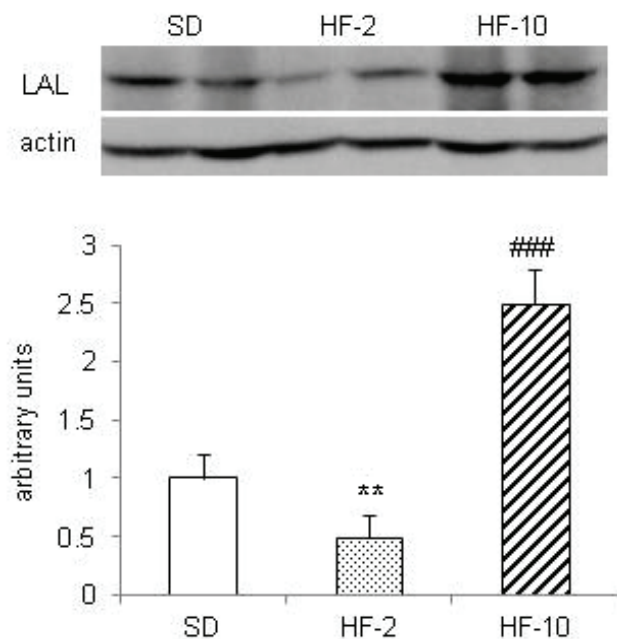


Fig. 3. Effect of HF diet on lysosomal lipase protein content in homogenate. Results are expressed as mean $\pm$ S.E.M., $n=6$. ** $p < 0.01$ HF-2 vs. SD; ### $p < 0.005$ HF-10 vs. SD

Discussion

In this study, we bring the evidence that the intensity of autophagy-lysosomal pathway depends on the TAG liver content and on the duration of steatosis. Our data further suggests that in response to HF diet administration, autophagy flux itself and lysosomal enzymes expressions are regulated independently. Our conclusions are based on following findings: 1) the autophagy response to HF diet is biphasic - two weeks of HF administration resulted in the increased autophagy flux in the liver while the long-term HF feeding led to its significant attenuation; 2) total activity of lysosomal enzyme responsible for TAG degradation, LAL, was increasing along with the duration of HF administration and TAG liver accumulation; 3) the total activity of two other lysosomal enzymes was not uniformly influenced by HF diet. These results indicate that the regulation of the whole autophagy-lysosomal pathway in response to the increased supply of dietary fat occurs at the proximal step (autophagy flux).

The TAG metabolism in the liver is subject to a highly sensitive regulation in order to fulfil the actual needs of the organism. It has been shown that the liver is a site of continuous lipolysis of endogenous TAG stored in the form of cytosolic lipid droplets. The released FFA's are used for oxidation, VLDL synthesis or are re-

esterified back to the intracellular lipid storage pool (Lankester *et al.* 1998). This process must be carefully regulated as the insufficient lipolysis may lead to an energy substrate shortage while an excessive one may result in the production of surplus potentially toxic intermediates of lipid metabolism. There are some hints that steatosis is associated with the impairment of autophagy function (Singh *et al.* 2009) but exact experimental proof is still lacking.

This task is complicated by the difficulties associated with the *in vivo* quantification of the intensity of autophagy flux. Cellular LC3-II levels correlate with numbers of autophago(lyso)somes in cells at a snapshot in time. LC3-II measured in this way rise: a) if there is enhanced autophagosome synthesis or b) if there is a block in LC3-II degradation. Likewise, decreased snapshot levels of LC3-II can be associated with a drop in autophagosome formation but can also occur when autophagy flux, including lysosomal degradation, is induced (Rubinsztajn *et al.* 2009). In order to overcome this problem, LC3-II protein must be measured in the presence of lysosomal activity inhibitor, i.e. chloroquine, which prevents LC3-II degradation in lysosome. We modified a standard *in vitro* method for measuring of autophagic flux *in vivo* using chloroquine assay. In our experimental setting, the animals were subjected to partial hepatectomy to obtain a sample of unaffected liver tissue. Then chloroquine was then applied i.p. and the intra-abdominal organs were exposed to this lysosomotropic agent. Autophagy flux intensity was assessed as the accumulation of autophagy marker LC3-II in chloroquine-treated vs. untreated samples from the same animal. The data presented in Fig. 1 show the importance of this approach. If only the untreated samples were evaluated, then the conclusion would be that autophagy is comparably stimulated both after short- and long-term HF diet feeding. Nevertheless, the determination of autophagy flow revealed that autophagy is actually stimulated only in HF-2 group while in HF-10 is substantially diminished.

Our results showed that in the liver the autophagic response to the increased fat supply in the diet is biphasic. At the beginning, autophagy flux is stimulated. We speculate that this transitional increase of autophagy may represent an adaptive mechanism in order to get rid of the fat overload. The total activity of lysosomal lipase, lysosomal TAG degrading enzyme, was comparable with SD fed animals but as we have previously shown, a short-term HF diet is associated with

a significant translocation of LAL activity as well as LAL protein into autophagolysosomal fraction enriched with TAG and with increased formation of TAG degradation products (Cahova *et al.* 2012). Rather surprisingly, total LAL protein content was decreased in the HF-2 group. We explain this discrepancy by the accentuated degradation of the enzyme in activated autophagolysosomes. The total LAL amount present in the cell exceeds several times the amount of the enzyme actually active so the degraded enzyme could be easily replenished from the inactive stores. The effect of long-term HF diet was quite opposite. In the liver of rats fed HF diet for 10 weeks, the autophagy flux was nearly completely impaired. In contrast, the activities (measured on artificial substrate under optimal conditions) of lysosomal enzymes LAL and AP were significantly higher than in the SD group and the GU activity was comparable. We suppose that this observation could be explained by their significantly attenuated degradation in lysosomes that are not activated by autophagy. In accordance with this hypothesis, we observed an elevation of LAL protein content in HF-10 group.

In the liver of animals chronically fed the high-fat diet, the autophagy could be down-regulated by several mechanisms. First, it has been reported that expression of some key autophagy genes (*vps34*, *atg12*, and *gabarapl1*) were suppressed in the presence of insulin resistance and hyperinsulinemia *via* FoxO1-dependent pathway (Liu *et al.* 2009). Second, one of the most important cellular inhibitors of autophagy is mTOR kinase which serves as a cellular sensor of nutrients. Data coming from several groups revealed that mTOR is chronically activated in the liver of high-fat diet administered mice (Korshennikova *et al.* 2006, Wang *et al.* 2010). And finally, Koga *et al.* (2010) showed that macroautophagy failure induced by chronic lipid

challenge or acute exposure to abnormally high lipid concentrations may be caused by the reduced fusion ability between autophagosomes and lysosomes.

In general, the attenuation of autophagy is considered to be deleterious as it may render cells particularly susceptible to stressors, such as oxidative stress, because of the inability to handle the damage associated with these stressors (Rodriguez-Navarro *et al.* 2012). Nevertheless, in this particular condition, the down-regulation of autophagy may also represent a sort of “protective” mechanism. It has been previously reported that LAL is capable of hydrolysing triacylglycerols and that the dominant products of its action on TAG molecule are diacylglycerol (DAG) and one molecule of fatty acid (Hayase and Tappel 1970). DAG is a known activator of several protein kinase C (PKC) isoforms of including PKC ϵ which was delineated a specific role in the development of fat-induced insulin resistance in the liver (Samuel *et al.* 2004, Samuel *et al.* 2007). We have previously shown that short-term HFD-induced TAG accumulation in the liver is associated with the elevated DAG production by LAL and rapid onset of hepatic IR *via* the PKC ϵ activation (Cahova *et al.* 2012). From this point of view, the down-regulation of autophagy-lysosomal pathway in steatosis may represent a protective mechanism against the increased formation of potentially deleterious lipid metabolism intermediates.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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PŘÍLOHA IV

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Research Article

The Increased Activity of Liver Lysosomal Lipase in Nonalcoholic Fatty Liver Disease Contributes to the Development of Hepatic Insulin Resistance

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We tested the hypothesis that TAG accumulation in the liver induced by short-term high-fat diet (HFD) in rats leads to the dysregulation of endogenous TAG degradation by lysosomal lipase (LIPA) via lysosomal pathway and is causally linked with the onset of hepatic insulin resistance. We found that LIPA could be translocated between qualitatively different depots (light and dense lysosomes). In contrast to dense lysosomal fraction, LIPA associated with light lysosomes exhibits high activity on both intracellular TAG and exogenous substrate and prandial- or diet-dependent regulation. On standard diet, LIPA activity was upregulated in fasted and downregulated in fed animals. In the HFD group, we demonstrated an increased TAG content, elevated LIPA activity, enhanced production of diacylglycerol, and the abolishment of prandial-dependent LIPA regulation in light lysosomal fraction. The impairment of insulin signalling and increased activation of PKC ϵ was found in liver of HFD-fed animals. Lipolysis of intracellular TAG, mediated by LIPA, is increased in steatosis probably due to the enhanced formation of phagolysosomes. Consequent overproduction of diacylglycerol may represent the causal link between HFD-induced hepatic TAG accumulation and hepatic insulin resistance via PKC ϵ activation.

1. Introduction

NAFLD (nonalcoholic fatty liver disease) is often associated with insulin resistance (IR) and type 2 diabetes [1]. High-fat diet-induced liver triacylglycerol (TAG) accumulation results in the hepatic IR even after three days of administration and without significant impairment of insulin-mediated peripheral glucose disposal [2]. However, the mechanism by which hepatic fat accumulation might lead to the hepatic insulin resistance is far from being clearly understood [3]. The TAG metabolism in the liver is subject to a highly sensitive regulation in order to fulfil the actual needs of the organism. It has been shown that the liver is a site

of continuous lipolysis of endogenous TAG and partial reesterification of released free fatty acids (FFA) back to the intracellular lipid storage pool [4]. The rate of intracellular lipolysis is 2-3 times greater than required to maintain the observed rate of TAG secretion [5].

Nevertheless, in spite of intensive research in this field, there are many uncertainties concerning the enzyme(s) responsible for intracellular TAG degradation. One possible candidate is lysosomal lipase (LIPA) [6]. It belongs to a group of more than 50 acid hydrolases that are characterised by low pH optimum (4.5–5). Because these enzymes require a pH range that is incompatible with the neutral cytoplasmic milieu, they are sequestered in specific cytoplasmic particles

termed lysosomes [7]. Due to a large variety of lysosomal enzymes (including proteases, lipases, glycosidases, and nucleases), lysosomes mediate complete breakdown of many types of molecules and confer upon this organelle its high degradative capacity [8]. Lysosomal enzymes are synthesized in endoplasmic reticulum, sequestered into specialised regions of Golgi apparatus, and bud out and detach as small vesicles called primary lysosomes [9]. Substrates can reach lysosomes via heterophagy (including exocytosis and phagocytosis), in which cargo originates at the plasma membrane or extracellularly, or via autophagy, for cargo located in the cytosol. Material designed for degradation is temporally stored in digestively inactive organelles termed phagosomes. Only after fusion of phagosome with primary lysosome and acidification of intralysosomal space could the internalized material be degraded and the degradation products released back into cytoplasm [10]. The lysosomal pathway was originally associated with removal of organelles and degradation of proteins [11]. Only recently the critical role of this pathway in metabolism and storage of intracellular lipids has been discovered [12]. Hayase and Tappel [13] showed that lysosomal lipase is capable of hydrolyzing triacylglycerols and that the dominant products of lysosomal lipase action on TAG molecule are diacylglycerol (DAG) and one molecule of fatty acid. DAG is a known activator of classic and novel isoforms of protein kinase C (PKC), and DAG concentrations have closely paralleled insulin resistance in other models [14, 15]. While PKCs, in general, have been implicated in the pathogenesis of insulin resistance in many tissues, Samuel et al. [16, 17] delineated the specific role of one particular isoform, PKC ϵ , in the development of fat-induced insulin resistance in the liver.

We hypothesized that steatosis-associated hepatic IR is causally linked with alteration of endogenous TAG degradation in NAFLD. To address this issue, the activity of LIPA and the production of TAG breakdown intermediates were determined in animals with normal insulin sensitivity and with hepatic IR induced by a two-week administration of a high-fat diet. We identified the steatosis-associated changes in the regulation of LIPA activity based on the alteration in its intracellular distribution, and we proposed the mechanism by which it can contribute to the establishment of hepatic IR.

2. Materials and Methods

2.1. Animals and Experimental Protocol. Male rats were kept in temperature-controlled room at 12:12 h light-dark cycle. Animals had free access to drinking water and diet if not stated otherwise. All experiments were performed in agreement with the Animal Protection Law of the Czech Republic 311/1997 which is in compliance with Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) and were approved by the ethical committee of the Institute for Clinical and Experimental Medicine. Starting at age 3 months (b.wt. 300 ± 20 g), all animals were fed either HFD (70 cal% as saturated fat, 20 cal% as protein, and 10 cal% as carbohydrate) or standard laboratory chow diet (SD) for 2 weeks. The groups labelled SD fed or HFD fed

had free access to the diet until decapitation (10–11 am), and the groups designated as SD fasted or HFD fasted were deprived of food for the last 24 hours. Glucose tolerance was determined as the rate of disappearance of glucose from circulation after a single dose of glucose (3 g/kg b.wt.) administered intragastrically to overnight-fasted animals.

2.2. Preparation of Lysosomal and Phagolysosomal Fractions. The lysosomes and phagolysosomes represent a heterogeneous population of organelles. 20% (wt/vol) homogenate was prepared by homogenization of liver tissue in 0.25 M sucrose; 0.001 M EDTA pH = 7.4; heparin 7 IU/m, 1 mM PMSE, leupeptin 10 μ g/mL, and aprotinin 10 μ g/mL by Teflon pestle homogenizer. The crude impurities were removed by brief centrifugation at 850 g. The fat cake was removed carefully in order to prevent contamination of liquid fraction. An aliquot of the homogenate was kept at 4°C until lipase assay (maximum 2 hour), the rest was centrifuged for 10 000 g 20 min 4°C, and the resulting pellet and supernatant were separated. The supernatant contains preferentially the less dense lysosomes with higher TAG content (light lysosomes), and the pellet is formed by more dense particles (dense lysosomes).

2.3. Assay of Triacylglycerol Lipase Activity on Exogenous Substrate. The optimal conditions for the lipase assay (substrate concentration, reaction temperature, and linear range of the assay) were determined in the pilot experiments. The data are provided in supplements (a–c). 4% liver homogenate or lysosomal subfractions prepared from the fresh tissue under iso-osmotic conditions were used for the assay. The reaction medium (92.5 kBq 3 H triolein, 100 μ M triolein, 110 μ M lecithin, 0.15 M NaCl, and 0.1 M acetate buffer pH 4.5) was emulsified by sonication (Hielsler sonicator UP200S). The assay itself was performed under hypoosmotic conditions (50 mM sucrose) in order to ensure the release of the enzyme sequestered within the lysosomes. The liver homogenate or isolated fractions were incubated for 60 minutes at 30°C. The released fatty acids were extracted according to [18] and counted for radioactivity.

2.4. Assay of Triglyceride Lipase Activity on Endogenous Substrate. This approach takes advantage of the coordinated changes in the intracellular localisation of LIPA and its intracellular substrate. The optimal conditions for the lipase assay were determined in the pilot experiments. The data are provided in supplements (e, f). The liver homogenate and subcellular fractions were prepared as described above under iso-osmotic conditions that prevent the disruption of lysosomes. The lysis of lysosomes was induced only after separation of fractions during the assay. 20% homogenate was mixed 1:1 with 0.2 M acetate buffer pH = 4.5 and incubated for 60 min in 30°C in shaking water bath. The reaction mixture was extracted in chloroform-methanol, and phases were separated by 1 M NaCl.

Aliquots of lower chloroform phase were separated for further determination of FFA and DAG content. An aliquot of chloroform phase was evaporated, and 100 μ L

of Krebs-Ringer phosphate buffer (pH = 7.6) containing 6% FFA-free BSA was added. The tubes were incubated in shaking incubator at 37°C for 2 hours. FFA concentration in final KRF/BSA solution was measured using commercially available kit. In order to check the efficiency of FFA solubilisation, the emptied tubes were washed with fresh KRB + 6% BSA, and then 100 μ L of chlorophorm was added. An aliquot was separated by TLC, but no substantial traces of FFA were detected.

2.5. Determination of DAG Content. This method is based on the phosphorylation of DAG in the sample to DAG-3-phosphate using γ^{35} -ATP followed by quantification of radioactivity in a chlorophorm extract. Lipids from liver tissue or incubation mixture were extracted in chlorophorm-methanol and an aliquot of chlorophorm phase was evaporated under the stream of nitrogen. The sample was then solubilised by sonication in detergent buffer (7.5% n-octyl- β -D-glucopyranoside, 5 mM cardiolipin, and 1 mM DETAPAC). Reaction buffer (50 mM imidazole/HCl, pH = 6.6, 50 mM NaCl, 12.5 mM MgCl₂, and 1 mM EGTA), diacylglycerol kinase, and γ^{35} -ATP were added and incubated 30 min in 25°C. Lipids were extracted into chlorophorm-methanol, phases were separated with 1% HClO₄, and the exact volume of lower chlorophorm phase was determined. An aliquot was evaporated, resolved in 5% chlorophorm-methanol, and separated by TLC. Individual populations of lipids were visualised by iodine vapours, the bands corresponding to DAG were scraped off, and the radioactivity was determined by scintillation counting.

2.6. Incubation of Liver Slices In Vitro. The production of β -hydroxybutyrate from liver slices *in vitro* was measured in the absence of exogenous FFA. Liver slices (width approximately 1 mm) were quickly dissected and incubated for 2 hours in Krebs Ringer bicarbonate buffer with 5 mmol/L glucose, 2% bovine serum albumin, gaseous phase 95% O₂, and 5% CO₂. All incubations were carried out at 37°C in sealed vials in a shaking water bath. The aliquots of the incubation medium were stored frozen until the further analysis.

2.7. Electrophoretic Separation and Immunodetection. The homogenate, light lysosomal fraction, and dense lysosomal fraction prepared as described above were used for the assessment of LIPA protein content. A separate group of rats were used to assess the impact of hepatic fat accumulation on the insulin signalling pathway. The animals were either deprived of food for 24 hours (fasted) or had free access to food, and insulin (6 U/kg i.p.) was administered 30 min prior decapitation (fed + insulin). Liver samples (200 mg) were harvested *in situ* and stored in liquid nitrogen until further utilization. The homogenate was prepared by Ultra-Turax homogenizer (IKA Werke, Staufen, Germany) in homogenization buffer (150 mM NaCl, 2 mM EDTA, 50 mM TRIS, 20 mM glycerolphosphate, 1 mM Na₃VO₄, 2 mM sodium pyrophosphate, 1 mM PMSF, leupeptin 10 μ g/mL, and aprotinin 10 μ g/mL). The homogenate was used for determination of mTOR and Akt phosphorylation. The proteins were

separated by electrophoretic separation under denaturing conditions and electroblotted onto PVDF membranes. The level of phosphorylation of Akt and mTOR kinases was assessed by immunodetection using specific phospho-Akt (Ser473) antibody and phospho-mTOR (Ser2448) antibody, respectively. The total expression of Akt and mTOR protein was determined on the same membrane after stripping and reblotting using specific antibodies. All these antibodies were purchased from Cell Signalling Technology, (Boston, MA). The immunodetection of LIPA protein was performed using mouse monoclonal (9G7F12) antibody to lysosomal acid lipase (Abcam, Cambridge, UK). The loading control was performed using rabbit polyclonal antibody to beta actin (Abcam, Cambridge, UK). The bands were visualized using ECL and quantified using FUJI LAS-3000 imager (FUJI FILM, Japan) and Quantity One software (Biorad, Hercules, CA).

2.8. PKC Membrane Translocation. The liver homogenate was prepared as described above. The total membrane and cytosolic fractions were prepared by centrifugation of the homogenate at 100 000 g. Solubilisation of membrane fraction was carried out in 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate. After electrophoretic separation and blotting, the PKC ϵ was detected using anti-PKC ϵ antibody (Sigma, St. Louis, USA). PKC translocation was expressed as the ratio of arbitrary units of membrane bands over the cytosol bands.

2.9. Biochemical Analysis. TAG content in liver homogenate or phagolysosomal fraction was determined after the extraction according to Folch et al. [19]. The glycogen content was determined in fat-free dry mass after hydrolysis in 30% KOH and expressed as a glucose equivalent (μ moles per g dry weight).

FFA, insulin, TAG and glucose serum content, and β -hydroxybutyrate production were determined using commercially available kits (FFA: FFA half microtest, Roche Diagnostics GmbH Mannheim, Germany; triglycerides and glucose: Pliva-Lachema, Brno CR; insulin: Mercodia, Uppsala, Sweden; β -hydroxybutyrate: RanBut, RANDOX Crumlin, UK).

2.10. Statistical Analysis. Data are presented as mean $\pm$ SEM. Statistical analysis was performed using Kruskal-Wallis test with multiple comparisons ($n = 5-7$). Differences were considered statistically significant at the level of $P < 0.05$.

3. Results

3.1. The Effect of HFD on Physical and Metabolic Parameters. The two-week period of HFD resulted in higher body weight and increased fat accumulation determined as epididymal fat pad : body weight ratio (Table 1). The impairment of glucose metabolism was indicated by increased fasting glycemia, increased fasting insulinemia, and impaired glucose tolerance measured by oral glucose tolerance test and expressed as AUC_{1-180 min}. The alterations in glucose metabolism were

TABLE 1: The effect of HFD on physical and metabolic parameters.

	Standard diet		High-fat diet	
	Fasted	Fed	Fasted	Fed
Body weight (g)		331 ± 6.9		379 ± 10.7 ^x
Epididymal fat pad/b.w. (g/100 g)	0.9 ± 0.07	1 ± 0.06	1.4 ± 0.07 [*]	1.4 ± 0.04 [#]
Glycemia (mmol/L)	5.1 ± 0.1	7.9 ± 0.5 ⁺	5.9 ± 0.2 [*]	8.1 ± 0.3 ⁺
Insulinemia (pmol/L)	56 ± 15	135 ± 21 ⁺	125 ± 10 [*]	127 ± 18
AUC ₀₋₁₈₀ (mmol glucose/L)		1168 ± 26.6		1325 ± 32.9 ^x
Serum Tg (mmol/L)	0.7 ± 0.1	1.4 ± 0.08 ⁺	0.7 ± 0.02	1.4 ± 0.1 ⁺
Serum FFA (mmol/L)	0.7 ± 0.05	0.4 ± 0.02 ⁺	0.6 ± 0.08	0.45 ± 0.07 ⁺
ALT (μkat/L)		1.2 ± 0.1		1.2 ± 0.1
AST (μkat/L)		4.3 ± 0.6		3.9 ± 0.3
β-hydroxybutyrate (μmol/L)	1.67 ± 0.05	0.05 ± 0.01 ⁺	3.2 ± 0.25 [*]	0.28 ± 0.05 ^{#,+}

Data are given as means ± SEM, *n* = 7. ^x*P* < 0.05 HFD versus SD group; ⁺*P* < 0.05 fasted versus fed animals; ^{*}*P* < 0.05 SD- versus HFD-fasted animals; [#]*P* < 0.05 SD- versus HFD-fed animals.

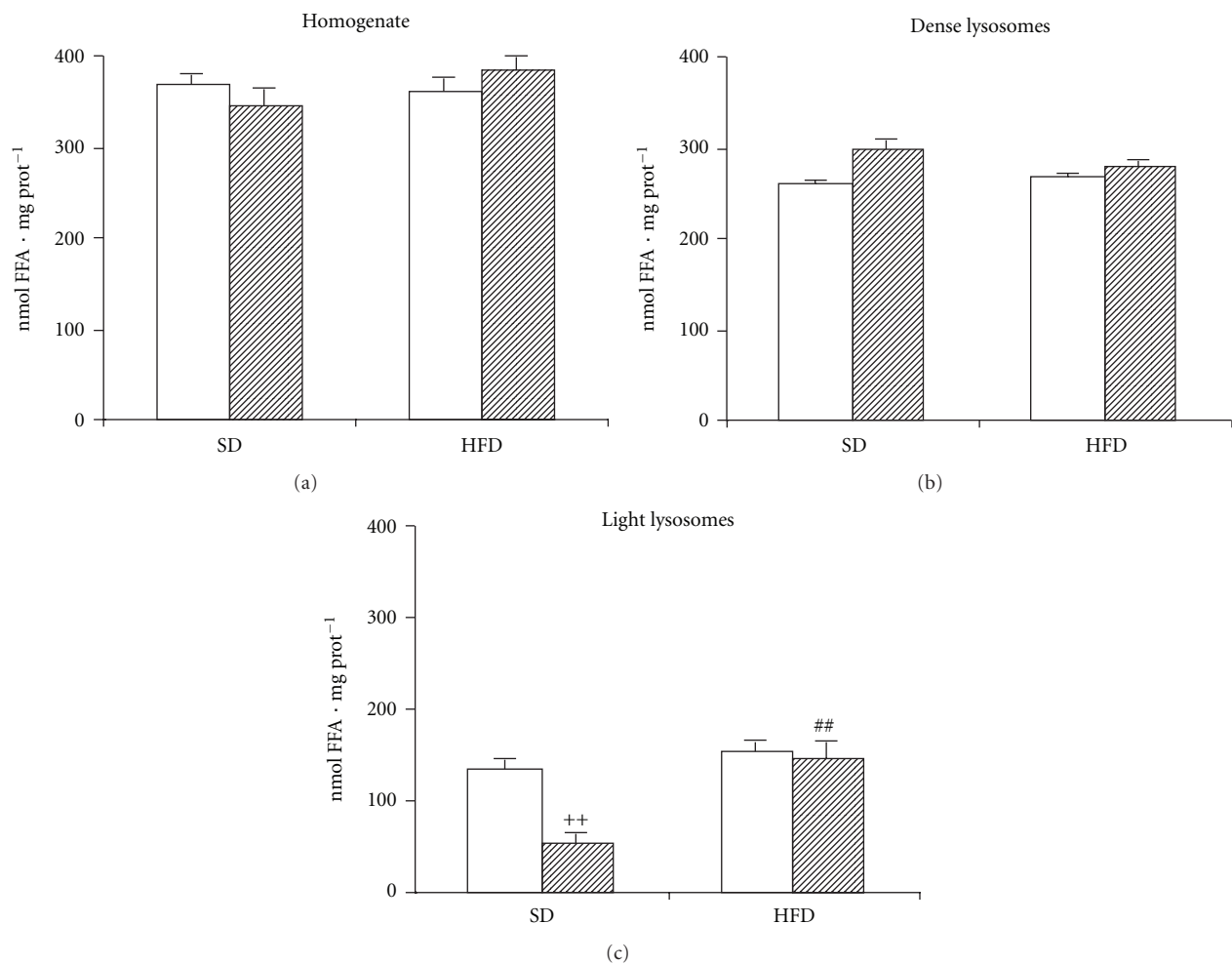


FIGURE 1: The effect of HFD on the LIPA activity measured as FFA release from artificial substrate (³H-triolein). (a) Homogenate; (b) dense lysosomes; (c) light lysosomes. The lipase activity was measured as the release of fatty acids at pH = 4.5 from ³H-triolein. Open bars = fasted animals; hatched bars = fed animals. ++*P* < 0.01 fed versus fasted; ##*P* < 0.01 HFD fed versus SD fed.

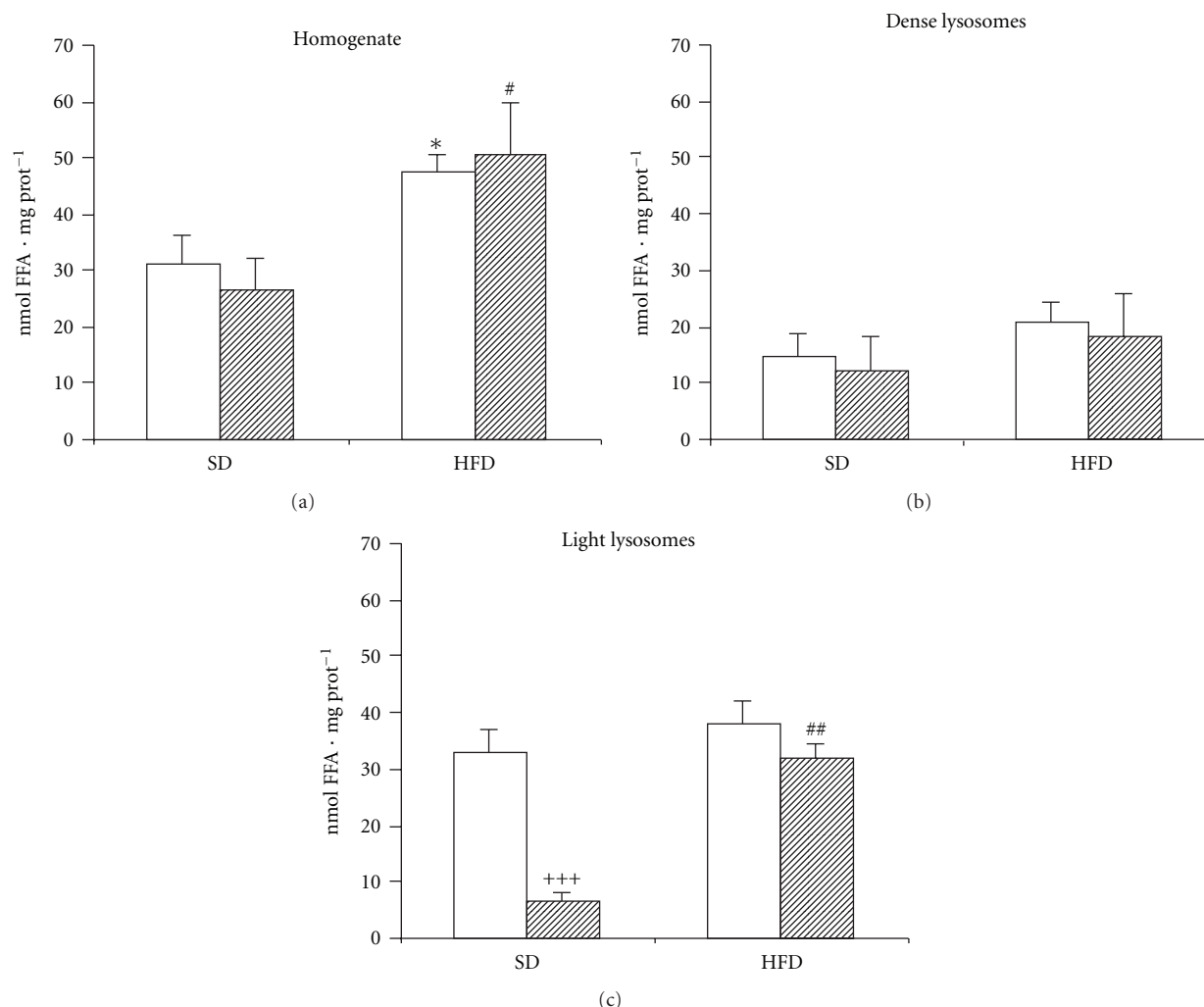


FIGURE 2: The effect of HFD on the LIPA activity measured as FFA release from endogenous TAG. (a) Homogenate; (b) dense lysosomes; (c) light lysosomes. 10% liver homogenate, light or dense lysosomal fraction were incubated 60 min at pH = 4.5. At the end of incubation, the released FFAs were quantified as described in Section 2. The graph shows the difference between FFA concentration in the sample at the beginning and at the end of the incubation. Open bars = fasted animal; hatched bars = fed animals. +++ $P < 0.001$ fed versus fasted; * $P < 0.05$ HFD fasted versus SD fasted; # $P < 0.05$, ## $P < 0.01$ HFD fed versus SD fed.

not accompanied by dyslipidemia. Serum β -hydroxybutyrate concentration was significantly elevated in both HFD-fed as well as HFD-fasted group compared to corresponding SD groups what indicates increased utilisation of fatty acids for ketogenesis in the liver. Short-term HFD administration did not alter ALT and AST serum concentrations.

As expected, compared to the SD group, the HFD-administered animals accumulated increased amount of TAG (fasted: 14.6 ± 1.4 versus 3.2 ± 0.2 ; $P < 0.001$; fed: 16.2 ± 2.5 versus 2.9 ± 0.2 $\mu\text{mol/g}$; $P < 0.001$) and DAG (fasted: 138 ± 17 versus 83 ± 12 ; $P < 0.01$; fed: 145 ± 19 versus 53 ± 9 nmol/g; $P < 0.001$) in the liver. The insulin-stimulated increase of glycogen content in liver was lower in HFD compared to SD animals (fasted: 27 ± 7 versus 41 ± 9 n.s.; fed: 123 ± 10 versus 261 ± 15 $\mu\text{mol/g}$; $P < 0.001$).

3.2. The Effect of HFD on Lysosomal Lipase Activity. In order to determine maximal LIPA activity in liver homogenate and in particular lysosomal subpopulations, we employed

emulsified ^3H -labeled triolein as a substrate. In this experimental setting, the substrate is present in excess, and the only limiting factor is the amount of enzyme. The total LIPA activity measured in the whole homogenate was not affected either by prandial status (fasted or fed state) or diet intervention (SD or HFD) (Figure 1(a)). Similar results were observed in the fraction of dense lysosomes that represent primary lysosomes (Figure 1(b)). On ^3H -triolein as a substrate, we found most of total LIPA activity in this fraction. The LIPA activity determined in light lysosomes represents only minor portion of total activity, but unlike homogenate or dense lysosomes, it responds to different metabolic states (Figure 1(c)). In the SD group, it is elevated in fasting and depressed in fed state. HFD feeding abolished the prandial regulation of LIPA activity especially due to its upregulation in fed state.

A separate set of experiment was designed in order to evaluate the contribution of LIPA associated with dense and

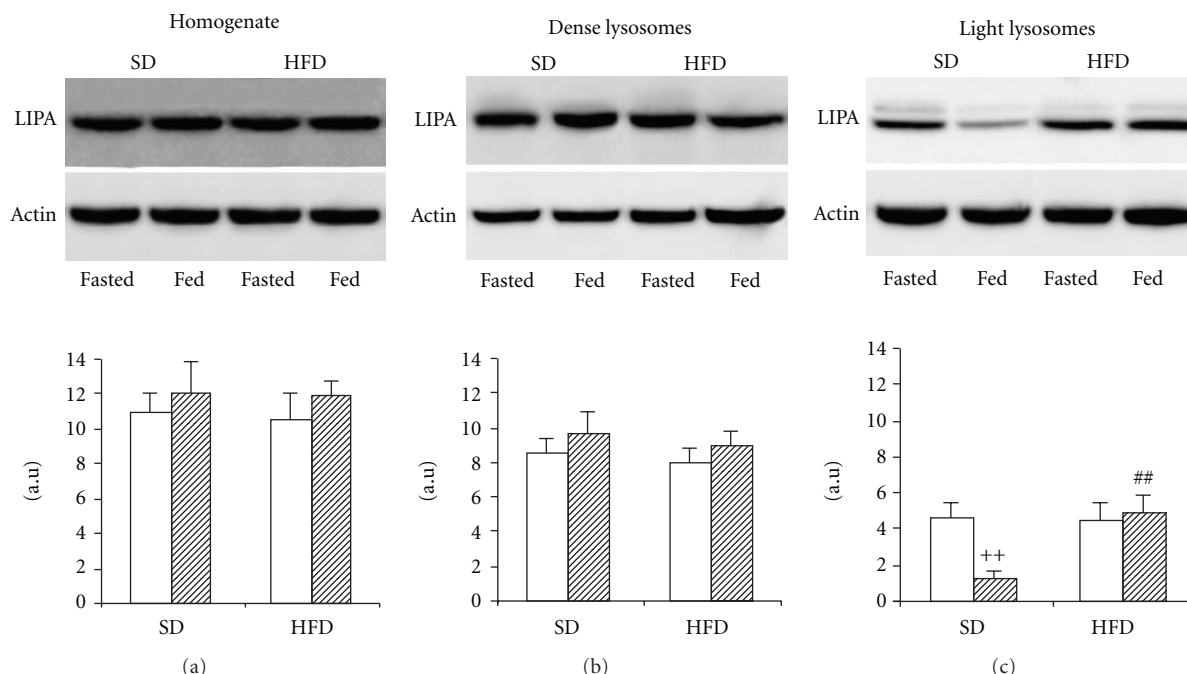


FIGURE 3: The effect of HFD on the LIPA protein expression. (a) Homogenate; (b) dense lysosomes; (c) light lysosomes. Representative Western blots are shown above each graph (f = fasted, F = fed). The results are expressed as arbitrary units after normalisation to the actin expression (loading control). Open bars = fasted animals; hatched bars = fed animals. ⁺⁺ $P < 0.01$ fed versus fasted; ^{##} $P < 0.01$ HFD fed versus SD fed.

light lysosomes to the degradation of intracellular TAG. In this experimental design, the intracellular TAGs contained in particular fraction are the only source of substrate, and the intensity of lipolysis depends not only on the amount of enzyme but also on the amount of substrate available in the sample (Figures 2(a), 2(b), and 2(c)). Compared with the same experiments carried on ^3H -triolein, we found two differences. First, HFD administration led to a significant increase of total LIPA activity measured in homogenate. Second, after separation of lysosomal subpopulations, LIPA activity associated with light lysosomes was higher than those associated with dense lysosomes. This observation could be explained by the previous “*in vivo*” translocation of both the substrate (TAG droplets) and the enzyme (LIPA) into light lysosomal fraction (phagolysosomes). In accordance with this presumption, we found higher TAG content in phagolysosomal fraction in HFD compared with SD group (fasted: 2.3 ± 0.2 versus 4.1 ± 0.7 ; fed: 1.02 ± 0.3 versus $5.6 \pm 0.48 \mu\text{mol} \cdot \text{mg prot}^{-1}$). Similarly with the results obtained on ^3H -triolein, the effect of fasting was manifested only in SD group and only in light lysosomal fraction. HFD feeding resulted into the elevation of LIPA activity in light lysosomes and into the abolishment of prandial regulation. Taken together, our results indicate that in the liver most of the enzyme is present in inactive form in dense (primary) lysosomes, and the physiologically active portion of the enzyme could be determined in light lysosomal fraction.

3.3. The Effect of HFD on Lysosomal Lipase Protein Distribution. In order to distinguish whether the higher LIPA

activity found in the light lysosomal fraction in HFD group is consequent to the increased amount of enzyme in this fraction or only to the increased availability of the substrate, we determined the amount of LIPA protein in liver homogenate and in particular fractions. We found that the LIPA protein content in homogenate (Figure 3(a)) and dense lysosomal fraction (Figure 3(b)) is similar in both fasted and fed animals and that it is not affected by short-term HFD administration. In contrast to these findings, the abundance of LIPA protein in light lysosomal fraction is lower than in homogenate or dense lysosomes, but it varies according to several factors (Figure 3(c)). In SD group, it strongly depends on prandial status. In SD-fasted rats, LIPA protein abundance in this fraction is significantly higher compared with their fed counterparts. Short-term HFD diet has no effect on the content of LIPA protein in fasted animals, but it significantly increases its amount in the fed ones. Consequently, the prandial-dependent regulation is completely abolished in HFD group.

3.4. Diacylglycerol Production in Incubated Liver Homogenate.

DAG is one of the major products of LIPA action on TAG molecule as this enzyme has lower affinity to DAG or monoacylglycerol compared with its affinity to TAG [13]. In our experimental conditions (incubation of liver homogenate or isolated fraction in $\text{pH} = 4.5$), DAG could not be further utilised for TAG biosynthesis, and the difference in DAG concentrations at the end and at the beginning of incubation represents the net DAG production from TAG degradation. Nevertheless, we cannot exclude some

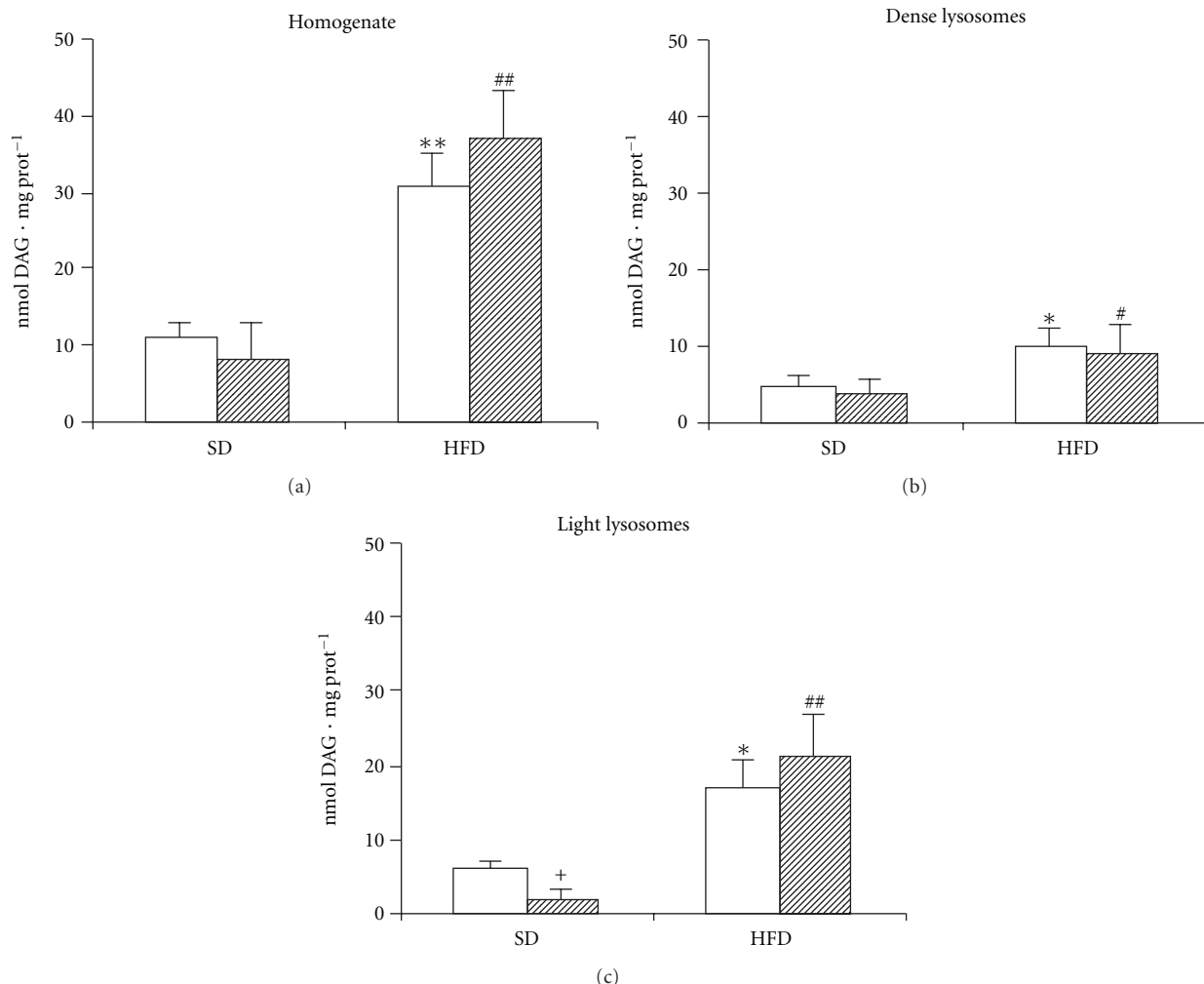


FIGURE 4: The effect of HFD on the DAG production from intracellular TAG *in vitro*. (a) Homogenates; (b) dense lysosomes; (c) light lysosomes. 10% liver homogenate, dense lysosomal or light lysosomal fractions were incubated 60 min at pH = 4.5. At the end of incubation, DAG was extracted into chlorophorm-methanol and quantified as described in Section 2. The graph shows the difference between DAG concentration in the sample at the beginning and at the end of the incubation. Open bars = fasted animals; hatched bars = fed animals. ⁺P < 0.05 fed versus fasted; *P < 0.05, **P < 0.01 HFD fasted versus SD fasted; #P < 0.05, ##P < 0.01 HFD fed versus SD fed.

degradation of DAG by lysosomal carboxylesterases. In homogenate, DAG production in SD group was significantly lower compared with those in HFD, and it was prandial dependent, that is, elevated in fasting and downregulated in fed state. In HFD group, a significant DAG production was detected in both fasted and fed animals (Figure 4(a)). The stimulatory effect of HFD on DAG production was found in both dense (Figure 4(b)) and light (Figure 4(c)) lysosomes. In HFD group, approximately 60% of DAG formation occurred in light lysosomal fraction, and in contrast to the SD group, it was independent of prandial status.

3.5. The Effect of HFD on Ketogenesis In Vitro. LIPA is expressed not only in hepatocytes but also in many other cell types including Kupffer cells present in the liver. In order to address the issue whether the above-mentioned changes in LIPA activity could be ascribed to hepatocytes, we measured ketone bodies production from liver slices *in vitro*

(Figure 5). Ketogenesis is the metabolic pathway occurring exclusively in hepatocytes and tightly reflects the intracellular TAG metabolism. We found an elevated ketogenesis due to the HFD administration what under these experimental set up implicates the accentuation of TAG hydrolysis. When liver slices were incubated in the absence of exogenous FFA, HFD-fasted group exhibited significantly higher β -hydroxybutyrate production compared with SD fasted. A similar trend was found also in fed animals.

3.6. The Effect of HFD on Key Components of Insulin Signalling Pathway. To determine the effect of HFD on hepatic insulin sensitivity, the activation of key components of insulin signalling pathway was measured by immunodetection of their phosphorylation status. As shown in Figure 6(a), the insulin-stimulated phosphorylation of Akt kinase was significantly impaired in HFD-compared to SD group. Similar

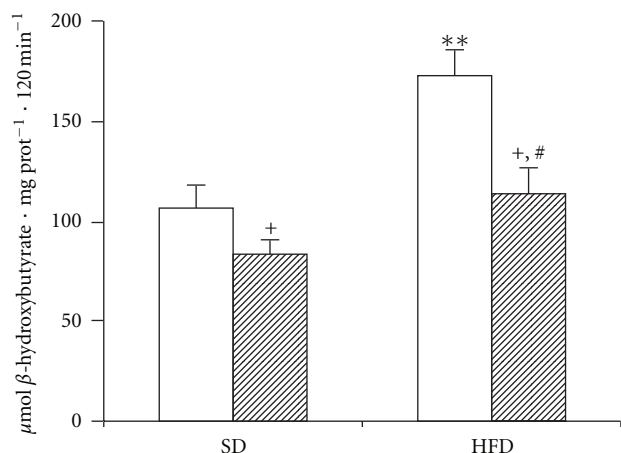


FIGURE 5: The effect of HFD on β -hydroxybutyrate production from liver slices *in vitro*. Liver slices were incubated in oxygenated KRB without exogenous fatty acids. Open bars = fasted animals; hatched bars = fed animals. $^+P < 0.05$ fed versus fasted; $^{**}P < 0.01$ HFD versus SD fasted; $^{\#}P < 0.05$; $^{\#}P < 0.05$ HFD fed versus SD fed.

results were obtained for mTOR (Figure 6(b)) suggesting an impairment of insulin signal transduction.

3.7. The Effect of HFD on PKC ϵ Activity. Determination of the relative abundance of the particular PKC isoform in the membrane and cytosol fractions reflects PKC ϵ activation. An increase in the membrane to cytosol fraction ratio was used as an indicator of PKC ϵ activation. As shown in Figure 7, PKC ϵ was significantly activated in the liver of the HFD-administered animals.

4. Discussion

In the present study, we provide evidence that in steatosis the increased degradation of TAG mediated by LIPA and associated with the increased production of DAG may be one of the mechanisms determining the rapid onset of hepatic IR. Our hypothesis is based on following findings. First, alterations in LIPA activity associated with different metabolic states are based on prandial-dependent translocation of the enzyme from the inactive pool of dense lysosomes into light lysosomal fraction, and it is upregulated in fasting and downregulated in the fed state. After short-term HFD administration, this prandial-dependent regulation of LIPA activity is abolished. The fed state-associated downregulation of LIPA activity is impaired, and the portion of the active enzyme is permanently increased. These changes were demonstrated on both endogenous TAG and exogenous substrate (emulsified ^3H -triolein). Second, in steatosis, the production of TAG degradation intermediates, FFA and DAG, by lysosomal lipase was significantly elevated. Finally, we proved an increased PKC ϵ activation together with the defects in the insulin-signalling cascade in the fatty liver. Taken together, these data indicate that the enhanced activity of LIPA in HFD-fed animals and following overproduction of

PKC ϵ activator DAG contribute to the establishment of HFD-induced IR. We have previously shown that LIPA is involved in the degradation of intracellular TAG in the liver [20]. The essential role of LIPA for hydrolysis of TAG is supported by findings of Du et al. [21] who reported that LIPA knock-out mice (*Lipa* $^{-/-}$) exhibited progressive hepatosplenomegaly and massive TAG accumulation in the liver.

Our data indicate that the principal factor regulating the LIPA activity is not the total amount of the enzyme itself but rather its intracellular localisation. We did not find any significant differences in LIPA mRNA expression in response to either fasting or diet intervention (not shown), and in accordance with this, we did not find any difference in total LIPA protein content determined in the whole homogenate. According to Seglen and Solheim [22], active phagolysosomes have a lower density than the small, inactive lysosomes, allowing their separation by differential centrifugation. Based on this observation, we separated the total lysosomes into two subpopulations according to their density. We expected that the active lysosomes containing the TAG substrate would remain in the less dense fraction (light lysosomes), while the inactive lysosomes would sediment (dense lysosomes). In our experimental setting, the effect of fasting or HFD was manifested predominantly in light lysosomal fraction what supports the physiological relevance of this methodology. In SD group, we observed a significant prandial-dependent regulation, LIPA activity being upregulated in fasted and downregulated in fed animals. HFD feeding was associated with a significant elevation of LIPA protein content and LIPA activity in light lysosomal fraction particularly in fed animals and consequently with the abolishment of prandial-dependent regulation of LIPA activity. Similar trends were observed on both exo- and endogenous substrates. The changes in LIPA activity were reflected by the corresponding changes in LIPA protein content in light lysosomal fraction. The interesting conclusions come from the comparison of LIPA activity in the light and dense lysosomal fractions determined on either ^3H -triolein or intracellular TAG. The activity measured on ^3H -triolein depends only on the amount of the enzyme present in the particular fraction as the substrate is available in excess. In contrast, when intracellular TAGs are the only source of substrate, the activity in particular fractions depends on the coordinated translocation of the enzyme and the substrate. The main difference in LIPA activity determined by these two approaches was found in the distribution of LIPA activity among dense and light lysosomal fractions. In dense lysosomes, we found high LIPA activity on ^3H -triolein but only low LIPA activity on intracellular TAG. This difference indicates that dense lysosomal fraction contains an enzyme that is not active in physiological situation but that could be activated after addition of the arteficial substrate. The LIPA activity determined in light lysosomes represented the bulk of total LIPA activity on intracellular TAG substrate but only minor portion of total activity determined on ^3H -triolein. It is possible to speculate that the LIPA activity on endogenous substrate quantitatively reflects the formation of activated lysosomes, that is particles containing both the substrate and the enzyme. Taken together, these data indicate that LIPA

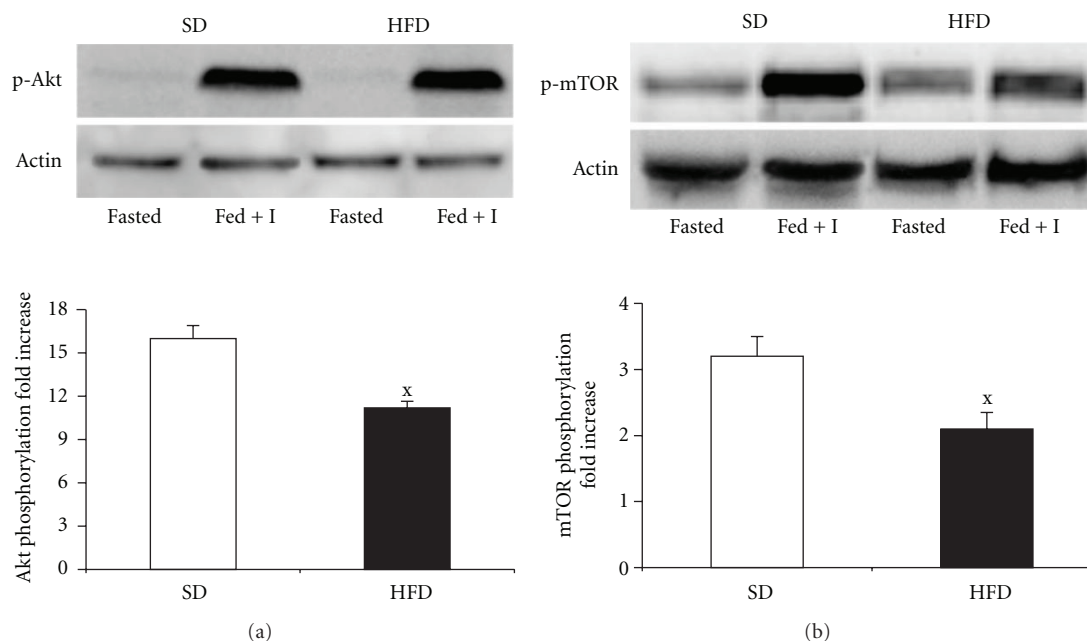


FIGURE 6: Alterations of insulin signalling cascade associated with hepatic fat accumulation. All results are expressed as a fold increase in the insulin-stimulated state relative to the basal state. Representative Western blots are shown above each graph. (a) Fold increase in Akt (Ser473) phosphorylation, (b) fold increase in mTOR (Ser2448) phosphorylation. The basal level of protein phosphorylation was determined in the homogenate prepared from the liver of 24 hours fasted animals. The effect of insulin was determined in identically processed samples from animals which had free access to food and 40 min prior to decapitation were administered insulin 6 U/kg. The total protein (Akt or mTOR) expression was determined after stripping the membrane and reblotting with anti-Akt or anti-mTOR antibody. Values represent means $\pm$ S.E.M. of 7 animals. ^x $P < 0.05$.

associated with light lysosomes represents the physiologically active enzyme.

We suppose that in NAFLD, characterised by high TAG intracellular content, one of the factors determining the phagolysosomal formation may be the substrate availability itself. The increased amount of intracellular lipid droplets in steatosis could promote the phagolysosome formation and stimulate the lysosomal lipolysis. Only recently, Singh et al. [23] described direct involvement of autophagy and lysosomal pathway in the degradation of intracellular lipid droplets in the liver. They found that lipid droplets can enter the autophagic degradation pathway in the same manner as proteins and damaged organelles via formation of autophago(lipo)somes that further fuses with primary lysosomes. As the only known lysosomal enzyme with lipolytic activity is LIPA, we believe that our results are in accordance with findings of Singh et al..

The ketone body formation tightly reflects the liver lipid metabolism. Debeer et al. [24] demonstrated that both ketogenesis and FFA oxidation are a particularly good markers of lysosomal TAG degradation. We observed higher β -hydroxybutyrate concentration in serum and higher ketone body production from isolated liver slices in the absence of exogenous fatty acids in HFD group. We conclude that these data provide indirect evidence that confirms the stimulatory effect of short-term HFD on lysosomal lipolysis.

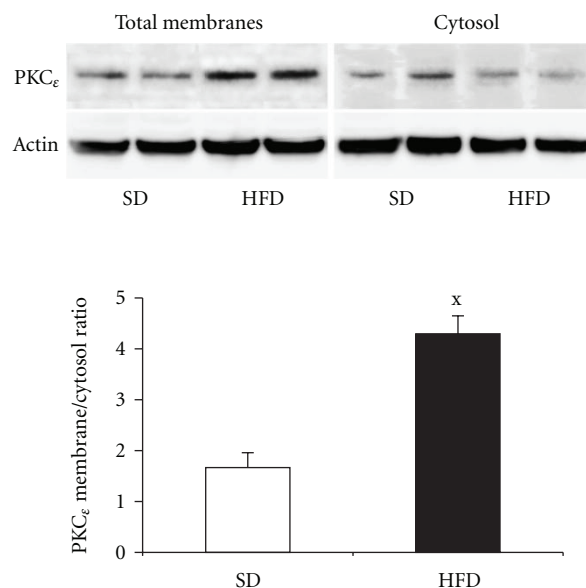


FIGURE 7: The effect of hepatic fat accumulation on PKC ϵ activation. Representative Western blot is shown in the upper part of the figure; TM, total membrane fraction, C, cytosol fraction. The PKC ϵ membrane to cytosol ratio is shown in the graph. The relative densities of the bands in the membrane fraction were compared with corresponding ones in cytosol fraction in order to obtain the measurable parameter of activation. Values represent means $\pm$ S.E.M. of 7 animals. ^x $P < 0.05$.

Concomitantly occurring stimulation of lipolysis and the accumulation of endogenous TAG after HFD administration seem to be contradictory. However, in hepatocytes, a significant portion of FFA released from intracellular TAG (approximately 70%) is reesterified back [25]. HFD impairs VLDL secretion [26], and most of FFA reenter the intracellular storage pool. We have previously reported that DGAT-1 expression is increased in fatty liver what indicates enhanced esterification of fatty acids and may result in the intensification of lipolytic/reesterification cycle in hepatocytes [20]. The increased lipolysis thus does not result in decreased TAG content but only in higher TAG turnover.

In the liver, PKC ϵ , member of novel PKCs subfamily, is involved in the development of HFD-induced IR [16, 17]. Samuel et al. showed that fat-induced hepatic IR may result from activation of PKC ϵ and its downstream targets. Nevertheless, the nature of the signal that activates PKC ϵ has not been fully explained. Systemic increase in FFA serum levels, as one possible underlying factor, has not been described after HFD administration. Another candidate, 1,2-sn-DAG, is an important intracellular signalling molecule, and it is the known activator of novel PKCs isoform family [27]. The increased DAG content due to the increased flux through TAG synthetic pathway and the following PKC ϵ activation was described in skeletal muscle in HFD-administered animals [28]. However, DAG is also an intermediate in TAG degradation pathway that, in contrast to muscle, is quite active in the liver. Our findings suggest that DAG originating from the increased lipolytic activity of LIPA and accentuated TAG breakdown could act as PKC ϵ activator in fatty liver. This hypothesis is supported by the fact that in fatty liver LIPA is activated specifically in the fed state, and possible PKC ϵ activator is available during the period of insulin action.

In conclusion, we found that short-term HFD-induced TAG accumulation in the liver is associated with the increased degradation of intracellular TAG by lysosomal lipase and with higher production of lipolytic products—DAG and FFA. Our findings suggest that the elevated DAG production by LIPA activated by increased supply of dietary lipids may represent the causal link between dietary fat-induced hepatic TAG accumulation and hepatic IR via the PKC ϵ activation. In the light of these findings, lysosomal lipolysis may represent a new promising therapeutic target.

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PŘÍLOHA V

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Original Article

The Autophagy-Lysosomal Pathway Is Involved in TAG Degradation in the Liver: the Effect of High-Sucrose and High-Fat Diet

(lysosomal lipase / high-fat diet / high-sucrose diet / steatosis / rat)

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Abstract. This study was designed to test the role of liver lipases in the degradation of liver triacylglycerols (TAG) and to determine the effect of dietary induced TAG accumulation in the liver on regulation of their lipolysis. Male Wistar rats were administered high-fat or high-sucrose diet for two weeks. Individual lipases (HL; TGH; LAL) were identified according to their different pH optimum. Administration of both diets resulted in liver TAG accumulation (HFD >>> HSD). The only lipase capable to hydrolyse intracellular TAG was LAL. On standard diet, LAL activity towards both endogenous and exogenous substrates was up-regulated in fasting and down-regulated in fed state. The intensity of autophagy determined according to the LC3-II/LC3-I protein ratio followed a similar pattern. HFD led to an increase of this ratio, elevation of LAL activity in phagolysosomal fraction and abolishment of fasting/fed-dependent differences. LAL activity significantly correlated with ketogenesis in all groups ($r = 0.86$; $P < 0.01$). In the HFD group, we determined the enhanced release of lysosomal enzymes (glucuronidase, LAL) into the cytosol. Dgat-1 expression was up-regulated in HFD- and HSD-fed groups, which indicates increased

FFA esterification. We demonstrated that LAL is a dominant enzyme involved in degradation of intracellular TAG in the liver and its translocation into the fraction of active (auto)phagolysosomes is stimulated by diet-induced TAG accumulation. Autophagy is stimulated under the same conditions as LAL and may represent the mechanism ensuring the substrate-enzyme contact in autophagolysosomes. In fatty liver, destabilization of (auto)phagolysosomes may contribute to their susceptibility to further stress factors.

Introduction

Non-alcoholic fatty liver disease (NAFLD) defined as fat accumulation in the liver exceeding 5–10% of wet weight (Mensink et al., 2008) is alarmingly spreading around the Western world. It clusters with other disorders associated with metabolic syndrome and increases the risk of development of insulin resistance, type 2 diabetes, cardiovascular and liver diseases. Recent data indicate that dietary induced liver triacylglycerol (TAG) accumulation induces hepatic insulin resistance (IR) before the onset of whole body IR (Samuel et al., 2004). Hepatic steatosis also represents a serious risk factor for further development of progressive stages of liver injury. There are several conditions contributing to the development of fatty liver, i.e. impaired very-low-density lipoprotein (VLDL) secretion or increased supply of FFA or remnant chylomicrons from circulation, either as a result of the increased supply of dietary lipids or as the consequence of increased free fatty acid (FFA) release from adipose tissue. The FFA entry into hepatocytes is not actively regulated and depends only on FFA concentration in the serum. Nevertheless, the TAG liver content is not merely a function of FFA serum levels but the induction of steatosis is the result of concerted action of several intrahepatic mechanisms. Changes in the FFA endogenous utilization, increased *de novo* FFA synthesis, changes in the secretion of VLDL and genetic predispositions rank among the most important factors. In the past, substantial progress was made in identifying the molecular and physiologic changes that cause hepatic steatosis.

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Abbreviations: DAG – diacylglycerol, Dgat-1 – diacylglycerol-acyl transferase 1, FA – fatty acid, FFA – free fatty acid, HFD – high-fat diet, HL – hepatic lipase, HSD – high-sucrose diet, IR – insulin resistance, LAL – lysosomal lipase, LD – lipid droplets, NAFLD – non-alcoholic fatty liver disease; SD – standard diet, TAG – triacylglycerols, TGH – triacylglycerol hydrolase, VLDL – very-low-density lipoproteins.

Nevertheless, one important feature of intrahepatocyte fatty acid metabolism – the regulation of breakdown of intracellular TAG – is still poorly understood.

Several groups using different experimental approaches have quantitatively determined that the TAG droplets are not a metabolically inactive storage pool but undergo an intensive cycle of lipolysis followed by re-esterification within hepatocytes (Yang et al., 1995; Lankester et al., 1998). Approx. 70 % of exogenous FFA taken up by the hepatocyte are not directly utilized for VLDL secretion but enter the intracellular storage pool as TAG (Gibbons and Wiggins, 1995). The necessary condition for TAG further utilization is their breakdown catalysed by one of the hepatic lipases. Three main distinct lipolytic activities were identified in the liver. Lysosomal acid lipase (LAL) is an intracellular enzyme with pH optimum 4–5 located in the lysosomes (EC 3.1.1.13) (Vavřínková and Mosinger, 1971). Triacylglycerol hydrolase (TGH) belongs to the carboxylesterase family of enzymes (EC 3.1.1.1), is most active at neutral pH (optimum 7–8) and is associated with endoplasmic reticulum (Lehner et al., 1997). Hepatic lipase (HL) (EC 3.1.1.3) is a heparin-releasable enzyme with alkaline optimum (pH = 9.5) and is associated with the exterior face of the plasma membrane of hepatocytes and liver sinusoidal cells (Assmann et al., 1973). In spite of the fact that these enzymes were described long ago, their exact role in physiological TAG mobilization and in the development of hepatic steatosis is still imperfectly understood and the results obtained are sometimes contradictory.

Also the intracellular localization of the TAG degradation process is still controversial. Previous reports suggested that TAG breakdown is associated with endoplasmic reticulum (Lehner et al. 1999; Dolinsky et al., 2004). An alternative to this concept was published recently by Singh et al. (2009), who described a previously unknown function for autophagy in degradation of intracellular lipid droplets – macrolipophagy. According to their report, the degradation of lipid droplets occurs in the cytoplasm, particularly in autophagolysosomes. The lysosomal degradation pathway, autophagy, is the main physiological pathway for the degradation of intracellular macromolecules such as proteins, organelles, etc., and provides metabolic substrates during the period of starvation. Very little information is available concerning the role of macrolipophagy in NAFLD and associated disorders.

The aim of this study was to determine the role of three liver lipases (TGH, HL, LAL) under different physiological situations (fasted vs. fed animals) and after different dietary manipulations (high-sucrose or high-fat diet) with respect to their possible role in the degradation of intracellular TAG in the liver.

Material and Methods

Animals and experimental protocol

Male Wistar rats were kept in a temperature-controlled room at 12:12-h light-dark cycle. Animals had free

access to drinking water and diet if not stated otherwise. All experiments were performed in agreement with the Animal Protection Law of the Czech Republic 311/1997, which is in compliance with European Community Council recommendations for the use of laboratory animals 86/609/ECC, and were approved by the ethical committee of the Institute of Clinical and Experimental Medicine.

Starting at age of 3 months (b. wt. 300 ± 20 g), all animals were fed either high-sucrose diet (HSD: 70 cal % as sucrose; 20 cal % as protein, 10 cal % as fat), high-fat diet (HFD: 70 cal % as saturated fat, 20 cal % as protein, 10 cal % as carbohydrate) or standard laboratory chow diet (SD) for 2 weeks (N = 14 animals per group). The groups labelled “fed” had free access to the diet until decapitation (10–11 a.m.), the groups designated as “fasted” were deprived of food for the last 24 h (N = 7 animals in each subgroup). A separate experiment was designed to assess the contribution of Kupffer cells to the lysosomal lipase activity determined in whole liver homogenate. The rats were fed SD or HFD according to the above-described protocol. Kupffer cells were depleted using gadolinium chloride (GdCl_3) as described previously (Krohn et al., 2009). GdCl_3 was applied i.v. in three doses (10 mg/kg each dose) 48 h, 24 h and 2 h prior to the beginning of the experiment.

Preparation of subcellular fractions

Lysosomes and phagolysosomes represent a heterogeneous population of organelles sedimenting in a wide range of relative centrifugation force. Active (auto)phagolysosomes have a lower density than the small, inactive lysosomes, allowing their separation by differential centrifugation (Seglen and Solheim, 1985). Twenty-percent (wt/vol) homogenate was prepared by homogenization of liver tissue in 0.25 M sucrose; 0.001 M EDTA, pH = 7.4; heparin 7 IU/ml, 1 mM PMSF, leupeptin 10 $\mu\text{g}/\text{ml}$, aprotinin 10 $\mu\text{g}/\text{ml}$ by Teflon pestle homogenizer. Crude impurities were removed by brief centrifugation at 850 g. Fat cake and all traces of fat remaining on the tube walls were carefully removed in order to prevent contamination of the homogenate. An aliquot of the homogenate was kept at 4 °C until lipase assay (max. 2 h), the rest was centrifuged for 10 000 g 20 min 4 °C and the resulting pellet and supernatant were separated. The supernatant preferentially contains the less dense lysosomes with higher TAG content (“(auto)phagolysosomes”), the pellet is formed by more dense particles (“dense lysosomes”). The cytosolic fraction was obtained after centrifugation of the initial homogenate at 100 000 g.

Assay of triacylglycerol lipase activity using exogenous substrate

The use of exogenous substrate enables determination of maximal releasable enzyme activity using a standard amount of the substrate. The reaction medium for all lipase assays was prepared identically except for the buffer used. ^3H triolein (92.5 kBq) in toluene was

added to 100 μ M of cold triolein and 100 μ M lecithin in chloroform, and solvents were evaporated under a stream of nitrogen. Three-percent FFA-free BSA was dissolved in 0.1 M buffer (acetate buffer pH = 4.5 for LAL, TRIS buffer pH = 8.0 for TGH or glycine buffer pH = 9.5 for HL), 0.15M NaCl. The whole mixture was emulgated by sonication in Hielscher sonicator UP200S (Hielscher Ultrasonic GmbH, Teltow, Germany) 20 min continuously and incubated with the homogenate or subcellular fractions for 60 min at 30 °C in a shaking water bath. The released fatty acids were extracted according to Belfrage and Vaughan (1969) and counted for radioactivity.

Assay of triglyceride lipase activity using endogenous substrate

This approach takes advantage of the coordinated changes in the intracellular localization of LAL and its intracellular substrate. The liver homogenate was prepared as described above under iso-osmotic conditions that prevent disruption of lysosomes. The lysis of lysosomes was induced only after separation of fractions during the assay. Twenty-percent homogenate was mixed 1 : 1 with 0.2 M acetate buffer and incubated for 60 min in 30 °C in shaking water bath. The reaction mixture was extracted in chloroform-methanol and phases were separated by 1 M NaCl. Aliquots of lower chloroform phase were separated by TLC. The lipid subclasses (FFA, DAG) were visualized by Coomassie blue staining (Sigma-Aldrich, St. Louis, MO) and quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA). Palmitic acid, 1,2-dioleoylglycerol and triolein of known concentrations were used as standards.

Real-time RT-PCR

The samples of liver tissue were dissected immediately after decapitation and frozen in liquid nitrogen. Total RNA was extracted from tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA) according to a standard protocol as described previously (Brinkhof et al., 2006). A DNAase step was included to avoid possible DNA contamination. A standard amount of total RNA (1600 ng) was used to synthesize first-strand cDNA (High Capacity RNA-to-cDNA kit, Applied Biosystems, Foster City, CA). No template control was included to test for contamination. RT-PCR amplification mixtures (25 μ l) contained 1 μ l template cDNA, SYBR Green master mix buffer (Quanti-Tect, Qiagen, Hilden) and 400 nM (10 pmol/reaction) forward and reverse primers. Reactions were run in an Applera 7300H Fast Real-Time PCR detector (Applied Biosystems). The results were analysed by SDS software vs2.3 (Applied Biosystems). *DGAT1* primers were designed using Primer3 software (left primer: TGATGGCTCAGGTCCCACTG; right primer: GTTAGGGAGGCTGGCCTTTG).

Incubation of liver slices in vitro

Production of β -hydroxybutyrate from liver slices *in vitro* was measured in the absence of exogenous FFA.

Liver slices (width approx. 1 mm) were quickly dissected and incubated for 1 h in Krebs Ringer bicarbonate buffer (Sigma-Aldrich) with 5 mmol/l glucose, 2% bovine serum albumin, gaseous phase 95% O₂ and 5% CO₂. All incubations were carried out at 37 °C in sealed vials in a shaking water bath. The aliquots of the incubation medium were stored frozen until further analysis.

Extraction, separation and immunodetection of LC3

Twenty-percent liver homogenate was prepared as described above and kept frozen at -50 °C until analysis. The samples were lysed by 2% SDS at 100 °C and the proteins were immediately separated by SDS-PAGE (15% gel). LC3 in the lysates was recognized using rabbit polyclonal to LC3A/B (Abcam, Cambridge, UK). LC3-I and LC3-II were distinguished by difference in molecular weight (18 and 16 kDa, respectively).

Biochemical analysis

The TAG content in the liver was determined in chloroform extract after the extraction according to Folch et al. (1957). FFA, insulin, triglyceride and glucose serum content and β -hydroxybutyrate production were determined using commercially available kits (FFA: FFA half micro test, Roche Diagnostics GmbH, Mannheim, Germany; triglycerides and glucose: Pliva-Lachema, Brno, Czech Republic; glucuronidase: Sigma-Aldrich; insulin: Mercodia, Uppsala, Sweden; β -hydroxybutyrate: Ran-But, Randox Laboratories Limited, Country Antrim, UK).

Chemicals

All materials were reagent grade. ³H-triolein was purchased from Amersham Biosciences (Amersham, Little Chalfont, UK), FFA-free bovine serum albumin (fraction V) was purchased from Serva (Heidelberg, Germany), palmitic acid and triolein from Fluka (Buchs, Switzerland), all other chemicals were purchased from Sigma Czech Republic (Prague, Czech Republic).

Statistical analysis

Data are presented as mean $\pm$ S.E.M. Statistical analysis was performed using Kruskal-Wallis test with multiple comparisons (N = 7). Differences were considered statistically significant at the level of P < 0.05. The correlations between LAL activity and hepatic TAG content or LAL activity and serum β -hydroxybutyrate concentration were evaluated using Spearman's correlation coefficient.

Results

Characteristics of experimental groups

The animals fed the SD, HSD or HFD did not differ in their final body weight, but the relative weight of epididymal fat pads was higher in HSD and HFD groups. Both diets induced fasting hyperinsulinaemia, but the

Table 1. Characteristics of experimental groups

	SD		HSD		HFD	
	fasted	fed	fasted	fed	fasted	fed
weight (g)	-	290 ± 10	-	295 ± 15	-	299 ± 12
adiposity index	1.00 ± 0.04	1.24 ± 0.04*	1.40 ± 0.05 ^x			
glycaemia (mmol/l)	4.60 ± 0.10	7.90 ± 0.30	4.50 ± 0.05	8.90 ± 0.10	5.40 ± 0.05 ^{&}	7.80 ± 0.10
insulinaemia (pmol/l)	56 ± 15	135 ± 21	125 ± 10 [#]	281 ± 32 [@]	115 ± 12 ^{&}	127 ± 18
serum TAG (mmol/l)	0.70 ± 0.05	1.10 ± 0.08	1.00 ± 0.10 [#]	1.60 ± 0.10 [@]	0.65 ± 0.08	0.85 ± 0.09 ⁺
serum FFA (mmol/l)	0.70 ± 0.05	0.40 ± 0.02	1.00 ± 0.09 [#]	1.30 ± 0.10 [@]	0.60 ± 0.08	0.45 ± 0.07
serum β -hydroxybutyrate (μ mol/l)	1.67 ± 0.05	0.05 ± 0.01	2.47 ± 0.14 [#]	0.07 ± 0.01	3.20 ± 0.25 ^{&}	0.28 ± 0.05 ⁺

Data are given as mean $\pm$ S.E.M., N = 7. The adiposity index is expressed as the relative weight of epididymal adipose tissue per 100 g of total body weight. * SD vs. HSD $P < 0.05$; ^x SD vs. HFD $P < 0.05$; [#] HSD fasted vs. SD fasted $P < 0.05$; [&] HFD fasted vs. SD fasted $P < 0.05$; [@] HSD fed vs. SD fed $P < 0.05$; ⁺ HFD fed vs. SD fed $P < 0.05$.

elevated fasting glycaemia was found only in the HFD group. The effect of HSD and HFD on serum TAG levels was the opposite – HSD increased fed triglyceridaemia by 45 % compared with standard diet, while HFD decreased the serum TAG content by 25 %. The changes in serum FFA content followed a similar trend as TAG. Both diets significantly increased the ketogenesis in fasting, but only HFD led to increased production of

β -hydroxybutyrate in the fed state. The data are shown in Table 1.

Triacylglycerol content in the liver

Both diets resulted in TAG accumulation in the liver. The effect of HSD was less pronounced (fasted +60 %, fed +37 %) compared with HFD (fasted +258 %, fed +434 %). In SD and HFD groups, the liver TAG content was higher in fasted compared with fed animals. In the HFD group this relationship was reversed, the TAG content was higher in fed than in fasted liver (Table 2).

Table 2. Liver triacylglycerol content

diet	fasted (μ mol/g)	fed (μ mol/g)
SD	4.1 $\pm$ 0.8	3.2 $\pm$ 0.3
HSD	6.6 $\pm$ 0.6 [#]	4.4 $\pm$ 0.2 ^{•,@}
HFD	14.7 $\pm$ 1.1 ^{&}	17.1 $\pm$ 0.4 ^{•,+}

Data are expressed in μ mol triacylglycerol/g wet weight as mean $\pm$ S.E.M., N = 7. [•] fasted vs. fed $P < 0.05$; [#] HSD fasted vs. SD fasted $P < 0.05$; [&] HFD fasted vs. SD fasted $P < 0.05$; [@] HSD fed vs. SD fed $P < 0.05$; ⁺ HFD fed vs. SD fed $P < 0.05$.

The effect of HSD and HFD on lipase activities in liver homogenate: exogenous substrate

In the liver homogenate, we were able to distinguish three lipolytic activities with distinct pH optimum *in vitro* using exogenous triolein substrate: TGH (pH = 8.0), HL (pH = 9.5) and LAL (pH = 4.5) (Fig. 1). TGH was responsible for production of approx. 10 % of the

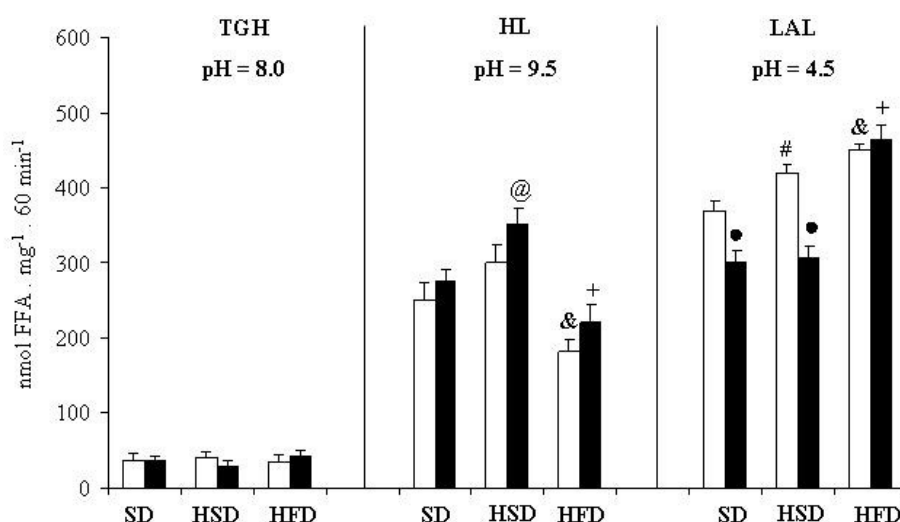


Fig. 1. Liver lipase activities measured as the release of fatty acids from exogenous substrate: the effect of HSD and HFD. Four-percent liver homogenate was incubated in appropriate buffer with 100 μ M cold triolein labelled by 3 H-triolein and the release of 3 H-FFA was determined. LAL activity was measured at pH 4.5 (0.1 M acetate buffer); TGH activity was measured at pH = 8.0 (0.1 M Tris buffer); HL activity was measured at pH = 9.5 (0.1 M glycine-HCl buffer). Values represent means $\pm$ S.E.M. of 7 animals. [•] fasted vs. fed $P < 0.05$; [#] HSD fasted vs. SD fasted $P < 0.05$; [@] HSD fed vs. SD fed $P < 0.05$; [&] HFD fasted vs. SD fasted $P < 0.05$; ⁺ HFD fed vs. SD fed $P < 0.05$. $\square$ fasted animals $\blacksquare$ fed animals.

sum of FFA released by all three lipases and its activity was not affected by any dietary manipulation or by the fasted/fed state. HL activity accounted for approx. 40 % of total FFA production. HSD increased the activity of this enzyme (HL) only in fed animals, while HFD diet led to a significant decrease of its activity independently of the nutritional status. LAL exhibited the highest activity among all three lipases tested. On SD, the activity of LAL in the homogenate was elevated in fasting and significantly lower in fed animals. Both diets increased the activity of LAL in fasting, but only HFD resulted in the elevation of LAL activity in the fed animals. In the HFD group the difference between fasted and fed state was blunted.

We determined the correlation between lipase activities and hepatic TAG content (possible source of substrate) and between lipase activities and serum β -hydroxybutyrate (possible end product of intrahepatic lipid metabolism). The LAL activity strongly correlated with ketogenesis ($r = 0.86$; $P < 0.01$) and with hepatic TAG content (fed: $r = 0.97$; $P < 0.001$; fasted: $r = 0.65$; $P < 0.05$). We found no correlation in the case of TGH and HL (data not shown).

The effect of HFD on lipase activities in liver homogenate: endogenous substrate

A separate set of experiments was designed in order to identify the roles of HL, TGH and LAL in the breakdown of endogenous TAG stores. Animals were fed either SD or HFD and production of FFA and DAG from liver homogenate at pH = 4.4; 8.0 and 9.5 during 60 min incubation was determined without any addition of exogenous substrate. These experimental conditions en-

sured that intracellular TAG were the only available source of substrate. We found no detectable amount of fatty acids released at pH = 8.0 and 9.5 (data not shown). The results obtained at pH = 4.5 are shown in Fig. 2. We were able to identify two main metabolites – FFA and DAG. In the SD group, the LAL activity pattern was similar as those observed with the exogenous substrate – LAL was activated in fasting and significantly less active in fed animals. In the HFD group, the fasting LAL activity was comparable with those in the SD group but in contrast to the latter, the LAL activity in the fed state remained elevated. The net production of DAG during incubation was found only in the HFD group.

The effect of HSD and HFD on lysosomal lipase intracellular distribution

Within the cell, LAL can degrade the substrate only when it co-localizes with the substrate (TAG droplets) in activated (i.e. acidified) (auto)phagolysosomes. Centrifugation of the liver homogenate at 10 000 g allowed us to separate the fraction containing dense lysosomes and the fraction containing (auto)phagolysosomes. The enzyme activity in individual fractions was determined with a standard amount of ^3H -triolein. We found that the type of the diet significantly affects the portion of LAL activity detected in the (auto)phagolysosomal fraction (Fig 3A). In the SD group, LAL activity in this fraction is significantly higher in fasting compared with fed state. In the HSD-fed group, this pattern of LAL activity regulation (up-regulation in fasting, down-regulation in the fed state) is preserved with an elevation found in fasted animals. HFD led to a significant increase of LAL activity, and no difference between fasted and fed animals

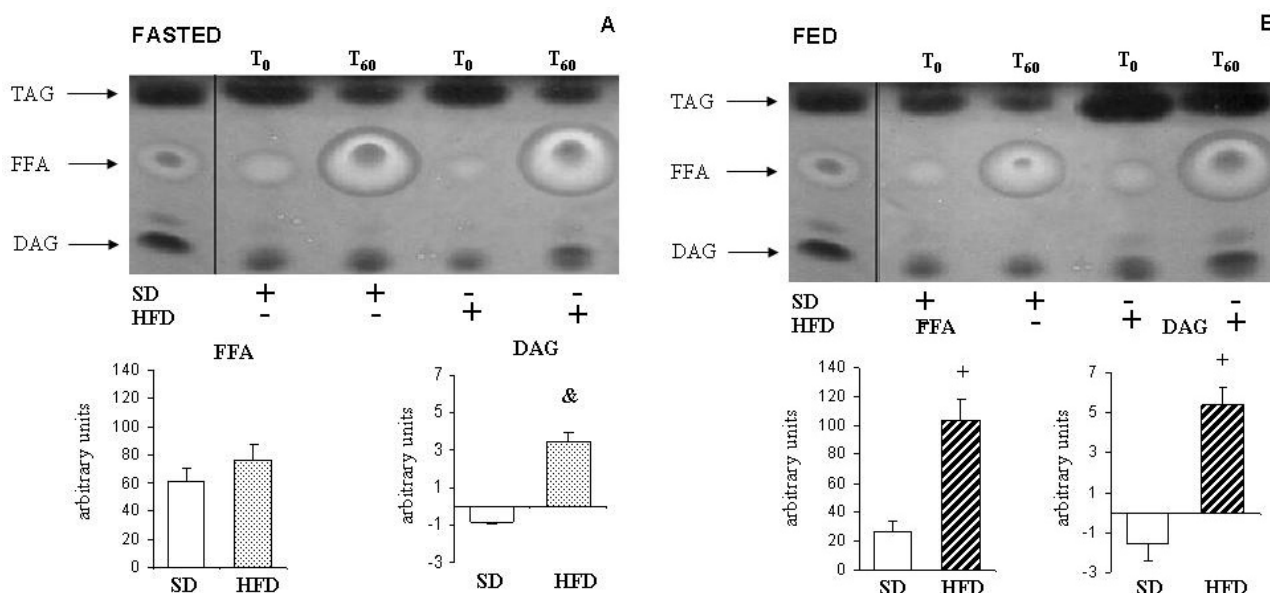


Fig. 2. Production of fatty acids and diacylglycerol from endogenous substrate in fasted (A) and fed (B) state

A typical result of TLC separation is shown in the upper part of the figure. Ten-percent liver homogenate was incubated for 60 min at pH 4.5 and the released FFA and DAG were determined by TLC and visualized by Coomassie blue staining. T_0 : state at the beginning of incubation, T_{60} : state at the end. Bars represent the difference $T_{60} - T_0$. Values represent means $\pm$ S.E.M. of 7 animals. & HFD fasted vs. SD fasted $P < 0.05$; + HFD fed vs. SD fed $P < 0.05$.

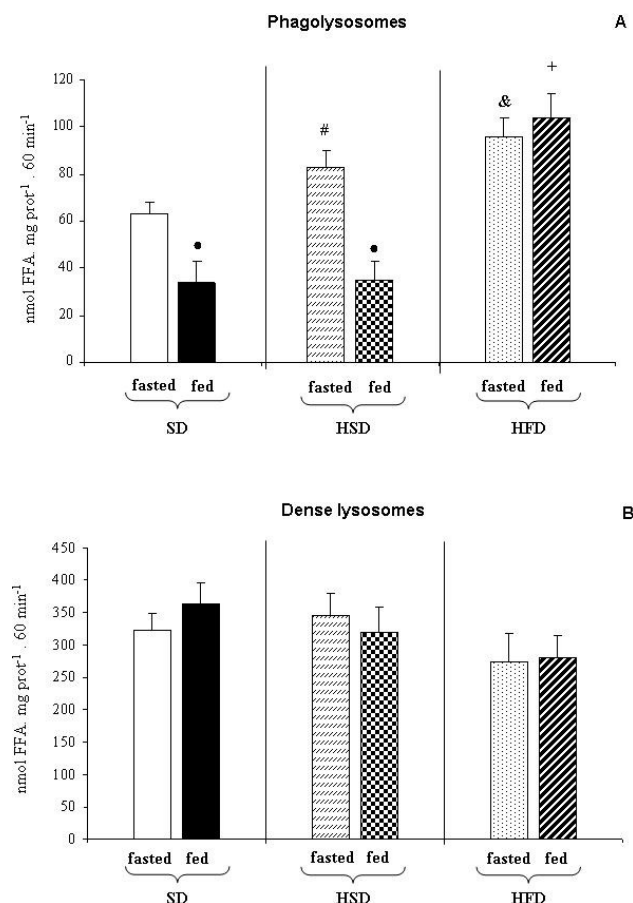


Fig. 3. Effect of HSD and HFD on the distribution of LAL activity between fractions of (auto)phagolysosomes (A) and dense lysosomes (B) prepared from rat liver homogenates

The subcellular fractions prepared from fresh liver homogenate were incubated with emulgated ³H-triolein and LAL activity was measured as the release of fatty acids at pH = 4.5. Values represent means ± S.E.M. of 7 animals. * fed vs. fasted $P < 0.05$; # HSD fasted vs. SD fasted $P < 0.05$; & HFD fasted vs. SD fasted $P < 0.05$; + HFD fed vs. SD fed $P < 0.05$.

was found. The changes in (auto)phagolysosomal LAL activity were partly mirrored by the corresponding fluctuations in the fraction of dense lysosomes (Fig 3B).

The effect of HSD and HFD on the intensity of autophagy

The ratio of lipidated, membrane-bound (LC3-II) to the cytosolic (LC3-I) form of LC3 protein is considered to be an indicator of the intensity of autophagy. As shown in Fig. 4, the LC3-II/LC3-I ratio was elevated in the SD group after starvation and low in fed condition. HSD had no significant effect either on the LC3-II amount or on the LC3-II/LC3-I ratio. The HFD diet affected LC-II formation in fed and starved animals differently. In the fed animals, HFD administration resulted in approximately three-fold elevation of the LC3-II/LC3-I ratio compared with the SD group. LC3-II formation in fasting was not affected by the diet. Consequent-

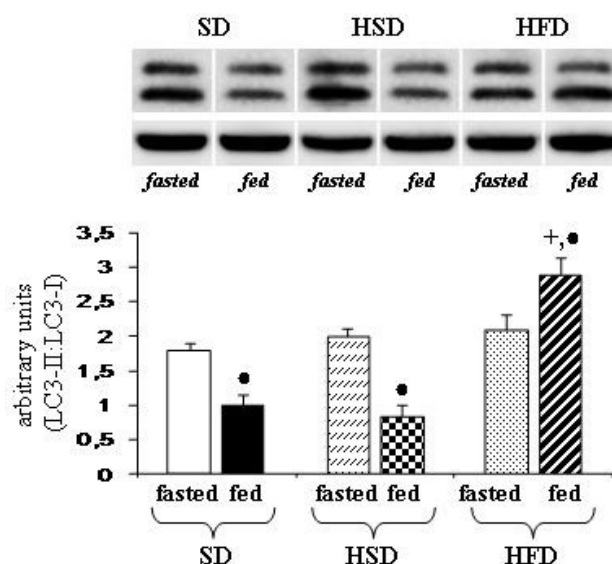


Fig. 4. Effect of HSD and HFD on autophagy in the liver. The intensity of autophagy was determined according to the LC3-II/LC3-I ratio in phagolysosomal fraction. Representative Western blots are shown above the graph. Values represent means ± S.E.M. of 7 animals. * fed vs. fasted $P < 0.05$; + HFD fed vs. SD fed $P < 0.05$.

ly, the effect of the prandial status was completely reversed by high-fat feeding being higher in the fed than in the fasted animals.

The effect of HSD and HFD on the release of lysosomal enzymes into the cytosol

Compared with SD fed animals, an elevated LAL (Fig. 5A) and glucuronidase (Fig. 5B) activity was found in the cytosol of both fasted and fed animals in the HFD group. HSD had no effect. This finding may indicate increased lysosomal fragility in the HFD group.

The effect of HSD and HFD on FFA esterification

The expression of *Dgat1* specific mRNA was determined by RT-PCR (Fig. 6). This enzyme catalyses the final step of TAG biosynthesis, i.e. translocation of the acyl moiety to DAG. HSD increased *Dgat1* expression both in fasted and fed animals. HFD led to significant up-regulation of *Dgat1* expression in the fed animals, but we found no differences between the HFD and SD groups in fasting.

The effect of HFD and HSD on ketogenesis in vitro

Liver slices harvested from the fasted animals from both HSD and HFD groups exhibited significantly higher β -hydroxybutyrate production into incubation medium as compared to SD-fast rats (HSD < HFD). When liver slices from fed animals were used for the incubation, ketone body production was potentiated only by HFD (Fig. 7). Our results confirmed that ketogenesis mirrors changes in the LAL activity, i.e. the nutritional

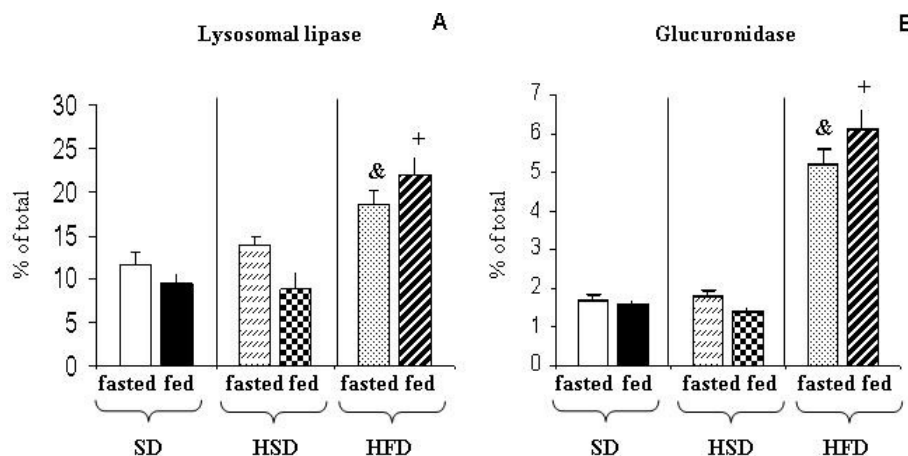


Fig. 5. Effect of HSD and HFD on the release of lysosomal enzymes into the cytosol

The LAL activity (A) and the glucuronidase activity (B) were determined in freshly prepared cytosolic fraction. The results are expressed as % of total enzyme activity released from homogenate used for preparation of the cytosolic fraction. Values represent means $\pm$ S.E.M. of 7 animals. & HFD fasted vs. SD fasted $P < 0.05$; + HFD fed vs. SD fed $P < 0.05$.

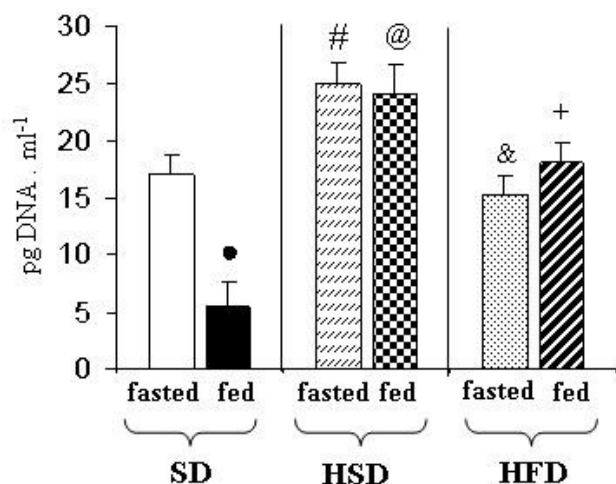


Fig. 6. Effect of HSD and HFD on *Dgat1* expression

Dgat1 mRNA expression was determined by RT-PCR. Values represent means $\pm$ S.E.M. of 7 animals. • fed vs. fasted $P < 0.05$; # HSD fasted vs. SD fasted $P < 0.05$; @ HSD fed vs. SD fed $P < 0.05$; & HFD fasted vs. SD fasted $P < 0.05$; + HFD fed vs. SD fed $P < 0.05$.

status-dependent regulation (stimulation in fasting/depression in the fed state) and the stimulatory effect of HFD.

The effect of gadolinium chloride treatment on lysosomal lipase activity

Gadolinium chloride was applied *in vivo* in three doses immediately prior to the experiment in order to deplete resident liver macrophages (Kupffer cells). As shown in Fig. 8, depletion of Kupffer cells led to an approx. 25% decrease of LAL activity determined in whole liver homogenates (Fig. 8A). In contrast, in the phagolysosomal fraction, no significant changes in the LAL activity due to the gadolinium treatment were detected either in the SD or in the HFD group (Fig. 8B).

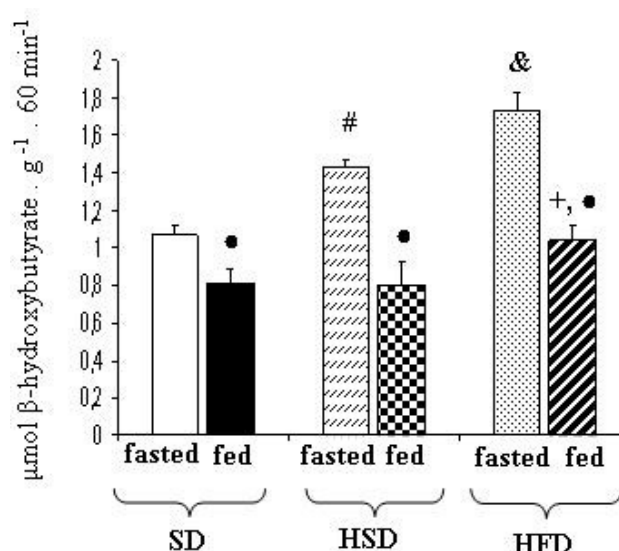


Fig. 7. Effect of HFD and HSD on the β -hydroxybutyrate production from liver slices *in vitro*

The liver slices harvested from fasted and fed animals of each group were incubated in KRB buffer for 60 min. Values represent means $\pm$ S.E.M. of 7 animals. • fed vs. fasted $P < 0.05$; # HSD fasted vs. SD fasted $P < 0.05$; & HFD fasted vs. SD fasted $P < 0.05$; + HFD fed vs. SD fed $P < 0.05$.

Discussion

Most fatty acids entering the liver are esterified to TAG and their further utilization depends on intracellular TAG breakdown. In this study, we provided evidence that lipolysis of endogenous TAG is actually stimulated in the liver by dietary induced steatosis. Our results indicate that LAL is involved in lipolysis and mobilization of the stored TAG in liver cells and that the autophagy-lysosomal pathway is involved in the degradation of intracellular TAG. We further demonstrated that HFD blunted the physiological down-regulation of both au-

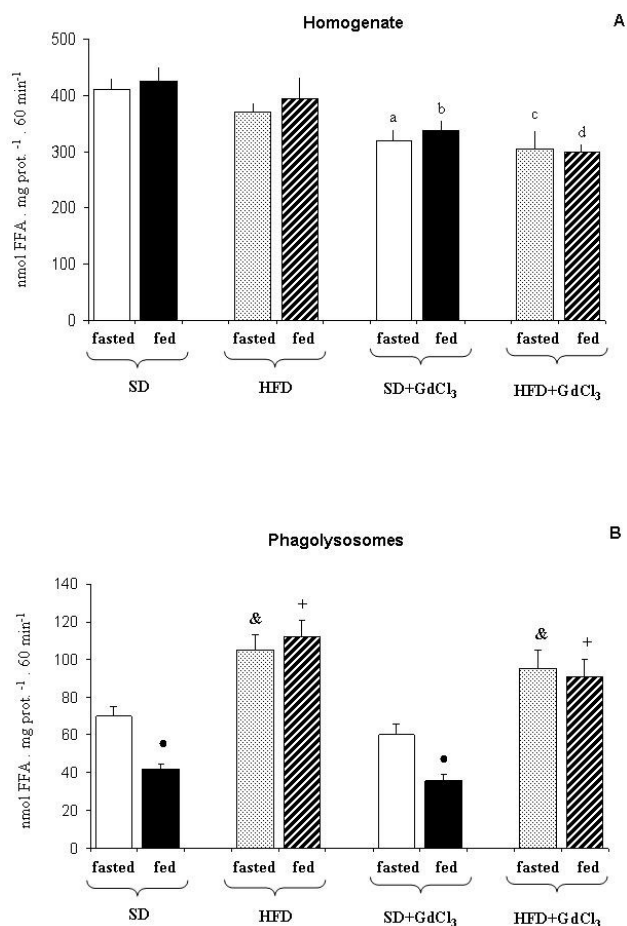


Fig. 8. Effect of Kupffer cell depletion on liver lysosomal lipase activity

The fresh 4% liver homogenate and phagolysosomal fraction prepared from the same homogenate were incubated with emulgated ³H-triolein, and LAL activity was measured as the release of fatty acids at pH = 4.5. Values represent means ± S.E.M. of 7 animals. * fed vs. fasted $P < 0.05$; & HFD fasted vs. SD fasted $P < 0.05$; + HFD fed vs. SD fed $P < 0.05$; ^a SD+GdCl₃ fasted vs. SD fasted $P < 0.05$; ^b SD+GdCl₃ fed vs. SD fed $P < 0.05$; ^c HFD+GdCl₃ fasted vs. HFD fasted $P < 0.05$; ^d HFD+GdCl₃ fed vs. HFD fed $P < 0.05$.

tophagosome formation and LAL activity in the fed state. Expression of Dgat-1 was increased in both HSD and HFD groups, which indicates enhanced esterification of fatty acids. Finally, stimulation of lysosomal activity in the HFD group resulted in lysosomal destabilization measured as the release of lysosomal enzymes into the cytosol.

HSD and HFD had profoundly different effects on the development of hepatic steatosis. HSD led to elevation of serum FFA and to increased accumulation of liver TAG in fasting. On the other hand, these processes were compensated by accentuated oxidation of FFA originating from endogenous TAG (ketogenesis) in fasting and by enhanced postprandial VLDL output from the liver (Yamamoto et al., 1987). Taken together, the result was only a mild elevation of liver TAG in the HSD

group. HFD actually led to lowering of TAG and FFA serum levels and this is what made questionable the increased uptake of FFA as the main cause of steatosis on this diet. Nevertheless, the hepatic TAG content rose in rats on HFD more than five times compared to SD and two or four times (fasted or fed animals, respectively) compared to the HSD group. In the light of these results, the only possible explanation of the significant accumulation of TAG in the liver and the normal TAG concentration in the serum in HFD-fed animals is the impaired output of VLDL. This effect of HFD has already been described by several authors (Francone et al., 1992; Oussadou et al. 1996).

As reported by Wiggins and Gibbons (1992), almost all FFA entering the hepatocyte are esterified and must be released from the TAG molecule prior to any utilization. It remains an open question which lipase(s) are involved in this process and whether manipulations leading to the accumulation of liver TAG influence its (their) activities.

It has been suggested that lipase mobilizing intracellularly stored TAG should be associated with endoplasmic reticulum (ER) in order to channel lipolytic products towards resynthesis to TAG at the site of VLDL assembly. These demands could be met by TGH with optimum at pH = 8 found nearly exclusively in the microsomal fraction. Nevertheless, TGH has high specific activity towards soluble short-chain triacylglycerol substrates and to esters, but much lower specificity towards insoluble TAG containing long-chain fatty acids – the specific activity of TGH towards tributyrin substrate was 240 $\mu\text{mol FFA/mg protein}$ and only 0.2 $\mu\text{mol FFA/mg protein}$ towards triolein substrate, i.e. 1000 times lower (Lehner et al., 1997). Recent results (Wei et al., 2007) documented that TGH may catalyse one of the important steps in the mobilization of lipids for lipoprotein assembly and secretion, but TGH activity has been reported to be associated preferentially with lipids found within the ER lumen (Gilham et al., 2003). This localization makes its contact with cytosolic lipid droplets rather complicated. Our results indicate that LAL is a dominant enzyme involved in the degradation of intracellular TAG stores. LAL was responsible for the lipolysis of a major part of the totally available exogenous substrate and this portion was further significantly increased in the HFD group. These findings are in accordance with those of Debeer et al. (1979), who measured the triacylglycerol lipase activity in liver homogenates from livers perfused with heparin prior to homogenization and found maximal activity at pH = 4.4. The essential role of LAL in the TAG hydrolysis was also demonstrated in LAL knock-out mice (*Lal*^{-/-}), which developed progressive hepatosplenomegaly and exhibited massive TAG accumulation in the liver (Du et al., 2001).

Production of fatty acids exclusively from an endogenous source of substrate (during incubation of 10% liver homogenate) was determined as this experimental setup might be closer to the real processes occurring *in vivo*. When using intracellular TAG as the exclusive

substrate for hydrolysis, the only lipolytic activity was detected at pH = 4.5. In contrast, the lipolytic activity towards endogenous substrate in pH range corresponding to TGH optimum (pH = 7–8) or HL (pH = 9.5) was very low. The activity of LAL in the (auto)phagolysosomal fraction positively correlates with the liver TAG content. Concomitantly occurring stimulation of lipolysis and accumulation of endogenous TAG after HFD administration seem to be contradictory. However, in hepatocytes a significant portion of FFA released from intracellular TAG (approx. 70%) is re-esterified (Gibbons et Wiggins, 1995). HFD impairs VLDL secretion (Francone et al., 1992) and most FFA re-enter the intracellular storage pool. The increased expression of Dgat-1, an enzyme catalysing the final step of triacylglycerol synthesis, in the HFD-fed group supports this idea. Thus, the increased lipolysis in the HFD group does not result in decreased liver TAG content but rather in higher TAG turnover.

As a lysosomal enzyme, LAL is synthesized in endoplasmic reticulum, transported into the trans-Golgi network and packed into vesicles termed primary lysosomes (Tanaka et al., 1990). Lysosomal enzymes can adopt the active form only after primary lysosomes fuse with autophagosomes or endosomes carrying the cargo destined for degradation and form (auto)phagolysosomes with acidic pH. Within the cell, most of LAL is present in inactive form. Singh et al. (2009) have recently found that lipid droplets in hepatocytes can enter the autophagic degradation pathway in the same manner as damaged proteins or organelles via formation of autophago(lipo)somes that further fuse with primary lysosomes. As the only known lysosomal enzyme with lipolytic activity is LAL and as it shares a similar regulatory pattern with autophagy (stimulation in response to starvation), we believe that our results are complementary to those of Singh et al. It is possible that the LAL activity determined in the (auto)phagolysosomal fraction quantitatively reflects the autophagosome formation.

LC3 protein is an important member of the autophagy metabolic pathway. During the formation of autophagosomes, the cytosolic form of LC3 protein (LC3-I) is conjugated to phosphatidylethanolamine (LC3-II). LC3-II is incorporated into the autophagosomal membrane and remains there until the stage of late autophagolysosome (Rubinshtein et al., 2009). The LC3-II/LC3-I ratio is considered to be an indicator of the intensity of autophagy. In the SD group, fasting condition was associated with the increase of LC3-II/LC3-I ratio (stimulated autophagy) while in the fed state this ratio decreased (depressed autophagy). In contrast, in the HFD group the LC3-II/LC3-I ratio in the fed state remained elevated and the intensity of autophagy estimated according to this parameter was stimulated and not prandially-dependent. Since part of LC3-II is processed by lysosomal hydrolases, the rise in LC3-II abundance may indicate either the increased rate of autophagosome formation or block in lysosomal degradation (Klionsky et al., 2007). However, concomitant stimula-

tion of lysosomal lipolysis makes the second possibility rather improbable. We suggest that increased expression of the LC3-II protein in HFD groups indicates higher intensity of autophagy.

It is important to note that Singh et al. (2009) reported data indicating that formation of autophagosomes carrying a lipid cargo may be decreased in mice fed HFD for 16 weeks. Apart from the difference in the duration of HFD administration, they measured the LC3-II content in lipid droplets (LD) and found an inhibitory effect of the diet only in starved animals, while in the fed ones the LC3-II content in the LD fraction was higher in HFD compared with the SD group. This finding, i.e. the stimulatory effect of HFD manifesting itself in the fed state, is in accordance with our results.

Our data indicate that the activation of lysosomes, i.e. increased formation of (auto)phagolysosomes, is associated with destabilization of the (auto)phagosomal membrane and with release of lysosomal enzymes into the cytosol. The lysosomes contain a number of enzymes, e.g. proteases (cathepsins) that are able to damage subcellular organelles such as mitochondria (Li et al., 2008). We propose the hypothesis that not steatosis itself but rather the accelerated TAG lipolysis/re-esterification cycle accompanied by lysosomal destabilization may be one of the causes of NAFLD-associated liver injury.

LAL is present in a wide variety of cell types including hepatocytes (Debeer et al., 1979) and resident macrophages (Kupffer cells) (Du et al., 2001). Unfortunately, determination of the LAL activity in whole liver homogenates does not allow the exact determination of its source. To confirm that the described findings are valid for hepatocytes we performed the following experiment based on two presumptions: 1. hepatocytes are the only mammalian cells capable of FFA conversion to ketone bodies and 2. intracellular TAG are the only endogenous source of FFA for ketogenesis (McGarry et al., 1980). *In vitro* ketogenesis in liver slices incubated in the absence of exogenous FFA thus reflects the intra-hepatocyte TAG breakdown. Our results confirmed that production of ketone bodies mirrors the changes in the LAL activity, i.e. the nutritional status-dependent regulation (stimulation in fasting/depression in the fed state) and the stimulatory effect of HFD. Another approach to the assessment of the Kupffer cells contribution to the LAL activity measured in homogenate is the employment of GdCl_3 , which allows acute depletion of liver resident macrophages. The total LAL activity in the liver of GdCl_3 -treated rats was 20–25 % lower compared with the untreated ones, but no significant effect was found in phagolysosomal fraction. Further, GdCl_3 treatment did not affect the nutritional status-dependent or dietary-induced changes of LAL activity. We conclude that these findings provide evidence that the changes in LAL activity reported in this study can be ascribed to changes occurring in hepatocytes.

In conclusion, we found that fat accumulation in the liver is associated with the increased lipolytic activity towards intracellular substrate and with higher produc-

tion of lipolytic products. LAL was identified as an important enzyme responsible for the breakdown of intracellular TAG. The increased LAL activity was associated with its translocation into the (auto)phagolysosomal fraction. Our data indicate that autophagy may represent the mechanism responsible for the transportation of the substrate (lipid droplets) to the site of degradation (autophagolysosomes). We further demonstrated that the increased lipolytic activity is accompanied by increased Dgat-1 expression in fatty liver, which may result in intensification of the lipolytic/re-esterification cycle in hepatocytes. Finally, our results showed that lysosomal activation is associated with destabilization of (auto)-phagolysosomes, which may contribute to the increased susceptibility of fatty liver to further stress factors.

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PŘÍLOHA VI

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Research Article

The Opposite Effects of High-Sucrose and High-Fat Diet on Fatty Acid Oxidation and Very Low Density Lipoprotein Secretion in Rat Model of Metabolic Syndrome

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Aims. To determine the effect of two different diets (high-sucrose (HS) and high-fat (HF)) on the main metabolic pathways potentially contributing to the development of steatosis: (1) activity of the liver lysosomal and heparin-releasable lipases; (2) fatty acid (FFA) oxidation; (3) FFA synthesis *de novo*; (4) VLDL output *in vivo* in a rat model of metabolic syndrome (MetS), hereditary hypertriglyceridemic (HHTg) rats fed HS or HF diets. **Results.** Both diets resulted in triacylglycerol (TAG) accumulation in the liver (HF > HS). The intracellular TAG lipolysis by lysosomal lipase was increased in both groups and positively correlated with the liver TAG content. Diet type significantly affected partitioning of intracellular TAG-derived fatty acids among FFA-utilizing metabolic pathways as HS feeding accentuated VLDL secretion and downregulated FFA oxidation while the HF diet had an entirely opposite effect. FFA *de novo* synthesis from glucose was significantly enhanced in the HS group (fed $\gg$ fasted) while being completely eradicated in the HF group. **Conclusions.** We found that in rats prone to the development of MetS associated diseases dietary-induced steatosis is not simply a result of impaired TAG degradation but that it depends on other mechanisms (elevated FFA synthesis or attenuated VLDL secretion) that are specific according to diet composition.

1. Introduction

Metabolic syndrome (MetS) also known as insulin resistance syndrome is characterized as a combination of cardiometabolic risk determinants including insulin resistance, glucose intolerance, dyslipidemia, nonalcoholic fatty liver disease, and hypertension [1] and is associated with a significantly increased probability of type 2 diabetes development [2]. The liver is partially susceptible to ectopic fat accumulation, one of the most important causal components of MetS, and nonalcoholic fatty liver disease (NAFLD) is now considered to be the hepatic manifestation of MetS.

Hepatic steatosis arises from imbalance in TAG acquisition and removal. The conventional explanation of hepatic triglyceride accumulation is that obesity and insulin resistance result in an increased release of FFAs from adipocytes.

Increased adipocyte mass and increased hydrolysis of triglycerides through enhanced activity of a hormone-sensitive lipase contributes to elevated plasma levels of FFAs. Up to date no specific regulation of FFA transport into hepatocytes has been described and hence it is supposed that the rate of hepatic FFA uptake is unregulated and therefore directly proportional to plasma FFA concentrations. Nevertheless detailed studies performed by Kalopissis and her coworkers showed that in fat-fed rats the cellular uptake of ^{14}C -oleate by hepatocytes *in vitro* is decreased despite significant TAG accumulation in the liver. Qualitatively this phenomenon was observed on different metabolic backgrounds (Wistar, Zucker lean, Zucker obese) and differs only in the extent of its manifestation [3–5]. These observations indicate that the regulation of liver triacylglycerol content is not merely a function of plasma FFA delivery alone but that other

intrahepatic mechanisms (i.e., regulation of intracellular TAG breakdown, partitioning of the FFA between oxidation and esterification, *de novo* fatty acid synthesis, regulation of TAG secretion) determine steatosis development.

Without any doubt dietary factors are one of the significant contributors to the NAFLD phenotype and dietary recommendations are a significant tool in current trends in health promotion. From this point of view a detailed understanding of the impact of different diets on the network of metabolic pathways involved in liver TAG metabolism is essentially necessary. Special attention should be given to the interaction of dietary composition with particular genetic/metabolic background.

Hereditary hypertriglyceridemic (HHTg) rats that were the subject of our study display a majority of the MetS symptoms including hypertriglyceridemia, impaired glucose tolerance, hyperinsulinemia, insulin resistance, and increased blood pressure (see Supplementary Material S1 available at doi:10.1155/2012/757205). This phenotype is manifested even without nutritional stimuli but high sucrose feeding aggravates these symptoms further [6]. The aim of the present study was to determine the effect of two different diets (high-sucrose and high-fat) on the main metabolic pathways potentially contributing to the development of steatosis specifically on genetic background that is particularly prone to the onset of diabetes symptoms. We focused on following metabolic processes: (1) mechanisms regulating intracellular TAG degradation in the liver specifically on the activities of liver lysosomal (LIPA; EC3.1.1.13) and heparin-releasable (HL; EC 3.1.1.3) lipases; (2) partitioning of the released FFA between oxidation and secretion as TAG; (3) on the FFA *de novo* synthesis.

2. Materials and Methods

2.1. Animals and Experimental Protocol. Male hereditary hypertriglyceridemic rats (HHTg) were kept in a temperature-controlled room at a 12:12-h light-dark cycle, the dark phase from 6 pm till 6 am. Animals had free access to drinking water and diet unless stated otherwise. The strain of HHTg rats was originally selected from Wistar strain rats in our laboratory [7]. All experiments were performed in agreement with the Animal Protection Law of the Czech Republic 311/1997 which is in compliance with European Community Council recommendations for the use of laboratory animals 86/609/ECC and were approved by the ethical committee of the Institute for Clinical and Experimental Medicine.

Starting at 3 months of age (b. wt. 281 ± 3 g), all animals were fed either a high sucrose diet (HS: 70 cal% as sucrose; 20 cal% as protein, 10 cal % as carbohydrate), a high-fat diet (HF: 70 cal% as saturated fat, 20 cal% as protein, 10 cal % as carbohydrate) or a standard laboratory chow diet (SD) for 4 weeks. All diets were isocaloric. (see Supplementary Material S2). Groups designed as “fed” had free access to the diet until termination and the groups designed as “fasted” were food deprived for the last 24 hours.

2.2. Determination of Fatty Acid Synthesis De Novo from Glucose in Liver Slices In Vitro. The fasted or fed rats were euthanized between 9–10 am and their liver slices (approx. 1 mm thickness, 150 ± 25 mg) were rapidly dissected. The tissues were incubated for 2 hours in a Krebs-Ringer bicarbonate buffer supplemented with 5 mmol/L unlabelled “cold” glucose, D-[U- ^{14}C] glucose (specific activity $20 \mu\text{Ci}/\text{mmol}$) and 2% bovine serum albumin, gaseous phase 95% O_2 and 5% CO_2 . All the incubations were carried out at 37°C in sealed vials using a shaking water bath. The estimation of the ^{14}C -glucose incorporation into total lipid content was carried out as described previously [8]. Briefly, liver slices were removed from incubation medium, rinsed in physiological solution, and immediately put into CH_3Cl . The pieces of tissue were dissolved using a teflon pestle homogeniser, methanol was added (CH_3Cl :methanol 2:1) and lipids were extracted at 4°C overnight according to Folch et al. [9]. Next day the residual tissue was removed and the clear extract was taken for further analysis. An aliquot was evaporated, reconstituted in scintillation liquid and its radioactivity was measured by scintillation counting.

To determine the site (glycerol versus acyl moiety) of glucose incorporated into neutral lipids, an aliquot of clear extract was evaporated and saponified in ethanolic 15% potassium hydroxide at 70°C . Saponification was terminated by adding 5.4 M H_2SO_4 . After cooling to room temperature the released fatty acids were extracted repeatedly into petroleum ether. The pooled petroleum ether fractions were evaporated, reconstituted in scintillation liquid and the radioactivity was measured by scintillation counting. The amount of radioactivity incorporated into the glycerol residue was calculated as the difference of total activity incorporated into neutral lipids and the petroleum ether fraction of the same aliquot.

2.3. Determination of the Metabolism of Intracellular TAG-Derived Fatty Acid in Liver Slices In Vitro. The labelling of cytoplasmic TAG *in vivo* was performed as described by Francone et al. [10]. The rats received an intravenous injection of $20 \mu\text{Ci}$ ^{14}C -palmitic acid complexed to 4% albumin under light ether anaesthesia. The animals were euthanised 90 min later and the preparation of liver slices was carried out as described above. For determination of ^{14}C -palmitic acid oxidation to CO_2 , the experiment was carried out in glass vials with central wells. The vials were capped with rubber stoppers and the reaction was terminated by addition of 0.5 mL of 0.5 M H_2SO_4 whereas strips of filter paper soaked with hyamine hydroxide were added to the central wells for collection of $^{14}\text{CO}_2$. TCA (tricarboxylic acid cycle) intermediate content was measured in the incubated liver slices after homogenisation by UltraTurax (IKA Werke, Staufen, Germany) in 150 mM NaCl. The homogenate was extracted into petroleum ether and radioactivity remaining in the water fraction was counted by scintillation counting. According to Kawamura and Kishimoto [11] this fraction represents mostly TCA cycle intermediates (>80%) and a minor part are amino acids derived from FFA via the TCA cycle (<20%). The ^{14}C -palmitic acid incorporation into secreted TAG was determined in a chloroform extract of

the incubation medium. The conversion of ^{14}C -palmitic acid into secreted water-soluble oxidation products (i.e., predominantly ketone bodies) was assessed according to the radioactivity remaining in the aqueous fraction of the incubation medium following the chlorophorm extraction.

2.4. Lipase Assay: Exogenous Substrate. Lipase activities were determined in 2% homogenates or subcellular fractions prepared from fresh tissue. 0.5 g of liver was homogenised in 2.5 mL of homogenisation buffer (0.25 M sucrose; 0.001 M EDTA pH = 7.4; heparin 7 IU/mL) on a teflon pestle homogeniser. The homogenate was passed through a nylon mesh, centrifuged briefly in order to remove crude impurities (800 g, 5 min, 4°C) and kept on ice until lipase assay was performed (max. 1 hour). The subcellular fractions were obtained by centrifugation at 10 000 g for 15 min.

The reaction medium for acid or alkaline lipase assay was prepared identically except for the buffer used. ^3H triolein in toluen was added to 88 mg (100 μM) of cold triolein and the solvent was evaporated under a nitrogen stream. 3% FFA free BSA was dissolved in 4 mL 0.1 M buffer (acetate buffer pH = 4.5 for lysosomal lipase or glycine buffer pH = 9.5 for hepatic lipase) with 150 mM NaCl and 0.05% Triton X-100. The whole mixture was emulsified on a Hielsler sonicator UP200S, amplitude 1 on an ice/water bath 3 min continuously. 40 μL of the homogenate or subcellular fractions were incubated for 60 min at 30°C with the 160 μL of reaction medium in a shaking water bath. After termination of the reaction, the released fatty acids were extracted according to Belfrage and Vaughan [12] and counted for radioactivity.

2.5. Lipase Assay: Endogenous Substrate. This approach takes advantage of the coordinated changes in the intracellular localisation of lysosomal lipase and its intracellular substrate. The liver homogenate and subcellular fractions were prepared as described above under iso-osmotic conditions that prevent lysosome disruption. The lysis of lysosomes was induced only after fraction separations during the assay. 20% homogenate was mixed 1:1 with 0.2 M acetate buffer pH = 4.5 and incubated for 60 min at 30°C in a shaking water bath. The reaction mixture was extracted into chloroform-methanol and phases were separated by 1 M NaCl. An aliquot of the chlorophorm phase was evaporated and 100 μL of Krebs-Ringer phosphate buffer (pH = 7.6) containing 6% FFA-free BSA was added. The tubes were incubated in a shaking incubator at 37°C for 2 hours. FFA concentration in final KRF/BSA solution was measured using commercially available kit. In order to check the efficiency of FFA solubilisation the emptied tubes were washed with fresh KRB + 6% BSA and then 100 μL of chlorophorm was added. An aliquot was separated by TLC but no substantial traces of FFA were detected.

2.6. Determination of TAG Entry Rate into Plasma. The TAG entry rate into circulation was estimated according to Otway and Robinson [13]. Briefly, rats were starved

for 24 hrs and then given 1 mL of 10% Triton WR-1339 in 0.9% NaCl or 0.9% NaCl alone intravenously via the tail vein under the light ether anaesthesia. Triton WR-1339 is an alkaryl polyether anionic detergent that blocks the removal of intravascular $d < 1.006$ g/mL lipoproteins. The animals were sacrificed 90 min after receiving either Triton or 0.9% NaCl by exsanguination via aortic puncture. It has been demonstrated that the concentration of triglyceride in plasma is linear up to 3 hrs after the intravenous injection of WR 1339 hence the time point 90 min after application lies within the linear range. Triglyceride entry (secretion) rate into the plasma was calculated from the following formula:

$\text{TAG entry rate } (\mu\text{mol. } 100 \text{ g b. wt.}^{-1} \cdot \text{hr}^{-1}) = [(T_{90} - T_0)/1.5] \times V \times (W/100)$, where T_0 is plasma TAG concentration ($\mu\text{mol/mL}$) before Triton administration, T_{90} is plasma TAG concentration ($\mu\text{mol/mL}$) at the end of the study, V is plasma volume (mL) and W is body weight. Plasma volume was determined as 3.86 mL/100 g body wt [14].

2.7. Triglyceride Content in Tissues. Lipids were extracted from 1 g of fresh tissue homogenised in 1 mL of H_2O . 0.2 mL of the homogenate was extracted in 15 mL of 2:1 chloroform:methanol for 24 hours. The organic and aqueous phases were separated by adding of 6 mL KH_2PO_4 and centrifugation at 3000 rpm for 20 min. 1 mL of the organic phase was completely dried, resuspended in 100 μL of isopropylalcohol and 10 μL were used for the analyses. The triglyceride concentration in this aliquot was determined using a commercially available kit (Pliva-Lachema Diagnostics, Czech Republic).

2.8. Biochemical Analyses. Nonesterified fatty acids, insulin, triglyceride and glucose serum content and β -hydroxybutyrate were determined using commercially available kits (FFA: FFA half microtest, Roche Diagnostics, GmbH Germany; triglycerides: Pliva-Lachema Diagnostics, Czech Republic; glucose: Pliva-Lachema Diagnostics, Czech Republic; insulin: Mercodia, Sweden; β -hydroxybutyrate: RanBut, RANDOX, UK).

2.9. Chemicals. All materials were reagent grade. ^{14}C -palmitic acid and ^3H -triolein were purchased from Amersham, D-[U- ^{14}C -] glucose was purchased from UUVVR, Prague. FFA free bovine serum albumin (fraction V) was purchased from Serva, palmitic acid and triolein from Fluka, all other chemicals were purchased from Sigma Czech Republic.

2.10. Statistical Analyses. Data are presented as mean $\pm$ SEM of multiple determinations. Statistical analyses were performed using ANOVA and the Tukey-Kramer multiple comparisons test ($n = 5-7$). Differences were considered statistically significant at the level of $P < 0.05$. Pearson's correlation coefficients were calculated to assess possible relationships between lipase activities and liver triglyceride content and lipase activities and ketone bodies production.

3. Results

3.1. Characteristics of Experimental Groups. The weight of the animals on HS and HF diet (HF > HS) rose rapidly during the first two weeks, then the rate of the weight gain significantly decreased. All tested diets were isocaloric and the more rapid increase in body weight in the HF and HS groups reflects the higher food intake during the first two weeks of diet administration (see Supplementary Material S3). In animals on standard diet this parameter was even throughout the whole experiment (Figure 1). During the last two weeks of diet administration the food intake and the rate of the weight gain in all three groups was comparable. Both final body weight and the weight of epididymal fat pads was higher in HS and HF diet fed animals in comparison with the SD group (HF > HS > SD) (Table 1). The fasting glycaemia and insulinemia were increased only in HF group. The effect of HS and HF diets on serum triacylglycerol levels was the opposite—HS diet significantly increased both fasted and fed triglyceridemia compared to the standard diet while HF diets has slight hypolipidemic effect (fed s-TAG decreased by 30%, $P < 0.05$). The changes in serum FFA content followed a similar trend. Both diets significantly increased the ketogenesis in fasting (HF > HS) but only the HF diet led to the increased production of ketone bodies in a fed state. The hepatic triacylglycerol content in fasting animals was increased by 105% and by 280% after HS and HF feeding, respectively. On standard diet, the liver triacylglycerol content was the same in the fed and fasted states but in the HS group liver TAG content in fed state was significantly lower than in fasting. In contrast, in the HF group the trend was the opposite, hepatic triacylglycerol content being actually higher in fasted than fed animals (Table 2).

3.2. FFA Synthesis De Novo. The liver of rats fed both HS and HF diets contained more triglyceride compared to the SD group. In order to assess the contribution of *de novo* FFA synthesis from glucose to the elevated TAG content, we incubated liver slices prepared from the liver of rats fed each particular diet in the presence of ^{14}C -labeled glucose without exogenous FFA and measured the incorporation of glucose into total lipids, into the glycerol part of TAG molecules (i.e., glucose esterification) and into the acyl moiety of TAG molecule (i.e., FFA synthesis *de novo*). As shown in Figure 2, FFA *de novo* synthesis was significantly enhanced in the HS group (fed $\gg$ fasted) while being completely eradicated in the HF group.

3.3. The Utilisation of Intracellular TAG-Derived Fatty Acids in Liver Slices In Vitro. In order to estimate the accessibility of FFA derived from intracellular liver TAG for further metabolic utilisation we prelabelled cytosolic TAG by the injection ^{14}C -palmitic acid *in vivo* and measured the radioactivity incorporation into TCA intermediates, CO_2 and ketone bodies (FFA oxidation) and into TAG secreted into medium (VLDL secretion) in liver slices *in vitro* 90 min later. As reported by Francone et al. [15], nearly 90% of

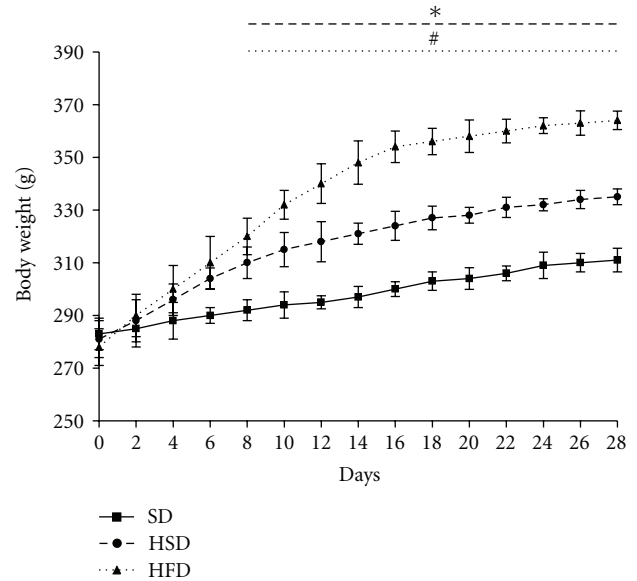


FIGURE 1: Evolution of body weight in SD, HSD, or HFD fed rats. Body weight was measured three times a week from the beginning of the feeding period till the end of week 4. Mean values $\pm$ s.e. obtained in each group are represented. *Significant difference between SD and HSD with $P < 0.05$ or more; #Significant difference between SD and HSD with $P < 0.05$ or less.

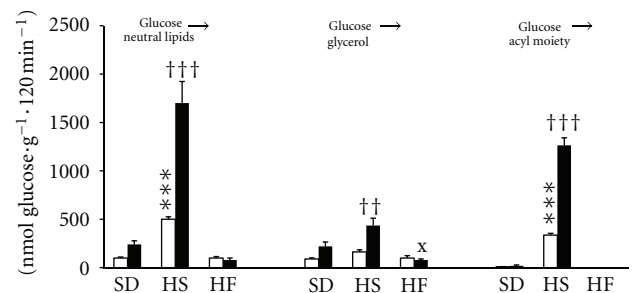


FIGURE 2: Glucose incorporation into lipids in liver slices *in vitro*. The utilisation of glucose for esterification and *de novo* fatty acid synthesis was determined in the same sample as glucose incorporation into total lipids (described in Section 2). open bars = fasted animals; closed bars = fed animals. All data are means SEM, $n = 6$ individual incubations for each bar. *** $P < 0.001$ HS-fasted versus SD-fasted; †† $P < 0.01$, ††† $P < 0.001$ HS-fed versus SD-fed; * $P < 0.05$ HF-fed versus SD-fed.

the radioactive label is found in cytosolic TAG 90 min after the radioactivity administration into the venous blood, so we expect that under this experimental setting most of the FFA incorporated into oxidation products or VLDL had to be released from intracellular TAG by lipolysis. In SD fed animals, both oxidation and VLDL production was lower in liver slices prepared from fed animals compared with those from the fasted ones. The administration of HSD resulted in a significant attenuation of TCA intermediates and CO_2 production but the fasting ketogenesis was somewhat accentuated in this group ($P < 0.05$) compared to SD fed animals. The TAG secretion was significantly higher in HS fed rats than in the two other groups and the prandial

TABLE 1: Characteristics of experimental groups.

		Prediet values	SD (post-diet)	HS (post-diet)	HF (post-diet)	P HS versus SD	P HF versus SD
Body weight (g)		281 ± 3	311 ± 4***	335 ± 15***	364 ± 10***	<0.05	<0.001
Epididymal fat (g)		—	2.8 ± 0.2	3.8 ± 0.3*	5.5 ± 0.5***	<0.05	<0.001
s-glucose (mmol/L)	Fasted	4.5 ± 0.2	4.6 ± 0.1	4.5 ± 0.05	5.4 ± 0.05*	N.S.	<0.05
	Fed	6.2 ± 0.2	6 ± 0.1	11.4 ± 0.1***	7.6 ± 0.4*	<0.001	<0.05
s-insulin (pmol/L)	Fasted	126 ± 18	135 ± 21	155 ± 12	204 ± 20*	N.S.	<0.05
	Fed	155 ± 20	158 ± 15	342 ± 29***	127 ± 18	<0.001	N.S.
s-TAG (mmol/L)	Fasted	1.3 ± 0.25	1.5 ± 0.3	4.9 ± 0.5***	1.4 ± 0.3	<0.001	N.S.
	Fed	2 ± 0.4	2.4 ± 0.2	7.2 ± 0.4***	1.8 ± 0.2	<0.001	<0.05
s-FFA (mmol/L)	Fasted	0.65 ± 0.03	0.7 ± 0.05	1 ± 0.09*	0.6 ± 0.08	<0.05	N.S.
	Fed	0.38 ± 0.02	0.4 ± 0.02	1.3 ± 0.1***	0.45 ± 0.07	<0.001	N.S.
s-β hydroxyl butyrate (μmol/L)	Fasted	1.2 ± 0.04	1.3 ± 0.05	2.2 ± 0.14**	3.8 ± 0.2***	<0.01	<0.001
	Fed	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.45 ± 0.05***	N.S.	<0.001

Data are given as mean ± SEM, $n = 6$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ before versus after diet.

TABLE 2: Liver triacylglycerol content.

Diet	Fasted	Fed
SD	3.7 ± 0.3	3.7 ± 0.2
HS	7.6 ± 0.6**	4.8 ± 0.2 [†]
HF	14 ± 1.1 ^{###}	20.8 ± 2.3 ^{xxx}

Data are given in $\mu\text{mol} \cdot \text{g}^{-1}$ wet weight and expressed as mean ± SEM, $n = 6$.

** $P < 0.01$ HS fasted versus SD fasted; [†] $P < 0.05$ HS fed versus SD fed;

$P < 0.001$ HF fasted versus SD fasted; xxx $P < 0.001$ HF fed versus SD fed.

dependent regulation was eradicated. In liver slices from the HFD group, we did not find any effect of the diet on TCA intermediates and CO_2 production but the ketone body production was significantly elevated in both fasting and fed animals ($P < 0.001$). In contrast, TAG secretion into medium was significantly decreased ($P < 0.001$). Taken together, this data indicates that the diet type does not profoundly affect the total availability of FFA for metabolic utilisation but that it rather influences their partitioning between oxidation and VLDL secretion (Table 3).

3.4. The Activity of Alkaline and Acid Lipase. As demonstrated above, intracellular TAG's were degraded *in vivo* in the liver of both HS and HF administered animals. In order to analyse the effect of the diets on liver lipolysis in more detail, we directly measured the activity of two liver lipases on exogenous substrate. In liver homogenate, we were able to prove two distinct lipases with optimum pH 4.5 and 8-9. The acid lipase can be identified as "lysosomal" (EC 3.1.1.13) while the alkaline is termed "hepatic" (EC 3.1.1.3). These two enzymes differ in their intracellular distribution and regulation and are affected differently by the administered diets. The effect of the diets on lipase activities in homogenate is shown in Figures 3(a) (lysosomal) and 3(b) (hepatic). On standard diet, the activity of lysosomal lipase is higher in fasting and depressed postprandially, whereas the hepatic lipase activity did not differ between fed and fasted animals. Both diets increased the activity of lysosomal

lipase but by different amount. On the HS diet, the lysosomal lipase activity increased on fasting but remained unchanged in the fed state. The stimulatory effect of the HF diet on lysosomal lipase activity was significantly higher and was found both in the fasting and fed states. The regulation due to fasting was preserved in rats fed the HS diet but it was completely eradicated in the HF group. The hepatic lipase responded to the tested dietary manipulations differently. HS diet increased the activity of this enzyme both in fasting and postprandially while the HF diet led to significant decrease of its activity.

Centrifugation of liver homogenate for 10 000 g allowed us to separate fractions containing dense lysosomes (pellet) and a fraction containing cytosol, total membranes and light lysosomes (supernatant). On the standard diet, most of the lysosomal lipase activity (85%) was localised in the 10 000 g pellet while most of the hepatic lipase activity (fasting 70%; fed 77%) was found in the 10 000 g supernatant. Neither the HS nor HF diet influenced the subcellular distribution of hepatic lipase, approx. 75% of the activity was found in the 10 000 g supernatant in all experimental settings. As far as the lysosomal lipase is concerned, the HF diet redistributed a significant portion of its activity into the 10 000 g supernatant (Table 4). This data indicate that lysosomal lipase is significantly affected by the HF diet both with regards to the total activity as well as the intracellular localisation.

The activity of lysosomal lipase determined on endogenous substrate *in vitro* was positively correlated with hepatic triacylglycerol content. This correlation was stronger in the fed ($r^2 = 0.97$) than in the fasting state ($r^2 = 0.65$) (Figure 4). We found no significant correlation between the hepatic triacylglycerols and hepatic lipase activity (fed: $r^2 = -0.4$; fasting: $r^2 = -0.35$) (data not shown). Furthermore the lysosomal lipase is activity positively correlated with serum β -hydroxybutyrate concentration both in fasting ($r^2 = 0.81$) and fed ($r^2 = 0.62$) states (Figure 5). These positive correlations in the case of lysosomal lipase suggest that this enzyme is affected by the availability of the intracellular

TABLE 3: The incorporation of intracellular TAG-derived ^{14}C palmitic acid into CO_2 , TCA intermediates, secreted β -hydroxybutyrate, and secreted triacylglycerols from liver slices *in vitro*.

	SD		HS		HF	
	Fasted	Fed	Fasted	Fed	Fasted	Fed
CO_2 (released)	16 ± 1.2	11.9 ± 1	$11 \pm 1.5^*$	$8.3 \pm 0.3^\dagger$	15 ± 0.7	9.8 ± 0.8
TCA intermediates (intracellular)	55.3 ± 1.6	41 ± 3.4	$46.6 \pm 1.8^{**}$	$25.7 \pm 1.5^{\dagger\dagger}$	54.2 ± 4.2	40.3 ± 1
β -hydroxybutyrate (secreted)	269 ± 9	204 ± 8	$380 \pm 18^{**}$	178 ± 15	$452 \pm 22^{***}$	480 ± 11^{xxx}
TAG (secreted)	581 ± 35	401 ± 44	$713 \pm 29^{**}$	$825 \pm 29^{\dagger\dagger}$	$314 \pm 16^{***}$	286 ± 35^{xx}

Data are expressed in nmol palmitic acid per g tissue and given as mean $\pm$ SEM $n = 6$. * $P < 0.05$, ** $P < 0.01$ HS fasted versus SD fasted; $^\dagger P < 0.05$, $^{\dagger\dagger} P < 0.001$ HS fed versus SD fed; $^{***} P < 0.001$ HF fasted versus SD fasted; $^{xxx} P < 0.01$, $^{xx} P < 0.001$ HF fed versus SD fed.

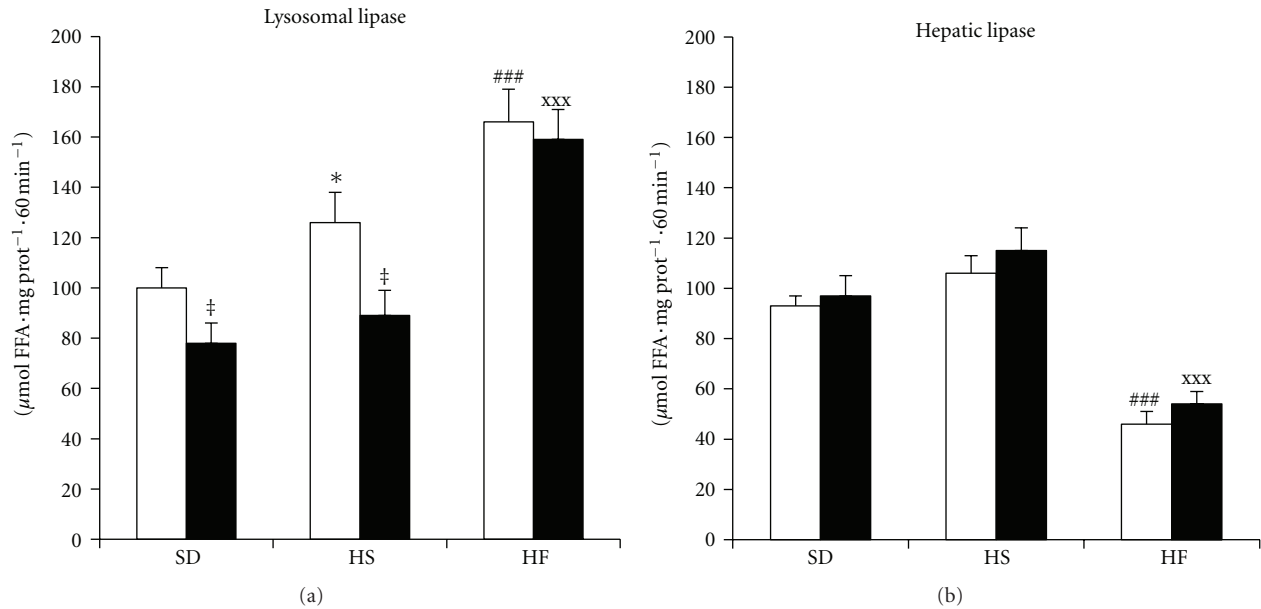


FIGURE 3: The activity of lysosomal (a) and hepatic (b) lipase in liver homogenate measured as FFA release from artificial substrate (^3H -triolein). The lipase activity was measured as the release of fatty acids at pH = 4.5 from ^3H triolein. Open bars = fasted animals; closed bars = fed animals. $^\dagger P < \text{fed versus fasted}$; * $P < 0.05$ HS-fasted versus SD-fasted; $^{***} P < 0.001$ HF-fasted versus SD-fasted; $^{xxx} P < 0.001$ HF-fed versus SD-fed.

substrate and that FFA's released by lysosomal lipase action are available at least for ketogenesis.

3.5. VLDL Production. The rate of VLDL production *in vivo* was estimated using Triton WR-1339. This detergent effectively blocks the lipoprotein clearance from the blood due to its inhibitory effect on LPL and thus it seems that under such circumstances the accumulation of TAG in plasma could be a valid measure of the rate of VLDL secretion from the liver. As shown in Table 5, TAG entry rate into the circulation is strongly accentuated in the HS group while being significantly diminished in animals administered with HF diet.

4. Discussion

The aim of the present study was to contribute to the understanding of the role of different metabolic pathways in the development of hepatic steatosis induced by two different dietary manipulations (HS and HF diet) in the model of the

metabolic syndrome. The diet rich in simple carbohydrates or in fat rapidly promotes the TAG accumulation in the liver. It has been suggested that the development of steatosis is associated with the attenuation of intracellular TAG hydrolysis. In contrast to this hypothesis our data indicate that lipolysis, the first step in the intracellular TAG utilisation, is not negatively affected by HS or HF diets. We showed that the important enzyme involved in the intracellular TAG degradation is lysosomal lipase. Furthermore we provided evidence that the mechanism underlying the dietary induced hepatic steatosis depends on the prevailing component of the diet. The HS diet induced steatosis is associated with a significant stimulation of FFA synthesis *de novo*, decreased FFA oxidation and with enhanced VLDL output from the liver. In contrast HF diet associated steatosis is characteristic by downregulated FFA synthesis *de novo*, increased FFA oxidation and significantly impaired VLDL output.

In spite of decades devoted to the study of TAG metabolism in the liver consensus is still not reached in identification of the exact contribution of particular lipases

TABLE 4: The intracellular distribution of lipases activities in liver.

Diet	Lysosomal lipase				Hepatic lipase			
	10 000 g sediment		10 000 g supernatant		10 000 g sediment		10 000 g supernatant	
	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed
SD	86 ± 9.2	85 ± 2	14 ± 1.5	15 ± 2	30 ± 4	23 ± 2	70 ± 5	77 ± 9.5
HS	87 ± 6.8	86 ± 7.3	13 ± 0.8	14 ± 3.6	40 ± 8.3	38 ± 7.3	60 ± 8.2	72 ± 5
HF	82 ± 4	75 ± 3 ^{xx}	18 ± 2 [#]	25 ± 2 ^{xx}	38 ± 5.2	24 ± 3.1	62 ± 4.5	76 ± 7

Data are given in % of the sum of the activities in 10 000 g sediment and supernatant prepared from liver homogenates, *n* = 6. [#]*P* < 0.05 HF fasted versus SD fasted; ^{xx}*P* < 0.01 HF fed versus SD fed.

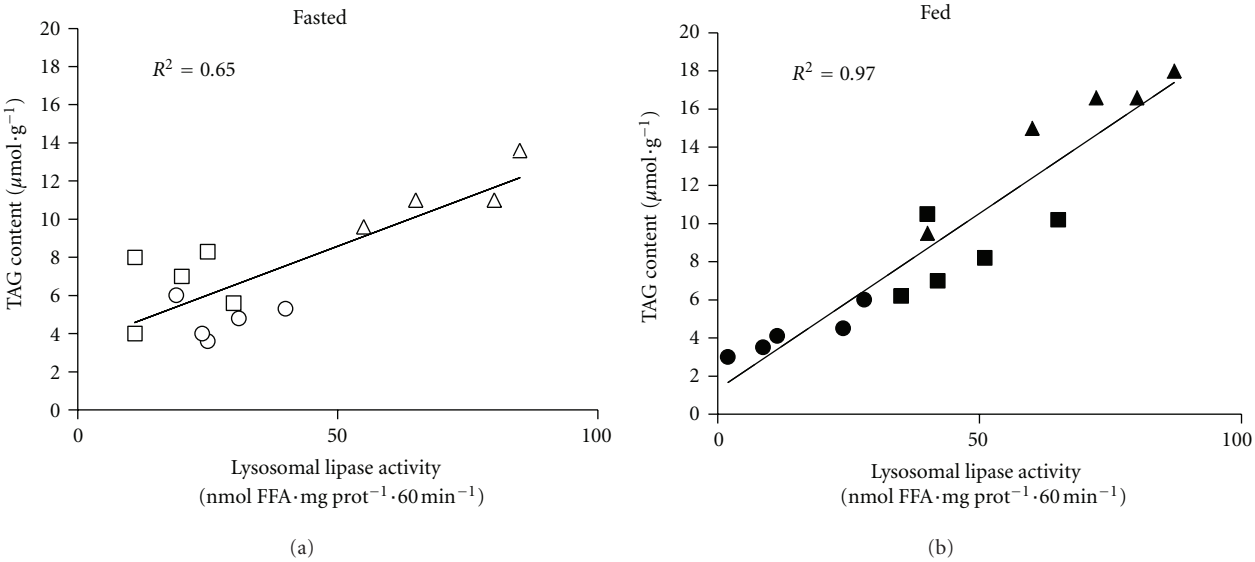


FIGURE 4: The correlation between lysosomal lipase activity and hepatic triacylglycerol content in fasted (a) and fed (b) animals. Lysosomal lipase activity was determined as the FFA release from endogenous TAG. Open circle = SD-fasted; open square = HS-fasted; open triangle = HF-fasted; closed circle = SD-fed; closed square = HS-fed; closed triangle = HF-fed.

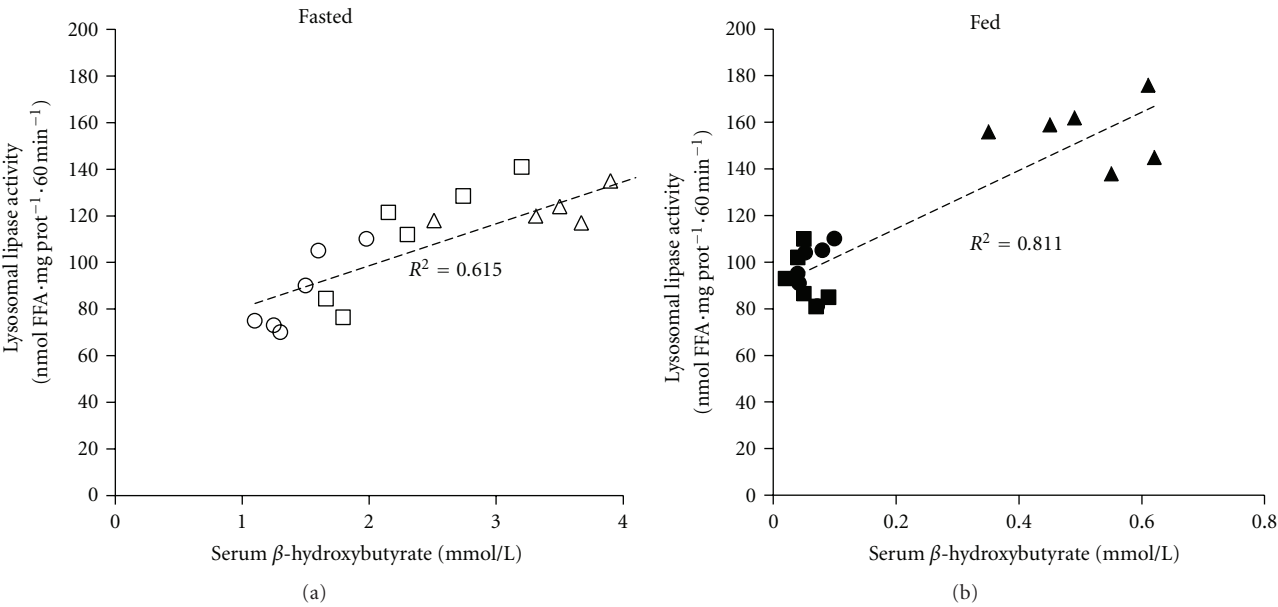


FIGURE 5: The correlation between lysosomal lipase activity and β -hydroxybutyrate content in serum. Lysosomal lipase activity was determined as the FFA release from endogenous TAG. Open circle = SD-fasted; open square = HS-fasted; open triangle = HF-fasted; closed circle = SD-fed; closed square = HS-fed; closed triangle = HF-fed.

TABLE 5: Effect of HS and HF diet on TAG entry rate.

	SD	HS	HF
TAG ₀ ($\mu\text{mol/mL}$)	3.9 ± 0.2	$6.4 \pm 0.5^{+++}$	$3.0 \pm 0.1^{**\#\#\#}$
TAG ₉₀ ($\mu\text{mol/mL}$)	7.4 ± 0.4	$11.7 \pm 0.75^{+++}$	$5.2 \pm 0.44^{**\#\#\#}$
TAG entry rate ($\mu\text{mol} \cdot 60 \text{ min}^{-1} \cdot \text{kg}$)	11.2 ± 0.7	$45.7 \pm 6.1^{+++}$	$8.2 \pm 0.5^{**\#\#\#}$

TAG₀: triacylglycerol serum concentration before WR 1339 administration; TAG₉₀: triacylglycerol serum concentration 90 min after WR 1339 administration. TAG entry rate was calculated according to the formula TAG entry rate ($\mu\text{mol} \cdot 100 \text{ g b. wt.}^{-1} \cdot \text{hr}^{-1}$) = $[(T_{90} - T_0)/1.5] \times V \times (W/100)$. Data are given as mean $\pm$ S.E.M. $n = 6$. $+++P < 0.001$ HS versus SD; $###P < 0.001$ HF fasted versus SD fasted; $**P < 0.01$ HF versus SD; $\#\#\#P < 0.001$ HF versus HS.

to the intracellular TAG degradation. In our study, we focused on two lipases present in the liver-heparin-releasable hepatic lipase with pH optimum 8-9 and acid lysosomal lipase with pH optimum 4.5. The activity of hepatic lipase in liver homogenate was increased by HS diet but depressed by HF feeding. Hepatic lipase seems to be induced by chronic hyperinsulinemia [16] which is associated with high sucrose as well. Numerous studies indicate that the expression and activity of hepatic lipase is increased in an insulin resistant state, mostly accompanied by visceral obesity [17]. Nevertheless, most of the activities attributed to the hepatic lipase are oriented out of the hepatocyte, that is, facilitation of the interaction of LDL and remnant chylomicrons with an LRP receptor or participation in HDL metabolism [18–21]. Furthermore, we have previously reported that alkaline lipase is not able to degrade endogenous TAG in the liver homogenate and the only lipolytic activity towards intracellular substrate was found in the acidic range [22]. In perspective of these findings, hepatic lipase is probably not involved in the mobilisation of endogenous triacylglycerols.

Lysosomal lipase, first described by Vavřínková and Mosinger [23], is localised in lysosomes and its activity is elevated in fasting under physiological conditions [24]. In our experiments, this pattern of regulation was preserved only in HHTg rats on standard and HS diets but it was completely eradicated by HF feeding. Both HF and HS diets had a stimulatory effect on the activity of lysosomal lipase but the effect of HF diet was significantly higher than the effect of the diet rich in sucrose. HF feeding led to the translocation of part of the lysosomal lipase activity to supernatant (up to 25%) at the expense of the activity found in pellets. This observation is in accordance with the recently proposed mechanism of lysosome-dependent TAG degradation in the hepatocytes [25, 26]. According to this hypothesis, lipid droplets are incorporated into the autophago(lipo)lysosomes by the process of autophagy, similarly to the autophagy of cytosolic proteins or damaged organelles. The translocation of lysosomal activity from the 10 000 g pellet to supernatant may thus reflect the formation of less dense autophagolipolysomes containing TAG [27]. We further found a strong positive correlation between acid lipase activity and TAG content in the liver homogenate. Taken together, these data indicate that TAG degradation is actually enhanced in steatosis and that it is mediated by lysosomal lipase.

In contrast to previously published hypotheses, our results based on direct measurements of acid lipase activity indicate that accumulation of intrahepatic lipids is not the

consequence of the impaired mobilisation of intracellular triacylglycerol stores. Despite this, we found positive correlation between the lysosomal lipase activity and oxidation of endogenous triglycerides (ketogenesis) even in the HF fed group which implies that the stored triglycerides are accessible at least for some metabolic pathways. As the capacity of hepatocytes to breakdown intracellular triglycerides is actually raised in both diet groups another factor must explain the different effects of HS and HF diets on triacylglycerol accumulation in the liver and circulation.

Extensive literature concerning the effect of diet composition (carbohydrate versus fat) on liver TAG metabolism is available. There is a fair consensus that a diet rich in carbohydrates and low in fat results in elevation of serum TAG levels, increased VLDL production rate, elevation of TAG amount per particle and attenuation of carbohydrate/fatty acid oxidation [28–32]. However the sensitivity to high carbohydrate intake is highly variable. Numerous studies were performed in order to identify subject characteristics that may be useful in predicting sensitivity to carbohydrate feeding. Characteristics such as sex, TAG concentration when on HF diet, BMI, and insulin concentration have been variably shown to individually predict the effect of the diet and no single variable has a significant predictive value. In contrast, a highly significant effect of the type of carbohydrate has been demonstrated, the effect of monosaccharides being much higher in comparison to polysaccharides, and this effect was similar in both hyper- and normotriglyceridemic subjects [33]. Previous studies performed in our laboratory showed that HHTg rats are significantly more sensitive to a HS diet but that the effects imposed by this diet on the TAG metabolism are principally similar in HHTg and their normotriglyceridemic controls although they differ in their magnitude [34, 35].

The data concerning the effect of high fat diet on liver TAG metabolism, particularly on VLDL secretion, are more diverse. Several detailed studies focused on this issue and performed on lean and obese Zucker rats were published by Kalopissis group in the 90's. They demonstrated on primary hepatocytes that previous HF feeding (60 cal% as fat, lard) decreases the uptake and utilization of exogenous fatty acids and profoundly influences the intracellular partitioning of FFA released from intracellular TAG in favor of FFA oxidation at the expense of VLDL secretion (40–50% decrease) and lipogenesis (80% inhibition) [3–5, 10]. Of note, qualitatively the effect of HFD on liver TAG metabolism was the same both in lean and obese Zucker rats, but the degree of the changes were more pronounced in the latter.

In spite of the fact that the HF diet-induced inhibition of VLDL secretion has been repeatedly observed the cause of this phenomenon has not been fully elucidated yet. The availability of glucose is severely lowered in HF diet compared to the standard and HS diets. One of the direct effects of glucose at the hepatic level is the increased secretion of VLDL triacylglycerols [36, 37]. Brown et al. [38] in their study on primary hepatocytes showed that glucose mediated effects are numerous and involve enhanced transport of triacylglycerols out of the cells as VLDL together with an increase in the net synthesis of apoB-48 in the intestine and apoB-100 in the liver. Based on this data, we could speculate that the limiting condition determining the lipid accumulation in liver is the rate of lipoprotein output from the liver which depends on the availability of glucose. Nevertheless, we found a slight increase in fasting serum glucose in HF fed rats which indicates the increased endogenous glucose production. Whether this glucose is available for VLDL production still remains an open question.

In the presented study we focused on the mechanisms underlying the development of hepatic steatosis in a model that is particularly prone to the dietary manipulation. In conclusion, we provided evidence that in the HHTg rat model of metabolic syndrome hepatic steatosis (both HS- or HF-induced) is not associated with the impairment of intracellular TAG lipolysis. On the contrary, the lysosomal TAG breakdown was actually enhanced under these conditions. Although the effect of both tested diets was qualitatively, but not quantitatively, the same—TAG accumulation in the liver—the underlying mechanisms were different. High sucrose diet was associated with the depression of fatty acid oxidation in parallel with increased TAG secretion and *de novo* FFA synthesis. In contrast in HF diet administered animals the intracellular TAG-derived FFA were channelled predominantly to the oxidative utilisation, namely, ketogenesis, at the expense of a secretory pathway. This finding stresses the importance of understanding exact mechanisms responsible for particular cases in order to choose an efficient therapeutic approach.

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PŘÍLOHA VII

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Biguanides Inhibit Complex I, II and IV of Rat Liver Mitochondria and Modify Their Functional Properties

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Summary

In this study, we focused on an analysis of biguanides effects on mitochondrial enzyme activities, mitochondrial membrane potential and membrane permeability transition pore function. We used phenformin, which is more efficient than metformin, and evaluated its effect on rat liver mitochondria and isolated hepatocytes. In contrast to previously published data, we found that phenformin, after a 5 min pre-incubation, dose-dependently inhibits not only mitochondrial complex I but also complex II and IV activity in isolated mitochondria. The enzymes complexes inhibition is paralleled by the decreased respiratory control index and mitochondrial membrane potential. Direct measurements of mitochondrial swelling revealed that phenformin increases the resistance of the permeability transition pore to Ca²⁺ ions. Our data might be in agreement with the hypothesis of Schäfer (1976) that binding of biguanides to membrane phospholipids alters membrane properties in a non-specific manner and, subsequently, different enzyme activities are modified *via* lipid phase. However, our measurements of anisotropy of fluorescence of hydrophobic membrane probe diphenylhexatriene have not shown a measurable effect of membrane fluidity with the 1 mM concentration of phenformin that strongly inhibited complex I activity. Our data therefore suggest that biguanides could be considered as agents with high efficacy but low specificity.

Key words

Metformin • Phenformin • Mitochondrial enzymes • Mitochondrial permeability transition pore • Membrane fluidity

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Introduction

Metformin (N,N-dimethylimidodicarbonimidic diamide) has been used for the treatment of type 2 diabetes since late 50's in most European countries and in the United States (Bailey and Turner 1996, Campbell *et al.* 1992). Metformin is currently the first drug of first choice in type 2 diabetes treatment and it is highly efficient in lowering blood glucose levels. Initially, it was suggested that one of its key actions is the stimulation of the muscle glucose uptake (Goodarzi and Bryer-Ash 2005, Klip and Leiter 1990). Recently, there is a growing body of evidence suggesting that the primary function of metformin is to decrease hepatic glucose production, mainly by inhibiting gluconeogenesis (Hundal *et al.* 2000). Numerous reports indicate that metformin

treatment leads to the diminution of ATP reserves in the liver (Argaud *et al.* 1993, Owen *et al.* 2000), decreased ATP/ADP ratio and to a reduction in cellular energy charge. Quite reasonably, AMP-activated protein kinase (AMPK) was therefore considered to be a major mediator of the glucose-lowering effects of metformin (Boyle *et al.* 2010, Zhou *et al.* 2001). Surprisingly, Foretz *et al.* (2010) demonstrated that the hypoglycemic effect of metformin was fully maintained in mice lacking AMPK. Moreover, they showed that the control of hepatic glucose production by metformin is linked to the inhibition of gluconeogenesis in response to a decrease in hepatic energy charge. In spite of long-lasting metformin application in clinical practice, its precise mechanism of action is still unknown (Campbell *et al.* 1996, Gonzalez-Barroso *et al.* 2012).

The primary cellular target for metformin is believed to be complex I of mitochondrial oxidative phosphorylation. However, the data obtained on hepatocytes, mitochondria or cultured fibroblasts showed that high metformin concentrations (10 mM) and long-term preincubation (2-24 hours) are required for demonstration of its inhibitory effect on oxidation of NADH-dependent substrates and also its direct effect on complex I (NADH dehydrogenase) activity was not completely confirmed (Owen *et al.* 2000). These data indicate that the inhibition of mitochondrial respiratory chain complex I may be one, but not the only, effect of metformin. Regarding the mechanism of action it has become likely that the hypoglycemic effect of biguanides results from a combination of a number of modulations of membrane-linked metabolic reactions, depending on the particular experimental conditions.

In our last study (Palenickova *et al.* 2011), we have shown that 10 mM metformin inhibits oxidation of glutamate + malate or palmitoylcarnitine + malate by about 60 % both in homogenate and in mitochondria after 3-5 min preincubation and that cell integrity is not required for its action. A significant inhibitory effect (about 20 %) can be detected also at 2.5-5 mM concentration; however, these concentrations are much above those detected in blood after *in vivo* application as well.

Phenformin, another biguanide, has qualitatively similar effects as metformin (Owen *et al.* 2000) but it is a more potent inhibitor of mitochondrial oxidation and it has been excluded from clinical practice because of an unacceptable incidence of severe lactate acidosis. Nonetheless, it is highly eligible for experimental studies

focused on biguanide mechanisms of action due to its more pronounced inhibitory effect at lower concentrations.

Under physiological conditions metformin and phenformin only can exist in the positively charged protonated form. The positively charge on both drugs could account for their generation of positive surface potential at phospholipid membranes and their accumulation within matrix of mitochondria. Phenformin is more hydrophobic, lipid-soluble molecule, than metformin. The phenyl and ethyl groups on phenformin make it to permeate biological membranes readily. While phenformin is accumulated transitorily in liver, kidney, pancreas and muscle, metformin is concentrated mainly in the walls of esophagus, stomach, duodenum and salivary glands as well as in kidney. (Owen *et al.* 2000, Schäfer 1983)

In this paper, we thus evaluated the phenformin effect on activity of mitochondrial enzymes, on respiratory control index, on mitochondrial membrane potential and on function of mitochondrial membrane permeability transition pore. We further focused on the question whether the biguanides inhibitory effect is dependent on cell structural integrity.

Methods

Animals

Male Wistar rats (6-8 months old) were kept in a temperature-controlled environment ($25 \pm 2^\circ\text{C}$) with a 12 h light/dark cycle on standard laboratory diet and tap water supply *ad libitum*. All the experiments were conducted in accordance with the Animal Protection Law of the Czech Republic and according to the Institute for Clinical and Experimental Medicine Animal Care and Use Committee.

Preparation of rat liver homogenate and isolation of liver mitochondria

Liver mitochondria were prepared by differential centrifugation as described by Bustamante *et al.* (1977) with some modifications. Rat liver tissue was homogenized at 0°C by a teflon-glass homogenizer as a 10 % homogenate in medium containing 220 mM mannitol, 75 mM sucrose and 1 mM HEPES, pH 7.2. For homogenization, 0.5 g/l fatty acid free bovine serum albumin (BSA) and 1 mM EGTA were added. Homogenate was centrifuged for 10 min at 800 g. The supernatant was filtered through nylon mesh and

centrifuged for 10 min at 8 000 g. Pelleted mitochondria were re-suspended in the medium without BSA and EGTA and washed twice by 10 min centrifugation at 8 000 g. The final mitochondrial pellet was re-suspended in the medium without BSA and EGTA to a concentration of 20-30 mg protein/ml. Mitochondrial proteins were determined according to the method of Bradford (1976) using BSA as a standard.

Determination of mitochondrial respiration

Oxygen consumption was measured at 30 °C using Oxygraph-K2 (OROBOROS, Austria). Measurements were performed in 2 ml of K-medium containing 80 mM KCl, 10 mM Tris-HCl, 3 mM MgCl₂, 1 mM EDTA, 5 mM KH₂PO₄, pH 7.4. The rate of oxygen consumption was calculated using Oroboros DatLab-4 software and expressed as nmol oxygen/s/mg protein.

Determination of mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was determined using safranin O dye according to Akerman and Wikstrom (1976). Fluorescence changes were measured in 3 ml of K-medium containing 3 µM safranin, Ex 495 nm/Em 586 nm wavelengths, 30 °C, and using ISS-Vinci software in ISS Inc.-PC1 Spectrofluorimeter (Illinois U.S.A.).

Determination of mitochondrial swelling

Mitochondrial swelling was measured as described before (Drahota *et al.* 2012) as a decrease of mitochondrial suspension absorbance at 520 nm after the addition of Ca²⁺. The measurements were carried out at 30 °C using a Shimadzu spectrophotometer. The swelling medium contained 125 mM sucrose, 65 mM KCl, 5 mM succinate, 10 mM HEPES and 1 mM KH₂PO₄, pH 7.2. Mitochondria were added to yield an absorbance of approximately 1. After 1 min of incubation, CaCl₂ solution was added to the suspension of mitochondria. The decrease in the absorbance was determined at 0.1-min intervals for 5 min. The extent of swelling was calculated as the difference of the optical density at the beginning and the end of the measurement period. Maximum swelling rate was calculated from the curve obtained after derivation of the original swelling curve and expressed as absorbency change per 0.1-min interval. This derivation was used as a source to obtain the third parameter of swelling process – the time at which the maximum swelling rate was reached after calcium

addition. These three parameters than can better characterize the effects of biguanides on the kinetics of the swelling process.

Determination of anisotropy of fluorescence hydrophobic membrane probe diphenylhexatriene

The steady-state anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence was measured as described previously (Brejchova *et al.* 2011). Frozen-thawed rat liver mitochondria were labelled with DPH by the fast addition of 1 mM DPH in acetone to the mitochondrial suspension (0.1 mg protein/ml, final concentration 1 µM) under mixing. After 30 min at 25 °C, DPH fluorescence was measured at Ex 365 nm/Em 425 nm wavelengths with a ISS PC1 spectrofluorometer and the steady-state anisotropy (r_{DPH}) determined by ISS Vinci software. Both excitation and emission spectra of 1 µM DPH were determined in the presence of increasing concentrations of phenformin with the aim of detecting a possible quenching effect on DPH fluorescence.

Results

Phenformin inhibits complex I of mitochondrial respiratory chain in a dose-dependent manner

As previously reported (El-Mir *et al.* 2000, Owen *et al.* 2000, Palenickova *et al.* 2011), metformin inhibits complex I of mitochondrial respiratory chain. In this study, we focused our attention on the action of other representative of biguanide family, phenformin, on rat liver mitochondria. In order to obtain detailed information about the effect of phenformin on different respiratory chain components, we determined the oxygen consumption of isolated liver mitochondria using different substrates known to provide reducing equivalents specifically to the particular mitochondrial complexes. Figure 1 demonstrates the typical result of the oxygen consumption curve by rat liver mitochondria shortly (5 min) incubated with 1 mM phenformin after sequential additions of NADH- and flavoprotein-dependent substrates compared with control, i.e. untreated sample. When using only glutamate and malate as substrates, the respiration was very low and we did not observe any difference between phenformin-treated and control mitochondria. In contrast, when the respiratory chain was coupled to ATP synthesis by the addition of ADP, we discovered a strong inhibitory effect of phenformin on glutamate and malate respiration. Added

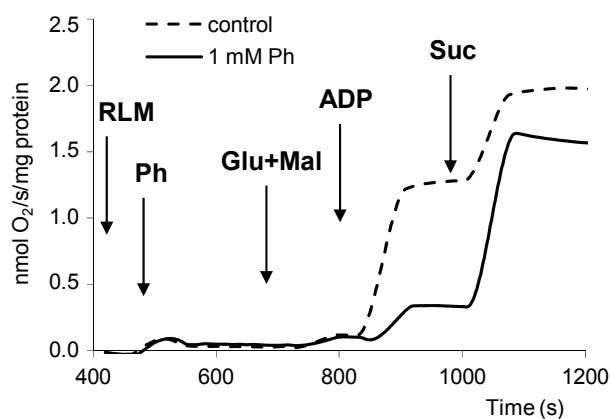


Fig. 1. Oxygenographic measurement of rat liver mitochondria respiration *in vitro*. Oxygen consumption measurement is described in Methods. Dashed trace indicate the respiration of rat liver mitochondria (RLM) incubated in the absence of phenformin (control), solid trace indicate the respiration of mitochondria to which 1 mM phenformin (Ph) was added where indicated. Other additions to both curves were: 10 mM glutamate and 2.5 mM malate (Glu+Mal), 1.5 mM ADP and 10 mM succinate (Suc). Mitochondrial protein concentration was 0.2 mg/ml. This figure represents a typical result of three independent experiments.

succinate then significantly increased mitochondrial respiration in both control and phenformin-treated mitochondria but the respiration rate of both substrates was still lower in the presence of phenformin compared to controls (Fig. 1).

As we have shown previously (Palenickova *et al.* 2011), the effect of metformin on glutamate + malate + ADP respiration was dose dependent with the maximum inhibition to 30 % at 10 mM metformin, EC_{50} was 5 mM. When we have measured under the same experimental conditions inhibitory effect of phenformin, maximum inhibition to 30 % of control values was obtained at 1 mM concentration and EC_{50} was 0.25 mM (Fig. 2A). At 1 mM phenformin glutamate respiration was inhibited to 30 %, after addition of succinate to 55 % only (Fig. 2A, B). Respiratory control index (RCI) was calculated as a ratio of oxygen consumption with and without ADP. In coupled mitochondria, phenformin decreased RCI in dose-dependent manner (Fig. 2C) and the course of the inhibitory curve was in parallel to its effect on glutamate + malate + ADP oxidation (Fig. 2A). We may thus conclude that both biguanides have the same inhibitory effect on glutamate oxidation and on the decrease of respiratory control, however, at different concentrations. Succinate oxidation under these experimental conditions was not much inhibited and could thus partially compensate the inhibition of glutamate + malate + ADP oxidation (Fig. 2B).

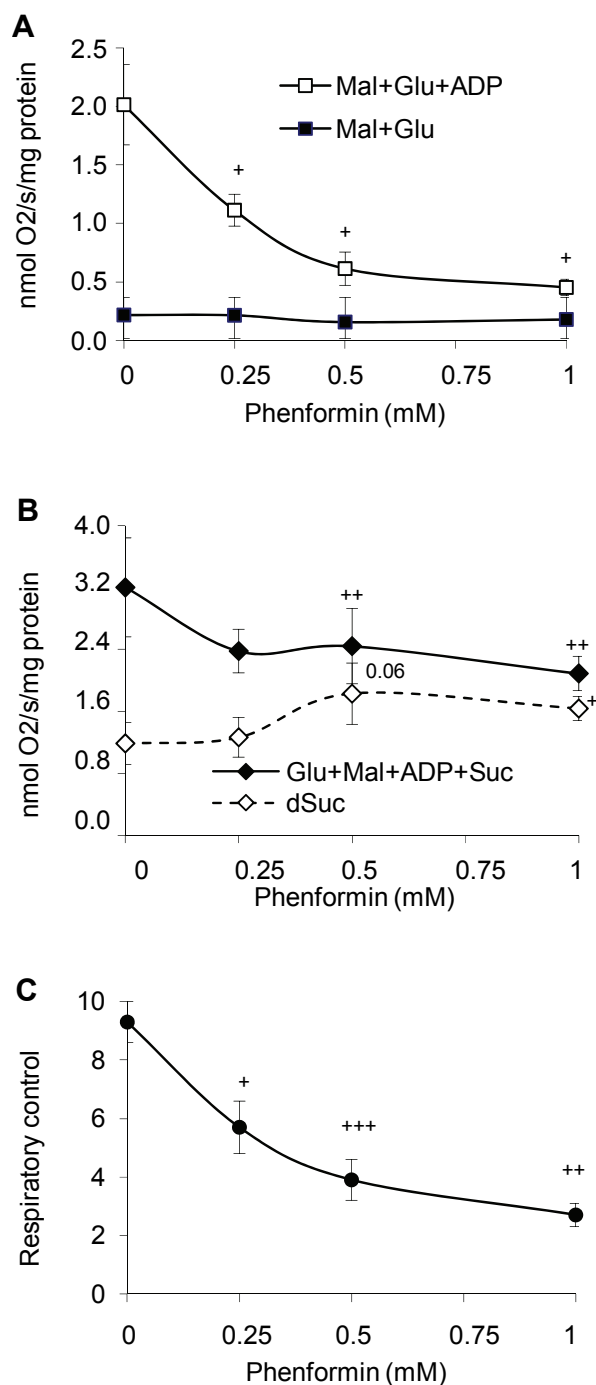


Fig. 2. Dose-dependent effect of phenformin on the oxidation of glutamate + malate and glutamate + malate + ADP (A), on the FADH-dependent (B) substrates and respiratory control index (C) in rat liver mitochondria. Respiration with glutamate + malate (black squares) (A) and glutamate + malate + ADP (white squares) as substrates (B). The effect of phenformin on glutamate + malate + ADP + succinate respiration (black rhombus) and on the increase of respiration induced by addition of succinate after glutamate + malate + ADP (white rhombus). dSuc was calculated as the difference of the oxygen consumption before and after the addition of succinate to glutamate + malate + ADP. (C) Respiratory control index (RCI) is calculated as a ratio of oxygen consumption with glutamate + malate with and without ADP. Data are given as mean of three independent experiments $\pm$ S.E.M. $p < 0.05$; $++ p < 0.01$; $+++ p < 0.001$ phenformin-treated vs. control mitochondria.

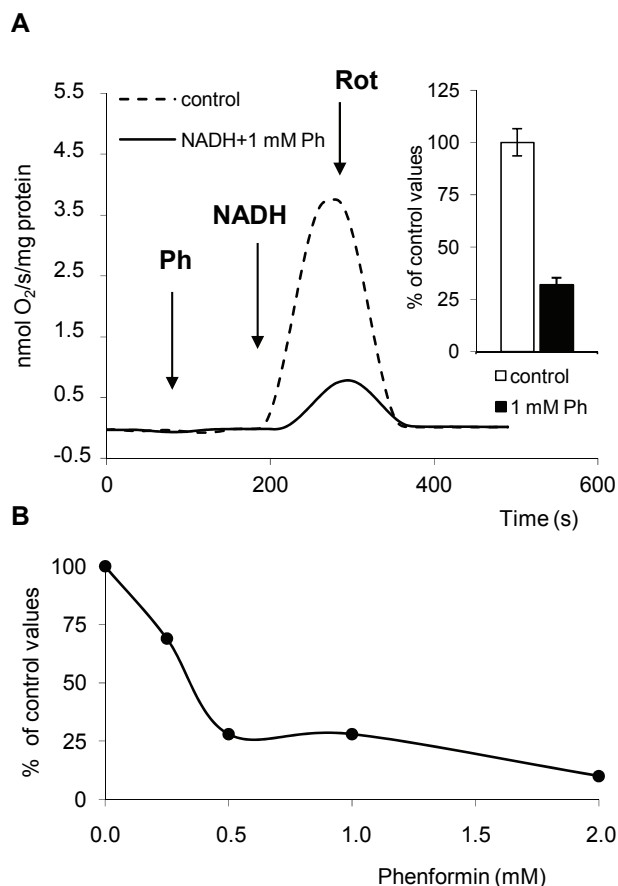


Fig. 3. The effect of phenformin on rotenone-sensitive NADH oxidation by frozen-thawed liver mitochondria. **(A)** The effect of 1 mM phenformin on the NADH oxidation in the absence (dashed line) or in the presence (solid line) 1 mM phenformin (Ph). 100 μ M NADH and 2 μ M rotenone (ROT) were added as indicated. This figure represents a typical result of three independent mitochondrial preparations. The extent of the inhibitory effect of Ph in % of control values is shown in the insert. **(B)** Concentration-dependent effect of phenformin on NADH oxidation.

Phenformin inhibitory effect on NADH oxidation

The observed biguanide-induced inhibition of NADH-dependent substrates oxidation may be explained either by the inhibition of dehydrogenases producing NADH or by inhibition of complex I (NADH-oxidoreductase), i.e. by diminution of electron flux from complex I through the respiratory chain. In order to distinguish between these possibilities, we directly measured NADH oxidation using frozen-thawed liver mitochondria as in this model NADH had free and direct access to complex I. Figure 3A demonstrates that in the presence of 1 mM phenformin rotenone-sensitive NADH oxidation in disrupted mitochondria is decreased to the same extent as the respiration of glutamate + malate + ADP in intact mitochondria. Furthermore, the curve describing the dependence of O₂ consumption on the phenformin concentration (Fig. 3B) had a similar value of

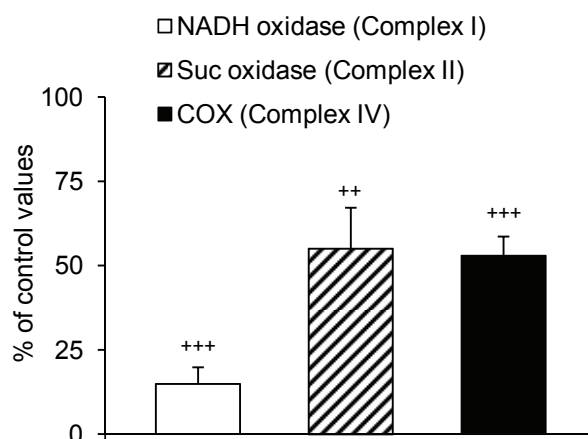


Fig. 4. Effect of phenformin on the oxidation of complex I, complex II and complex IV substrates by rat liver mitochondria *in vitro*. The effect of 6 mM phenformin was determined on substrates of NADH oxidase (complex I), succinate oxidase (complex II) and cytochrome c oxidase (complex IV). Medium was supplemented with 0.15 mM uncoupler 2,4-dinitrophenol and 0.02 mM cytochrome c. Data are given as mean of five independent experiments $\pm$ S.E.M. ++ $p < 0.01$; +++ $p < 0.001$ phenformin-treated vs. control mitochondria.

50 % inhibition (0.25 mM) as for glutamate + malate + ADP oxidation (Fig. 2A). These data indicate that phenformin site of action is complex I.

Phenformin in high concentration inhibits not only complex I but also other complexes of mitochondrial respiratory chain

In our previous study, we were not able to find any inhibitory effect of metformin on complex II at concentrations required for the maximum inhibition of complex I (Palenickova *et al.* 2011). In contrast, phenformin is a 10 times more potent inhibitor of complex I compared with metformin and also exhibits some inhibitory action on succinate oxidation when complex I is fully inhibited (Fig. 2B). We have therefore tested the inhibitory effect of higher concentrations of phenformin on succinate and cytochrome c oxidases and we found that at 6 mM concentration phenformin displays significant inhibitory effect not only on complex I but also on complex II and IV (Fig. 4). Our data thus have shown that biguanides do not specifically inhibit complex I activity, but also other complexes of the mitochondrial respiratory chain.

Phenformin inhibitory effect on mitochondrial membrane potential is dependent on complex I function

As demonstrated in Figure 5, phenformin inhibits glutamate + malate oxidation in uncoupled as in

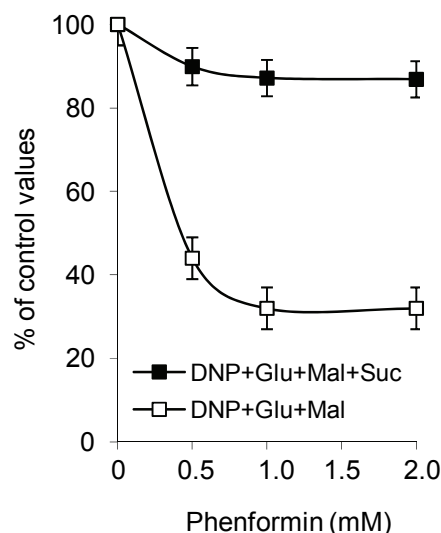


Fig. 5. Effect of phenformin on the oxidation of FADH-dependent and NADH-dependent substrates by uncoupled rat liver mitochondria. The inhibition of oxidation of glutamate + malate (Glu+Mal, white squares) and for the increase of respiration induced by succinate addition (black squares) after phenformin treatment is shown. 0.15 mM 2,4-dinitrophenol (DNP) was added prior to the other substrates in order to dissipate the electron transport from oxidative phosphorylation. Data are expressed in % of control, i.e. untreated mitochondria, values and are given as means from two independent experiments.

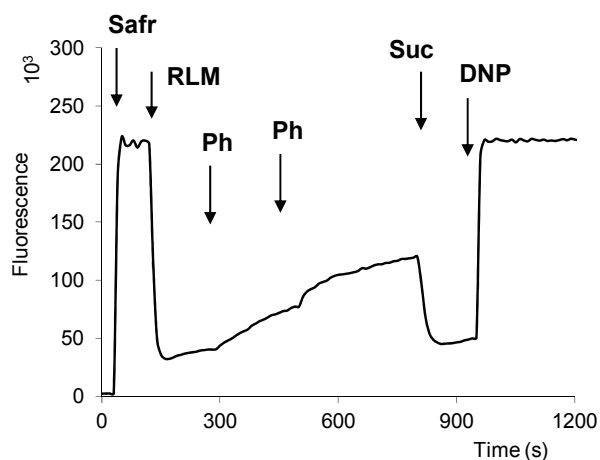


Fig. 6. Effect of phenformin on the mitochondrial membrane potential of rat liver mitochondria. Safranin O (Safr) was added to the K-medium containing 10 mM glutamate and 2.5 mM malate. Then intact rat liver mitochondria (RLM) were added (0.12 mg of protein/ml) followed by addition of 0.25 mM and 0.5 mM phenformin (Pf), 10 mM succinate (Suc) and 0.05 mM 2,4-dinitrophenol (DNP). The curve demonstrates a typical representative of three independent experiments.

coupled mitochondria (Fig. 2A). As the final step to confirm these findings, we measured the effect of phenformin on mitochondrial membrane potential. Membrane potential measured by fluorescent probe safranin O in the presence of glutamate and malate was slowly discharged by the addition of phenformin,

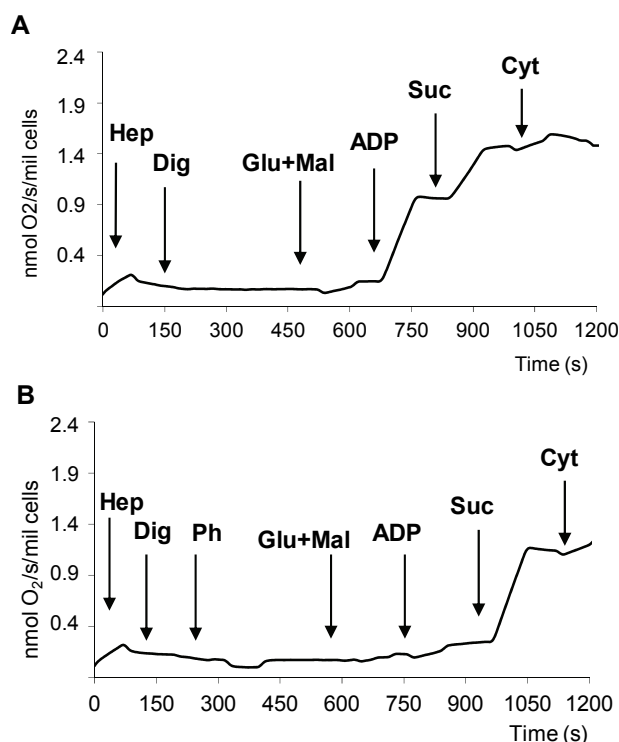


Fig. 7. Respiration of digitonin-permeabilised hepatocytes in the absence (A) and presence (B) of 1 mM phenformin. Hepatocytes (Hep; 2.5×10^5 cells/ml) were incubated in K-medium. Additions to both curves were as follows: 0.03 mg/ml digitonin (Dig), 1 mM phenformin (Ph, Fig. 7B), 10 mM glutamate and 2.5 mM malate (Glu+Mal), 1.5 mM ADP, 10 mM succinate (Suc) and 0.02 mM cytochrome c (Cyt). This figure represents a typical result of three independent experiments.

however, when succinate was added, the membrane potential was again recovered and could be released by an addition of uncoupler (Fig. 6). Taken together, we may conclude that phenformin has no uncoupling effect and impairment of glutamate + malate + ADP respiration and the decrease of the respiratory control index as well as the mitochondrial membrane potential is the consequence of the mitochondrial complex I inhibition.

Phenformin exhibits similar inhibitory effect both on isolated mitochondria and permeabilized hepatocytes

Several lines of evidence indicate that mitochondrial function may be modified in isolated mitochondria due to the destruction of mitochondrial filament structures as well as the disruption of contacts between mitochondria and other cell structures that may have an important role in their functional properties (Kondrashova *et al.* 2001, 2009). There are also discussions whether there are differences in biguanides action on intact cells and isolated mitochondria (El-Mir *et al.* 2000). We have consequently tested the inhibitory effect of phenformin on the activity of mitochondrial

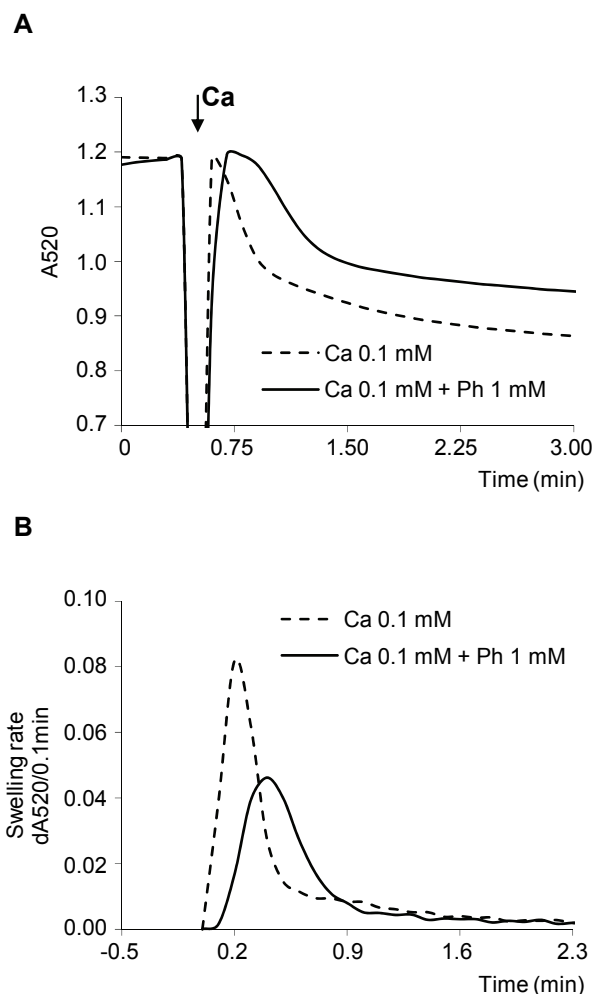


Fig. 8. Effect of phenformin on the calcium-induced swelling of rat liver mitochondria. Mitochondria were shortly (3 min) pre-incubated with 1 mM phenformin followed by the addition of 0.1 mM Ca^{2+} . The decrease of absorbance at 520 nm was detected in 0.1 min intervals. The extent of swelling (**A**) was calculated as the change in the optical density during the measurement period. Swelling rate (**B**) was calculated from the curves obtained after as the original data derivation. This figure represents a typical result of three independent experiments.

enzymes in permeabilized hepatocytes. Under these conditions, mitochondria are still in contact with other intracellular particles and mitochondrial filament structures are preserved (Kondrashova *et al.* 2001, 2009). As shown in Figure 7, in the presence of 1 mM phenformin, we obtained the same inhibitory effects with isolated hepatocytes as with isolated mitochondria (see Fig. 1). Phenformin affected only the glutamate and malate oxidation in the presence of ADP similarly as in isolated mitochondria and the inhibition of electron flux through complex I was partly compensated by electron supply from succinate. No significant increase was observed after cytochrome c addition which confirmed

that the mitochondria were not damaged by digitonin. Similar data could be also obtained with liver homogenate (not shown). We may thus conclude that the inhibitory effect of phenformin can be detected under the same experimental conditions in liver hepatocytes, in homogenate as well as in isolated mitochondria.

Phenformin increases the resistance of mitochondrial permeability pore to Ca^{2+}

Several reports indicate that biguanides can modify the function of the mitochondrial permeability transition pore (Detaille *et al.* 2005, Guigas *et al.* 2004). These data have shown that in isolated cells, metformin can change the mitochondrial calcium retention capacity as well as the accompanying release of mitochondrial cytochrome c.

We have therefore tested the effect of phenformin on the calcium induced swelling on isolated mitochondria. Three parameters characterizing the kinetics of the membrane permeability pore function were evaluated – the extent of swelling, the maximum rate of swelling and the time interval between the calcium addition and the time, when the swelling rate reaches its maximum rate (Drahota *et al.* 2012). As demonstrated in Figure 8, the extent of swelling and the maximum rate of swelling after addition of 0.1 mM CaCl_2 decreased due to the phenformin treatment by 20 % from 0.36 to 0.29 A_{520} , the maximum swelling rate decreased by 44 % from 0.0819 to 0.0462 $dA_{520}/0.1 \text{ min}$ and the time interval preceding the maximum swelling rate after CaCl_2 addition was prolonged two-fold from 0.2 to 0.4 min. All these results confirm that phenformin can increase the mitochondrial transition pore resistance to calcium and influence mitochondrial membrane bound complex which is not involved in the energy transformation process.

The effect of phenformin on anisotropy of fluorescence of hydrophobic membrane probe DPH in liver mitochondria

Our previous data are in agreement with the hypothesis that modification of various mitochondrial functions by biguanides could be explained by their unspecific modifications of protein-lipid interactions (Schäfer 1976, 1983, Wiernsperger 1999). We have therefore tested to what extent inhibition of various mitochondrial enzymes and functions by phenformin could be explained by its non-specific change of the hydrophobic interior of the mitochondrial membrane. This has been performed by determination of steady-state anisotropy of DPH (r_{DPH}) fluorescence. Fluorescence of

Table 1. Effect of increasing concentrations of phenformin on fluorescence anisotropy of hydrophobic membrane probe DPH in frozen rat liver mitochondria.

Phenformin (mM)	0	0.125	0.25	0.5	1	2
Mean	0.199	0.197	0.201	0.198	0.200	0.197
s.e.	0.001	0.001	0.002	0.001	0.005	0.003

Mitochondria were labeled with DPH as described in methods and the effect of increasing concentrations of phenformin on anisotropy of DPH fluorescence was determined at Ex 365 nm/Em 425 nm wavelengths. The data are expressed as mean $\pm$ standard error of means.

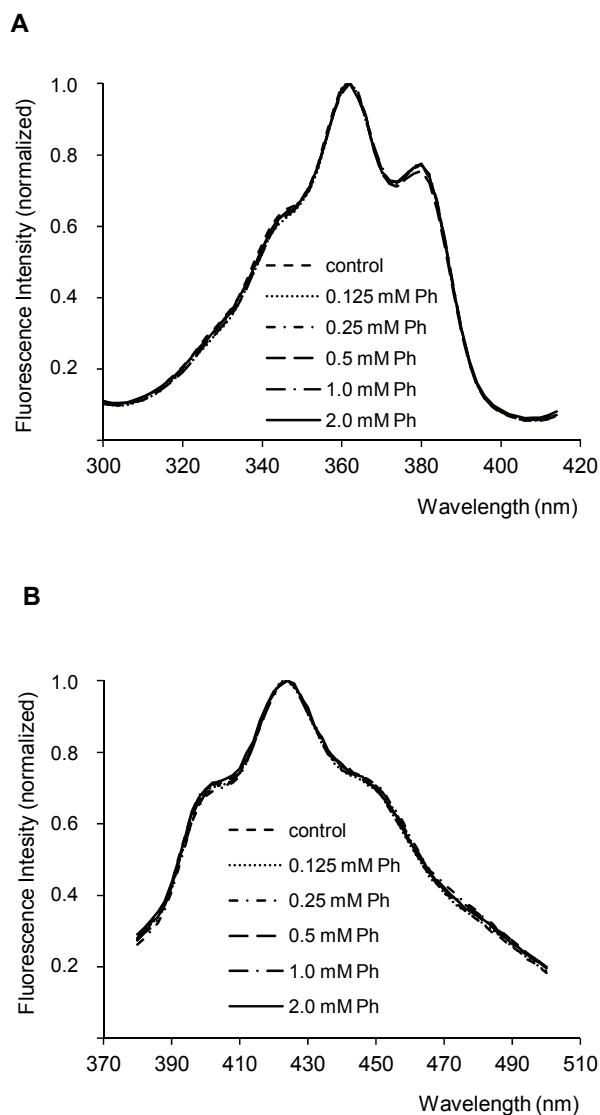


Fig. 9. Quenching of DPH fluorescence intensity by increasing concentrations of phenformin. DPH-labelled mitochondria were exposed to increasing concentrations of phenformin and the decrease of fluorescence intensity was determined up to the 2 mM concentration of this inhibitory agent. Fluorescence intensity values for each excitation wavelength (300–415 nm) were normalized as fraction of the fluorescence intensity of excitation maximum at 365 nm (**A**) and values for each emission wavelength (380–500 nm) were normalized as fraction of the fluorescence intensity of emission maximum at 425 nm (**B**). Both emission curve and a curve of excitation spectra represent 5 measurements with 0 mM, 0.125 mM, 0.25 mM, 0.5 mM, 1.0 mM and 2.0 mM phenformin.

this highly hydrophobic probe is based exclusively on the membrane-bound signal and r_{DPH} reflects readily the changes of the hydrophobic membrane interior altered by temperature, hydrostatic and osmotic pressure, membrane lipid composition (cholesterol level, state of saturation of fatty acids, hydration), cell cycle, ageing and wide range of pharmacological interferences (Shinitzki 1984, Cossins and Sinenski 1984, de Laat *et al.* 1984, Hitzemann *et al.* 1984, Amler *et al.* 1986, 1990).

Surprisingly, we found that anisotropy of DPH fluorescence was unchanged at 1 mM and 2 mM concentration of phenformin (Table 1) under experimental conditions when the activity of complex I was strongly inhibited (Fig. 3 and 4). Simultaneously, determination of both emission and excitation spectra of DPH-labeled membranes indicated no significant change of intensity of DPH fluorescence which might obscure r_{DPH} data (Fig. 9).

Discussion

In our study, we present data indicating that biguanides (metformin and phenformin) seriously affects more than one aspect of mitochondrial function. Our conclusion is based on the following findings: 1) Phenformin, as well as metformin, inhibits mitochondrial respiration in dose-dependent manner, phenformin being 10 times more efficient than metformin; 2) both the phenformin and the metformin inhibitory effect is not dependent on cellular integrity but manifests itself in liver homogenates and isolated mitochondria as well as in permeabilized cells; 3) phenformin and metformin inhibit most effectively the activity of mitochondrial complex I but phenformin in higher concentration affects also complex II and complex IV; 4) in isolated mitochondria, phenformin decreases respiratory control index and releases the mitochondrial membrane potential; 5) phenformin *in vitro* decreases the sensitivity of mitochondrial Ca^{2+} -activated pore to calcium and reduces

the rate and extent of mitochondrial swelling.

From all the data presented in this study, we may conclude that the contradictory results relating to metformin inhibitory action on mitochondrial respiration resulted from its very low efficacy to affect mitochondrial complex I in *in vitro* experiments. On the contrary, another biguanide, phenformin, was known as a substance with many side effects when used for clinical treatments and as a more potent inhibitor in *in vitro* experiments. For this reason, for experimental studies, phenformin is evidently a better choice when we try to understand mechanism through which biguanides affect mitochondrial functions.

Guanidines and biguanides cause a wide variety of metabolic effects on many types of organisms and species and this rather extreme variability is mirrored by the heterogeneity of proposed explanations of their actions. Nevertheless, we can find several common features across all of the seemingly unrelated desired and undesired biguanide effects: 1) there are no biguanide-specific receptors which could transmit specific signals; 2) the glucose lowering effect of biguanides is not associated with the increased insulin secretion; 3) all blood glucose lowering biguanides exist only as positively charged species in physiological environment and 4) they bind readily to biological membranes.

It was proposed that membrane changes largely represent a common denominator explaining metformin effects on various systems involved in receptor signaling and related functions (Schäfer 1976, Wiernsperger 1999). Indeed, no effects of biguanides at therapeutic doses are known on soluble enzymes. As outlined in Schäfer (1983), the interaction of phospholipid membrane with biguanides may have several consequences on its physiological properties which may in turn induce a variety of disorders including changes at the membrane surface of ion activities, K_m of membrane enzymes, V_{max} of catalysis, protein conformation, receptor properties, fluidity of lipids, water structure at interphase, etc. Nevertheless, the exact character of this interaction remains unclear.

Based on our results, the direct interference of phenformin on the modification of hydrophobic lipid interior of mitochondrial membrane may be excluded as a mechanism of action of this potent inhibitory agent. The

possibility of interference with the polar-head group region of the membrane bilayer cannot be excluded at present time, but it is not to be expected as analysis of the effect of different salts on isolated plasma membranes prepared from the brain indicated no change of r_{DPH} values even at very high concentrations of NaCl or KCl (Vosahlikova, unpublished data). The role of the membrane surface-membrane water interface and the net-negative surface charge should be thus considered in this respect as a non-specific biophysical parameters increasing concentration of this inhibitor in the near vicinity of mitochondrial membrane and the changes of numerous enzyme activities participating in regulation of glucose metabolism and genesis of increased sensitivity of peripheral tissues to insulin (DeFronzo and Goodman 1995).

The data presented in our study indicate that biguanides act on various processes occurring in mitochondria. All these processes are connected with the inner mitochondrial membrane and thus the observed response of mitochondrial function to phenformin and metformin is compatible with the above mentioned concept. In fact, 36 years ago a paper entitled “Some new aspects on the interaction of hypoglycemia-producing biguanides with biological membranes” was published (Schäfer 1976). We thus propose that the conclusion of Prof. Schäfer that, “the specific consequences of biguanide – membrane phospholipids interactions provide a unique molecular basis for an understanding of the large variety of biguanide effects and explain their hypoglycemic effect as an accidental result rather than a principal of drug action” should be re-evaluated in further studies as it offers the most plausible explanation of the observed variety of biguanide effects.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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PŘÍLOHA VIII

Páleníčková E, Cahová M, Drahota Z, Kazdová L, Kalous M. Inhibitory effect of metformin on oxidation of NADH-dependent substrates in rat liver homogenate. *Physiol Res*. 2011;60(5):835-9. doi: 10.33549/physiolres.932193.

SHORT COMMUNICATION

Inhibitory Effect of Metformin on Oxidation of NADH-Dependent Substrates in Rat Liver Homogenate

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Summary

Metformin is widely used in the treatment of Type 2 diabetes, however, mechanisms of its antihyperglycemic effect were not yet fully elucidated. Complex I of mitochondrial respiration chain is considered as one of the possible targets of metformin action. In this paper, we present data indicating that the inhibitory effect of metformin can be tested also in liver homogenate. Contrary to previous findings on hepatocytes or mitochondria under our experimental conditions, lower metformin concentrations and shorter time of preincubation give significant inhibitory effects. These conditions enable to study the mechanism of the inhibitory effect of metformin in small samples of biological material (50-100 mg wet weight) and compare more experimental groups of animals because isolation of mitochondria is unnecessary.

Key words

Liver homogenate • Glutamate and Palmitoyl carnitine oxidation • Metformin

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Type 2 diabetes is a progressive metabolic disorder associated with abnormal glucose and lipid metabolism. Metformin (N,N-dimethylimidodicarbonimidic diamide) has been used for the treatment

of diabetes since the late 1950's (Bailey and Turner 1996) and until now is the drug of the first choice. Metformin decreases the hepatic glucose production mainly by inhibiting gluconeogenesis (Hundal *et al.* 2000). We have previously also shown that metformin decreases fatty acids oxidation in liver (Cahova *et al.* 2010).

Recently it was reported that metformin inhibits gluconeogenesis through mechanisms linked to perturbation of mitochondrial function (Foretz *et al.* 2010). Its effect was explained as an inhibition of mitochondrial complex I activity. The inhibitory effect of metformin was demonstrated in hepatocytes incubated with 10 mM metformin for 180 min (El-Mir *et al.* 2000). In isolated mitochondria incubated for 255 min in the presence of 10 mM metformin the oxidation of glutamate+malate+ADP was decreased by 40 % but in disrupted mitochondria the inhibitory effect of metformin was only 10 % (Owen *et al.* 2000). El-Mir *et al.* (2000) could find inhibition by metformin only in intact cells but not in isolated mitochondria. Recently it was demonstrated that metformin attenuates the generation of oxygen reactive species (Ouslimani *et al.* 2005) and inhibits the opening of the mitochondrial membrane permeability transition pore activated by cytosolic Ca²⁺ and ROS, which was associated with prevention of necrotic processes (Carvalho *et al.* 2008, Guigras *et al.* 2004). Despite of these findings, the detailed mechanism of metformin action on mitochondrial respiration and on complex I in particular has not been well defined yet.

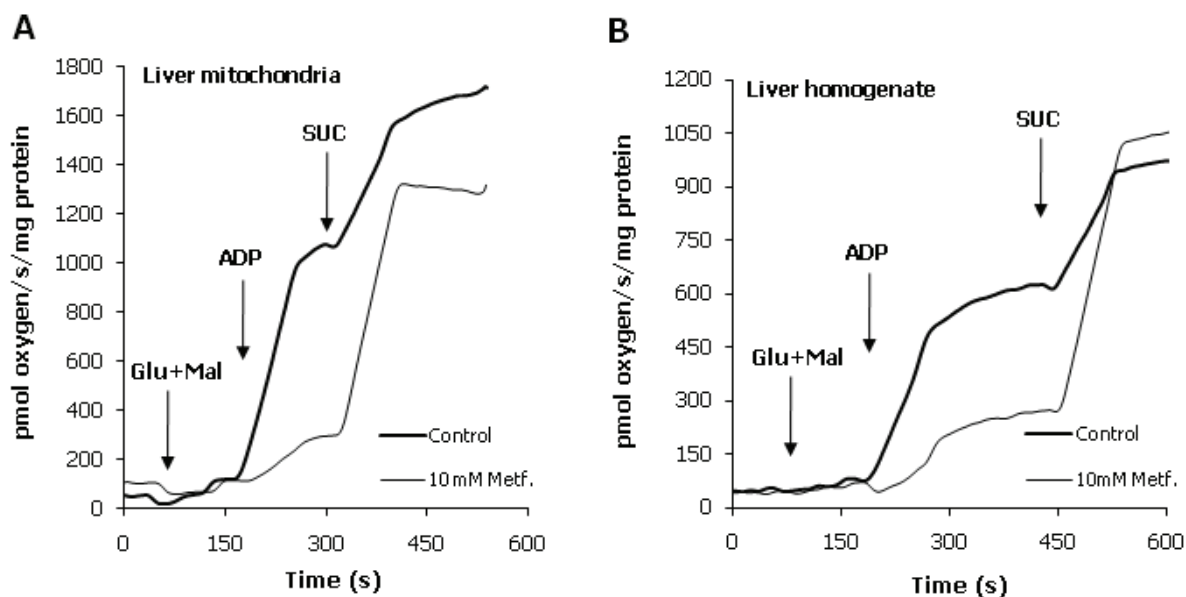


Fig. 1. The effect of 10 mM metformin on the oxidation of glutamate and malate by isolated liver mitochondria (**A**) and liver homogenate (**B**). Respiration of liver mitochondria (0.2 mg protein/ml) or liver homogenate (0.6 mg protein/ml) was measured with or without 10 mM metformin (5 min preincubation) in potassium medium supplemented with 0.5 mg fatty acid free bovine serum albumin per ml and 20 μ M cytochrome c. 10 mM glutamate, 2.5 mM malate, 1.5 mM ADP and 10 M succinate were added as indicated.

More data are thus required to understand the mechanisms through which metformin can inhibit mitochondrial respiratory chain activity.

We tried therefore to compare the effect of metformin on substrate oxidation of mitochondria and liver homogenate and to define conditions for such in vitro studies and we found that the metformin inhibitory effect can be well evaluated also in tissue homogenate which has not been used up to now. In our experimental design we were able to detect significant metformin inhibitory effect after short (3–5 min) preincubation at a lower concentration range (2.5–5 mM) compared with previously published data (Owen *et al.* 2000).

For the oxygen consumption measurements we used High Resolution Oxygraph K2- OROBOROS (Austria). Wistar male rats 200–250 g fed standard laboratory diet were used. Liver homogenate was 10 % (w/v) prepared in 0.25 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 medium and liver mitochondria were isolated by differential centrifugation according to Schneider and Hogeboom (1950). Oxygen consumption was registered in a medium (potassium medium) containing 100 mM KCl, 10 mM Tris-HCl, 4 mM KH_2PO_4 , 3 mM MgCl_2 , 1 mM EDTA, pH 7.3 at 30 °C, which was prepared with modification according to Gnaiger *et al.* (1995). Palmityl carnitine ((3R)-3-hexadecanoyloxy-4-trimethylazaniumylbutanoate) oxidation was determined as described in our previous papers

(Křiváková *et al.* 2008, Červinková *et al.* 2008, Endlicher *et al.* 2009). The protein concentration was determined according to Bradford (1976).

The data presented in Fig. 1 confirmed that metformin inhibitory effect does not require measurement in intact cell. The inhibitory effect of 10 mM metformin could be demonstrated both in mitochondria and in homogenate after a short 3–5 min preincubation. The sensitivity of both homogenate and mitochondria to metformin was higher compared with previously published data (Owen *et al.* 2000).

In further experiments, we tested the concentration dependence of the inhibitory effect of metformin using glutamate+malate+ADP and palmitylcarnitine+malate+ADP as substrates. We found a significant inhibitory effect of metformin for glutamate+malate+ADP respiration and for respiratory control index already at 5 mM concentration both for homogenate and mitochondria. Maximum inhibitory effect of metformin (about 80 %) was found in a range between 10–20 mM concentration (Fig. 2). Inhibition of “state 3” respiration was parallel with the inhibition of the respiratory control index (Fig. 2B). Decrease of respiratory control may represent another indicator of metformin action on mitochondria connected with decreased ATP production, however, further studies are necessary to elucidate this issue.

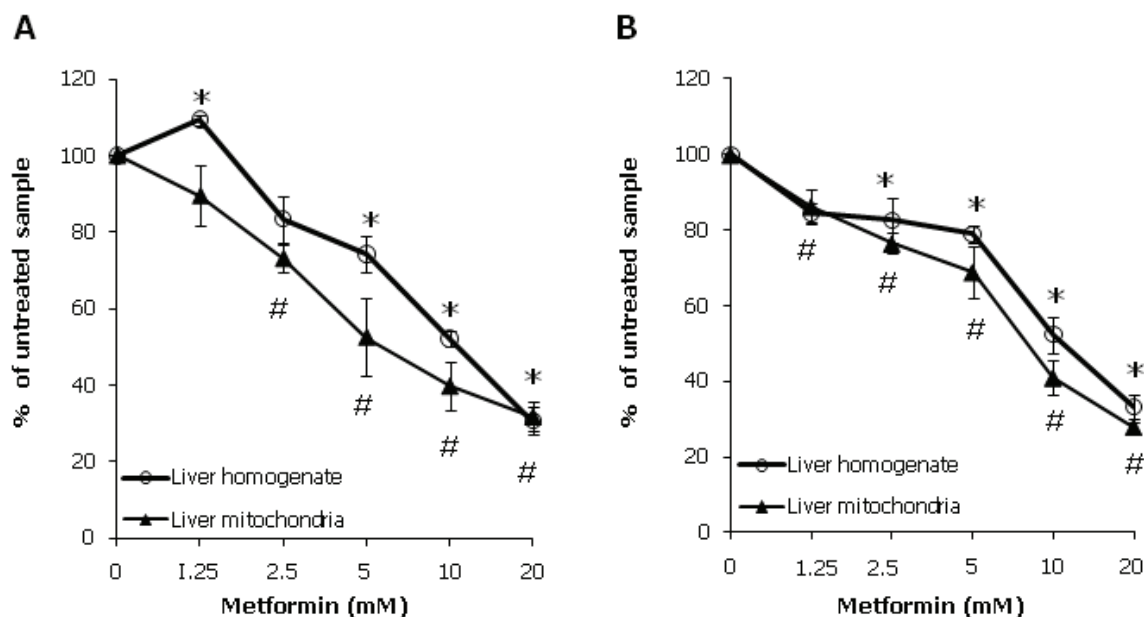


Fig. 2. The effect of metformin concentration on glutamate+malate oxidation (**A**) and respiratory control index (**B**) in the rat liver homogenate and mitochondria. Glutamate and malate-dependent respiration of rat liver homogenate and mitochondria was measured as described in Fig. 1 after 5 min preincubation with 0, 1.25, 2.5, 5, 10, 20 mM metformin. Respiratory control index was calculated from values obtained in Fig. 2A as the ratio of respiration rate in the presence and absence of ADP. * $P < 0.05$ vs. untreated sample (homogenate). # $P < 0.05$ vs. untreated sample (mitochondria). The presented data represent average from 4 independent experiments $\pm$ S.E.M. and they are expressed as percent of untreated sample values. The oxygen consumption of liver homogenate and mitochondria were 609 pmol/s/mg protein and 1100 pmol/s/mg protein, resp. Control values of the respiratory control index of liver homogenate and mitochondria were 7.7 and 7.6, resp.

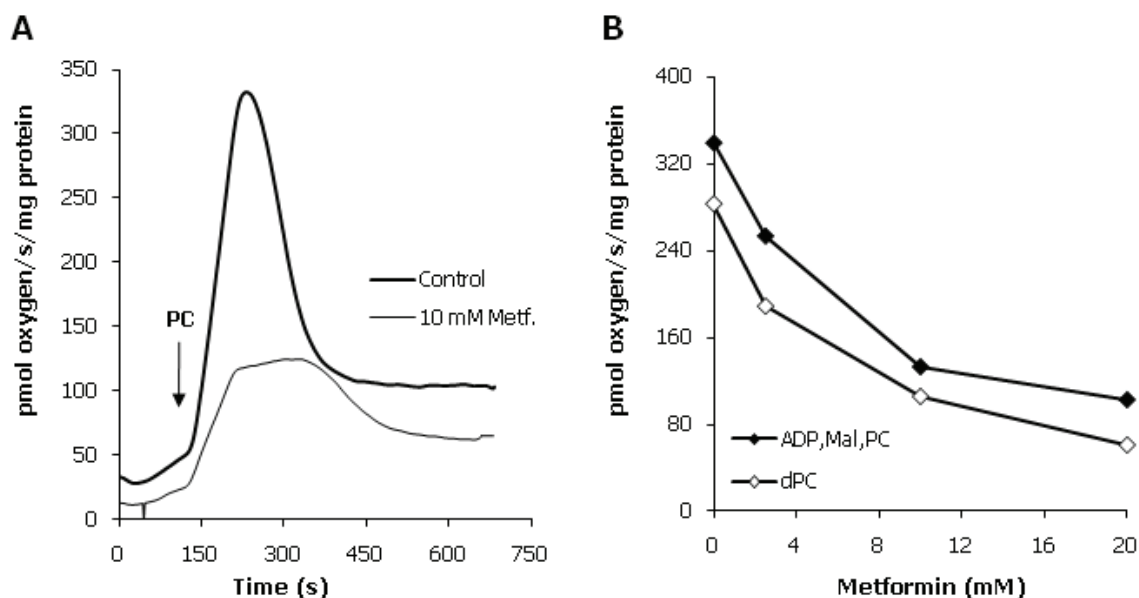


Fig. 3. The palmityl carnitine oxidation in rat liver homogenate **A**: the effect of single dose of metformin; **B**: concentration-dependent effect of metformin on palmityl carnitine oxidation. Palmityl carnitine oxidation was determined under condition described at Fig. 1, but BSA was not added. 12.5 μ M palmityl carnitine was added to the incubation medium with homogenate malate and ADP after 5 min equilibration with metformin. dPC were calculated as the difference of oxygen consumption in the presence of malate+ADP with and without palmityl carnitine. Data are representative values from two independent experiments.

Palmityl carnitine oxidation which involves both NADH- and flavoprotein-dependent dehydrogenases was inhibited at the same concentration range as oxidation of

glutamate+malate (Fig. 3). Similarly, 60 % inhibition of palmityl carnitine oxidation by 10 mM metformin after 5 min preincubation, as with glutamate+malate was found

(Fig. 3A). We evaluated both the maximum rate of palmityl carnitine oxidation in the presence of malate and ADP and the net increase of respiration induced by palmityl carnitine addition (Fig. 3A). Data in Fig. 3B show that both these parameters are inhibited by metformin similarly as glutamate+malate oxidation.

In agreement with previous findings, we found that succinate oxidation is not affected by metformin (see Fig. 1AB). Our data show that combination of two substrates, NADH-dependent glutamate and flavoprotein-dependent succinate, can fully saturate respiratory chain activity by electrons. Glutamate+malate (NADH-dependent substrate) alone can not saturate respiratory chain capacity and succinate (flavoprotein-dependent substrate) was used to reach maximum values of the respiratory rate. However, when the complex I activity is decreased by metformin complex II activity can fully compensate this decrease of electron flow (see Fig. 1AB), both in liver homogenate and isolated mitochondria.

We may thus conclude that liver homogenate

represents a very useful system for evaluation of metformin inhibitory effect on mitochondrial function. The results are comparable with those obtained on isolated mitochondria and the whole procedure is less labour- and time-consuming. Our measurements on homogenate also confirm previous findings indicating that intact cells are not required for the manifestation of metformin inhibitory effect. We further show that the respiratory control index, an important indicator of mitochondrial function, could also be well detected on samples of tissue homogenates.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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PŘÍLOHA IX

Cahova M, Palenickova E, Dankova H, Sticova E, Burian M, Drahota Z, Cervinkova Z, Kucera O, Gladkova C, Stopka P, Krizova J, Papackova Z, Oliyarnyk O, Kazdova L. *Metformin prevents ischemia-reperfusion induced oxidative stress in the fatty liver by attenuation of reactive oxygen species formation*. Am J Physiol Gastrointest Liver Physiol. 2015 Jul 15;309(2):G100-11. doi: 10.1152/ajpgi.00329.2014.

Metformin prevents ischemia reperfusion-induced oxidative stress in the fatty liver by attenuation of reactive oxygen species formation

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²Department of Cell Biology, Faculty of Science, Charles University, Prague, Czech Republic; ³Clinical and Transplant Pathology Department, Charles University, Prague, Czech Republic; ⁴Department of Diagnostic and Interventional Radiology, Institute for Clinical and Experimental Medicine, Prague, Czech Republic; ⁵Institute of Physiology, Czech Academy of Sciences, Prague, Czech Republic; ⁶Department of Physiology, Charles University in Prague, Faculty of Medicine in Hradec Kralove, Hradec Kralove, Czech Republic; ⁷Department of Chemistry, University of Cambridge, Cambridge, United Kingdom; and ⁸Institute of Inorganic Chemistry Academy of Science CR, Husinec-Rez, Czech Republic

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Cahova M, Palenickova E, Dankova H, Sticova E, Burian M, Drahota Z, Cervinkova Z, Kucera O, Gladkova C, Stopka P, Krizova J, Papackova Z, Oliyarnyk O, Kazdova L. Metformin prevents ischemia reperfusion-induced oxidative stress in the fatty liver by attenuation of reactive oxygen species formation. *Am J Physiol Gastrointest Liver Physiol* 309: G100–G111, 2015. First published June 4, 2015; doi:10.1152/ajpgi.00329.2014.—Nonalcoholic fatty liver disease is associated with chronic oxidative stress. In our study, we explored the antioxidant effect of antidiabetic metformin on chronic [high-fat diet (HFD)-induced] and acute oxidative stress induced by short-term warm partial ischemia-reperfusion (I/R) or on a combination of both in the liver. Wistar rats were fed a standard diet (SD) or HFD for 10 wk, half of them being administered metformin (150 mg·kg body wt⁻¹·day⁻¹). Metformin treatment prevented acute stress-induced necroinflammatory reaction, reduced alanine aminotransferase and aspartate aminotransferase serum activity, and diminished lipoperoxidation. The effect was more pronounced in the HFD than in the SD group. The metformin-treated groups exhibited less severe mitochondrial damage (markers: cytochrome *c* release, citrate synthase activity, mtDNA copy number, mitochondrial respiration) and apoptosis (caspase 9 and caspase 3 activation). Metformin-treated HFD-fed rats subjected to I/R exhibited increased antioxidant enzyme activity as well as attenuated mitochondrial respiratory capacity and ATP resynthesis. The exposure to I/R significantly increased NADH- and succinate-related reactive oxygen species (ROS) mitochondrial production in vitro. The effect of I/R was significantly alleviated by previous metformin treatment. Metformin downregulated the I/R-induced expression of proinflammatory (TNF- α , TLR4, IL-1 β , Ccr2) and infiltrating monocyte (Ly6c) and macrophage (CD11b) markers. Our data indicate that metformin reduces mitochondrial performance but concomitantly protects the liver from I/R-induced injury. We propose that the beneficial effect of metformin action is based on a combination of three contributory mechanisms: increased antioxidant enzyme activity, lower mitochondrial ROS production, and reduction of postischemic inflammation.

metformin; oxidative stress; mitochondrial respiration; liver injury; ³¹P MR spectroscopy

METFORMIN IS AN ORAL ANTIHYPERGLYCEMIC drug widely used in the treatment of Type 2 diabetes (T2D) (7). There is evidence that metformin also protects against oxidative stress in various tissues, although the antioxidant activity of metformin is not well understood. It has been shown that hyperglycemia is associated with increased reactive oxygen species (ROS) formation due to superoxide overproduction from the mitochondrial electron transport chain as a consequence of increased glycolysis (8). It has been hypothesized that the antioxidative effect of metformin is simply the consequence of its glucose-lowering effect and subsequent decrease in superoxide anion production (3). Nevertheless, metformin has also displayed antioxidative characteristics in several models of oxidative stress without hyperglycemia (2, 19, 20, 21, 62).

Increased production and/or ineffective scavenging of ROS play an important role in the pathogenesis of many diabetic complications (14). Nonalcoholic fatty liver disease, a component of a cluster of pathophysiological conditions preceding the manifestation of overt T2D, is often associated with chronic oxidative stress. Surprisingly, there is not much data regarding the effect of metformin on oxidative stress in the fatty liver in the context of insulin resistance.

Liver ischemia-reperfusion (I/R) injury is a major contributor to tissue damage during liver transplantation, liver resection procedures, hypovolemic shock, and trauma. It is a major cause of primary nonfunction after liver transplantation (11). I/R injury is a phenomenon in which damage to a hypoxic organ is accentuated following the return of oxygen delivery. The immediate response involves the disruption of the cellular mitochondrial oxidative phosphorylation and accumulation of metabolic intermediates during the ischemic period and oxidative stress following the resumption of blood flow (42). As a result, direct cellular damage occurs due to the ROS impact on lipids and other biomacromolecules. Moreover, oxidative stress impairs mitochondrial function, which leads to further production of ROS and amplification of oxidative stress and tissue injury (37). ROS produced during oxidative stress may also act as signaling molecules, which activate inflammatory pathways and which in turn accentuate primary injury (32). Thus the extent of early ROS formation represents a critical event in terms of the magnitude of the final injury.

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The aim of our study was to assess the effect of metformin on both chronic oxidative stress induced by high-fat diet (HFD) feeding and acute stress caused by short-term warm I/R, or on the combination of both. We recorded the effects of metformin on whole body, tissue, and cellular levels. We further focused on the possible mechanisms of metformin antioxidant action, particularly on those related to metformin interaction with the mitochondrial respiratory chain, ROS production, and inflammatory processes.

MATERIALS AND METHODS

Animals and experimental design. Male Wistar rats aged 4 mo were kept in a temperature-controlled environment with a 12-h light-dark cycle on either standard diet (SD; protein 33cal%, starch 58cal%, fat 9cal%) or HFD (protein 28cal%, starch 12cal%, fat 60cal%) diets. Half of the animals in both groups were provided metformin in a dose of 150 mg/kg body wt for 10 wk by gavage. This dose corresponds to a human dose of 15 mg·kg⁻¹·day⁻¹, according to the normalization of body surface area (18), and results in comparable serum metformin concentrations (1–10 µM). The animals in groups labeled I/R were subjected to short-term (20 min) warm partial ischemia induced during anesthesia by portal vein ligation. After restoration of circulation, the abdominal cavity was closed and animals were left to recover 48 h prior to decapitation. Sham-operated animals were subjected only to the opening of the abdominal cavity without portal vein ligation. All experiments were performed in accordance with the Animal Protection Law of the Czech Republic 311/1997, which is in compliance with the Principles of Laboratory Animal Care (NIH Guide to the Care and Use of Laboratory Animals, 8th edition, 2013) and were approved by the Ethical Committee of the Institute for Clinical and Experimental Medicine.

Histological evaluation of liver injury. Liver tissue was fixed in 4% paraformaldehyde, embedded in paraffin blocks, and routinely processed. Sections cut at 4–6 µm were stained with hematoxylin and eosin and examined with an Olympus BX41 light microscope.

Electrophoretic separation and immunodetection. The homogenate and postmitochondrial fractions were prepared from freshly excised liver tissue. The protein concentration was determined by the BCA method (Thermo Scientific, Waltham, MA). The proteins were separated by electrophoretic separation under denaturing conditions and electroblotted to PVDF membranes. Proteins of interest were detected by using specific antibodies (cytochrome c: ab53056 Abcam, Cambridge, UK; caspase 3: no. 9662, caspase 9: no. 9506, Cell Signaling, MA). The loading control was performed with rabbit polyclonal antibody to β-actin (ab 8227, Abcam, Cambridge, UK). Bands were visualized by using ECL and quantified by using the FUJI LAS-3000 imager (FUJI FILM, Japan) and Quantity One software (Bio-Rad, Hercules, CA).

Citrate synthase specific activity. Citrate synthase (CS) activity was measured according to Rustin et al. (52). Briefly, the cells were lysed with 1% lauryl maltoside and CS activity was measured according to the conversion of acetyl-CoA and oxaloacetate to citrate and CoA. This rate-limiting reaction catalyzed by CS was coupled to the irreversible reduction of chromogenic substrate TNB (thionitrobenzoic acid). Citrate synthase activity was measured as an increase of absorbance at 412 nm per time interval (1 min) and was related to the total content of protein determined by the BCA Protein Assay Reagent (Thermo Scientific).

mtDNA copy number estimation. mtDNA copy number per cell was quantified as the ratio of mitochondrial DNA to nuclear DNA as previously described (70). In brief, quantitative PCR analysis using SYBR green (Solis BioDyne, Tartu, Estonia) was performed with 25 µg of isolated DNA (Qiagen). Mitochondrial DNA was assessed according to the expression of mitochondrial-encoded *Nd5* gene. Nuclear DNA level was determined by amplifying the genomic *Ucp2*.

The relative amount of mitochondrial to nuclear DNA was determined by normalized *Nd5* to *Ucp2* levels.

Isolation of liver mitochondria and preparation of submitochondrial particles. Liver mitochondria were prepared by differential centrifugation as described by Bustamante et al. (10) with some modifications. Rat liver tissue was homogenized at 0°C by using a Teflon-glass homogenizer as a 10% homogenate in a medium containing 220 mM mannitol, 70 mM sucrose, and 1 mM HEPES, pH 7.2 (MSH medium). Crude impurities were removed by centrifugation at 800 g, 10 min, and the remaining supernatant was centrifuged for 10 min at 8,000 g. Pelleted mitochondria were resuspended in the MSH medium, washed twice via 10 min centrifugation at 8,000 g, and finally resuspended in a concentration of 20–30 mg protein/ml. Mitochondrial proteins were determined by the BCA method (Thermo Scientific, Waltham, MA). To determine ROS production, submitochondrial particles (SMP) were prepared according to Ide et al. (27). Briefly, the isolated mitochondria were sonicated and pelleted by centrifugation at 21,000 g, 10 min. The resultant pellet was washed three times in MSH buffer to get rid of matrix components and stored at –80°C.

Measurement of mitochondrial respiration by the Seahorse technique. An XF 24 extracellular flux analyzer was used to determine mitochondrial function (XF 24, Seahorse Bioscience, <http://www.seahorsebio.com/company/about.php>). The validity of this method compared with classical Clark electrode oxygraph measurements has been proved recently (22). Freshly isolated liver mitochondria (10 µg per well) in 50 µl of MAS buffer (220 mM mannitol, 70 mM sucrose, 10 mM KH₂PO₄, 5.5 mM MgCl₂, 2 mM HEPES, 1 mM EGTA, pH 7.4) were delivered to each well on ice and the 24-well plate was centrifuged at 20,000 g for 20 min at 4°C to enhance the attachment of the mitochondria to the plastic. After centrifugation, 450 µl of prewarmed MAS buffer was added to each plate and the first two measurements were performed in the absence of any added substrate. Subsequently, the following substances were added in the indicated order (final concentrations): 10 mM glutamate + 5 mM malate; 4 mM ADP; 2 µM rotenone; 10 mM succinate. The data are given as the oxygen consumption rate (OCR) in picomoles O₂ per minute.

Parameters of oxidative stress. The activities of superoxide dismutase (SOD), catalase, glutathione peroxidase 1 (GPx1) and thiobarbituric acid reactive substances (TBARS) content were determined as described previously (50). The content of reduced GSH was determined by using the Glutathione HPLC diagnostic kit (Chromsystems, Munich, Germany), 4-hydroxynonenal (4-HNE) concentration by OxiSelect HNE Adduct Competitive ELISA Kit (Cell Biolabs, San Diego, CA).

Fluorometric determination of reactive oxygen species production. ROS production in SMPs in vitro was measured by using a DCFDA (Cell Biolabs, San Diego, CA) probe as described previously (67). Briefly, the assay was performed with ~0.2 mg of mitochondrial protein per milliliter in an MAS buffer supplemented with either 10 mM NADH, 10 mM glycerol phosphate (sn-glycerol 3-phosphate), or 10 mM succinate. The final concentration of DCFDA was 10 µM and the excitation/emission wavelength was 485/528 nm. The fluorescence signal rose linearly from 0 until the 45th minute of the assay. The presented data were obtained 45 min after the start of the assay. All experiments were repeated in the absence of mitochondria and background fluorescence changes were subtracted. The obtained values were normalized per milligram of protein.

EPR spin-trapping measurements. Measurement of free radicals by electron paramagnetic resonance (EPR) is based on the ability of radical compounds to absorb microwave energy in strong magnetic fields. To demonstrate the effect of I/R and metformin on mitochondrial ROS production, an aliquot of the SMP suspension (2 mg protein) was reacted with 10 mM NADH and the spin-trapping agent 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) in a Quartz flat cell with a thickness of 0.5 mm. Immediately after the start of the reaction, EPR spectra were recorded at an ambient temperature (25°C) using an

E-540 spectrometer operating at X-band (9.7 GHz). Microwave power was 20 mV and magnetic field modulations were 1 and 4 Gauss. The quantitation of the signals' intensities was performed by comparing the amplitude of the observed signal with the standard Mn^{2+}/ZnS and Cr^{3+}/MgO markers. EPR spectra were recorded as the first derivation and the main parameters [g -factor values, hyperfine coupling constant A , line widths ΔH_{pp} (peak-to-peak distance) and ΔA_{pp} (peak-to-peak amplitude)] were calculated according to Weil and Bolton (68). The DMPO adducts were identified according to g factor, and diphenyl-1-picrylhydrazyl (DPPH) served as an internal standard ($g = 2.0036$). The Bruker and Origin (Bruker, Rheinstetten, Germany) software interface was used for spectra recording, handling, and evaluation (51).

^{31}P -NMR studies. Rats were anesthetized with ketamine/dormitor, the abdominal cavity was opened and a vessel loop was loosely positioned around the porta hepatica. Spectra were scanned on the Bruker Biospec 47/20 (4.7-T field strength) animal scanner (Ettlingen, Germany) by using a custom-made 10-mm loop diameter remotely tuned $^{31}P/^1H$ coil. The coil consisted of two parts: a sole probe head lying on top of the exposed liver to get rid of muscle tissue signals and access the portal vein, and a remote block of tuning capacitors set ~10 cm apart from the probe head to reduce the influence of the weight and tuning torque reaction forces of the bulky tuning capacitors on the observed liver. During the nuclear magnetic resonance (NMR) experiment, the animals lay on the back in a holder rig with the coil on the top of the exposed liver. After acquisition of the first spectrum, the portal vein was occluded and the subject was repositioned to the same position in the magnet. Spectra were then acquired for the next 20 min. The occlusion was then released and 18 more spectra were acquired in 5-min intervals. A single-pulse sequence without proton decoupling was used to acquire the ^{31}P spectra with the following parameters: TR = 500 ms, 512 averages. Relative ATP levels were then evaluated from intensities of β -ATP peaks using the jMRUI software package (63).

Real-time quantitative PCR analysis. Total RNA was extracted from rat liver tissue by using an RNeasy Mini Kit (Qiagen, Courtaboeuf, France); 500 ng of total RNA was reverse-transcribed by use of a High Capacity Reverse Transcription Kit (Life Technologies, Forest City, CA) and real-time quantitative PCR analysis (RT-qPCR) was performed (viiA7, Life Technologies, Forest City, CA) with EvaGreen qPCR Mix (Solis BioDyne). The primers were designed with Primer3 software; details are given in Table 1. Gene expression values were normalized to the expression value of the housekeeping genes *Gusb* and *B2m*. Calculations were based on the comparative cycle threshold C_t method ($2^{-\Delta\Delta C_t}$).

Biochemical analyses. Nonesterified fatty acids (FFA), triacylglycerol, glucose, lactate, and β -hydroxybutyrate serum content were

determined with commercially available kits (FFA: FFA half micro test, Roche Diagnostics, Mannheim, Germany; triglycerides, cholesterol, and glucose: ERBA-Lachema, Brno, Czech Republic; β -hydroxybutyrate: Cayman Chemical, Ann Arbor, MI; lactate: Biovision, San Francisco, CA). Serum glucose was measured using an Accu-Chek glucometer (Roche, Czech Republic). Glycogen and triacylglycerol content in the liver were determined as described previously (12).

Statistical analyses. Data are presented as means $\pm$ SE of multiple determinations. Statistical analyses were performed with SigmaStat (Systat Software, San Jose, CA) or Microsoft Excel statistical packages. One-way ANOVA with Bonferroni or two-way ANOVA with multiple determinations was employed to test the differences between more than two groups. The Student's t -test was used to detect the effect of I/R within groups. Differences were considered statistically significant at the level of $P < 0.05$.

RESULTS

Metabolic characteristics of the experimental groups. Table 2 summarizes the effects of HFD administration and metformin treatment on selected metabolic characteristics. As expected, HFD led to the elevation of serum concentrations of lactate and nonesterified fatty acids and to the increase in ketogenesis. Metformin had no significant effect on most of the parameters despite the enhanced lactate production in both SD and HFD groups. However, metformin administration resulted in diminished weight gain in the HFD group. Liver triacylglycerol content was elevated and glycogen content reduced in the HFD group, but the effect of the diet was not reversed by metformin. To estimate the degree of liver injury induced by oxidative stress, we measured the serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities after I/R (Table 3). As expected, I/R led to a significant increase of serum AST and ALT, and this effect was more pronounced in HFD-fed animals. Metformin had no significant effect on I/R injury in SD-fed rats, but it significantly alleviated I/R-induced AST and ALT release in the HFD group.

Metformin alleviates acute oxidative stress-induced liver injury. As demonstrated by histological findings (Fig. 1A), SD-administered animals exposed to I/R developed confluent predominantly centrilobular necroses in 10% of the liver parenchyma. In HFD-fed rats, there were unevenly distributed necroses in 10–15% of the centrilobular hepatocytes in the I/R group. SD- and HFD-fed animals treated with metformin and

Table 1. Primer sequences

Gene	NCBI Ref. Seq.	Forward Primer	Reverse Primer
Arg 1	M_017134.1	CTGCTGGGAAGGAAGAAAG	TTCCCAAGAGTTGGGTTTCAC
Cd68	NM_001031638.1	CAAGCAGCAGATGGACATTC	GGCAGCAAGAGAGATTGGTC
Emr1	NM_001007557.1	CAACCGCCAGGTACGAGATG	TGCCGCCAACTAACGATACC
Mrc1	NM_001106123.1	CAACCAAAGCTGACCAAAGG	AGGTGGCCTCTTGAGGTATG
TLR4	NM_019178.1	GGATTATCCAGGTGTGAATTG	GCTCTCCACAGCCACCAGATT
Osp	NM_012881.2	TGATGAACAGTATCCCGATG	TGGTTCATCCAGCTGACTTG
Ccr 2	NM_021866.1	TTTGATCCTGCCCTACTTG	AACGCAGCAGTGTGTCATTC
IL10	NM_012854.2	CTGCAGGACTTTAAGGGTTACTTG	TTCTCAGAGGGAGAAATCG
Cd11b	NM_012711.1	CTTCTCCCAGAACCTCTCAAG	ATGCCGAGTCGTTAAAGAGG
IL1 β	NM_031512.2	AGCTGCACAGTTCGCCAAC	GAGACCTGACTTGGCAGAGG
Ly6-C	NM_020103.1	GTGTGCAGAAAGAGGTCAGG	ATTGCAACAGGAAACCTTCG
Tgfb1	NM_021578.2	ACTGCTTCAGCTCCACAGAG	TGGTTGTAGAGGGCAAGGAC
TNFA	NM_012675.3	CAAGGAGGAGAAGTCCCAAATG	GCTTGGTGGTTTGCTACGAC
mtNd5	NC_001665.2	GACTACTAATTGCAGCCACAGGAA	GTAGTAGGGCAGAGACGGGAGTTG
Ucp 2	NC_005100.4	AGAACGGGACACCTTAGAGA	TGCCCCAGTTTCCATCACAC

Ref. Seq., reference sequence.

Table 2. *Metabolic characteristics of the experimental groups*

	SD	SD + Met	HFD	HFD + Met
Body weight, g	482 ± 9	466 ± 12	490 ± 7 ⁱ	437 ± 10 ⁱ
Serum				
Metformin, $\mu\text{mol/l}$		6.8 ± 0.8		6.1 ± 1
Glycemia, mmol/l	6.4 ± 0.1	6.2 ± 0.2	6.1 ± 0.1	6.3 ± 0.1
Lactate, mmol/l	3.5 ± 0.2 ^{a,b}	4.7 ± 0.3 ^a	5.9 ± 0.5 ^{b,j}	6.7 ± 0.3 ^j
Triacylglycerol, mmol/l	1.8 ± 0.09 ^c	1.8 ± 0.2	1.4 ± 0.12 ^c	1.5 ± 0.2
FFA, mmol/l	0.75 ± 0.05 ^d	0.81 ± 0.04	1.02 ± 0.05 ^d	0.91 ± 0.1
Cholesterol, mmol/l	1.6 ± 0.11	1.4 ± 0.13	1.5 ± 0.17	1.6 ± 0.1
β -OH butyrate, mmol/l	0.17 ± 0.2 ^e	0.23 ± 0.01	0.94 ± 0.2 ^e	1.03 ± 0.03
Liver				
Triacylglycerol, $\mu\text{mol/g}$	12.0 ± 1.3 ^{f,g}	9.1 ± 1.1 ^f	60.5 ± 11.3 ^g	53.9 ± 8.6
Glycogen, $\mu\text{mol glucose/g}$	280 ± 13 ^h	277 ± 20	219 ± 28 ^h	185 ± 14

All parameters were determined in the serum or liver of fed animals. Data are expressed as means ± SE. Serum parameters: $n = 20$ per group (blood was collected prior operation); liver parameters: $n = 10$ per group (only sham-operated animals). SD, standard diet; Met, metformin; HFD, high-fat diet; FFA, free fatty acids. Values marked with the same letter are statistically significantly different; ^{a,c,d,j} $P < 0.05$; ^{b,h,i} $P < 0.01$; ^{e,f,g} $P < 0.001$.

subjected to I/R injury showed mild predominantly centrilobular steatosis without any significant necroinflammatory reaction. The HFD itself increased lipid peroxidation measured as TBARS or 4-HNE content in the liver (Fig. 1, B and C). For TBARS, I/R comparably increased lipoperoxide formation in both groups. Importantly, metformin administration diminished TBARS formation by 30 and 25% in the SD and HFD groups, respectively. 4-HNE content was increased only in the HFD, but not in the SD group subjected to I/R. Metformin mitigated the combined effect of the HFD and I/R and reduced 4-HNE content ~2.5 times. These results indicate that metformin protects liver tissue against acute oxidative stress in vivo, probably by preventing excessive ROS formation or strengthening the antioxidant defense.

Metformin reduces oxidative stress-induced mitochondrial damage and apoptosis in vivo. Lower oxidative stress may result in less severe mitochondrial damage in metformin-treated animals exposed to stress conditions compared with untreated ones. In the liver exposed to I/R, we found increased cytochrome *c* content in cytosol (SD << HFD), suggesting its leakage from the outer mitochondrial space (Fig. 2A). This was substantially diminished in metformin-treated animals, thus indicating less mitochondrial injury. Cytosolic cytochrome *c* is known to trigger apoptotic cell death, and we further wanted to confirm whether the reduced/increased mitochondrial damage would be reflected by the changes in the intensity of cell death executed by the apoptotic pathway. Therefore, we determined the activation of caspase 9 and its downstream target caspase 3 in liver homogenates. I/R resulted in caspase 9 and caspase 3 activations in both SD and HFD groups, the effect being more pronounced in the latter. Metformin administration eradicated (SD) or significantly attenuated (HFD) proapoptotic signaling in both groups (Fig. 2,

B and C). To assess whether the antiapoptotic effect of metformin is translated into the quantitative preservation of mitochondria, we determined the activity of citrate synthase which is an exclusive mitochondrial matrix enzyme. Figure 2D demonstrates that I/R resulted in the reduction of citrate synthase activity in both the SD and HFD groups and that the effect of I/R was prevented by metformin. The presence of mitochondrial damage and the protective effect of metformin were independently confirmed by the direct determination of mtDNA copy number in all experimental groups (Fig. 2E). In the HFD group, the effect of metformin and I/R on mitochondrial function was assessed by measuring mitochondrial respiration of NADH-dependent (Fig. 3A) and FADH-dependent substrates (Fig. 3B). In the basal state, i.e., without addition of any substrates and in the presence of malate and glutamate only (NADH-dependent substrates), we did not find any difference between the groups. In contrast, the maximal respiration rate (OCR), measured after the addition of ADP, was significantly diminished both by I/R and metformin administration, but this effect was not additive. In other words, although mitochondria from metformin-treated animals exhibited lower OCR compared with the untreated ones, they were not further damaged by the exposure to I/R. With succinate used as a substrate (FADH dependent), the OCR was not affected by metformin treatment or I/R injury. Taken together, these data indicate that metformin treatment protects liver mitochondria from I/R-induced mitochondrial damage, which further ameliorates apoptosis and partly preserves mitochondrial function.

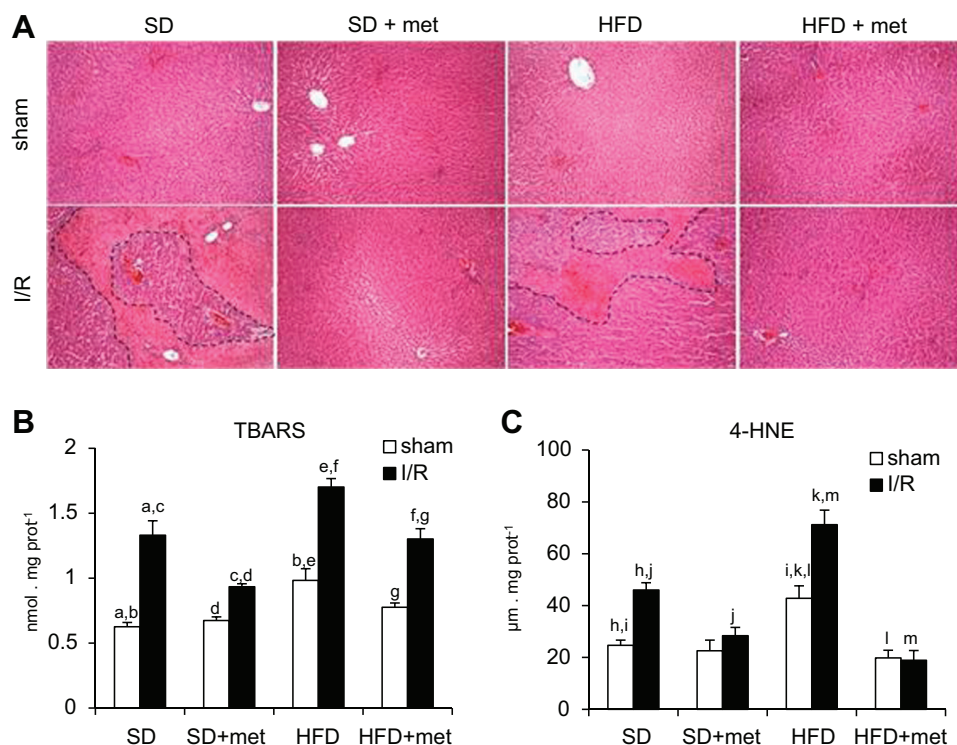
Effect of metformin on antioxidative defense status in vivo. The previous data demonstrate that metformin significantly alleviates oxidative stress evoked by the HFD itself or, more pronouncedly, in combination with I/R. Subsequently, we tested whether the effect of metformin is mediated by the increased activity of antioxidative mechanisms. In the liver, HFD administration itself was associated with diminished GSH stores and decreased activities of GPx1 and SOD, whereas catalase activity was upregulated (Table 4). Metformin treatment ameliorated some of these parameters (GSH content and SOD activity) affected by the diet alone. In the HFD group, oxidative stress evoked by I/R resulted in further exhaustion of liver GSH content and in reduction of antioxidant enzyme activity by 25–50% compared with the values in nonischemic animals. Metformin treatment led to partial restoration of GSH content and enzyme activity. We did not observe any effect of metformin on the expression of relevant genes that encode antioxidative enzymes (not shown). The presented data dem-

Table 3. *Effect of ischemia reperfusion on the markers of liver injury*

	SD	SD + Met	HFD	HFD + Met
AST, $\mu\text{kat/l}$				
Control	3.7 ± 0.2 ^a	3.8 ± 0.1 ^b	4.1 ± 0.3 ^c	3.9 ± 0.5 ^c
I/R	7.0 ± 0.3 ^a	6.8 ± 0.3 ^b	12.6 ± 1.5 ^{c,d}	7.0 ± 0.8 ^{d,e}
ALT, $\mu\text{kat/l}$				
Control	0.8 ± 0.1	0.7 ± 0.1	1.0 ± 0.1 ^f	0.7 ± 0.1 ^h
I/R	1.1 ± 0.2	1.0 ± 0.3	4.1 ± 0.8 ^{f,g}	2.0 ± 0.7 ^{g,h}

Data are expressed as means ± SE, $n = 10$. AST, aspartate aminotransferase; ALT, alanine aminotransferase; I/R, ischemia-reperfusion. Values marked with the same letter are statistically significantly different; ^a $P < 0.05$; ^{d,e} $P < 0.01$; ^{a,b,c,f} $P < 0.001$.

Fig. 1. Effect of metformin (met) on liver injury induced by ischemia-reperfusion. A: confluent necroses of hepatocytes were observed in animals fed a standard diet (SD) and high-fat diet (HFD) after ischemia-reperfusion (I/R) injury (dashed lines delimit the necrotic areas); no necroinflammatory activity was observed in sham-operated controls and in the SD+I/R and HFD+I/R groups treated with metformin. Hematoxylin and eosin staining, original magnification $\times 100$. The lipoperoxide formation was determined according to thiobarbituric acid reactive substances (TBARS; B) and 4-hydroxynonenal (4-HNE; C) content. Data are presented as means $\pm$ SE, $n = 10$. Bars marked with the same letter are statistically significantly different, $b,c,d,f,h,j P < 0.05$; $a,e,g,k,l P < 0.01$.



onstrate that the protective effect of metformin against oxidative damage is associated with increased activity of antioxidant systems in some stress situations.

Metformin decreases reactive oxygen species formation from succinate and NADH, but not from glycerol-3-phosphate. Electron transport through the mitochondrial respiratory chain is associated with potential ROS generation. This risk is further exacerbated during reoxygenation after ischemia. In vitro studies identified three potential targets of the antioxidant effect of metformin: forward electron flux through complex I, reverse electron flux from complex II to complex I and mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH). In vitro, utilization of NADH as the sole substrate stimulates forward electron flux, while utilization of succinate in the absence of NADH promotes reverse electron flux. To characterize the combined effect of in vivo metformin treatment and HFD administration on ROS formation, we used a model of SMP. ROS production in vitro was measured by using a fluorescent probe DCFDA on three different substrates: NADH as a source of electrons for complex I, glycerol-3-phosphate (G-3-P) for mGPDH, and succinate for succinate dehydrogenase (Fig. 4). SMPs isolated from rats subjected to I/R without metformin treatment exhibited significantly increased ROS production from NADH and succinate (succinate $\gg$ NADH) when compared with sham-operated controls. Previous metformin administration reduced ROS production from both these sources. No effect of metformin was observed in animals that were not exposed to I/R. Rather surprisingly, we observed no effect of either I/R or metformin on ROS production from G-3-P.

EPR spectroscopy is the only technique that can directly detect and identify different types of free radicals. We employed this method to investigate NADH-dependent ROS generation in vitro by SMPs prepared from HFD-fed groups. As shown in Fig. 5, after the addition of NADH to the SMP

suspension, we were able to detect the following free radicals: organic free radical OR $\cdot$, showing a symmetric singlet signal; nitroxide radicals NO $\cdot$: triplet spectrum 1:1:1; superoxide radical OOH $\cdot$: quartet 1:1:1:1; and hydroxyl radical OH $\cdot$: quartet 1:2:2:1, as adducts of these radicals with DMPO. The signal intensity was quantified by comparing the amplitude of the observed signal to the standard Mn²⁺/ZnS and Cr³⁺/MgO markers, the results of which are presented in Table 5. We found a significant increase in OH formation in animals subjected to I/R compared with sham-operated controls. A similar trend was observed for OOH $\cdot$, but it did not reach statistical significance ($P = 0.061$). Metformin treatment had no effect on sham-operated animals but completely prevented I/R-induced generation of OOH $\cdot$ and OH radicals ($P = 0.008$ and 0.045 , respectively). Other radical species were not affected by any of the interventions. In conclusion, our data indicate that long-term metformin administration in vivo effectively prevents I/R-associated ROS production from NADH-dependent substrates and succinate.

Metformin slows down ATP synthesis in the liver in vivo. The evidence given in the previous paragraph strongly indicates that metformin administered in vivo attenuates mitochondrial respiration. If so, metformin should also affect ATP synthesis. To confirm this hypothesis, we measured the extent and rate of ATP repletion during reperfusion after partial liver ischemia using ³¹P MR spectroscopy (Fig. 6). The restraint of blood supply led to a rapid fall of ATP levels in all groups. In the SD group, ATP rapidly (within 15 min of reperfusion) resumed original values. In the HFD group, ATP content did not fully recover within examination time, reaching only $\sim 80\%$ of preischemic values. The rate of ATP resynthesis was not significantly different from the SD group. The SD and HFD curves were significantly different ($P < 0.05$) from the 60th min until the end of the experiment. Metformin had a nonsig-

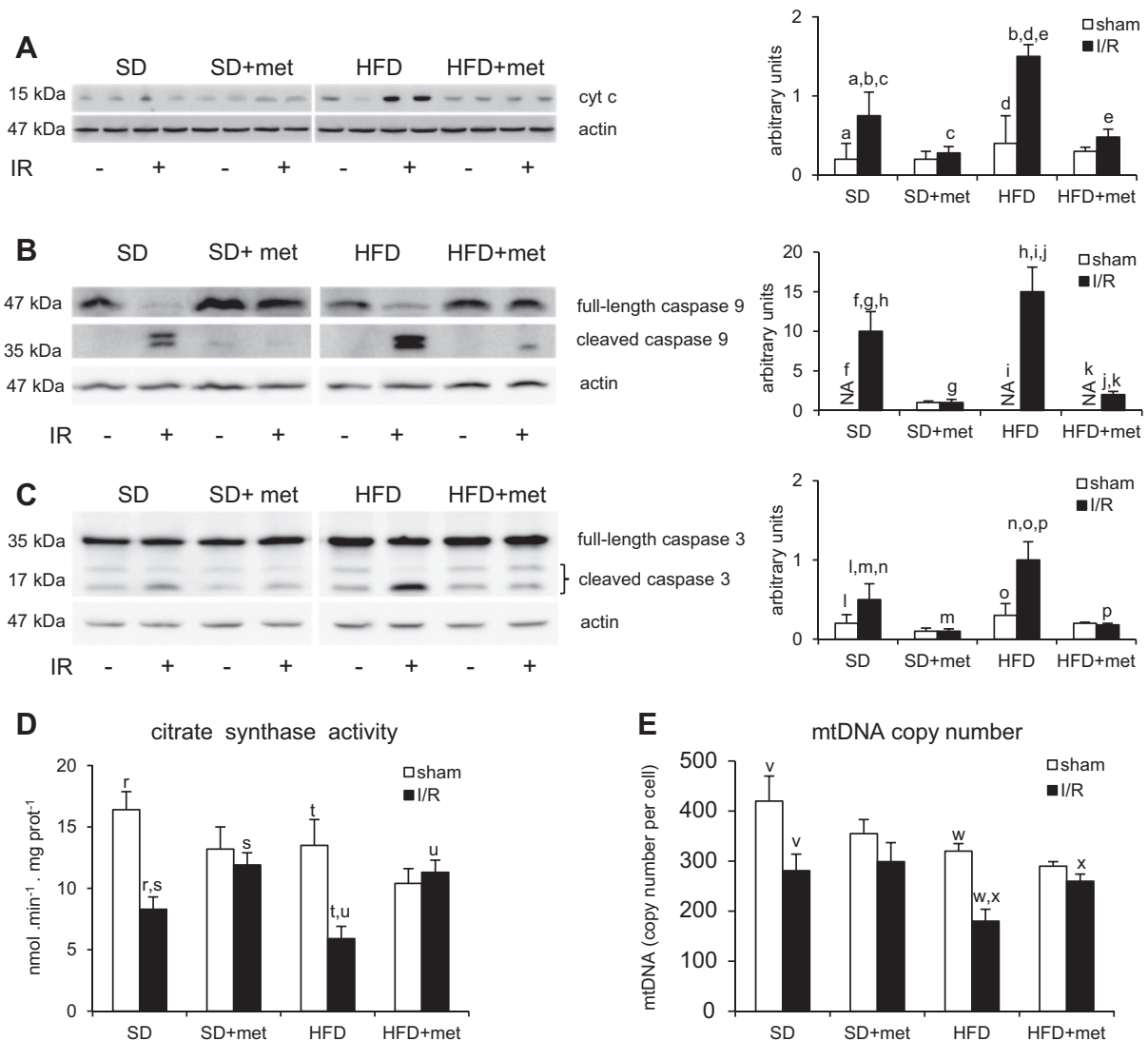


Fig. 2. Effect of metformin on oxidative stress-induced mitochondrial damage and apoptosis in rats subjected to ischemia-reperfusion (IR). **A:** cytochrome *c* (cyt *c*) release to cytosol estimated according to the abundance of cyt *c* in the freshly prepared postmitochondrial fraction. Cyt *c* band intensity was normalized to actin and expressed as cyt *c*-to-actin ratio. **B** and **C:** caspase 9 (**B**) and caspase 3 (**C**) activation. The signal intensities corresponding to the full-length proteins and cleaved fragments (caspase 9: 51, 40, and 38 kDa; caspase 3: 35 and 17 kDa) were assessed by densitometry. Graphs show the ratio of cleaved fragments to full-length protein density. **D:** citrate synthase activity in the liver homogenate. **E:** mitochondrial DNA copy number per cell in the liver. Data are presented as means $\pm$ SE, $n = 10$. Bars marked with the same letter are statistically significantly different; $a,b,c,d,h,i,n,o,s,u,v,x P < 0.05$; $e,m,p,r,t,w P < 0.01$; $f,g,i,j,k P < 0.001$.

nificant effect in the SD group, but the drop in ATP concentration was further emphasized in the HFD+metformin group compared with animals fed only a HFD. ATP content reached only 60% of initial values after reperfusion and ATP repletion in the HFD+metformin group was also significantly slower than for both other groups. The HFD+metformin and HFD curves were significantly different ($P < 0.05$) during the first 40 min of reperfusion ($t_{20} - t_{60}$).

Metformin attenuates the expression of proinflammatory markers in the liver. I/R injury is associated with the proinflammatory activation of immune cells in the liver, which is considered to be secondary to direct cellular damage resulting from ischemic insult. As we have shown, metformin reduced the I/R-induced liver injury. We further wanted to know whether this protection translated into an amelioration of proinflammatory status. Using RT-qPCR, we determined that the hepatic expression of proinflammatory markers TNF- α , TLR4,

IL-1 β , and Ccr2 was significantly higher in the liver of HFD-fed animals subjected to I/R compared with sham-operated controls and that it was significantly attenuated by metformin treatment (Fig. 7). With the exception of TLR4, we did not find this I/R-induced proinflammatory activation in rats fed a standard diet. We did not observe any effect of the diet, metformin treatment, or I/R injury on the expression of alternative activation markers (Arg1, Mrc1, IL-10).

The liver accommodates two different subsets of macrophages: resident macrophages [Kupffer cells (KCs)] and macrophages that infiltrate the liver by circulation. The expression of markers characteristic of KCs (CD68, F4/80) was not different among the groups and did not depend on the diet or I/R injury. In contrast, the expression of Ly6c and CD11b, infiltrating monocyte and macrophage markers, respectively, were significantly increased in the liver of both SD+I/R and HFD+I/R groups. Metformin treatment significantly decreased

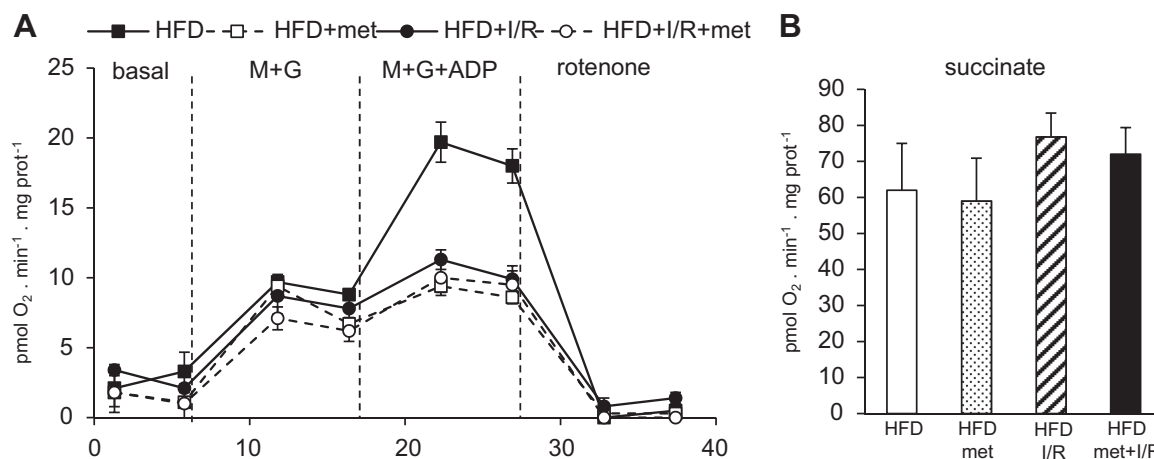


Fig. 3. Mitochondrial respiration of NADH-dependent (A) and FADH-dependent (B) substrates. Liver mitochondria were isolated from rats fed HFD without or with metformin, half of the animals in each group ($n = 5$) being subjected to ischemia-reperfusion (I/R) as described in MATERIALS AND METHODS. The oxygen consumption rate of isolated liver mitochondria ($10 \mu\text{g}$ protein per well) was monitored on an XF 24 Analyzer in the presence of chemicals added at time intervals shown on the graph. The additions included 5 mM malate (M), 10 mM glutamate (G), 4 mM ADP, 2 μM rotenone, and 10 mM succinate (final concentrations). Data are presented as means $\pm$ SE. The maximal respiration rate (M+G+ADP) was statistically significantly higher in HFD compared with all other groups.

their expression; in the case of Ly6c, even below the levels in controls. Our data suggest that long-term metformin administration alleviates I/R-induced inflammation, particularly in animals fed a HFD.

DISCUSSION

Our data show that in vivo, metformin protects both the steatotic and normal liver from oxidative stress-related hepatotoxic injury caused by I/R. This effect is, at least in part, mediated by decreased mitochondrial ROS production resulting in reduced mitochondrial damage, attenuation of apoptotic/necrotic cell death, and elimination of inflammation.

It has been previously shown that several tissues endangered by oxidative imbalance associated with T2D, particularly the myocardium (38, 44), endothelial cells (5, 48), and the brain (13), could be protected by metformin administration. Surpris-

ingly, there are scarce data regarding the antioxidant effect of metformin in the fatty liver, despite the fact that steatosis is a hallmark of T2D and that the fatty liver is highly susceptible to oxidative stress. One study conducted on 208 Indian diabetic patients showed that metformin restores antioxidant status in plasma (24). A hepatoprotective effect of metformin (decreased serum ALT and AST levels, improved histological parameters) was proved in two small studies focused on NASH patients (41, 65). Our results show that long-term metformin treatment not only alleviates chronic (HFD-induced) oxidative stress directly in liver tissue, but it also effectively protects the liver against acute and massive oxidative injury, particularly in the fatty liver. Nevertheless, the mechanism of metformin antioxidant activity is still far from clear.

The degree of oxidative stress results from the balance between the ROS formation rate and antioxidant defense capacity. Metformin is not able to scavenge superoxide radicals and is unlikely to engage in direct scavenging activity (6, 33, 49). In some models, antioxidative activity of metformin has been associated with its hypoglycemic effect, but in the presented study HFD-fed rats did not exhibit hyperglycemia. Numerous studies have demonstrated that both T1D (Type 1 diabetes) and T2D are associated with decreased activity and expression of antioxidant enzymes and that metformin treatment can restore normal conditions (13, 19, 62). In our experimental design, HFD-fed rats exhibited augmented markers of oxidative stress (lipoperoxides) accompanied by a significant decrease of liver GSH content and SOD activity and elevated catalase activity. I/R further exacerbated both oxidative injury and HFD-induced dysfunction of antioxidant mechanisms. Long-term metformin treatment normalized all these markers, both in sham-operated controls and in ischemic animals.

By directly measuring ROS production using a fluorometric assay and EPR, we showed that metformin attenuates mitochondrial ROS production. In reperfused liver, ROS can be generated by several mechanisms, including xanthine/xanthine oxidase, cytosolic NADPH oxidase, and the electron transport chain of mitochondria. Earlier work with rat hepatocytes using selective inhibitors has suggested that xanthine oxidase is the

Table 4. GSH concentration and antioxidant enzyme activities in the liver of rats exposed to ischemia/reperfusion

	SD	SD + Met	HFD	HFD + Met
GSH, mM/mg protein				
Control	7 $\pm$ 0.14 ^a	6.8 $\pm$ 0.2	4.3 $\pm$ 0.1 ^{a,j}	6.4 $\pm$ 0.13 ^j
I/R	4.3 $\pm$ 0.4 ^b	4.6 $\pm$ 0.3	1.7 $\pm$ 0.2 ^{b,k}	3.5 $\pm$ 0.1 ^k
GSH-Px, μM				
GSH $\cdot$ min ⁻¹ $\cdot$ mg protein ⁻¹				
Control	540 $\pm$ 30 ^c	583 $\pm$ 45	414 $\pm$ 22 ^c	476 $\pm$ 40
I/R	428 $\pm$ 22 ^d	429 $\pm$ 25	258 $\pm$ 20 ^{d,l}	435 $\pm$ 41 ^l
Catalase, μM				
H ₂ O ₂ $\cdot$ min ⁻¹ $\cdot$ mg protein ⁻¹				
Control	758 $\pm$ 26 ^e	820 $\pm$ 51	947 $\pm$ 13 ^e	893 $\pm$ 30
I/R	693 $\pm$ 33 ^f	729 $\pm$ 49	508 $\pm$ 29 ^{f,m}	764 $\pm$ 37 ^m
SOD, μU /mg protein				
Control	143 $\pm$ 10 ^g	156 $\pm$ 5	91 $\pm$ 3 ^{g,n}	129 $\pm$ 10 ⁿ
I/R	95 $\pm$ 5 ^{h,i}	138 $\pm$ 6 ^h	73 $\pm$ 5 ^{h,o}	130 $\pm$ 8 ^o

GSH content was determined in the isolated mitochondria; all other parameters were determined in the whole liver homogenate. Data are expressed as means $\pm$ SE, $n = 10$. Values marked with the same letter are statistically significantly different; ^{a,f,i,j,n} $P < 0.05$; ^{b,c,g,h,l,o} $P < 0.01$; ^{a,d,h,m} $P < 0.001$.

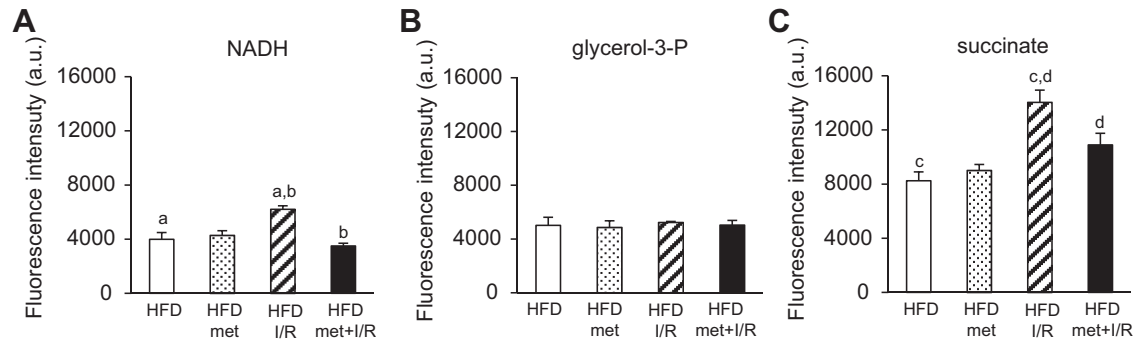


Fig. 4. Reactive oxygen species (ROS) production from different substrates in submitochondrial particles (SMPs). SMPs were prepared from liver mitochondria of rats fed HFD without or with metformin, half of the animals in each group being subjected to I/R as described in MATERIALS AND METHODS. ROS production in mitochondria was measured by DCFDA (10 μ M) in the presence of either 10 mM NADH (A), 10 mM glycerol-3-P (B), or 10 mM succinate (C). Values represent fluorescence units and are normalized per 1 mg of mitochondrial protein. Data are presented as means $\pm$ SE, $n = 5$. Bars marked with the same letter are statistically significantly different; $a,b,dP < 0.05$; $cP < 0.01$.

main generator of ROS (1). However, xanthine oxidase inhibitors employed in these studies were later recognized as mitochondrial inhibitors as well and mitochondria are now considered to be an important source of ROS within hepatocytes (28). Estimating the relative contribution of mitochondria to total ROS production is difficult, because it depends on actual physiological conditions, such as the ratio of resting to phosphorylating mitochondria and the capacity of reverse electron transport. In the case of the ethanol-stressed liver, where nonmitochondrial ROS production from CYP2E1 is accentuated, mitochondrial contribution has been estimated at ~ 22 –46% (30). In hypoxia/ischemia, when the portion of nonphosphorylating mitochondria is increased and reverse electron flow accentuated, this contribution may be significantly higher.

Several production sites for ROS are recognized at the respiratory chain, including complex I (36) and complex III (35), complex I being considered crucial for the regulation of mitochondria-related ROS production (66). At complex I, the superoxide radical may be generated either by reverse electron

transfer (e^- transport from succinate to NAD^+ through complex I) upon succinate oxidation at complex II, or in lower amounts during forward electron transport from NADH-linked substrates (36).

In a model of a liver perfused with 10^{-2} M metformin, Batandier et al. (4) demonstrated that metformin powerfully inhibits reverse flux-related ROS production. However, these data were obtained using extremely high metformin concentrations. We are the first to demonstrate that metformin administered in vivo has the same effect, as we observed significantly decreased ROS production from succinate in submitochondrial particles isolated from metformin-treated rats subjected to I/R compared with untreated animals. Importantly, this effect persisted even when no metformin was added to the reaction in vitro. The contribution of reverse flux-related ROS generation under normoxic conditions remains questionable, but it seems to be highly relevant in hypoxia (30). This is in accordance with our results as we found the inhibitory metformin effect on ROS production from succinate only in rats that underwent I/R. Batandier et al. have shown that reverse flux-related ROS generation is very sensitive to changes in membrane potential. We have previously shown that biguanides decrease membrane potential as a consequence of mitochondrial complex I inhibition (16). We hypothesize that this phenomenon may, at least

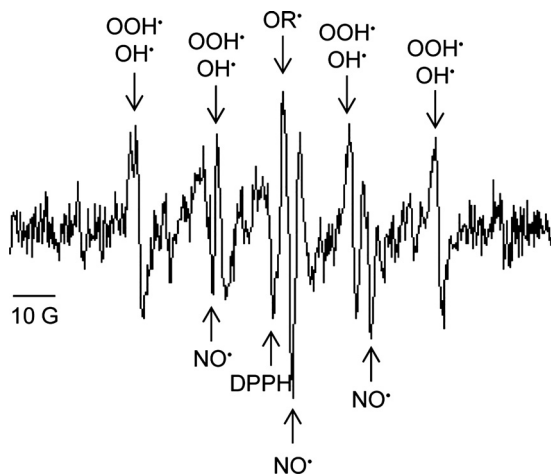


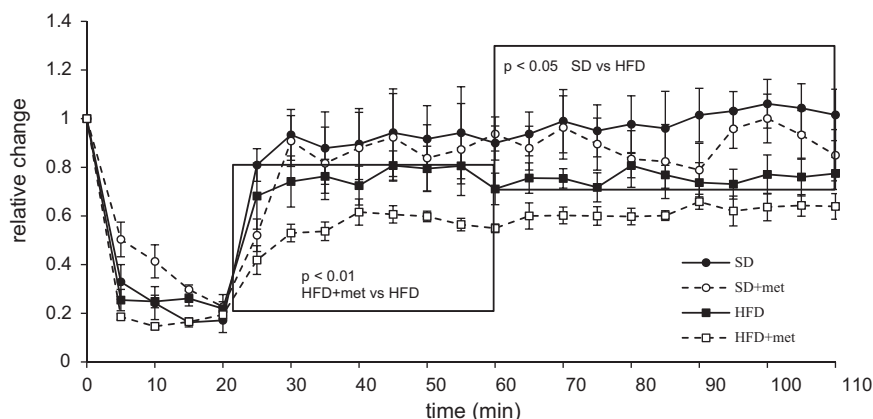
Fig. 5. Typical electron paramagnetic resonance spectra obtained after addition of NADH (10 mM) to the suspension of SMP by using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin-trapping agent. The DMPO adducts were identified according to their g factor and DPPH served as an internal standard ($g = 2.0036$). OOH and OH radicals were distinguished by detailed analysis of overlapping OOH and OH signals using computer simulation. OR, organic free radical; NO, nitroxide radicals; OOH, superoxide radical; OH, hydroxyl radical; DPPH, diphenyl-1-picrylhydrazyl.

Table 5. Free radical generation in submitochondrial particles in vitro

	HFD	HFD + Met	HFD+I/R	HFD + Met+I/R
Free organic radical	7.9 $\pm$ 1.1	7.9 $\pm$ 0.3	7.6 $\pm$ 0.8	6.9 $\pm$ 0.3
Nitrosyl radical NO·	3.8 $\pm$ 0.8	4.1 $\pm$ 0.2	4.3 $\pm$ 0.5	4.2 $\pm$ 0.6
Superoxide radical OOH·	4.1 $\pm$ 0.2 ^a	4.0 $\pm$ 0.25	5.1 $\pm$ 0.3 ^{a,b}	3.3 $\pm$ 0.4 ^b
Hydroxyl radical OH·	1.2 $\pm$ 0.03 ^c	1.1 $\pm$ 0.1	1.7 $\pm$ 0.2 ^{c,d}	1.2 $\pm$ 0.05 ^d

Submitochondrial particles prepared from liver mitochondria were isolated from rats fed HFD without or with metformin, half of the animals in each group being subjected to I/R as described in MATERIALS AND METHODS. Reactive oxygen species production in mitochondria was measured by electron paramagnetic resonance spectroscopy with 10 mM NADH as a substrate. Data are expressed as means $\pm$ SE, $n = 5$. Values marked with the same letter are statistically significantly different; $a,b,c,dP < 0.05$.

Fig. 6. Effect of long-term metformin administration on ATP resynthesis during reperfusion after partial ischemia. Partial ischemia was induced by portal vein ligation (*time 0*) and released 20 min later (*time 20*). The measurement continued in 5-min intervals. Liver ATP content was determined by ^{31}P -NMR spectroscopy. Data are expressed as the relative change compared with the respective value in *time 0* and presented as means $\pm$ SE, $n = 10$. The statistical significance of the course of the curves was tested by 2-way ANOVA with repeated measurements.



partly, underlie metformin-dependent attenuation of reverse flux-related ROS production.

Inhibition of forward electron flux at the level of complex I by the specific inhibitor, rotenone, results in increased ROS production (46) and thus it can be assumed that metformin as a mild inhibitor of complex I may have the same effect. In contrast, we observed the inhibitory effect of metformin on NADH-dependent ROS production. This apparent contradiction could be reconciled in light of recent findings published by Matsuzaki and Humphries (43). Complex I has two reversible conformational states: active and deactivated (23). In its deactivated state, ROS production at complex I is significantly attenuated. Biguanides selectively inhibit the deactivated form of complex I. Consequently, this deactivation greatly enhances the sensitivity of complex I toward biguanides, forming a feedback loop that “locks” complex I in a deactivated state leading to decreased superoxide production capacity (43). Furthermore, ischemia represents a condition that promotes deactivation of complex I *in vivo*, thereby increasing the sensitivity to biguanide-mediated inhibition. On the basis of these findings, we suggest that metformin could serve as an antioxidant, particularly during oxidative stress associated with ischemia, and also following reperfusion.

Another potential source of ROS may be mGPDH, a key component of the glycerophosphate shuttle, which ensures the transport of reduced equivalents from cytosol to complex II (45). Quite recently, mGPDH was recognized as a new target of metformin (40). Its contribution to ROS production has been documented in brown adipose tissue (65), but no data concerning liver mitochondria are available. In our study, the contribution of ROS produced by G-3-P was significantly lower than that from succinate. It also failed to respond to I/R and metformin. Given that mGPDH expression in the liver is very low (34), we do not consider this enzyme to be an important target of metformin antioxidant activity.

The accumulation of intracellular ROS induces cell death and, during hepatic I/R, hepatocytes undergo both apoptosis and necrosis (56). Some studies have suggested apoptosis to be the primary mode of death (55), whereas others have reported necrosis (61). In the present study, we found signs of both necroinflammation and ongoing apoptosis in the liver tissue of rats subjected to I/R, where the effect was more pronounced in the HFD group. We can explain the beneficial effect of metformin on both forms of cell death as the consequence of lower mitochondrial ROS production.

In the liver of metformin-treated animals, we observed lower expression of proinflammatory as well as infiltrating monocyte/macrophage markers 2 days after short-term ischemia, which points to the possible anti-inflammatory effect of metformin. The direct anti-inflammatory action of metformin has been reported in several animal experimental studies, both in the liver (69, 54, 47) and in circulating polymorphonuclear cells (9). In contrast to these experimental findings, metformin efficiency in human NASH treatment is the subject of open debate (58, 60). The beneficial effect of metformin in our study could be explained either by the direct effect of metformin on KCs or by the attenuation of intracellular ROS production in hepatocytes, resulting in diminished necrosis and suppressed inflammation.

Many reports demonstrated that ROS are important modulators of signaling pathways and the intensity of their production seems to be a key factor in discriminating between cell death and survival (53, 25, 39). It has been suggested that ROS act as second messengers that promote sustained activation of c-Jun NH₂-terminal kinase (JNK) and apoptotic signaling (59). Furthermore, they also have been shown to inhibit NF- κ B by preventing transcription of survival genes (26). During reperfusion after ischemia, Kupffer cells generate ROS, which in turn activate JNK at least in part through the ROS-dependent ASK1 (apoptosis signal-regulating kinase 1) pathway and enhance secretion of various chemokines and cytokines including TNF- α (57). TNF- α may serve as a potent activator of either prosurvival or proapoptotic pathways (15), and some authors even report that it is a critical mediator in warm hepatic I/R injury (64). TNF- α released from Kupffer cells may activate TNF receptors on hepatocytes, which induce JNK activation as well as ROS production. JNK activation results in the activation of its downstream targets, i.e., AP-1 family members c-Jun, ATF-2, and JunD, which are involved in the regulation of inflammation, proliferation, and cell death (57). In addition, ROS oxidize and inactivate MAPK phosphatases, which dephosphorylate JNK, leading to its prolonged activation and augmentation of apoptosis (31). In the present study we showed data indicating that metformin reduces mitochondrial ROS production and we hypothesize that the attenuation of ROS signaling may explain, at least partly, the protective effect of metformin in IR. Another mechanism contributing to the reduction of inflammation in metformin-treated animals may be the reduction of ROS-induced lipoperoxide products formation during early reperfusion phase. These products are potent

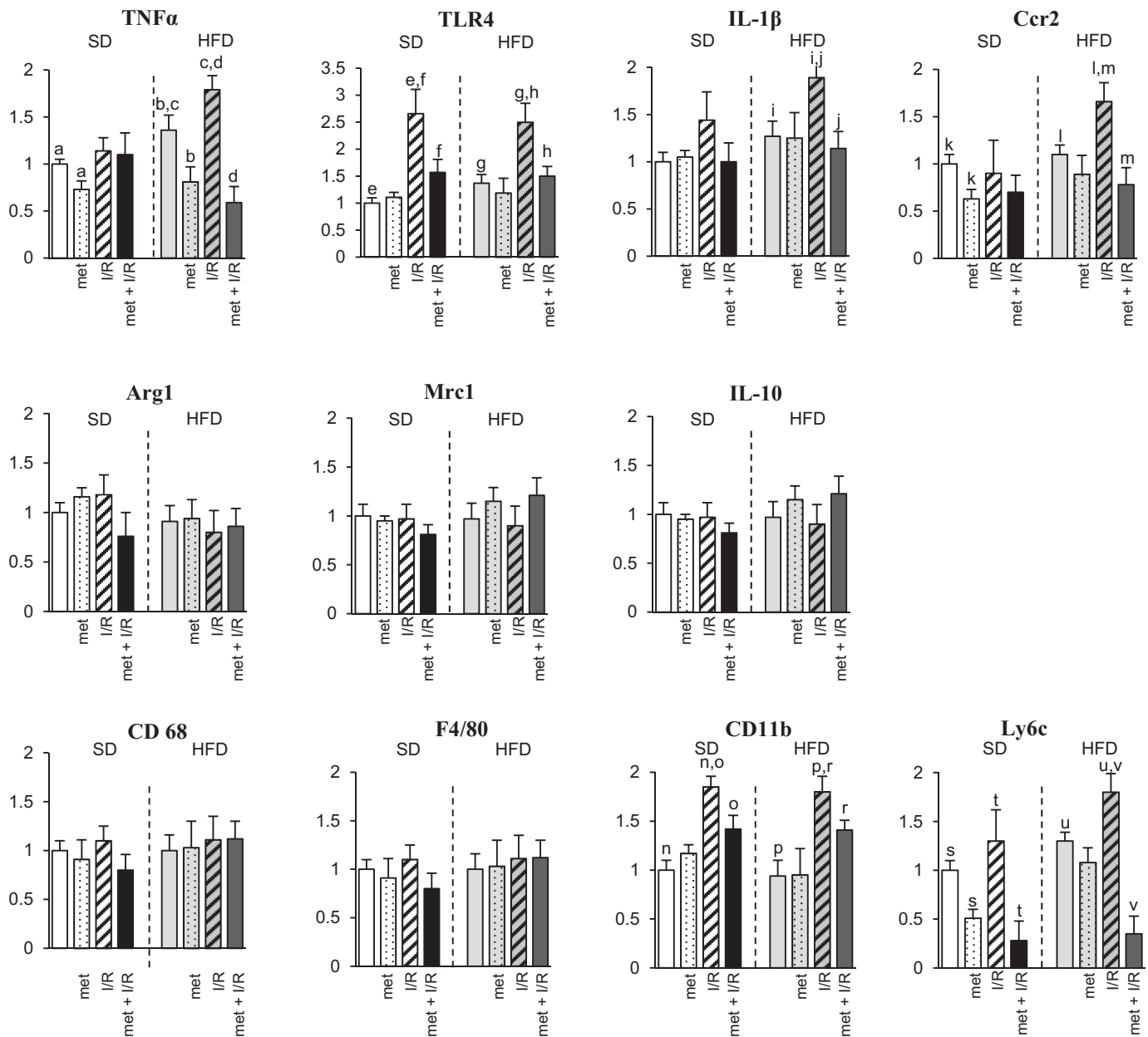


Fig. 7. Effect of metformin on the relative expression of pro and anti-inflammatory markers in the liver. The expression of individual mRNAs was determined by RT-qPCR. Data are expressed as a fold change related to the untreated SD group. Values are given as means $\pm$ SE, $n = 5$. Bars marked with the same letter are statistically significantly different; a,b,c,f,g,h,i,j,k,l,o,r,s,t,u $P < 0.05$; d,e,m,n,p $P < 0.01$; v $P < 0.001$.

chemotactic factors for neutrophils and are the determining factor for the continuation of neutrophil recruitment and aggravation of injury (29). In accordance with this supposition we observed a significantly lower expression of infiltrating macrophages markers in metformin-treated animals subjected to IR.

Attenuation of mitochondrial respiration compromises the hepatocyte energy state. Foretz et al. (17) have shown that metformin decreases ATP content in a dose-dependent manner, both in primary hepatocytes and in the liver after metformin administration in vivo. Using direct ^{31}P -NMR measurements in vivo, we demonstrated that long-term metformin treatment decreased ATP repletion during reperfusion, in terms of both rate and relative quantity. As we have previously shown (16),

metformin does not function as an uncoupler and thus the decrease in ATP content reflects the weaker performance of the mitochondrial respiratory chain. Low ATP availability is generally an unfavorable condition and may contribute to high-dose metformin toxicity, but we suggest that under conditions of acute oxidative stress the beneficial consequences of diminished electron flux through the respiratory chain, i.e., lower ROS formation, outweighs the disadvantages of compromised energy production.

In conclusion, we demonstrate that metformin protects the fatty liver from acute oxidative stress-related mitochondrial injury and cell death. We propose that the beneficial effect of metformin action is based on the combination of three contributory mechanisms: increase of antioxidant enzyme activity,

lower mitochondrial ROS production at complex I, and reduction of postischemic inflammation. The presented data support the extension of the therapeutic application of metformin as an antioxidant, particularly during I/R-related oxidative stress.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

M.C. and E.P. conception and design of research; M.C., E.P., H.D., E.S., M.B., Z.D., C.G., P.S., J.K., Z.P., O.O., and L.K. performed experiments; M.C., E.P., H.D., E.S., M.B., Z.D., Z.C., O.K., C.G., Z.P., O.O., and L.K. analyzed data; M.C., E.P., Z.D., Z.C., and O.K. interpreted results of experiments; M.C., E.P., H.D., E.S., and P.S. prepared figures; M.C., E.P., Z.P., and O.O. drafted manuscript; M.C., E.P., H.D., Z.C., O.K., P.S., and L.K. edited and revised manuscript; M.C., E.P., H.D., E.S., M.B., Z.D., Z.C., O.K., C.G., P.S., J.K., Z.P., O.O., and L.K. approved final version of manuscript.

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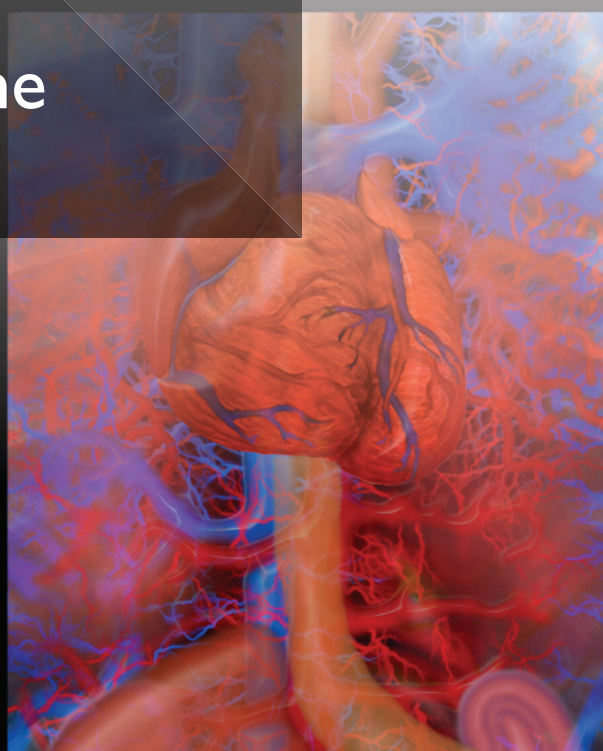
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Carnitine supplementation alleviates lipid metabolism derangements and protects against oxidative stress in non-obese hereditary hypertriglyceridemic rats

Monika Cahova, Petr Chrastina, Hana Hansikova, Zdenek Drahota, Jaroslava Trnovska, Vojtech Skop, Jana Spacilova, Hana Malinska, Olena Oliarnyk, Zuzana Papackova, Eliska Palenickova, and Ludmila Kazdova

Abstract: The aim of this study was to estimate the effect of carnitine supplementation on lipid disorders and peripheral tissue insulin sensitivity in a non-obese animal model of insulin resistance, the hereditary hypertriglyceridemic (HHTg) rat. Male HHTg rats were fed a standard diet, and half of them received daily doses of carnitine (500 mg·kg⁻¹ body weight) for 8 weeks. Rats of the original Wistar strain were used for comparison. HHTg rats exhibited increased urinary excretion of free carnitine and reduced carnitine content in the liver and blood. Carnitine supplementation compensated for this shortage and promoted urinary excretion of acetylcarnitine without any signs of (acyl)carnitine accumulation in skeletal muscle. Compared with their untreated littermates, carnitine-treated HHTg rats exhibited lower weight gain, reduced liver steatosis, lower fasting triglyceridemia, and greater reduction of serum free fatty acid content after glucose load. Carnitine treatment was associated with increased mitochondrial biogenesis and oxidative capacity for fatty acids, amelioration of oxidative stress, and restored substrate switching in the liver. In skeletal muscle (diaphragm), carnitine supplementation was associated with significantly higher palmitate oxidation and a more favorable complete to incomplete oxidation products ratio. Carnitine supplementation further enhanced insulin sensitivity *ex vivo*. No effects on whole-body glucose tolerance were observed. Our data suggest that some metabolic syndrome-related disorders, particularly fatty acid oxidation, steatosis, and oxidative stress in the liver, could be attenuated by carnitine supplementation. The effect of carnitine could be explained, at least partly, by enhanced substrate oxidation and increased fatty acid transport from tissues in the form of short-chain acylcarnitines.

Key words: metabolic syndrome, insulin resistance, antioxidant, liver steatosis, mass spectrometry.

Résumé : Cette étude se propose d'estimer l'effet de la supplémentation en carnitine sur les troubles du métabolisme des lipides et sur la sensibilité des tissus périphériques à l'insuline chez des rats présentant une hypertriglycéridémie héréditaire (« HHTg »), un modèle animal non obèse d'insulinorésistance. On soumet des rats mâles HHTg à un régime normal et, à la moitié d'entre eux, on administre tous les jours durant 8 semaines de la carnitine à raison de 500 mg·kg⁻¹ de masse corporelle. Des rats de la lignée Wistar originale servent de comparaison. Les rats HHTg présentent une plus grande excrétion urinaire de carnitine libre et une diminution du contenu hépatique et sanguin en carnitine. La supplémentation en carnitine comble le déficit et favorise l'excrétion urinaire d'acétylcarnitine en l'absence de signes d'accumulation d'(acyl)carnitine dans le muscle squelettique. Les rats HHTg traités à la carnitine présentent un gain de poids inférieur, une atténuation de la stéatose hépatique, une plus faible triglycéridémie à jeun et une plus grande diminution sérique d'acides gras libres à la suite d'une charge glucidique, et ce, comparativement aux individus non traités dans la même portée. Le traitement à la carnitine est associé à l'augmentation de la biogenèse mitochondriale et à la capacité d'oxydation des acides gras, à l'amélioration du stress oxydatif et à la restauration du changement de substrat dans le foie. Dans le muscle squelettique (diaphragme), la supplémentation en carnitine est associée de façon significative à une oxydation de palmitate plus élevée et à un ratio plus favorable de l'oxydation complète/incomplète des produits. La supplémentation améliore davantage la sensibilité à l'insuline *ex vivo*. On n'observe pas d'effet sur la tolérance corporelle globale au glucose. D'après nos données, la supplémentation en carnitine pourrait atténuer des anomalies du syndrome métabolique telles que, notamment, l'oxydation des acides gras, la stéatose et le stress oxydatif dans le foie. L'effet de la carnitine pourrait s'expliquer, du moins partiellement, par une meilleure oxydation du substrat et un accroissement du transport des acides gras provenant des tissus sous la forme d'acylcarnitines à courte chaîne. [Traduit par la Rédaction]

Mots-clés : syndrome métabolique, insulinorésistance, antioxydant, stéatose hépatique, spectrométrie de masse.

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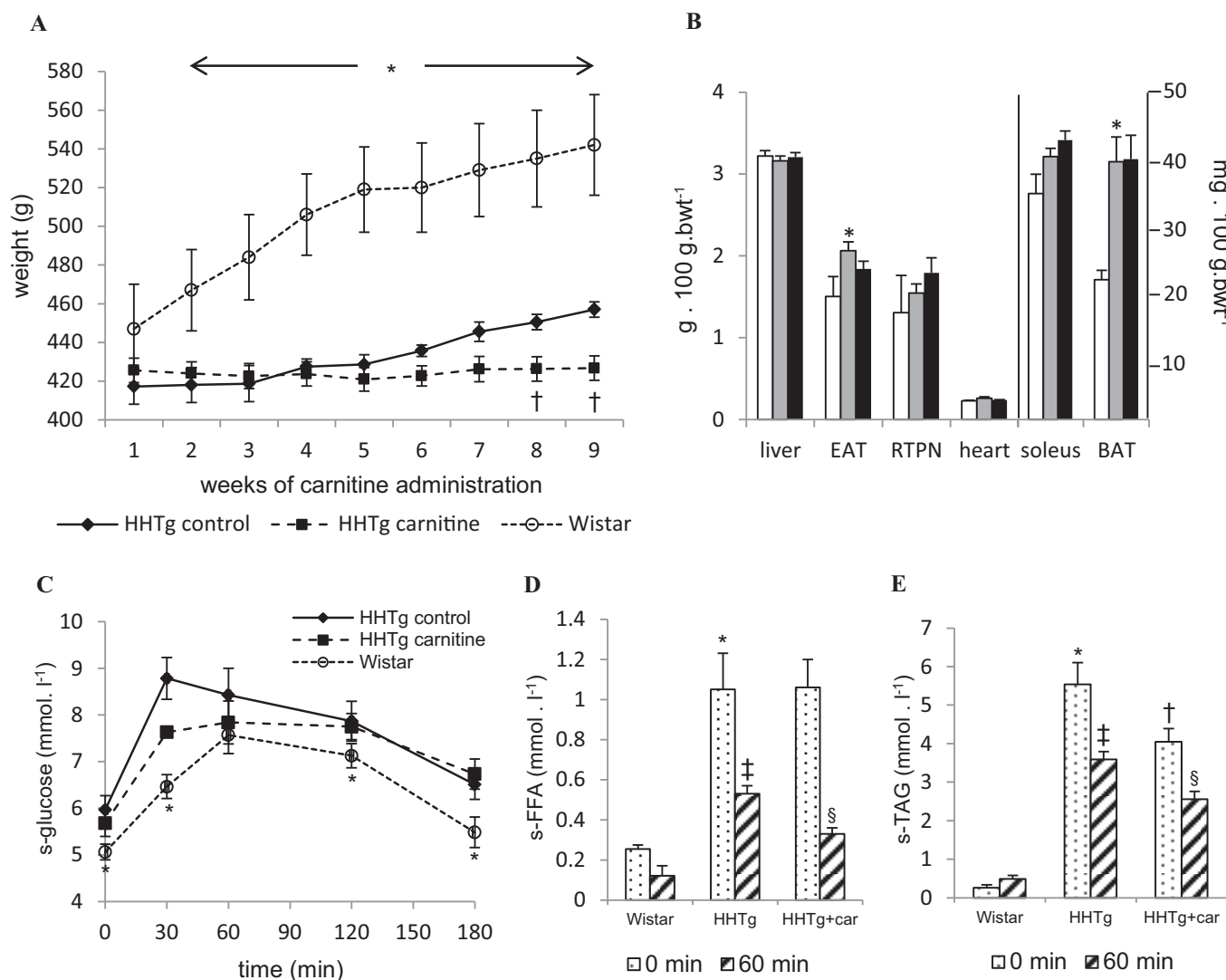
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Fig. 1. Basal growth and metabolic characteristics of Wistar, hereditary hypertriglyceridemic (HHTg) control, and carnitine-treated HHTg rats. (A) Total weight gain, (B) relative weight of individual organs, (C) glucose concentration during an oral glucose tolerance test (OGTT), (D) serum free fatty acid (FFA) concentration during the OGTT, and (E) serum triacylglycerol (TAG) concentration during the OGTT. The relative weight of individual organs is expressed per 100 g of body weight. Serum FFA and TAG concentrations were determined before (0 min) and 60 min after glucose administration. EAT, epididymal adipose tissue; RTPN, retroperitoneal adipose tissue; BAT, brown adipose tissue. Data are means $\pm$ SE, $n = 8-10$. Open bars: Wistar group; grey bars: HHTg group; closed bars: HHTg + carnitine group; dotted bars: determinations made prior to glucose administration (0 min); hatched bars: determinations made 60 min after glucose administration. *, $p < 0.05$, HHTg control vs. Wistar; †, $p < 0.05$, HHTg + carnitine vs. HHTg control; ‡, $p < 0.05$, HHTg control vs. Wistar (60 min after glucose administration); §, $p < 0.05$, HHTg + carnitine vs. HHTg control (60 min after glucose administration). bwt, body weight.



Introduction

Metabolic syndrome is a clinical disorder characterized by the clustering of multiple risk factors for diabetes and cardiovascular disease, including dyslipidemia, insulin resistance, and increased blood pressure (Kaur 2014). The use of macromolecules and nutrients that can correct relative or absolute deficiencies of critical metabolic components constitutes the basis of one possible therapeutic direction, metabolic medicine. Among many other candidates, L-carnitine and its analogs have been extensively studied with the objective of ameliorating metabolic abnormalities associated with mitochondrial dysfunction (Marcovina et al. 2012).

Carnitine supplementation has become very attractive as an adjunctive treatment for type 2 diabetes mellitus (T2DM) and related disorders. Many clinical studies have been performed, with variable outcomes (for reviews, see Ringseis et al. 2012; Vidal-Casariño et al. 2013). The diverse results may be due to the extreme variability

of tested individuals, the presence of other pharmacological treatments or regime interventions, differing doses of carnitine, or differing durations of treatment. In general, carnitine therapy is less effective in obese patients either with or without T2DM and no other intervention (Galloway et al. 2011; Liang et al. 1998; González-Ortiz et al. 2008) and in prediabetics (Bloomer et al. 2009) in which symptoms are less manifest. Carnitine supplementation has beneficial effects in patients with T2DM when supported by oral antidiabetics (Rahbar et al. 2005), a hypocaloric diet (Molfino et al. 2010), or moderate physical activity (Derosa et al. 2010a, 2010b). Carnitine is also effective in subjects newly diagnosed with T2DM (Derosa et al. 2003), who have probably suffered from diabetes-related metabolic disorders for a shorter period of time.

In contrast to clinical trials, animal studies provide more opportunities to change experimental conditions. To our knowledge,

only a few rodent studies have investigated carnitine in the treatment of diabetes. According to the type of animal model, these studies can be divided into several groups. First, genetically obese models (obese BAP-agouti mice, obese Zucker rats), in which carnitine treatment started when the obesity was already fully established (Power et al. 2007; Mingorance et al. 2009); second, rats fed a life-long (12-month) high-fat diet, in which carnitine treatment started at the age of 1 year and continued for 4 months together with the high-fat diet (Noland et al. 2009); and third, rodents (Black Swiss/129 mice selected according to their susceptibility to a short-term high-carbohydrate diet or Wistar rats) challenged for a short time (4 weeks) with a high-fructose diet, in which carnitine treatment started concomitantly with the diet intervention (Power et al. 2007; Rajasekar et al. 2005). In the “preventive” studies, when carnitine was administered concurrently with dietary manipulation, a rapid (within the range of weeks) and positive effect of carnitine on glucose homeostasis and lipid metabolism was observed. However, in the “reversion” studies, when the carnitine treatment started on the background of a fully developed metabolic disorder, results were not uniform. In BAP-agouti mice, carnitine had a significant positive effect on glucose tolerance but did not influence parameters of lipid metabolism (serum free fatty acids, β -hydroxybutyrate). In contrast, carnitine treatment of obese Zucker rats led to significant amelioration of lipid metabolism disorders but had no effect on glucose tolerance. In Wistar rats fed a life-long high-fat diet, perturbations in glucose and fatty acid metabolism were substantially reversed by short-term oral carnitine supplementation (Noland et al. 2009).

In our laboratory, a unique non-obese model of hypertriglyceridemia and insulin resistance, the hereditary hypertriglyceridemic (HHTg) rat, was developed from a colony of Wistar rats (Vrana and Kazdova 1990). A number of previously recognized facets of the metabolic syndrome, such as hypertriglyceridemia, hyperinsulinemia, resistance of muscle and adipose tissue to insulin action, impaired glucose tolerance, ectopic lipid accumulation, and elevated blood pressure, have been observed in this HHTg strain (Vrana et al. 1993). Although obesity is the main factor in pathophysiology of metabolic syndrome, 25% of humans suffering from this disease are not obese or overweight (Voulgaris et al. 2011).

The current study was undertaken to determine whether oral carnitine supplementation would improve glucose metabolism in a non-obese polygenic model of metabolic syndrome (HHTg rats). We further focused on the effect of carnitine on dyslipidemia in this model, particularly liver steatosis, fatty acid oxidation, and oxidative stress.

Materials and methods

Animals

The HHTg rat strain was originally selected from the Wistar strain in our laboratory (Vrana and Kazdova 1990) and since then has been kept in the breeding facility of the Institute for Clinical and Experimental Medicine, Prague, Czech Republic. Wistar rats were obtained from Charles River Laboratories, Germany. Male HHTg rats (4 months, 420 ± 10 g), fed a standard diet (VKS, Czech Republic) containing 20% protein, 43% carbohydrate, 3% fat, and 30% fibre, were randomly divided into 2 groups ($n = 10$). One group was provided with 1% carnitine in drinking water for 8 weeks (HHTg + carnitine), while the second group served as a control (HHTg control). The daily water intake was measured and ranged from 18–20 mL, i.e., approximately 500 mg carnitine per kilogram body weight. The animals had free access to drinking water and food and were kept in a temperature-controlled room with a 12-h light/12-h dark cycle. All experiments were performed in accordance with the Animal Protection Law of the Czech Republic (311/1997), which is in compliance with European Community Council recommendations for the use of laboratory animals

Table 1. Metabolic characteristics of experimental groups.

	State	Wistar	HHTg control	HHTg+ carnitine
Serum				
Glucose (mmol·L ⁻¹)	Fasted	5.1±0.2	6.0±0.3	5.6±0.28
	Fed	5.4±0.2	6.7±0.2*	6.5±0.2
Insulin (pmol·L ⁻¹)	Fed	257±47	231±20	298±30
TAG (mmol·L ⁻¹)	Fasted	1.3±0.2	5.5±0.4*	4.1±0.3†
	Fed	1.5±0.4	5.4±0.5*	5.7±0.3
FFA (mmol·L ⁻¹)	Fasted	0.7±0.1	1.7±0.15*	1.4±0.19
	Fed	0.33±0.04	0.69±0.06*	0.67±0.05
Liver				
TAG (mmol·g ⁻¹)	Fed	4.6±0.7	10.5±0.6*	7.9±0.7†
Soleus				
TAG (mmol·g ⁻¹)	Fed	3.5±0.4	8.9±1.3*	10.3±1.3
Heart				
TAG (mmol·g ⁻¹)	Fed	1.6±0.4	1.2±0.4	0.98±0.2

Note: Data are means $\pm$ SE, $n = 8$ –10. FFA, free fatty acids; HHTg, hereditary hypertriglyceridemic; TAG, triacylglycerols.

* $p < 0.05$, HHTg control vs. Wistar.

† $p < 0.05$, HHTg + carnitine vs. HHTg control.

(Directive 86/609/ECC), and were approved by the Ethical Committee of the Institute for Clinical and Experimental Medicine.

Oral glucose tolerance test

Rats were starved overnight and then given a single dose of glucose (3 g·kg⁻¹ body weight) dissolved in water by gavage. Blood was taken from the tail vein at 0, 30, 60, 120, and 180 min and glucose was measured using an Accu-Chek Performa glucometer (Roche Diagnostics, Germany). Blood samples for determination of non-esterified fatty acids (FFA) and triacylglycerols (TAG) in serum were collected immediately before and 60 min after glucose administration.

Carnitine and acylcarnitine determination

Free carnitine and acylcarnitines were analyzed in whole blood, urine, liver, musculus soleus, extensor digitorum longus (EDL), and diaphragm by electrospray ionization–tandem mass spectrometry with a derivatized MassChrom reagent kit (Chromsystems, Germany). All samples were investigated on an API 2000 triple quadrupole tandem mass spectrometer (AB SCIEX) with TurboIonSpray interface, used in combination with a PerkinElmer Series 200 Autosampler and a PerkinElmer Series 200 microgradient system. One 3-mm diameter dot was punched from a dried blood spot specimen into a single well of a 96-well microtiter filter plate, to which was added 200 μ L of a solution of internal deuterated standards. For tissue acylcarnitine measurements, 200 μ L of a solution of internal deuterated standards was added to a methanol tissue extract. A 40 μ L aliquot of the derivatized sample was injected directly into the tandem mass spectrometer at a solvent flow rate of 60 μ L·min⁻¹, resulting in a run time of 2.5 min for each sample. Detection of acylcarnitines was carried out using a precursor-ion scan of m/z 85 and scanning from m/z 200 to 550. Each acylcarnitine was quantified using the signal intensity ratio of the compound to its internal standard and related to concentrations using the slope derived from standard curves.

Real-time quantitative PCR analysis

Total RNA was extracted from rat liver, kidney, and skeletal muscle (diaphragm and EDL) tissue using an RNeasy Mini Kit (Qiagen, France). Five hundred nanograms of total RNA was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, Calif., USA), and real-time quantitative PCR was performed using a ViiA 7 Real-Time PCR System (Life Technologies) and HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne, Estonia). The primers were designed using Primer3 soft-

Table 2. Free carnitine (C0) and acetylcarnitine (C2) contents in whole blood and tissue.

	Whole blood ($\mu\text{mol}\cdot\text{L}^{-1}$)		Urine (mmol·mol ⁻¹ creatinine)		Liver (nmol·g ⁻¹)		Soleus (nmol·g ⁻¹)		EDL (nmol·g ⁻¹)		Diaphragm (nmol·g ⁻¹)	
	C0	C2	C0	C2	C0	C2	C0	C2	C0	C2	C0	C2
Wistar	31±0.9	3.9±0.3	2.6±0.4	0.9±0.2	63.5±3	18.3±2	121±8	56.9±6.6	250±55	78±9	274±33	77±6
HHTg control	25±0.7*	4.5±0.1	6.1±2.9*	0.7±0.03	43±5*	18.3±2	144±16	55.6±5.7	241±37	76±10	299±57	94±14
HHTg+carnitine	48±2.4†	5.9±0.2†	581±76†	220±29†	71±8†	23.2±2.3	180±19	82.4±9.6†	234±26	81±8	312±67	109±30

Note: Data are means ± SE, n = 10. EDL, extensor digitorum longus; HHTg, hereditary hypertriglyceridemic.

*p < 0.05, HHTg control vs. Wistar.

†p < 0.05, HHTg + carnitine vs. HHTg control.

ware; sequences are listed in Table S1.¹ Gene expression values were normalized to the expression value of the housekeeping gene β -2 microglobulin. Values were calculated using the comparative threshold cycle (Ct) method ($2^{-\Delta\Delta\text{Ct}}$).

Ex vivo tissue incubation procedure

The diaphragm (200 ± 20 mg) and liver slices (width approx. 1 mm) were rapidly dissected. Tissue sensitivity to insulin was measured in vitro for both the liver slices and diaphragm by measuring [^{14}C]glucose incorporation into glycogen. The effect of pyruvate on fatty acid oxidation was assessed by measuring conversion of [^{14}C]palmitate into CO_2 and tricarboxylic acid (TCA) cycle intermediates. The tissues were incubated for 2 h in Krebs–Ringer buffer with 5 mmol·L⁻¹ glucose, [^{14}C]glucose (7.4×10^6 Bq·mmol⁻¹ glucose) or [^{14}C]palmitate (1.85×10^7 Bq·mmol⁻¹ palmitate), 2% or 4% bovine serum albumin, and 95% O_2 and 5% CO_2 as the gaseous phase. Insulin ($250 \mu\text{U}\cdot\text{mL}^{-1}$) or pyruvate (5 mmol·L⁻¹) was added as indicated. All incubations were carried out at 37 °C in sealed vials in a shaking water bath. Glycogen synthesis in skeletal muscle and fatty acid oxidation in liver slices were measured as described previously (Cahova et al. 2004). The conversion of ^{14}C -palmitate into secreted water-soluble oxidation products (i.e., predominantly ketone bodies) in liver slices was assessed according to the radioactivity remaining in the aqueous fraction of the incubation medium after chloroform extraction (Skop et al. 2012). For determination of the amount of TCA cycle intermediates, tissues were homogenized by sonication (2×30 s, Hielscher UP200S ultrasonic homogenizer, Teltow, Germany) in 150 mmol·L⁻¹ NaCl and extracted with petroleum ether, and the radioactivity remaining in the water fraction was measured. According to Kawamura and Kishimoto (1981), this fraction represents mostly TCA cycle intermediates (>80%), and minor parts are amino acids derived from fatty acids via the TCA cycle (<20%).

Pyruvate dehydrogenase activity determination

Pyruvate dehydrogenase activity in the liver and muscle homogenates was determined by measuring $^{14}\text{CO}_2$ production by decarboxylation from [^{14}C]pyruvate (Dudkova et al. 1995). The incubation mixture contained 0.1–0.2 mg protein, 75 mmol·L⁻¹ Tris-HCl, 0.5 mmol·L⁻¹ EDTA, 4 mmol·L⁻¹ MgSO_4 , 2 mmol·L⁻¹ CaCl_2 , 7.5 mmol·L⁻¹ L-carnitine, 1.5 mmol·L⁻¹ β -NAD⁺, 1 mmol·L⁻¹ thiamine pyrophosphate, 2 mmol·L⁻¹ coenzyme A (CoA), and 0.5 mmol·L⁻¹ ^{14}C -pyruvate ($18.5 \text{ kBq}\cdot\mu\text{mol}^{-1}$) in a final volume of 0.5 mL. Assays were performed in sealed glass vials with filter paper soaked with 17 μL of 10% KOH. The mixture was pre-incubated for 10 min at 37 °C and the reaction was started by adding ^{14}C -pyruvate. After a 20-min incubation at 37 °C, reactions were stopped by the addition of 50 μL of 50% trichloroacetic acid and the radioactivity associated with KOH was measured by scintillation counting.

Determination of mitochondrial respiration

Liver mitochondria were prepared as described previously (Drahota et al. 2014). Oxygen consumption was measured at 30 °C using an

Oxygraph-K2 system (OROBOROS, Austria). Measurements were performed in 2 mL of K-medium containing 80 mmol·L⁻¹ KCl, 10 mmol·L⁻¹ Tris-HCl, 3 mmol·L⁻¹ MgCl_2 , 1 mmol·L⁻¹ EDTA, and 5 mmol·L⁻¹ K-PO₄, pH 7.4. The rate of oxygen consumption was calculated using OROBOROS DatLab 4 software and expressed as pmoles per minute per milligram of protein.

Determination of mitochondrial (mt)DNA copy number

The mtDNA copy number per cell was quantified as the ratio of amplicons from PCR with specific primers for the mitochondrial gene *Nd5* (bp 11 092 to 11 191, according to sequences from GenBank, National Center for Biotechnology Information, USA) and the nuclear gene *Ucp2* (intron 2 and exon 3). Total DNA was isolated from cells using an Invisorb Spin Forensic Kit (Stratag Molecular, Germany). Quantification of mitochondrial and genomic genes was performed by real-time quantitative PCR using HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne) on a ViiA 7 Real-Time PCR System (Life Technologies). Primer sequences are listed in Table S1. The mtDNA copy number was calculated as $2^{\Delta\text{Ct}}$ ($\Delta\text{Ct} = \text{Ct}_{\text{nuclear}} - \text{Ct}_{\text{mitochondrial}}$).

Parameters of oxidative stress

Tissue homogenate was prepared by homogenizing liver tissue in Tris-EDTA buffer (0.025 mol·L⁻¹ Tris-HCl, pH 7.4, 2 mmol·L⁻¹ EDTA, 0.025 mol·L⁻¹ sucrose) on a Potter–Elvehjem homogenizer. The activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione reductase and the levels of reduced (GSH) and oxidized (GSSG) glutathione were analyzed using commercially available kits (Superoxide Dismutase, Glutathione Peroxidase, and Glutathione Reductase Assay Kits, Cayman Chemical, Ann Arbor, Mich., USA; Glutathione HPLC reagent kit, Chromsystems) according to the manufacturers' instructions. Measurement of catalase activity was based on the ability of H_2O_2 to produce a colored complex with ammonium molybdate, detected spectrophotometrically (Aebi 1974). The levels of conjugated dienes were measured spectrophotometrically in the heptane layer after extraction of the tissue homogenate with heptane–isopropanol (2:1) (Ward et al. 1985). The levels of thiobarbituric acid reactive substances (TBARS) were determined by reaction with thiobarbituric acid.

Biochemical analyses

FFA, insulin, TAG, and glucose contents in serum were determined using commercially available kits (FFA: Free fatty acids, Half-micro test, Roche Diagnostics, Germany; TAG and glucose: Erba Lachema Diagnostics, Czech Republic; insulin: Mercodia, Sweden).

Chemicals

All materials were reagent grade. $\text{D-}[^{14}\text{C}]$ glucose (specific activity, 3.7 GBq·mmol⁻¹) and [^{14}C]palmitic acid (specific activity, 31.45 GBq·mmol⁻¹) were purchased from UVVR, Prague,

¹Supplementary data are available with the article through the journal Web site at <http://nrcresearchpress.com/doi/suppl/10.1139/apnm-2014-0163>.

Fig. 2. Acylcarnitine species content in whole blood (A), urine (B), liver (C), musculus soleus (D), extensor digitorum longus (E), and diaphragm (F). Data are means $\pm$ SE, $n = 8-10$. Open bars: Wistar rats; gray bars: hereditary hypertriglyceridemic (HHTg) control rats; closed bars: HHTg + carnitine rats. *, $p < 0.05$, HHTg control vs. Wistar; †, $p < 0.05$, HHTg + carnitine vs. HHTg control.

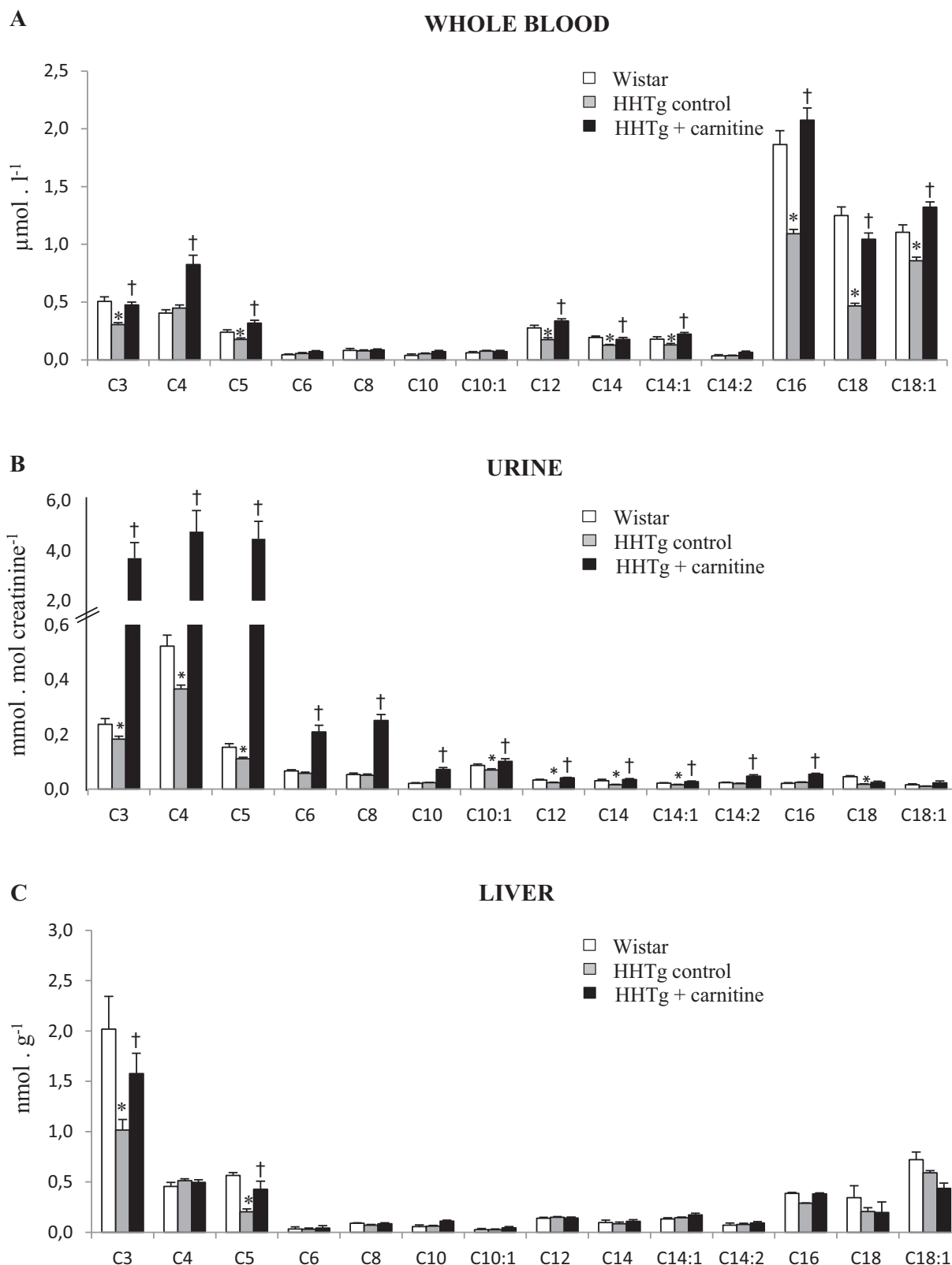
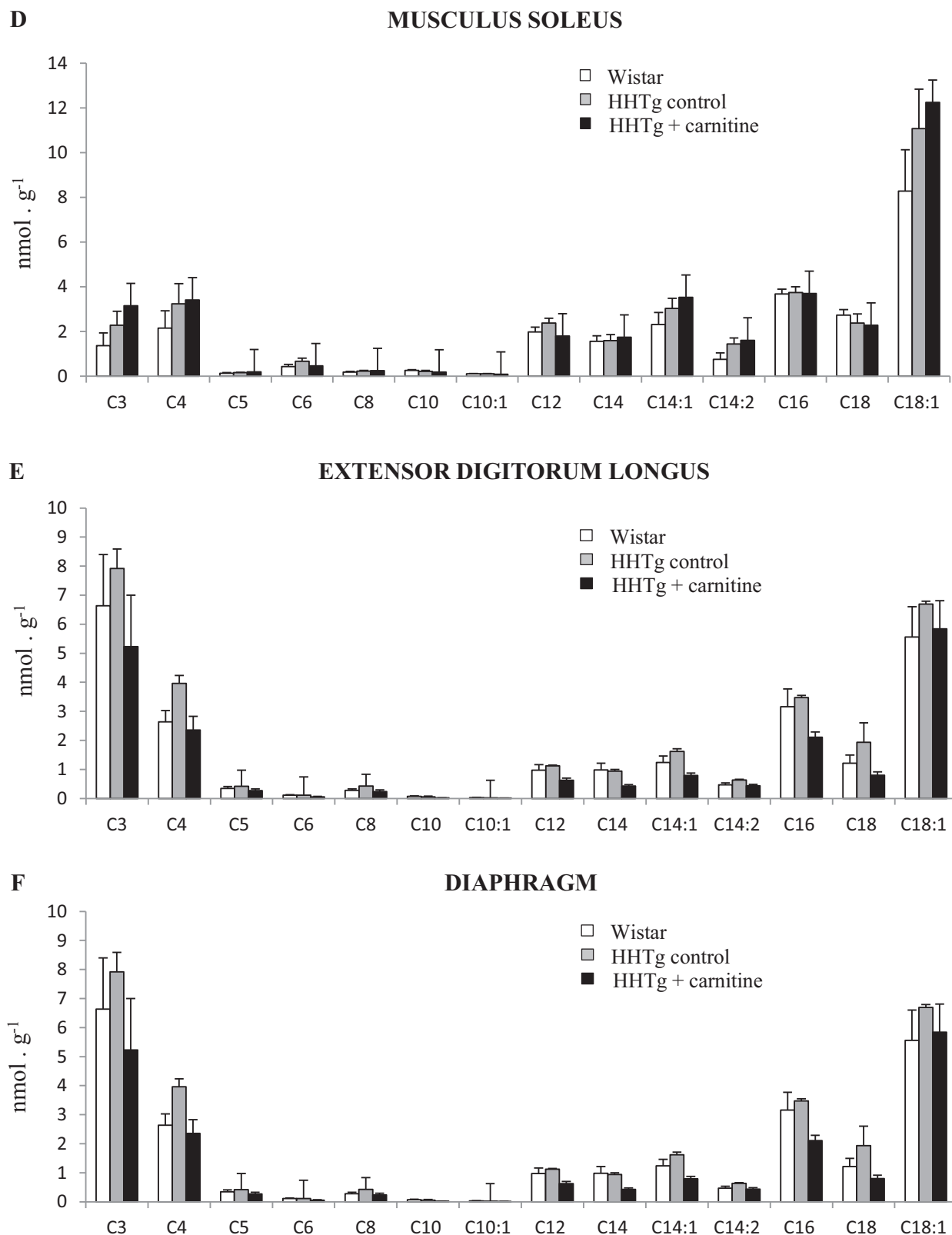


Fig. 2 (concluded).



Czech Republic, and PerkinElmer, Waltham, Mass., USA, respectively. FFA-free bovine serum albumin (fraction V) was purchased from Serva, palmitic acid was purchased from Fluka, and all other chemicals were purchased from Sigma, Czech Republic.

Statistical analyses

Data are presented as means $\pm$ SE ($n = 8-10$). Statistical analyses were performed using SigmaStat (Systat Software, San Jose, Calif., USA) or Microsoft Excel. One-way ANOVA with Bonferroni

correction was employed to analyze the differences among the Wistar, HHTg control, and HHTg + carnitine groups. Possible differences in the courses of the glycemic curves during the oral glucose tolerance test (OGTT) were tested using ANOVA with repeated measures (time intervals) and grouping factors (experimental groups). Student's *t* test was used to detect the effects of pyruvate or insulin within groups. Differences were considered statistically significant at the level of $p < 0.05$.

Results

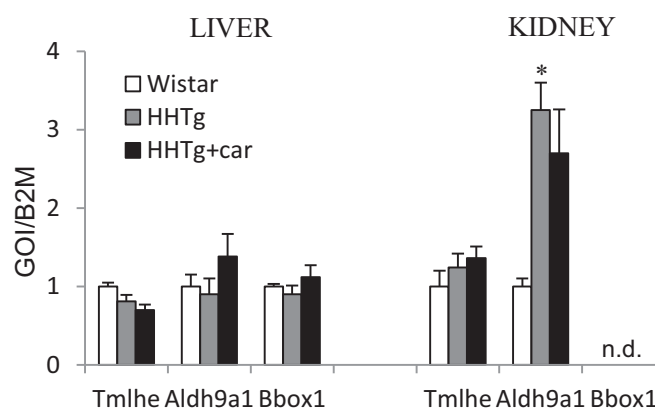
Metabolic characteristics

Throughout the experimental period (8 weeks), HHTg control and Wistar rats exhibited stable and significant weight gain, although this was more pronounced in Wistar rats (Fig. 1A). In contrast, the average weight in the HHTg + carnitine group remained unchanged. Food intake was not different among the groups. The differences in relative body composition are shown in Fig. 1B. In relation to total body weight, HHTg control rats exhibited a slightly increased amount of epididymal adipose tissue ($p = 0.028$) and a significantly increased amount of brown adipose tissue ($p = 0.009$). As shown in Table 1, HHTg control rats displayed significant deviations in glucose and lipid metabolism from Wistar rats. Glucose tolerance was measured by an OGTT, which revealed a significant difference between HHTg control and Wistar rats but no effect of carnitine (Fig. 1C). At the beginning of the test, the serum FFA content was comparable in the HHTg control and HHTg + carnitine groups, but 60 min after glucose administration, it was significantly lower in HHTg + carnitine rats than in HHTg controls (Fig. 1D). The same effect of carnitine was observed in relation to serum triglyceridemia (Fig. 1E). In conclusion, carnitine supplementation did not improve glucose tolerance, but the results concerning lipid metabolism parameters during the OGTT suggested an enhancement of the antilipolytic effect of insulin. Carnitine administration partially ameliorated fasting triglyceridemia and decreased liver TAG content while having no effect on glycemia, serum FFA content, or fed triglyceridemia.

Carnitine homeostasis is disturbed in HHTg rats

Data on the free carnitine and individual acylcarnitine species contents in whole blood, urine, liver, musculus soleus, EDL, and diaphragm are given in Table 2 and Fig. 2A–2F. In blood, there were significantly lower concentrations of free carnitine, some short-chain acylcarnitines (C3, C5), and long-chain acylcarnitines (C12–C18) in HHTg controls compared with Wistar rats. Medium-chain acylcarnitines were not influenced at all. Importantly, the urinary excretion of free carnitine, but not any of the other acylcarnitine species, was significantly higher in the HHTg control group compared with the Wistar group. In the liver, free carnitine and short-chain acylcarnitines (C3 and C5) were reduced in HHTg control rats, while both medium- and long-chain acylcarnitines were comparable between the strains. We did not observe any difference in (acyl)carnitine species between HHTg control and Wistar rats in any of the skeletal muscles tested, i.e., musculus soleus, EDL, and diaphragm. To test whether the observed shortage of free carnitine and some acylcarnitine species in serum and liver of HHTg rats could be explained by reduced carnitine biosynthesis, we determined the expression of mRNA for enzymes catalyzing different phases of carnitine biosynthesis – trimethyllysine hydroxylase- ϵ (Tmlhe, initial phase), aldehyde dehydrogenase-9 family member A1 (Aldh9, third step), and γ -butyrobetaine hydroxylase-1 (Bbox, final step) – in the liver and kidney (Fig. 3). Rather surprisingly, no differences between the strains were observed in the liver, and Aldh9 expression in kidney was increased (9-fold) in HHTg control rats compared with Wistar rats. We then looked at the expression of genes involved in carnitine transport (Fig. 4A–4C). Expression of the main plasmalemmal carrier of carnitine from circulation into cells, Octn2, was significantly

Fig. 3. Expression of genes involved in carnitine biosynthesis in liver and kidney of hereditary hypertriglyceridemic (HHTg) and Wistar rats. car, carnitine; Aldh9, aldehyde dehydrogenase-9 family member A1; B2M, β -2 microglobulin; Bbox, γ -butyrobetaine hydroxylase-1; GOI, gene of interest; n.d., not determined; Tmlhe, trimethyllysine hydroxylase- ϵ . Data are means $\pm$ SE, $n = 8$. Open bars: Wistar rats; gray bars: HHTg control rats; closed bars: HHTg + carnitine rats. *, $p < 0.05$, HHTg control vs. Wistar.



higher in the liver and diaphragm but was attenuated in the kidney in HHTg control rats compared with Wistar rats. The expression of 2 other transporters facilitating (acyl)carnitine transport across the inner mitochondrial membrane, Octn1 and Cact, was significantly increased in HHTg kidney. Our data indicate that the observed carnitine shortage in HHTg rats may be a consequence of higher urinary excretion. The increased inward transportation capacity in muscle may represent an adaptation to the compromised carnitine availability.

Carnitine supplementation increases (acyl)carnitine excretion

Dietary carnitine supplementation normalized the carnitine shortage in the blood and liver of HHTg rats (Table 2, Fig. 2A and 2C). Furthermore, carnitine supplementation significantly promoted the urinary excretion of free carnitine, acetylcarnitine, and other acylcarnitine species in HHTg rats compared with their untreated littermates (Fig. 2B). This effect was most notable in the case of acetylcarnitine (300-fold), C3–C5 acylcarnitines (200-fold), and free carnitine (95-fold). The tissue acylcarnitine content was unchanged in skeletal muscle (diaphragm, musculus soleus, and EDL). Carnitine supplementation did not affect the expression of genes involved in carnitine biosynthesis (Fig. 3) but did affect the expression of genes encoding carnitine transporters. The expression of Octn2, a transporter facilitating inward carnitine transport, was downregulated in the liver (Fig. 4A) and kidney (Fig. 4B), while the expression of Cact, which catalyzes the export of acetylcarnitine from mitochondria, was elevated in skeletal muscle (Fig. 4C). These data are in accordance with the hypothesis that carnitine supplementation facilitates the efflux and excretion of short-chain acylcarnitines and does not promote their accumulation within tissues, particularly muscles.

Fatty acid utilization in the liver

One of the possible explanations for the positive effect of carnitine treatment on TAG content in the liver of HHTg rats could be increased utilization of fatty acids. To test this hypothesis, we measured the oxidation of palmitate to CO_2 (Fig. 5A) or TCA cycle intermediates (Fig. 5B) and the production of ketone bodies in liver slices ex vivo (Fig. 5C). There was no difference in palmitate oxidation to CO_2 between HHTg control and Wistar rats. The formation of TCA cycle intermediates was higher in Wistar rats compared with the HHTg control group, while no significant differences

Fig. 4. Expression of genes involved in carnitine transport in the liver (A), kidney (B), and diaphragm (C) of hereditary hypertriglyceridemic (HHTg) and Wistar rats. B2M, β -2 microglobulin; Cact, solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase), member 20; GOI, gene of interest; car, carnitine; Octn1, solute carrier family 22 (organic cation/zwitterion transporter), member 4; Octn2, solute carrier family 22 (organic cation/carnitine transporter), member 5. Data are means $\pm$ SE, $n = 8$. Open bars: Wistar rats; grey bars: HHTg control rats; closed bars: HHTg + carnitine rats. *, $p < 0.05$, HHTg control vs. Wistar; †, $p < 0.05$, HHTg + carnitine vs. HHTg control.

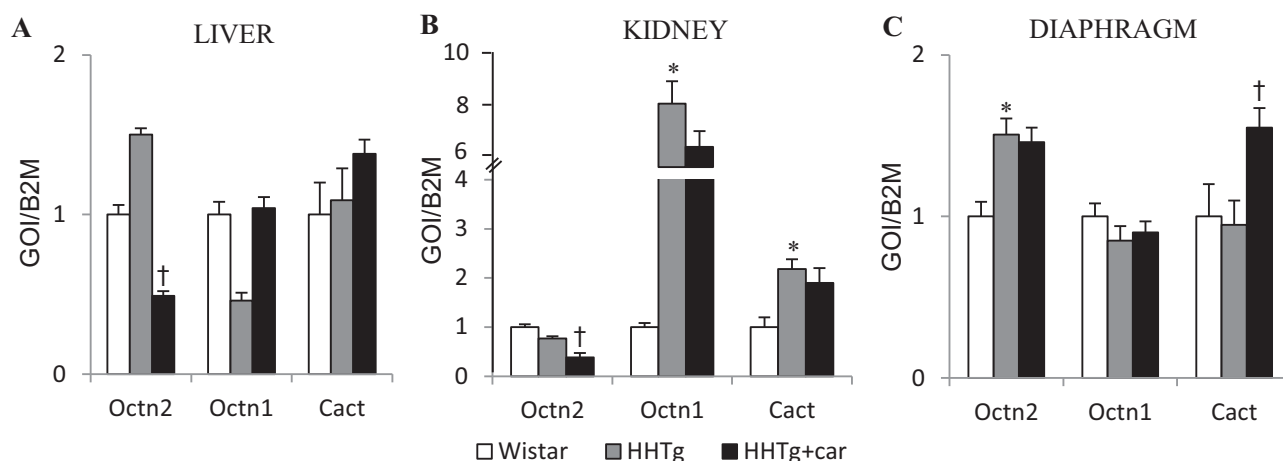


Fig. 5. Utilization of $[U-^{14}C]$ palmitate in liver slices ex vivo in the presence and absence of pyruvate. (A) Production of CO_2 , (B) formation of tricarboxylic acid (TCA) cycle intermediates, and (C) production of ketone bodies. car, carnitine. Open bars: basal Krebs–Ringer (KRB) medium; closed bars: KRB medium + $5 \text{ mmol} \cdot \text{L}^{-1}$ pyruvate. Data are means $\pm$ SE, $n = 8-10$. *, $p < 0.05$, hereditary hypertriglyceridemic (HHTg) control vs. Wistar (basal); †, $p < 0.05$, HHTg + carnitine vs. HHTg control (basal); ‡, $p < 0.05$, HHTg control vs. Wistar (+ pyruvate); §, $p < 0.05$, pyruvate vs. basal.

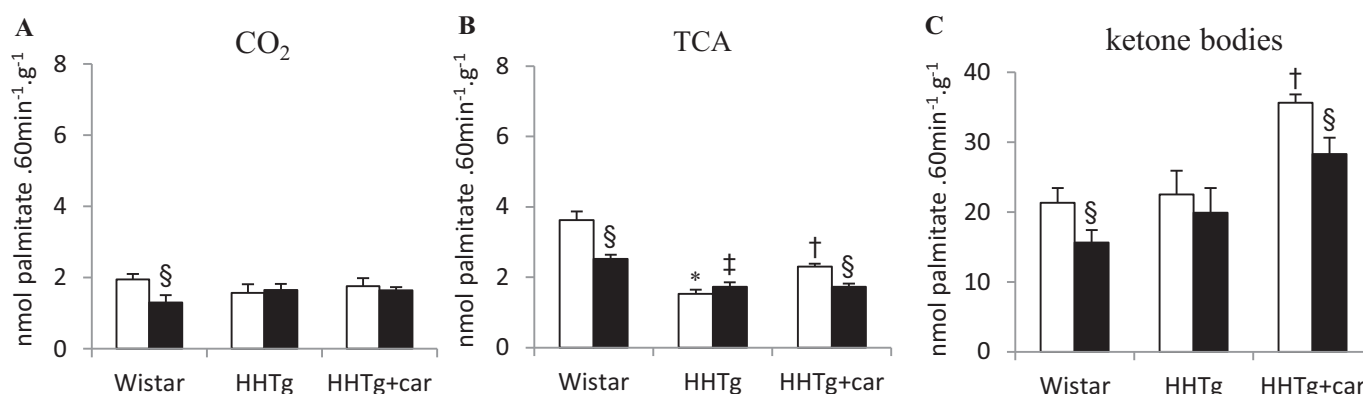


Fig. 6. Effect of carnitine supplementation on pyruvate dehydrogenase activity in the liver and diaphragm of Wistar, hereditary hypertriglyceridemic (HHTg) control, and HHTg + carnitine rats. car, carnitine; prot, protein. Data are means $\pm$ SE, $n = 8-10$. Open bars: Wistar rats; grey bars: HHTg control rats; closed bars: HHTg + carnitine rats. *, $p < 0.05$, HHTg control vs. Wistar; †, $p < 0.05$, HHTg + carnitine vs. HHTg control.

were found in ketogenesis. The inhibitory effect of pyruvate on palmitate utilization was preserved in Wistar but not HHTg rats. In HHTg rats, carnitine supplementation had no effect on CO_2 production but increased the production of TCA cycle intermediates in liver slices and significantly elevated ketogenesis. Furthermore, it partially restored the inhibitory effect of pyruvate on fatty acid utilization.

Substrate selection between glucose and fatty acids is mediated by pyruvate dehydrogenase (PDH). In accordance with the observed influence of carnitine supplementation on the inhibitory effect of pyruvate on palmitate utilization in liver slices, we found a significant increase of PDH activity in the liver of HHTg + carnitine rats compared with HHTg controls (Fig. 6).

The data obtained in tissues ex vivo were compared with direct measurements in isolated mitochondria (Table 3). We found no difference in mitochondrial respiration between the HHTg control and Wistar groups when succinate or malate + glutamate were provided as substrates. In contrast, palmitoylcarnitine utilization was significantly diminished in HHTg rats, and this defect was compensated by carnitine treatment.

Because we used palmitoylcarnitine and not only palmitic acid as a substrate for mitochondria, the positive effect of carnitine on palmitate respiration could not be explained only by improved

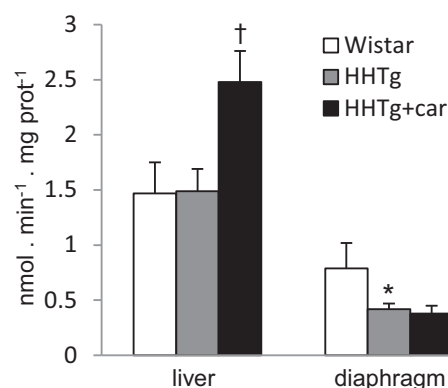


Table 3. Effect of in vivo carnitine treatment on respiration (pmol O₂·min⁻¹·mg⁻¹ protein) in isolated liver mitochondria using different substrates.

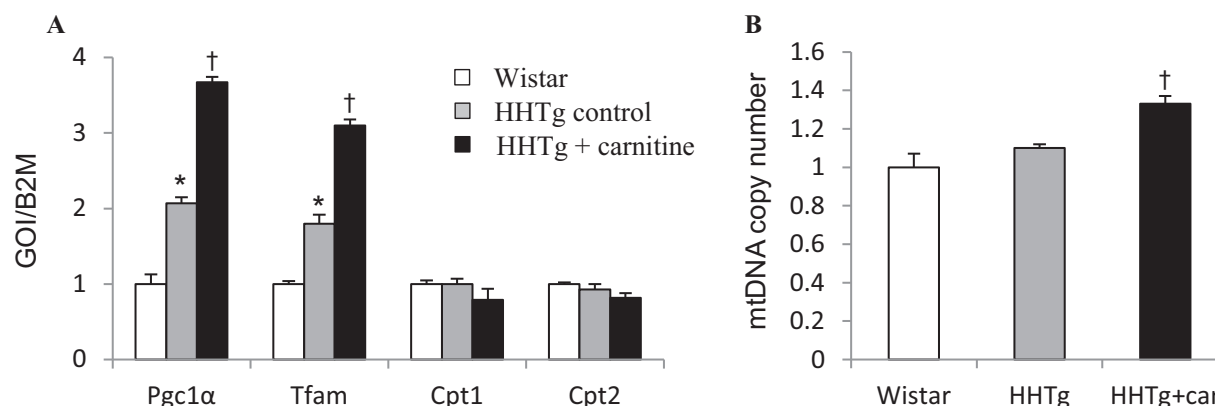
	Malate + glutamate	Palmitoylcarnitine	Succinate	Respiratory control
Wistar	363±32	261±19	473±41	3.4±0.2
HHTg control	347±48	214±14*	446±68	2.8±0.3
HHTg+carnitine	421±28	266±13†	555±35	3.5±0.1

Note: Data are means ± SE. All measurements were performed in the presence of ADP (state 3).

HHTg, hereditary hypertriglyceridemic.

*p < 0.05, HHTg control vs. Wistar.

†p < 0.05, HHTg + carnitine vs. HHTg control.

Fig. 7. Expression of genes involved in mitochondrial biogenesis and respiration (A) and mtDNA copy number (B). B2M, β-2 microglobulin; car, carnitine; GOI, gene of interest. Data are means ± SE, n = 8–10. Open bars: Wistar rats; grey bars: hereditary hypertriglyceridemic (HHTg) control rats; closed bars: HHTg + carnitine rats. *, p < 0.05, HHTg control vs. Wistar; †, p < 0.05, HHTg + carnitine vs. HHTg control.**Table 4.** Effect of carnitine supplementation on antioxidant systems and lipid peroxidation in the liver of HHTg rats.

	Wistar	HHTg control	HHTg + carnitine
GSH (μmol·g ⁻¹)	4±0.4	3.3±0.005*	3.1±0.25
GSSG (μmol·g ⁻¹)	0.13±0.04	0.21±0.02*	0.11±0.006†
GSH/GSSG ratio	28.9±4.3	14.9±0.5*	28.3±0.9†
Superoxide dismutase (U·mg ⁻¹)	168±0.03	137±0.01*	167±0.01†
Glutathione peroxidase (μmol NADPH·min ⁻¹ ·mg ⁻¹)	261±15	238±10*	278±12†
Glutathione reductase (μmol NADPH·min ⁻¹ ·mg ⁻¹)	162±14	174±20	156±20
Catalase (μmol H ₂ O ₂ ·min ⁻¹ ·mg ⁻¹)	1278±59	909±24*	1160±46†
Conjugated dienes (nmol·mg ⁻¹)	25.1±0.9	28±3	26±3
TBARS (nmol·mg ⁻¹)	0.769±0.02	0.974±0.03*	0.84±0.04†

Note: Data are means ± SE, n = 8–10. GSH, reduced glutathione; GSSG, oxidized glutathione; HHTg, hereditary hypertriglyceridemic; TBARS, thiobarbituric acid reactive substances.

*p < 0.05, HHTg control vs. Wistar.

†p < 0.05, HHTg + carnitine vs. HHTg control.

fatty acid transportation. To find another explanation, we measured the expression of several genes involved in mitochondrial metabolism (Fig. 7) in the liver. We found significantly higher expression of 2 genes involved in mitochondrial biogenesis, *Pgc1α* and *Tfam*, in HHTg rats that was substantially potentiated by carnitine treatment. In the HHTg + carnitine group, we also found an increased mtDNA copy number. The expression of 2 fatty acid transporter genes, *Cpt1* and *Cpt2*, was not different among the groups. In conclusion, our data support the hypothesis that carnitine supplementation results in increased fatty acid oxidation in the liver and that this effect could be mediated, at least partly, by increased mitochondrial biogenesis.

Oxidative stress in the liver

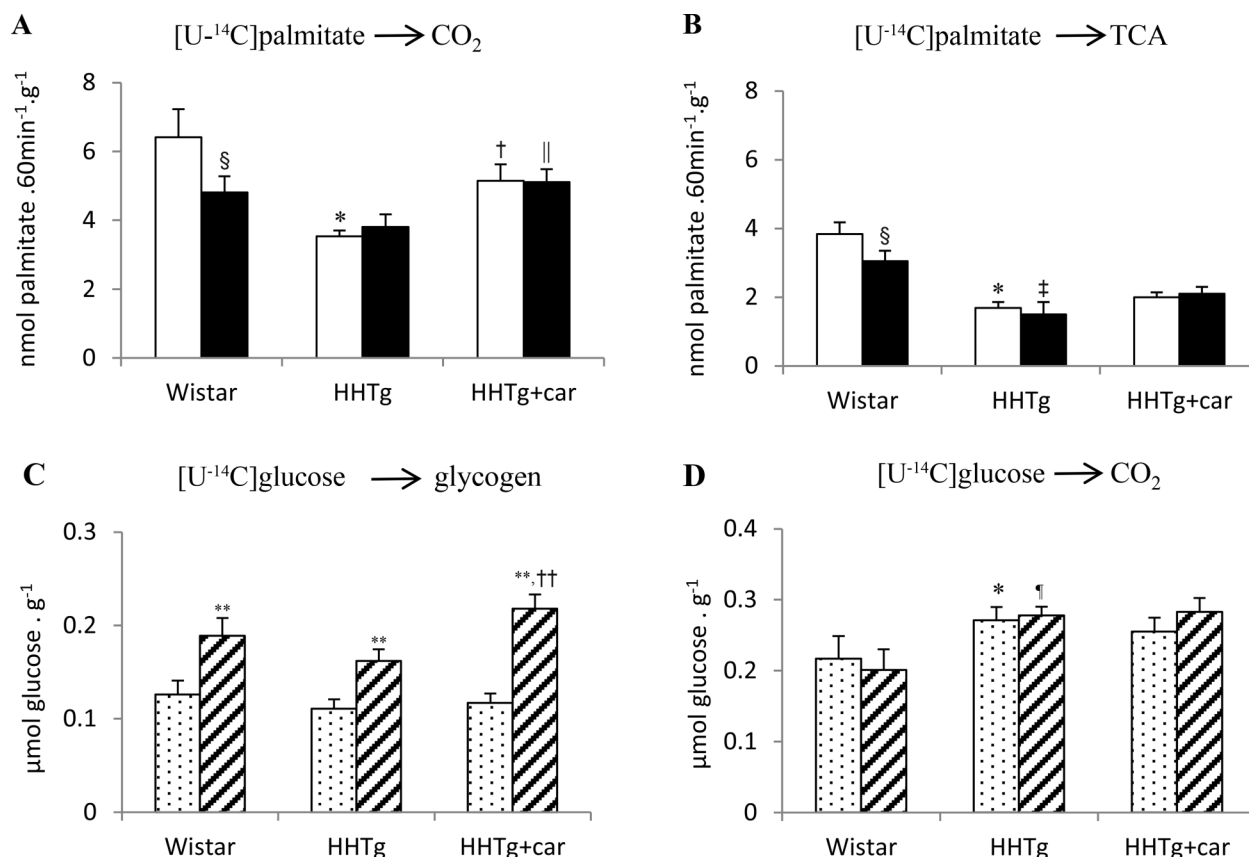
Theoretically, increased fatty acid oxidation in the liver of HHTg rats could be accompanied by an intensification of oxidative stress. To test this possibility, we measured the activities of the most important antioxidant enzymes as well as the concen-

trations of reduced and oxidized glutathione and lipid peroxides (Table 4). Most of these oxidative stress markers were significantly elevated in the liver of HHTg control rats compared with Wistar rats and were significantly ameliorated by carnitine treatment. The activities of GSH-Px, SOD, and catalase were significantly increased and the lipid peroxide (TBARS) content was significantly reduced in the liver of HHTg rats treated with carnitine compared with HHTg controls. The concentration of reduced glutathione was similar in both groups but the concentration of oxidized glutathione was markedly lower in the HHTg + carnitine group. Consequently, the GSH/GSSG ratio was higher in the carnitine-treated group. Taken together, these data indicate that the anti-oxidative defense capacity was elevated by carnitine supplementation, which resulted in lower oxidative stress.

Peripheral insulin sensitivity

Next, we examined whether carnitine supplementation affected glucose and fatty acid metabolism in skeletal muscle. Both

Fig. 8. Utilization of [U-¹⁴C]palmitate and [U-¹⁴C]glucose in diaphragm ex vivo. (A) Production of CO₂ from palmitate, (B) formation of tricarboxylic acid (TCA) cycle intermediates from palmitate, (C) incorporation of glucose into glycogen, and (D) production of CO₂ from glucose. car, carnitine. Open bars: basal Krebs–Ringer (KRB) medium + [U-¹⁴C]palmitate; closed bars: KRB medium + [U-¹⁴C]palmitate + 5 mmol·L⁻¹ pyruvate; dotted bars: basal KRB medium + [U-¹⁴C]glucose; hatched bars: KRB medium + [U-¹⁴C]glucose + insulin (250 μU·mL⁻¹). *, *p* < 0.05, hereditary hypertriglyceridemic (HHTg) vs. Wistar (basal); †, *p* < 0.05, HHTg + carnitine vs. HHTg (basal); ‡, *p* < 0.05, HHTg vs. Wistar (+ pyruvate); §, *p* < 0.05, pyruvate vs. basal; ||, *p* < 0.05, HHTg + carnitine vs. HHTg (+ pyruvate); ¶, *p* < 0.05, HHTg vs. Wistar (+ insulin); **, *p* < 0.05, basal vs. insulin; ††, *p* < 0.05, HHTg + carnitine vs. HHTg (+ insulin).



complete (CO₂) and incomplete (TCA cycle intermediates) palmitate oxidation ex vivo was reduced in HHTg rats compared with the Wistar strain. Carnitine supplementation led to significantly higher palmitate oxidation to CO₂ in HHTg rats but did not increase formation of TCA cycle intermediates, which resulted in a more favorable complete to incomplete oxidation products ratio (Fig. 8A and 8B). It also enhanced insulin sensitivity, measured as incorporation of ¹⁴C-glucose into glycogen in the diaphragm, in HHTg rats (Fig. 8C). Glucose oxidation was not affected (Fig. 8D). The inhibitory effect of pyruvate on fatty acid utilization was seen in the diaphragm of Wistar but not HHTg rats, and it was not affected by carnitine treatment. No differences were found in PDH activity in the diaphragm of carnitine-treated rats (Fig. 7). Taken together, these results show that carnitine supplementation enhances insulin sensitivity in muscle and that this effect may be associated with better substrate oxidation.

Discussion

In this study we have shown that the free carnitine content in whole blood and liver is significantly reduced in a rat model of genetically determined hypertriglyceridemia associated with insulin resistance, probably because of elevated urinary excretion. Oral carnitine supplementation led to full compensation of this shortage, significantly increased the urinary excretion of acetylcarnitine and other (acyl)carnitine species, and resulted in the amelioration of some aspects of dyslipidemia. Carnitine-treated HHTg rats exhibited increased utilization of fatty acids in the liver

(ketogenesis) and skeletal muscle (oxidation), a significant reduction of steatosis, alleviation of oxidative stress in the liver, and improved insulin sensitivity in skeletal muscle.

There are several possible mechanisms that could explain the beneficial metabolic effects of carnitine: (i) increased oxidation of fatty acids owing to their increased transport into mitochondria via carnitine palmitoyltransferase; (ii) an efflux of surplus acetyl carnitine from the mitochondria, resulting in the regeneration of free CoA and better oxidative capacity of the cell; (iii) a reduction in the accumulation of potentially toxic intermediates of lipid metabolism (i.e., long-chain acyl-CoA) owing to better oxidation.

The first proposed mechanism of carnitine action, enhancement of fatty acid utilization in mitochondria, may be responsible for the observed reduction of liver steatosis in HHTg rats supplemented with carnitine. In fact, we observed increased expression of transcription factors regulating mitochondrial biogenesis (PGC1α, Tfam) in carnitine-treated HHTg rats as well as an increased mtDNA copy number, which suggests stimulation of mitochondrial formation in the liver of carnitine-treated HHTg rats. In accordance with this, we observed increased utilization of fatty acids, provided in the form of palmitate bound to albumin, in the liver of carnitine-treated HHTg rats ex vivo, particularly for ketogenesis. This observation could be explained by the unique ability of hepatocytes to produce ketone bodies in mitochondria. Owing to our experimental conditions, the capacity of mitochondria to oxidize fatty acids was limited by the availability of ADP, i.e.,

respiratory control. Because we do not have any evidence indicating uncoupling in the liver mitochondria of carnitine-treated animals, the formation of ketone bodies is the most likely response to the increased fatty acid supply facilitated by enhanced carnitine availability. Rather surprisingly, we observed an increased capacity to oxidize palmitoylcarnitine in isolated liver mitochondria of HHTg rats supplemented with carnitine. Because fatty acids in this experiment were provided in the form of palmitoylcarnitine, the effect of previous carnitine treatment could not be explained by the activity of CPT1 and probably occurs in some post-transport step. Similar results were reported by Mingorance et al. (2012), who found that propionyl-L-carnitine supplementation in mice fed a high-fat diet was associated with elevated respiratory chain enzyme activities and increased citrate synthase activity. Their data were obtained by direct measurement of enzyme activities and thus avoided the fatty acid translocation step mediated by CPT1 and CPT2. Taken together, our data show that carnitine treatment increases the capacity for fatty acid utilization in the liver by a mechanism that involves increased mitochondrial biogenesis and an enhanced mitochondrial capacity for fatty acid utilization. We speculate that the effect would be even greater in combination with physical activity, which would allow fatty acid oxidation in the mitochondria to be used for ATP production.

An important feature of metabolic fitness is the ability to switch between substrates (glucose versus fatty acids) according to their actual supply and the metabolic needs of the organism. In our *ex vivo* experiments, the livers of HHTg control rats were completely insensitive to the presence of pyruvate, but carnitine treatment rescued the inhibitory effect of pyruvate on fatty acid utilization. PDH plays a key role in shifting mitochondrial substrate selection between glucose and fatty acids, its activity being inhibited by high acetyl-CoA/CoA ratios. In our study, carnitine treatment significantly increased liver PDH activity, indicating a positive effect of carnitine on substrate flux and suggesting a role of carnitine in regenerating free CoA in the liver.

The potential negative side effects of increased fatty acid oxidation, particularly elevated reactive oxygen species (ROS) production, were compensated for by elevated activity of the antioxidative defense system in the liver. An antioxidative effect of carnitine has also been described in other models of steatosis, i.e., steatosis induced by alcohol consumption (Sachan et al. 1984; Augustyniak and Skrzydlewska 2009), a high-fat diet (Kang et al. 2011), and some drugs (Bianchi et al. 1996; Yapar et al. 2007). Nevertheless, the mechanisms of the antioxidative action of carnitine have not been fully elucidated and probably involve more than one effect. One possible explanation is based on the ability of carnitine to directly scavenge both superoxide and H_2O_2 radicals (Gülçin 2006; Derin et al. 2004). In addition, several recent reports have documented an association between carnitine administration and increased SOD activity, as well as catalase and GSH-Px activities and expression (Yapar et al. 2007; Gómez-Amores et al. 2007; Cao et al. 2011). Interestingly, although we found a highly significant elevation of the GSH/GSSG ratio in carnitine-treated HHTg rats, this was due to decreased GSSG rather than increased GSH. We suggest that this reflects lower ROS formation in carnitine-treated animals and thus provides indirect evidence supporting the radical-scavenging function of carnitine.

The most common states of glucose intolerance, obesity and diabetes, have been shown to be tightly associated with mitochondrial dysfunction and high rates of incomplete fat oxidation (Koves et al. 2005, 2008), which occurs when the carbon flux through the β -oxidation machinery outpaces the entry of acetyl-CoA into the TCA cycle. The fluent flux of energetic substrates through the TCA cycle and electron transport chain is critically dependent on the availability of free CoA, among other factors. To avoid "mitochondrial collapse", mitochondria possess a mechanism that allows the regeneration of free CoA when there is an

excess supply of energy substrates for oxidation due to the action of carnitine, which could facilitate not only an influx of fatty acids into the mitochondria but also an efflux of excess carbon fuels. The crucial player in this process is the mitochondrial enzyme carnitine acetyltransferase, which converts acyl-CoAs to their acylcarnitine counterparts and simultaneously regenerates free CoA (Muio et al. 2012). Unlike acyl-CoAs, short-chain acylcarnitines are capable of diffusing across cellular membranes and may be eliminated in the urine. According to this hypothesis, carnitine supplementation might contribute to the amelioration of mitochondrial dysfunction and insulin resistance.

In our study, we found that carnitine supplementation was associated with significantly increased urinary excretion of short-chain acylcarnitine species, but we did not observe their accumulation in skeletal muscle. Furthermore, the complete to incomplete oxidation products ratio was increased in the muscle of HHTg rats supplemented with carnitine. The elevated expression of Cact, the transporter facilitating acylcarnitine transport across the mitochondrial membrane, in the diaphragm and EDL is in accordance with the anticipated enhancement of acetylacarnitine efflux from the skeletal muscle. Our finding of elevated insulin sensitivity in the skeletal muscle of HHTg rats treated with carnitine is in line with the observed improvement in mitochondrial performance in skeletal muscle. On the other hand, carnitine treatment neither restored metabolic flexibility in terms of pyruvate-induced substrate switching nor affected PDH activity in skeletal muscle.

In conclusion, we found that in a non-obese genetic model of insulin resistance associated with hypertriglyceridemia, there is a carnitine shortage in the liver and blood and some metabolic syndrome-related disorders could be reversed by carnitine supplementation. The effect of carnitine was most pronounced in the liver, where its administration enhanced fatty acid oxidation, diminished steatosis, and increased the capacity of the antioxidant defense system. The beneficial effect of carnitine could be explained, at least partly, by the enhanced removal of incomplete oxidation products from the tissues via increased urinary excretion.

Acknowledgements

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PŘÍLOHA XI

Bechynska K, Daskova N, Vrzackova N, Harant K, Heczková M, Podzimkova K, Bratova M, Dankova H, Berkova Z, Kosek V, Zelenka J, Hajslova J, Sedlacek R, Suttar J, Hlavackova A, Bartonova L, Cahova M. *The effect of ω -3 polyunsaturated fatty acids on the liver lipidome, proteome and bile acid profile: parenteral vs enteral administration*. Sci Rep. 2019 Dec 13;9(1):19097. doi: 10.1038/s41598-019-54225-8.

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The effect of ω -3 polyunsaturated fatty acids on the liver lipidome, proteome and bile acid profile: parenteral *versus* enteral administration

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Parenteral nutrition (PN) is often associated with the deterioration of liver functions (PNALD). Omega-3 polyunsaturated fatty acids (PUFA) were reported to alleviate PNALD but the underlying mechanisms have not been fully unraveled yet. Using omics' approach, we determined serum and liver lipidome, liver proteome, and liver bile acid profile as well as markers of inflammation and oxidative stress in rats administered either ω -6 PUFA based lipid emulsion (Intralipid) or ω -6/ ω -3 PUFA blend (Intralipid/Omegaven) via the enteral or parenteral route. In general, we found that enteral administration of both lipid emulsions has less impact on the liver than the parenteral route. Compared with parenterally administered Intralipid, PN administration of ω -3 PUFA was associated with 1. increased content of eicosapentaenoic (EPA)- and docosahexaenoic (DHA) acids-containing lipid species; 2. higher abundance of CYP4A isoenzymes capable of bioactive lipid synthesis and the increased content of their potential products (oxidized EPA and DHA); 3. downregulation of enzymes involved CYP450 drug metabolism what may represent an adaptive mechanism counteracting the potential negative effects (enhanced ROS production) of PUFA metabolism; 4. normalized anti-oxidative capacity and 5. physiological BAs spectrum. All these findings may contribute to the explanation of ω -3 PUFA protective effects in the context of PN.

Parenteral nutrition (PN) provides life-saving nutritional support in situations where caloric supply via the enteral route is either not possible or cannot cover the necessary needs of the organism¹. Nevertheless, PN does have serious adverse effects, one of which is the deterioration of liver function². While the liver function abnormalities are usually normalized after discontinuation of PN, it may represent a serious risk factor in individuals receiving long term PN³.

The etiology of parenteral nutrition-associated liver disease (PNALD) is not well understood and is likely multifactorial⁴. Among the risk factors, intravenous fat emulsions constituents play an important role⁵. The first well-tolerated lipid emulsion (Intralipid) introduced to parenteral nutrition mixtures was based on soybean oil rich in ω -6 polyunsaturated fatty acids (PUFA)⁶. Besides their undeniable benefits comprising the dense source of non-protein calories, prevention of essential fatty acid (FA) deficiency and minimization of respiratory and metabolic stress⁷, ω -6 PUFA serves as precursors for the synthesis of pro-inflammatory cytokines and eicosanoids and Intralipid administration was associated with serious adverse effects including inflammation and oxidative stress⁸.

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		control	ENIL	ENILOV	PNIL	PNILOV
serum	ALT $\mu\text{kat. l}^{-1}$	0.7 ± 0.2	0.7 ± 0.1	0.9 ± 0.3	0.4 ± 0.1	0.5 ± 0.2
	AST $\mu\text{kat. l}^{-1}$	2.5 ± 0.7	1.8 ± 0.3	1.9 ± 0.4	2.3 ± 0.8	2.1 ± 0.5
	Tg mmol. l^{-1}	0.7 ± 0.2	0.6 ± 0.2	$0.4 \pm 0.2^*$	0.6 ± 0.1	$0.5 \pm 0.3^*$
	total bilirubin $\mu\text{mol. l}^{-1}$	1.5 ± 0.3	1.4 ± 0.6	1.6 ± 0.7	$2.3 \pm 0.4^{*,\#}$	2.1 ± 0.6
	direct bilirubin $\mu\text{mol. l}^{-1}$	0.6 ± 0.2	0.6 ± 0.3	0.5 ± 0.1	$1.3 \pm 0.2^{*,\#}$	$0.7 \pm 0.2^{\dagger}$
	TNF α pg/ml	14.3 ± 6.3	12.5 ± 4.3	19.3 ± 8.7	24.4 ± 7.2	20.1 ± 6.3
	IL-6 pg/ml	17.1 ± 2.5	16.2 ± 4.3	10.5 ± 7.2	22.4 ± 6.5	19.3 ± 5.4
	IL-10 pg/ml	23 ± 31	97 ± 144	15 ± 37	142 ± 70	167 ± 142
liver	Tg $\mu\text{mol. mg prot}^{-1}$	3.3 ± 1.3	3.0 ± 2.0	4.1 ± 2.1	$47.8 \pm 21^{*,\#}$	$13.1 \pm 8.5^{\dagger}$

Table 1. Characteristics of the experimental groups. Data are expressed as a mean $\pm$ s.d. ALT alanine transaminase; AST aspartate transaminase; Tg triglyceride. TNF α tumor necrosis factor. * $p < 0.05$ vs control; $^{\#}p < 0.05$ vs ENIL; $^{\dagger}p < 0.05$ vs PNIL; $^{\ddagger}p < 0.05$ vs ENILOV.

The introduction of fish oil rich in ω -3 PUFA into nutrition mixtures was associated with several beneficial effects in the prevention and reversal of PNALD in both infant and adult patients^{9,10}. In spite of the ongoing research, the exact mechanisms that would explain why either lipid load or FA composition confers the optimal metabolic function and prevention of PNALD are not fully established yet. The proposed mechanisms include the absence of phytosterol occurring in high concentration in soybean oil and consequent normalization of bile acids (BAs) metabolism¹¹, improved lipid clearance due to the PPAR α activation¹² and modulation of inflammation due to the immunomodulatory potential of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids-derived eicosanoids^{13,14}.

Lipids have been recognized as essential cellular components and energy sources of living organisms. Nevertheless, recent data show that they are also a source of bioactive lipid species exerting pleiotropic effects¹⁵ and altered lipid composition is increasingly recognized as a signature of many disease states^{16–18}. We hypothesized that not only the composition of the lipid emulsion itself but also the parenteral route of administration bypassing the physiological mechanisms of dietary fat transport and distribution would specifically influence the composition of the liver lipidome and/or proteome and may constitute one of the first triggers towards final pathology.

Therefore, the present study aimed to identify liver lipid, protein and BAs signature associated with the lipid emulsion composition and the route of administration and to identify potentially significant processes/markers that may contribute to the PN-associated liver injury. To fulfill this task, we determined liver lipidome, proteome, and BAs profile as well as markers of inflammation and oxidative stress in rats administered either ω -6 PUFA based lipid emulsion (Intralipid) or ω -6/ ω -3 PUFA blend (Intralipid/Omegaven) via the enteral or parenteral route.

Results

Characteristics of the experimental groups. The animals were divided into five groups subjected to different nutrition regimes, i.e. control (Plasmalyte *i.v.*, granules *per os*); ENIL (Plasmalyte *i.v.* Intralipid *per os*, granules *per os*); ENILOV (Plasmalyte *i.v.*, Intralipid + Omegaven *per os*, granules *per os*); PNIL (nutrition mixture with Intralipid *i.v.*); PNILOV (nutrition mixture with Intralipid + Omegaven *i.v.*). Neither of the nutrition regimes resulted in the liver injury as evidenced by normal levels of serum aspartate transaminase (AST) or alanine transaminase (ALT). ω -3 PUFA administration, both enteral and parenteral, was associated with the reduction of serum triglyceride (Tg) concentration compared with controls or with animals provided only ω -6 PUFA. Parenteral administration of both emulsions led to the elevation of serum bilirubin. We did not observe any significant differences in the serum concentration of pro- (TNF α , IL-6) or anti-inflammatory (IL-10) cytokines among groups. Parenteral, but not enteral, administration of lipid emulsions resulted in the enhanced Tg accumulation in the liver but steatosis was significantly higher in PNIL than in the PNILOV group (Table 1). The histological evaluation confirmed the biochemistry data. We observed only a few cases of focal microvesicular steatosis in ENIL (2/6) or ENILOV (1/6) groups, and no signs of more severe injury. In the PNIL group, we detected liver pathology in four out of seven samples, particularly focal microvesicular steatosis (2/7), focal microvesicular steatosis combined with necrosis close to a portal tract with markers of inflammation (1/7) and metabolic changes with focal Mallory hyaline inclusions (1/7). In the PNILOV group, we found focal microvesicular steatosis (2/6) and metabolic changes with focal Mallory hyaline (2/7) (Supplementary Figure 1).

Oxidative stress and inflammatory markers. Oxidative stress was estimated according to the malondialdehyde (MDA) concentration in the liver homogenate, antioxidant capacity, and expression of genes associated with oxidative stress (Table 2). We did not find any markers of oxidative stress in the liver of animals fed

	control	ENIL	ENILOV	PNIL	PNILOV
MDA $\mu\text{mol. g}^{-1}$	1.5 $\pm$ 0.8	0.5 $\pm$ 0.2	1.6 $\pm$ 2.0	1.7 $\pm$ 1.6	1.8 $\pm$ 1.5
antioxidative capacity AUC	125 $\pm$ 13	127 $\pm$ 20	119 $\pm$ 12	96 $\pm$ 12 [*]	113 $\pm$ 23
Gclc mRNA	1.0 $\pm$ 0.2	1.2 $\pm$ 0.1 ^{†, &}	0.9 $\pm$ 0.3 [†]	1.6 $\pm$ 0.4 [*]	2.3 $\pm$ 0.3 [*]
Gclc protein	226 $\pm$ 42	253 $\pm$ 24 ^{†, &}	234 $\pm$ 12 ^{†, &}	415 $\pm$ 81 [*]	465 $\pm$ 153 [*]
Hmox1 mRNA	1.0 $\pm$ 0.1	0.7 $\pm$ 0.1 ^{*, &}	0.8 $\pm$ 0.1 ^{*, &}	2.3 $\pm$ 0.6 [*]	2.1 $\pm$ 0.7 [*]
Hmox1 protein	62.10 ⁶ $\pm$ 9	63 $\pm$ 5 ^{&}	63 $\pm$ 7 ^{&}	72 $\pm$ 8 ^{&}	106 $\pm$ 24 [*]
Nqo1 mRNA	1.0 $\pm$ 0.5	1.0 $\pm$ 0.7 ^{†, &}	1.0 $\pm$ 0.4 ^{†, &}	3.2 $\pm$ 0.9 [*]	4.3 $\pm$ 1.7 [*]
Nqo1 protein	128 $\pm$ 48	99 $\pm$ 54 ^{†, &}	95 $\pm$ 55 ^{†, &}	236 $\pm$ 91 [*]	199 $\pm$ 52 [*]
Ccr2 mRNA	1.0 $\pm$ 0.2	1.0 $\pm$ 0.3 [†]	0.9 $\pm$ 0.2 [†]	3.0 $\pm$ 1.7 [*]	1.1 $\pm$ 0.2 [†]
IL-1 β mRNA	1.0 $\pm$ 0.2	0.7 $\pm$ 0.1 [†]	0.9 $\pm$ 0.3 [†]	1.8 $\pm$ 0.2 [*]	0.9 $\pm$ 0.1 [†]
IL-6 mRNA	1.0 $\pm$ 0.4	0.7 $\pm$ 0.3 ^{&, †}	1.3 $\pm$ 0.4 ^{&}	2.2 $\pm$ 1.0 [*]	1.5 $\pm$ 0.5
IL-8 mRNA	1.0 $\pm$ 0.6	0.4 $\pm$ 0.4 ^{*, †, &}	0.4 $\pm$ 0.1 ^{*, †, &}	5.9 $\pm$ 2.8 [*]	2.6 $\pm$ 0.9 ^{*, †}
TNF α mRNA	1.0 $\pm$ 0.4	0.6 $\pm$ 0.3	0.7 $\pm$ 0.3	1.1 $\pm$ 0.5	0.9 $\pm$ 0.5
IL-4 mRNA	1.0 $\pm$ 0.3	1.0 $\pm$ 0.2	1.2 $\pm$ 0.5	1.0 $\pm$ 0.3	1.4 $\pm$ 0.4
Ptgs2 mRNA	1.0 $\pm$ 0.8	0.9 $\pm$ 0.4	1.2 $\pm$ 0.5	1.3 $\pm$ 0.6	0.9 $\pm$ 0.5
Ptges mRNA	1.0 $\pm$ 0.7	0.6 $\pm$ 0.4	0.7 $\pm$ 0.5	1.2 $\pm$ 0.7	0.9 $\pm$ 0.6
Ptgis mRNA	n.d.	n.d.	n.d.	n.d.	n.d.
Alox5 mRNA	n.d.	n.d.	n.d.	n.d.	n.d.

Table 2. Oxidative stress and inflammatory markers. Data are expressed as a mean $\pm$ s.d. mRNA data are expressed as fold change over control, protein abundance is expressed as protein intensity. n.d. not detected.

* $p < 0.05$ vs control; [†] $p < 0.05$ vs PNIL; [&] $p < 0.05$ vs PNILOV.

either ω -6 PUFA or ω -6/ ω -3 PUFA enterally (ENIL and ENILOV groups). MDA content tended to be even lower in the ENIL group although it did not reach statistical significance ($p = 0.062$). Parenteral nutrition itself, irrespective of the type of the emulsion, did not elevate the MDA content in the liver but resulted in the increased expression of Hmox-1, Nqo1, and Gclc mRNA and NQO1 and GCLC protein. All these genes are known to be positively regulated in response to oxidative stress. Antioxidant capacity of liver extract was significantly lower in PNIL but not in the PNILOV group compared with control, ENIL or ENILOV groups. The mRNA expression of pro-inflammatory cytokines Ccr2 and IL-1 β was significantly higher in PNIL but not in the PNILOV group compared with controls. IL-8 mRNA expression was increased in both PNIL and PNILOV groups compared with controls but it was significantly lower in PNILOV than in the PNIL group. The expression of key enzymes involved in prostaglandin synthesis Ptgs2 and Ptges did not differ among groups, the expression of Ptgis and Alox5 was below the detection limit (Table 2).

Liver bile acids profile. Analysis of the bile acid profile in the liver revealed 15 bile acid species (Table 3). Unconjugated BAs were present in concentrations lower by several degrees of magnitude compared with their conjugated derivatives. Ten of the BAs were not significantly different among groups but gCDCA, DCA, gDCA and LCA content was significantly higher while tUDCA content was significantly lower in PNIL compared with all other groups including PNILOV. Neither of the BAs tested significantly differ in PNILOV and control groups.

Serum lipidome. In serum, we detected 204 lipid signals confirmed by MS/MS fragmentation spectra. Using ANOVA, we filtered out 182 lipids significantly different ($p < 0.01$, FDR adjusted) between at least two groups. PCA analysis (Fig. 1A) identified separate clusters representing PNIL, ENILOV and PNILOV groups. Control and ENIL samples clustered into one cluster and were separated from the others. Hierarchical clustering (HC) confirmed a tendency to the separation of the groups albeit some exceptions occur (Fig. 1B).

Using supervised multivariate analysis (series of binary OPLS-DA models) based on significantly different (VIP > 1) lipids we identified eight sets of lipids most significantly contributing to the group separation. We identified partially overlapping subsets of 22, 27, 37 and 40 lipid species distinguishing ENIL, ENILOV, PNIL and PNILOV groups from controls, resp. (Supplementary Table 1). The parenteral route of lipid emulsion administration was associated with the increased content of choline- and ethanolamine-derived phospholipids containing C16 and C18 fatty acids (Supplementary Figure 2). As expected, the presence of ω -3 FA in the nutrition mixture was reflected by the increased content of EPA and DHA as well as of various lipid species containing these FAs (PNILOV > ENILOV) (Supplementary Figure 3).

Liver lipidome. In the liver extract, we detected 456 lipid signals confirmed by MS/MS fragmentation spectra. Using ANOVA, we filtered out 435 lipids significantly different ($p < 0.01$, FDR adjusted) between at least two groups. PCA scores plot (Fig. 2A) shows a separation of samples according to both the type of emulsion and the route of administration. Hierarchical clustering analysis confirmed excellent grouping of samples (Fig. 2B) with respect to these characteristics, the route of nutrition administration (enteral vs parenteral) being a superior discriminating parameter to the type of emulsion.

	control	ENIL	ENILOV	PNIL	PNILOV
CA	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
gCA	1.0 ± 0.8	0.8 ± 0.3	0.8 ± 1.1	1.0 ± 0.5	0.9 ± 0.8
tCA	78.2 ± 41.3	66.1 ± 27.3	78.1 ± 39.2	52.2 ± 19.8	32.9 ± 42.2
CDCA	0.2 ± 0.3	0.8 ± 1.0	0.3 ± 0.3	0.1 ± 0.04	0.2 ± 0.1
gCDCA	4.2 ± 3.1	1.6 ± 1.7	2.2 ± 2.1	8.6 ± 5.7 ^{*,#,&}	1.1 ± 0.7 [†]
tCDCA	9.6 ± 3.9	8.1 ± 6.3	6.6 ± 2.9	6.5 ± 5.6	9.6 ± 4.1
αMCA	0.02 ± 0.02	0.03 ± 0.02	0.01 ± 0.01 ^{&}	0.023 ± 0.02	0.04 ± 0.02
βMCA	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.01
DCA	0.0003 ± 0.0003	0.0002 ± 0.0003	0.0001 ± 0.0000	0.0008 ± 0.0013 ^{*,#,&}	0.0001 ± 0.0000 [†]
gDCA	2.6 ± 1.3	3.2 ± 2.4	1.5 ± 1.1	13.6 ± 6.5 ^{*,#,&}	6.8 ± 3.6 [†]
tDCA	6.7 ± 3.2	11.2 ± 7.8	5.4 ± 3.1	6.0 ± 1.2	4.7 ± 2.6
UDCA	0.03 ± 0.01	0.05 ± 0.01	0.03 ± 0.01	0.07 ± 0.01	0.06 ± 0.01
gUDCA	0.9 ± 0.5	0.5 ± 0.2	0.24 ± 0.13	2.1 ± 1.8	1.0 ± 0.8
tUDCA	1.7 ± 0.8	1.6 ± 0.8	1.1 ± 0.8	0.6 ± 0.2 ^{*,#}	1.0 ± 0.1 [†]
LCA	0.10 ± 0.05	0.15 ± 0.01	0.05 ± 0.01	0.30 ± 0.28 ^{*,#,&}	0.07 ± 0.02 [†]

Table 3. Bile acid profile in the liver. The values are given in μM and expressed as a mean $\pm$ s.d. CA, cholic acid; gCA, glycocholic acid; tCA, taurocholic acid; CDCA, chenodeoxycholic acid; gCDCA, glycochenodeoxycholic acid; tCDCA, taurochenodeoxycholic acid; α MCA, alpha muricholic acid; β MCA, beta muricholic acid; DCA, deoxycholic acid; gDCA, glycodeoxycholic acid; tDCA, taurodeoxycholic acid; UDCA, ursodeoxycholic acid; gUDCA, glyoursodeoxycholic acid; tUDCA, tauroursodeoxycholic acid; LCA, lithocholic acid. * $p < 0.05$ vs control; # $p < 0.05$ vs ENIL; & $p < 0.05$ vs ENILOV; † $p < 0.05$ vs PNIL; & $p < 0.05$ vs PNIL.

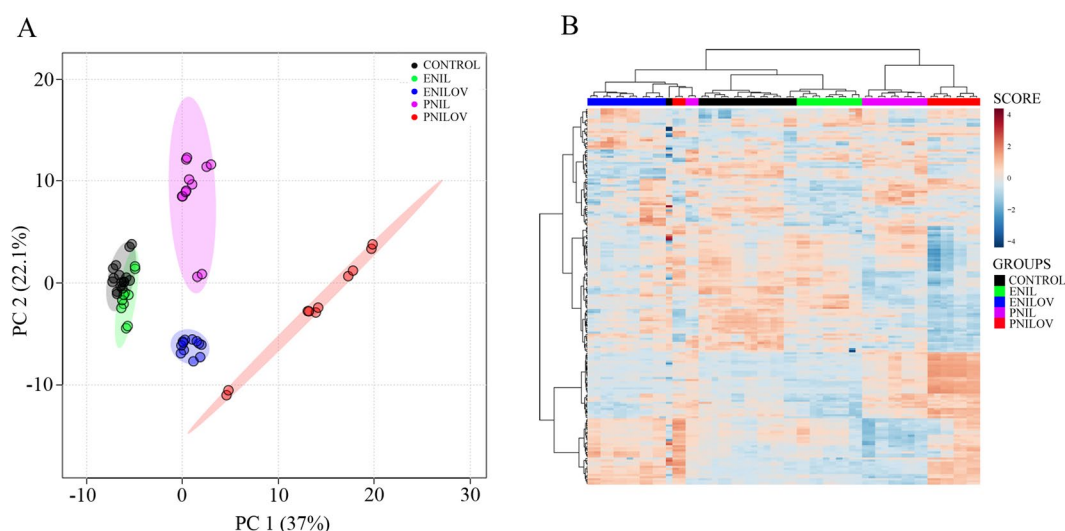


Figure 1. Lipidome composition in serum. (A) PCA score plot. Each sample was determined in a doublet. (B) Lipid heatmap with the clustering dendrogram of samples. Samples are colored according to the experimental groups.

We further analyzed the ω -3 and ω -6 FA abundance in individual lipid classes. Regarding phospholipids, both PCA and HC analyses revealed excellent separation of the groups that suggest the combined effect of both the lipid component of the nutrition mixture and the way of administration (Supplementary Figures 4,5). Phospholipids, the main constituents of plasma membranes, were particularly enriched with EPA and DHA in PNIL OV and ENILOV groups while phospholipids in the PNIL group were rich in linoleic acid, which is the prevailing component of soybean oil. Diacylglycerols and lysophospholipids containing ω -3 and ω -6 as well as respective free FA formed two separate clusters (PNIL OV + ENILOV vs PNIL + ENIL + controls) but within these clusters, we did not find clear distribution pattern (Supplementary Figures 6–8). This indicates that the composition of these lipid species is dictated mainly by the dominant lipid constituent of nutrition mixture independently on the enteral or parenteral route of administration. The experimental conditions exhibited only the weak influence on the composition of the least biologically active lipid species, TAGs, (Supplementary Figure 9).

Using OPLS-DA models based on significantly different (VIP > 1) lipids we identified eight sets of lipids most significantly contributing to the group separation. As expected, the administration of lipid emulsions resulted in a significant alteration of liver lipidome. We identified partially overlapping subsets of 131, 113, 70 or 118 lipid species distinguishing ENIL, ENILOV, PNIL and PNIL OV groups from controls, resp. (Supplementary Table 2).

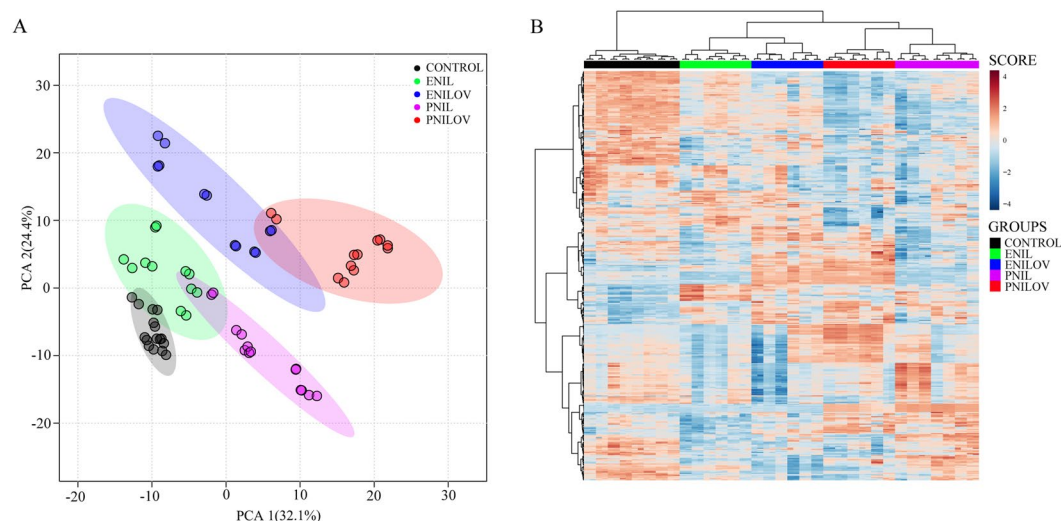


Figure 2. Lipidome composition in the liver. **(A)** PCA score plot. Each sample was determined in a doublet. **(B)** Lipidome heatmap with the clustering dendrogram of samples. Samples are colored according to the experimental groups.

As the next step, we wanted to identify significant lipids characterizing the different types of emulsion or administration strategies. Figures 3 and 4 show the relative content of significant lipids (without triglycerides) expressed as log(2) fold change over mean control value. First, we evaluated the effect of the route of administration, i.e. we compared PNIL vs ENIL and PNILOV vs ENILOV groups and looked for the unique and common features. Irrespective of the type of emulsion, parenteral feeding was associated with a significantly (100- to 464-times) increased content of six fatty acid esters of hydroxy fatty acids (FAHFA), i.e. FAHFA (16:2/20:1), FAHFA (18:0/18:3), FAHFA (18:2/18:2), FAHFA (16:1/20:3), FAHFA (18:2/18:1) and FAHFA (18:2/18:3) and FAHFA (18:3/18:1) (Fig. 3A). In both PNIL and PNILOV groups we found comparable (five times) decrease of PC(20:0/20:4). The dominant markers of ENIL compared with PNIL and control lipidome was the increased content of 18:4, 20:5 (EPA), 21:5 and 22:4 FAs, of the oxidized FAs (18:2 + O; 22:4 + O; 20:4 + O; 20:5 + O, 18:3 + O) as well as of phosphatidylglycerol (PG) containing arachidonic acid (20:4, AA). Parenteral Intralipid administration (PNIL group) was associated with significantly decreased content of phospholipids (phosphatidylethanolamines, PEs and phosphatidylcholines, PCs) containing AA mostly in combination with fatty acid with odd number of carbons and increased content of phospholipids containing linolenic acid, 22:5 FA, DHA and dihomo- γ -linoleic acid (20:3) (Fig. 3B). Parenteral feeding of ω -3 PUFA (PNILOV group) was associated with the increased concentration of diacylglycerols (DAG) containing DHA and with the decreased concentration of diacylglycerol containing AA and several other PC or PE lipid species compared with animals fed ω -3 PUFA enterally (ENILOV) or with controls (Fig. 3C).

We further examined the effect of the type of the lipid emulsion, i.e. we compared ENILOV vs ENIL and PNILOV vs PNIL groups. As expected, animals provided ω -3 PUFA exhibited the increased amount of lipid species containing EPA or DHA no matter if ω -3 PUFA was administered parenterally or *per os*. We also observed an increased amount of oxidized DHA (22:6 + O) in both ENILOV and PNILOV groups (Fig. 4A). The lipid species most discriminating between Omegaven and Intralipid emulsions administered enterally were three FAs (dihomo- γ -linoleic acid, EPA, 22:4) increased in ENIL group and PCs and plasmenyls containing AA and 22:4 FA higher in ENILOV group (Fig. 4B). In parenteral feeding, the difference between emulsions was most evident in FAHFA liver content. Albeit these compounds were dramatically increased in all parenterally fed animals, ω -3 PUFA administration resulted in their significant reduction compared with their content in the liver of animals provided only ω -6 PUFA (control $\ll$ PNILOV < PNIL). We also found the increased content of DHA and DAG or PG containing DHA in PNILOV compared with the PNIL group (Fig. 4C).

Liver proteome. In the liver, we distinguished 3439 proteins. Using ANOVA, we selected 1560 proteins which abundance significantly differed ($p < 0.01$, FDR adjusted) between at least two groups. PCA scores plot shows clear separation of control, PNILOV and ENIL groups from each other. ENILOV and ENIL groups formed one homogenous cluster that was separated from all other groups (Fig. 5A). HCA analysis performed on all significantly different proteins ($n = 1560$) identified the route of nutrients administration as the dominant parameter determining the similarity/dissimilarity of the liver proteome as control, ENIL and ENILOV groups grouped into one cluster while PNIL and PNILOV groups to the other one (Fig. 5B). At the next level, the samples clustered according to either the lipid presence itself or to the type of administered lipid.

Using supervised multivariate analysis based on significantly different proteins we identified eight sets of proteins most significantly ($VIP > 1$) contributing to the separation of the groups (Supplementary Table 3). We further employed the DAVID database to gain insight into the potential biological function of the differentially expressed proteins obtained from the group comparisons (Supplementary Table 4). Compared with controls,

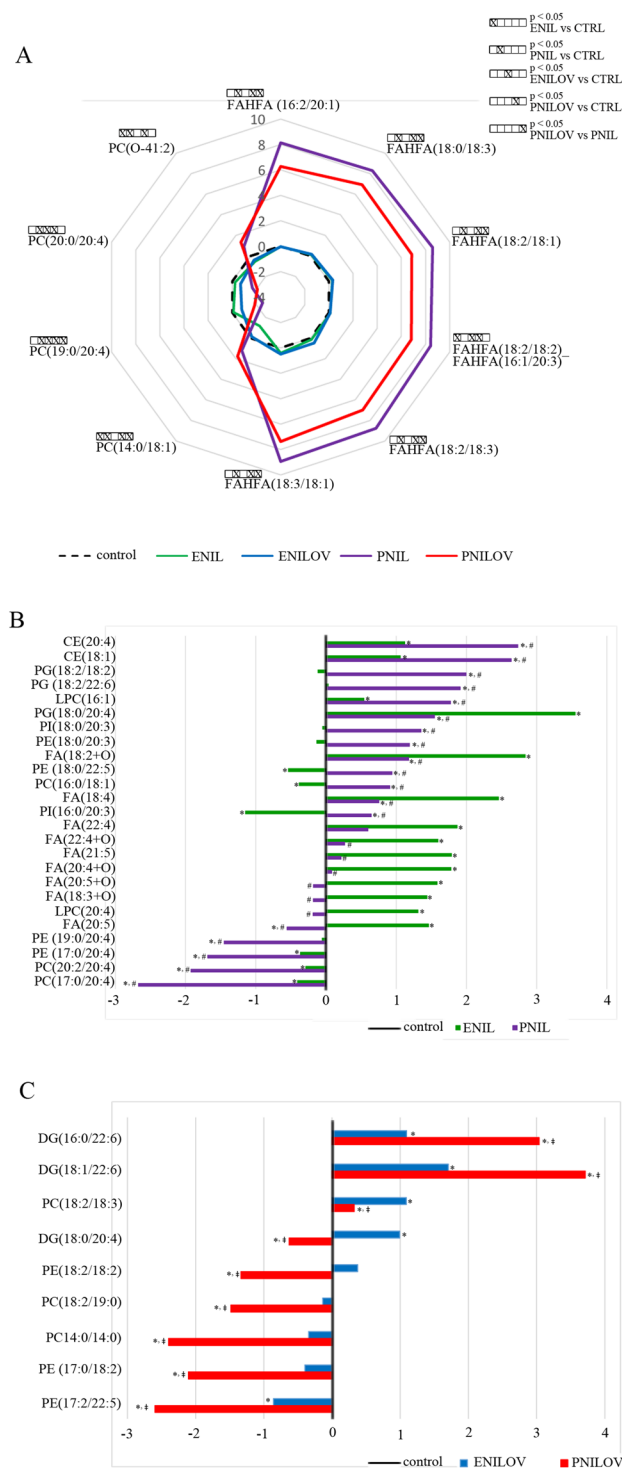


Figure 3. Effect of the route of administration on the relative content of selected lipid species in the liver. **(A)** all groups; **(B)** PN vs EN: Intralipid; **(C)** PN vs EN: Intralipid + Omegaven Data are expressed as log₂ fold change over median control value. **p* < 0.05 vs control; #*p* < 0.05 vs ENIL.

enteral administration of lipid emulsions affected liver proteome less than parenteral. In the ENIL group, we found 40 differently expressed proteins; eight of them were classified into four overlapping pathways (terpenoid backbone biosynthesis; synthesis and degradation of ketone bodies; butanoate metabolism; valine, leucine and isoleucine degradation). Four of these proteins (HMGCS1, HMGCS2, ACAT1, and ACAT2) were involved in the acetoacetyl-CoA synthesis. Thirty-three proteins distinguish the ENILOV group from controls but these proteins do not cluster into any metabolic pathway. PNIL group differed from controls in 134 proteins classified into a heterogeneous spectrum of metabolic pathways including the metabolism of carbohydrates (propanoate

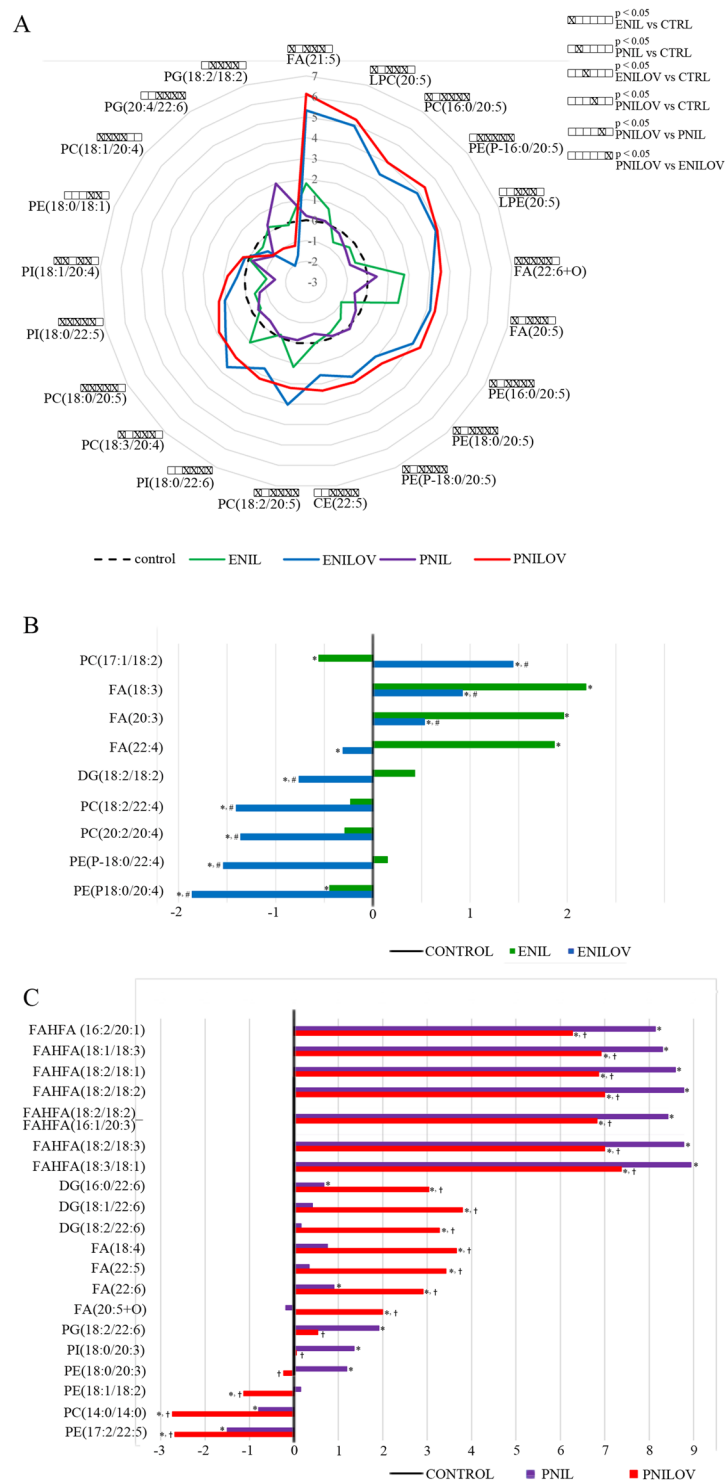


Figure 4. Effect of the composition of lipid emulsions on the relative content of selected lipid species in the liver. **(A)** all groups; **(B)** IL vs ILOV: enteral application; **(C)** IL vs ILOV: parenteral application. Data are expressed as log(2) fold change over median control value. * $p < 0.05$ vs control; # $p < 0.05$ vs ENIL.

and butanoate metabolism), amino acids (tryptophan, valine, leucine, isoleucine), fatty acids, steroid hormones, terpenoids, and retinol metabolism. Some of these pathways overlap with the ENIL group but the specific feature of the PNIL group is the deregulation (both up or down) of CYP 450 family members. In the PNILOV group, 305 proteins distinguish this group from controls. Within this subset, four metabolic pathways were identified (chemical carcinogenesis, glutathione metabolism, metabolism of xenobiotics by CYP450 and drug metabolism-CYP450). Dominating proteins in this set were isoforms of glutathione-S-transferase that were included in all these pathways. ENILOV and ENIL groups differed only in one protein (Acot1) what indicates

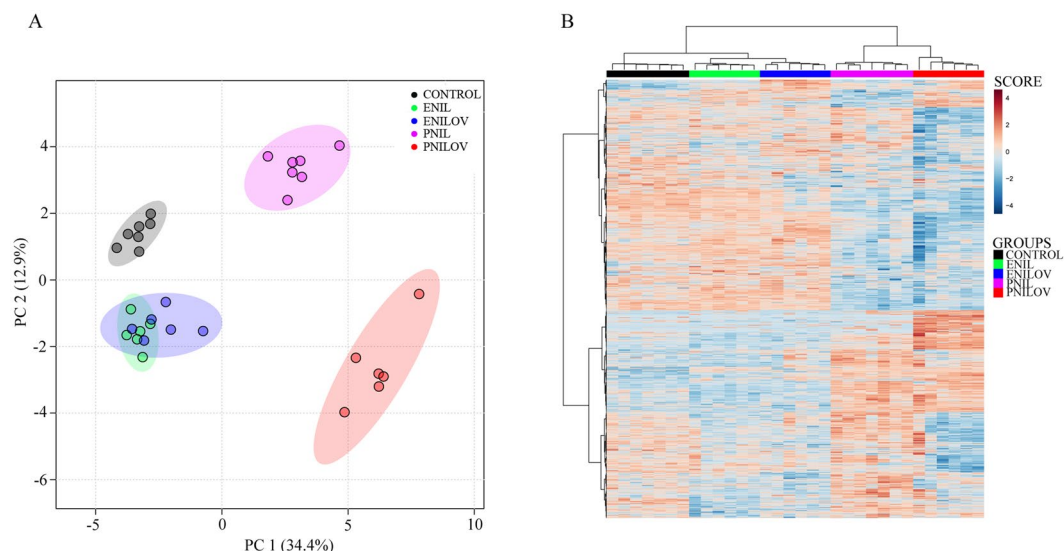


Figure 5. Proteome composition in the liver. **(A)** PCA score plot. **(B)** Proteome heatmap with the clustering dendrogram of samples. Samples are colored according to the experimental groups.

that when administered enterally, n-3 PUFA does not significantly affect the liver proteome. Parenteral vs enteral application of ω -6 PUFA resulted in a different expression of 147 proteins. Two pathways belonging to carbohydrate metabolism (glycolysis/gluconeogenesis; pyruvate metabolism) and steroid hormone biosynthesis were identified. The groups differing in the route of administration of ω -3 PUFA (PNILOV vs ENILOV) exhibited altered expression of 198 proteins and four partially overlapping metabolic pathways (FA metabolism, FA degradation, PPAR α signaling, and drug metabolism-CYP450). All these pathways were downregulated in PNILOV compared with ENILOV.

Figure 6 shows the quantitative changes in the expression of proteins included in the deregulated pathways (data are expressed as fold change over control). Most of the enzymes involved in FA degradation or FA synthesis was downregulated in PNILOV compared to other groups. Proteins involved in FA transport (CD36) and storage (PLIN2) were significantly upregulated in all experimental groups while lysosomal phospholipase A2 (PLA2G15) was upregulated only in groups provided ω -3 PUFA (PNILOV > ENILOV). Key enzymes involved in glucose metabolism (GCK, HK3, PCK2) were increased only in parenterally fed animals. All enzymes involved in the metabolism of xenobiotics or glutathione metabolism, i.e. glutathione-S-transferase isoforms, flavin-containing monooxygenases (FMO1, FMO5) and HSD11B1, were significantly downregulated in PNILOV group. Ten CYP 450 family members were deregulated due to the interventions studied in this experiment. Cyp17A1, CYP2B2, CYP4A10, and CYP4A14 were significantly upregulated in all groups (ENILOV = PNILOV >> ENIL > PNIL). CYP2C12 was upregulated and CYP1A2 downregulated only in parenterally fed groups but not influenced by enteral feeding. CYP2C11, CYP2D4, CYP3A9, and CYP51A1 were downregulated in all groups (control > EN feeding > PN feeding).

Discussion

Hepatobiliary dysfunction is one of the most common complications associated with long-term dependence on parenteral nutrition. The liver injury is more severe with fat-free than with fat-containing PN, but the type of fat matters as well. Our data show that both the way of nutrient administration (enteral vs parenteral) and lipid emulsion composition (pure ω -6 vs ω -6/ ω -3 mixture) significantly influence the composition of serum and liver lipidome, liver proteome and liver BAs profile in the rat model.

PUFA (AA, EPA, and DHA) are an essential source of biologically active lipids and several studies revealed that significant alterations in the content of PUFA and their metabolites occur in numerous pathophysiological conditions¹⁹. The first step in their synthesis is the release of FA from the *sn*-2 position of membrane phospholipid or DAG followed by enzymatic conversion mediated by one of three enzymatic systems - COX, LOX or CYP450 (CYP2C, CYP2J and CYP4A families). While EPA and DHA are considered to be relatively poor substrates for COX and LOX²⁰, virtually all CYP isoforms involved in AA metabolism accept a wide variety of other ω -6 and ω -3 PUFA as efficient alternative substrates²¹. Therefore, EPA and DHA compete with AA for binding and conversion by CYP 450 enzymatic system and the combination of precursor availability together with the activity of individual enzymatic systems leads to the unique combination of bioactive compounds determining the final effect.

Functionally, these compounds are extremely diverse and may exert a wide spectrum of biological properties. AA and EPA are precursors of pro-inflammatory eicosanoids (AA: 2-series prostaglandins, thromboxanes, 4-series leukotrienes; EPA: 3-series prostaglandins, 3-series thromboxanes, 5-series leukotrienes) albeit EPA gives rise to weaker pro-inflammatory agents than those derived from AA. On the other hand, all these fatty acids may be transformed into anti-inflammatory mediators like lipoxins (derived from AA), resolvins of the E-series (derived from EPA), and resolvins of the D-series, protectins, or maresins (derived from DHA)^{15,19,22}. These

Lipid metabolism

	ENIL	ENILOV	PNIL	PNILOV
Cyp1a2	1,1	1,1	-2	-1,3
Cyp17a1	11,9	26,1	8,1	18,4
Cyp2b2	9,6	29,8	4,9	20,5
Cyp2c11	-1,2	-1,3	-2,1	-4,3
Cyp2c12	-1,1	-1,2	6,1	3,2
Cyp2d4	-1,2	-1,7	-4,7	-4,4
Cyp3a9	1,9	-1,5	-2,2	-2,9
Cyp4a10	2,1	6,7	2,1	6,5
Cyp4a14	2,3	3,5	3,5	3,7
Cyp51a1	-1,4	-3,8	-23,8	-14,1
SULT1E1	1,3	-1,3	-2,1	-3,3
HSD17B7	-1,4	-2,0	-21,1	-21,1
STE2	-1,0	-2,0	-3,1	-3,9
PPT1	1,2	1,4	3,3	4,5
ACOX1	-2,1	1,8	1,1	-2,3
ACADSB	1,2	1,0	-1,6	-2,0
ACSL1	1,2	1,4	-1,4	-1,2
ACADM	1,1	1,4	-1,2	-1,1
EHHADH	1,3	3,4	-1,1	1,9
FADS1	-1,7	-1,4	-68,4	-10,9
FADS2	-2,8	-1,6	-3,5	-3,7
HADH	1,1	1,4	1,0	-1,4
TECR	-1,1	-1,4	-1,5	-2,2
ECI1	1,8	4,1	1,7	1,9
ACSL1	1,3	1,4	-1,4	-1,2
APOA1	1,5	1,5	-1,2	-1,5
APOA2	1,6	-1,0	-1,5	-2,4
APOC	1,6	1,7	1,0	-1,8
FABP5	-1,6	-2,9	-1,1	-1,0
CD36	8,9	17,2	9,9	15,1
PLA2G15	-1,0	2,2	-1,0	7,1
PLIN2	13,4	10,6	14,2	29,7

Carbohydrate metabolism

	ENIL	ENILOV	PNIL	PNILOV
HSD11B1	-1,4	-1,5	-1,3	-2,7
FMO1	1,8	1,4	-1,4	-1,9
FMO5	1,0	1,2	-1,1	-1,6
MGST3	1,4	1,4	-2,1	-1,8
GSTM1	-1,1	-1,3	-1,4	-2,2
GSTM2	1,1	-1,3	-1,8	-2,4
GSTM3	1,6	1,2	-1,2	-2,5
GSTA4	-1,1	-1,2	-1,4	-1,8
GSTA5	1,1	-1,2	-1,5	-1,7
GSTK1	-1,0	-1,2	-1,5	-1,9

Xenobiotic metabolism

	ENIL	ENILOV	PNIL	PNILOV
LDHA	1,3	1,4	1,5	1,5
ACACB	-1,2	-1,3	-24,9	-24,9
ACSS2	-1,5	-3,0	-3,7	-2,6
ME1	-1,0	-1,1	2,8	3,6
ALDH1B1	2,1	1,2	-1,3	1,4
PKLR	1,2	-1,3	2,9	2,0
PCK1	1,1	-1,2	-1,9	-1,6
PCK2	-1,2	-1,3	2,1	2,2
GCK	1,1	-1,3	2,4	2,2
HK3	-1,7	-1,7	1,5	1,3

Figure 6. Expression of selected proteins in the liver. The values (x) represent fold change of protein intensity (experimental group to control), if $x < 1$ we used the equation $-1/x$. Statistically significant values are shown in bold. Red color: increased vs control; blue color: decreased vs control. Gene names are listed according to HGNC guidelines.

lipokines are involved in the resolution of inflammation by several mechanisms including attenuation of the inflammatory response, induction of efferocytosis (clearance of apoptotic cells), and stimulation of macrophage migration from the site of inflammation to the peripheral lymph nodes²³.

CYP 450-catalyzed transformation of PUFA results in the formation of hydroxy- or epoxy-PUFA²⁴. These compounds serve as second messengers of various hormones and growth factors and play partially opposing roles in the regulation of vascular, renal or cardiac function and regulation of inflammation^{25–30}.

EPA, DHA, and AA are highly susceptible to the modification by exogenous supply. AA is derived from essential linoleic acid (LA, 18:2, n-6). EPA and DHA may be synthesized from another essential FA, α -linoleic acid (ALA, 18:3, n-3) but the conversion rate is very limited³¹. Arnold *et al.* showed that oral administration of diet rich in either ω -6 or ω -3 (EPA + DHA) PUFA resulted in dramatic enrichment of liver lipidome with LA or EPA + DHA, resp³². Similarly, EPA/DHA supplementation caused a profound shift in the endogenous CYP 450-derived eicosanoid profile.

Our data revealed that introducing of ω -3 PUFA into lipid emulsion results in a significant enrichment of liver lipidome with EPA- and DHA-containing lipid species that may serve as substrates for bioactive lipid synthesis. The content of oxidized DHA (22:6 + O) was increased in both PNILOV and ENILOV groups while significantly elevated content of oxidized EPA (20:5 + O) was found only in PNILOV compared with other groups. Even though our methodology does not allow us to differentiate what form of oxygen modification is present (hydroperoxydation/dihydroxylation or epoxidation/hydroxylation), it still describes the occurrence of oxidized forms of free fatty acids.

In PNILOV and ENILOV groups, we further confirmed the significant upregulation of two isoenzymes from the CYP4A family that catalyze microsomal ω -oxidation and are capable of synthesis of hydroxylated derivatives

of EPA or DHA^{33,34}. Therefore, our data suggest that introducing ω -3 PUFA into the nutrition mixture may significantly affect both substrate availability and the CYP 450 isoenzymes activities thus altering the final CYP 450-derived eicosanoid profile towards less pro-inflammatory phenotype. This conclusion is supported by the expression pattern of pro-inflammatory cytokines (IL-8, IL-1 β , Ccr2) where we found an anti-inflammatory effect of parenterally administered ω -3 PUFA. Nevertheless, it is necessary to mention that our experimental setting does not allow inferring any extensive conclusions regarding the anti-inflammatory effect of ω -3 PUFA. The general pro-inflammatory status of all animals was low what is evidenced by the normal levels of serum TNF α and IL-6 or comparable mRNA expression of TNF α and enzymes involved in prostaglandin synthesis in the liver among groups.

A characteristic feature, discriminating all PN patients from controls or enterally fed experimental groups, is an elevated content of FAHFAs in the liver. Only recently, this novel class of endogenous lipokines, branched fatty acid esters of hydroxy fatty acids, was identified³⁵. These FAs are synthesized in nearly all mammalian tissues being most abundant in adipose tissue and liver, and they are also present in the blood. The biological function of FAHFA is far from being completely understood. The first reports show their beneficial effects on glucose homeostasis³⁶ and in suppressing inflammation³⁷. Furthermore, Kuda *et al.* showed that FAHFA biosynthesis in white adipose tissue is involved in the adaptive Nrf2-regulated antioxidant system³⁸. At present, only some aspects of the mechanisms regulating FAHFA expression are known. FAHFA biosynthesis is positively regulated by carbohydrate responsive-element binding protein (ChREBP) and FAHFA levels are elevated also in fasting, probably due to the decreased degradation³⁵. In our study, FAHFAs (derived from palmitoleic, palmitolinoleic, linoleic, and linolenic fatty acyl moieties and oleic, linoleic, linolenic, icosanoic and icosatrienoic hydroxylated fatty acid moieties) were significantly elevated in the liver in both PNIL and PNILOV groups. Our study design does not allow identifying the mechanisms contributing to the PN-associated FAHFA elevation but we may speculate that it is the response to chronic oxidative stress or the sub-optimal saturation of energy needs. Interestingly, albeit the parenteral route of feeding was always associated with a dramatic increase in FAHFA content, the inclusion of ω -3 PUFA resulted in the significant reduction of FAHFA content in the liver. We have not sufficient data to speculate about the biological meaning of this observation but available information indicates that FAHFA are potent lipokines involved in metabolic homeostasis regulation and further research is needed to unravel their role in PN-associated metabolic adaptations.

Due to the high number of double bonds, n-3 PUFA may be more susceptible to lipid peroxidation and may increase the risk of oxidative stress³⁹. The expression of enzymes involved in xenobiotic or glutathione metabolism (HSD11B1, FMO1/5, CYP3A9, CYP2C11, GST isoforms) was significantly attenuated in PNIL and PNILOV groups, the effect being more pronounced in PNILOV. All reactions catalyzed by these enzymes are associated with electron transfer and potentially may be the source of reactive oxygen species. The main function of GSTs is to conjugate electrophilic compounds with glutathione, thereby enabling their excretion⁴⁰ but its activity is associated with the depletion of glutathione. The increased intake of PUFA prone to lipid peroxidation may enhance the risk of oxidative stress. Therefore, the decreased activity of these pathways may represent an adaptive mechanism to prevent some PUFA-mediated deteriorative effects such as ROS production. On the other hand, it may negatively affect the effectiveness of detoxification.

The published data support both pro- and antioxidant effect of ω -3 PUFA in the context of parenteral nutrition. Human studies are based mostly on the analysis of plasma lipids because of the inaccessibility of liver tissue. In animal studies, two authors confirmed the anti-oxidative effect of fish oil in mice⁴¹ or rats subjected to intestinal ischemia⁴². In contrast, Lavoie *et al.* described the increased oxidative stress in the lung tissue of newborn guinea pigs administered ω -3 PUFA (SMOFlipid) compared with ω -6 PUFA (Intralipid)⁴³. In our study, we did not find the elevated MDA content, the marker of lipoperoxidation, in any group. Nevertheless, we identified early markers of oxidative stress, i.e. the elevated mRNA and protein content of Nrf-regulated genes Hmox1, Nqo1, and Gclc in the liver of all parenterally fed animals, regardless of the type of the lipid emulsion. The anti-oxidant capacity of liver homogenate was significantly decreased in PNIL (ω -6; PN) but normal in the PNILOV group (ω -6/ ω -3; PN). These findings suggest that PN itself is associated with the pro-oxidant status in the liver but ω -3 PUFA protect the anti-oxidative defence capacity. Our study is limited by the short time of the treatment which is insufficient for the full development of the oxidative stress-related injury. Further studies are needed to fully explain the role of ω -3 PUFA in the PN-associated liver oxidative stress.

Cholestasis, i.e. the intrahepatic accumulation of bile acids, is one of the most common metabolic problems associated with PN especially in infants⁴⁴. Lipophilic bile acids, which are often increased in PNALD⁵, are known to cause cellular apoptosis. In contrast, hydrophilic bile acids, i.e. UDCA or tUDCA, have a rather protective effect through activation of mitogen-activated protein kinase survival pathway⁴⁵. gCDCA, the most abundant BA in serum and bile in cholestasis⁴⁶, is highly toxic. *In vitro*, rat and human hepatocytes or hepatic cell lines treated with gCDCA in high concentration ($\geq 100 \mu\text{M}$) develop severe mitochondrial dysfunction and apoptosis^{47–50}. The exposition to lower, physiological concentrations results in the expression of inflammatory mediators⁵¹ that stimulate the recruitment of hepatic neutrophils that in turn induce the oxidative injury of the liver tissue. Furthermore, the low-level BAs-induced mitochondrial dysfunction may contribute to stress the hepatocytes and worsen the ongoing inflammatory injury⁵². Recent clinical research demonstrated that pure ω -3 PUFA-based lipid emulsions reversed severe cholestasis in infants or adult patients when administered instead of soybean oil emulsion^{10,53,54}. Tillman *et al.* reported that EPA and DHA treatment attenuated the CDCA-induced hepatocellular apoptosis in HepG2 cells *in vitro*⁵. Our data confirmed the accumulation of four toxic BAs (gCDCA, DCA, gDCA and LCA) and depletion of protective tUDCA in the liver of animals administered parenterally ω -6 PUFA. In contrast, no such effect was observed when animals were provided ω -3/ ω -6 PUFA mixture what supports the hypothesis about the protective effect of ω -3 PUFA.

Conclusions. In this study, we employed -omics approach to describe characteristic features of lipid, protein and BAs liver profile associated with enteral or parenteral administration of ω -6 PUFA and ω -3/ ω -6 PUFA based lipid emulsions and possible implications for the development of PNALD. In general, we found that enteral administration of both lipid emulsions has less impact on the liver than the parenteral route. Compared with parenterally administered Intralipid, PN administration of ω -3 PUFA was associated with 1. increased content of EPA- and DHA-containing lipid species; 2. higher abundance of CYP4A isoenzymes capable of bioactive lipid synthesis and the increased content of their potential products (oxidized EPA and DHA) with potentially anti-inflammatory properties; 3. down-regulation of enzymes involved CYP450 drug metabolism what may represent an adaptive mechanism counteracting the potential negative effects (enhanced ROS production) of FA metabolism; 4. normalized anti-oxidative capacity; 5. physiological BAs spectrum. All these findings may contribute to the explanation of ω -3 PUFA protective effects in the context of PN. In the present state of knowledge, we cannot assess the physiological relevance of the ω -3 PUFA influence on FAHFA liver content.

Material and Methods

Animals and experimental design. Male Wistar rats (Charles River, initial weight 300–325 g) were kept in a temperature-controlled environment under a 12 h light/dark cycle. After the acclimatization period, all animals underwent the same operation procedure. The right jugular vein was cannulated with a Dow Corning Silastic drainage catheter (0.037 inches) as previously described⁵⁵. The catheter was flushed daily with TauroLock HEP-100 (TauroPharm GmbH, Waldbüttelbrunn, Germany). After the operation, the rats were housed individually and connected to a perfusion apparatus (Instech, PA, USA), which allows free movement. For the next 48 hours, the rats were given free access to a standard chow diet (SD, SEMED) and provided Plasmalyte (BAXTER Czech, Prague, CZ) via the catheter at increasing rates (initial rate: 1 ml/hr; goal rate: 4 ml/hr) in order to adapt to the increasing fluid load. Two days after the operation, the rats were randomly divided into five groups as follows: Control (Plasmalyte *i.v.* granules); ENIL (Plasmalyte *i.v.* Intralipid *per os*, granules); ENILOV (Plasmalyte *i.v.* Intralipid + Omegaven *per os*, granules); PNIL (nutrition mixture with Intralipid *i.v.*); PNILOV (nutrition mixture with Intralipid + Omegaven *i.v.*) (Supplementary Table 5). The composition of the *i.v.* nutrition mixture is described in Supplementary Table 6. The infusion was applied daily for 10 hrs (CTRL, ENIL, ENILOV, PNIL) or 11 hrs (PNILOV) in the light phase for 12 consecutive days. In ENIL and ENILOV groups, the lipid emulsion was administered three times a day by gavage. Each animal was provided the same amount of energy (60 kcal. day⁻¹) and had free access to water. All experiments were performed in accordance with the Animal Protection Law of the Czech Republic 311/1997 in compliance with the Principles of Laboratory Animal Care (NIH Guide for the Care and Use of Laboratory Animals, 8th edition, 2013) and approved by the Ethical Committee of the Ministry of Health, CR (approval no. 53/2014).

Histological evaluation. Liver tissue samples were fixed in 4% paraformaldehyde, embedded in paraffin blocks and routinely processed. Sections cut at 4–6 μ m were stained with hematoxylin/eosin and examined with an Olympus BX41 light microscope.

Biochemical analyses. Biochemical analyses were performed using commercially available kits (Roche Diagnostics, Basel, Switzerland; ALT-(ALAT/GPT) cat.no.: 10851132; AST-(ASAT/GOT) cat.no.: 10851124; TG-Triglycerides GPO-PAP cat.no.: 11730711; Bilirubin – BLT3 cat.no.: 05795320) on Hitachi analyzer 902. Statistical analyses for biochemical assays were performed using Graph PadPrism version 5.03 for Windows. Results were expressed as means $\pm$ standard deviation (SD). Kruskal–Wallis test with Bonferroni correction for multiple comparisons was performed to determine significant differences between the groups at a significance level of $p < 0.05$.

Determination of malondialdehyde concentration. The total malondialdehyde (MDA) content in liver homogenate was determined using stable isotope dilution assay based on liquid chromatography–tandem mass spectrometry. An internal standard of 1,3-dideuteriomalondialdehyde (MDA-D2) stock solution was prepared by acid hydrolysis of 1,3-D2-1,1,3,3-tetraethoxypropane (Cambridge Isotope Laboratories, Tewksbury MA, USA). 100 μ l of washed erythrocytes was mixed with 10 μ l of diluted internal standard MDA-D2 (10 μ M) and lysed with four volumes of cold distilled water in a refrigerator at 4 °C for 15 min; the cell debris was removed by centrifugation. The concentration of hemoglobin was measured at 540 nm using an extinction coefficient $E_{540}^{1\%} = 8.5$. For plasma samples, 100 μ l of EDTA plasma was mixed with 10 μ l of internal standard MDA-D2 (10 μ M). The supernatant was hydrolyzed with NaOH (1 M final concentration) for 30 min at 60 °C. To the hydrolysate, 3 M HClO₄ was added for protein precipitation and samples were centrifuged. The supernatant was derivatized by 5 mM 2,4-dinitrophenylhydrazine (DNPH). The reaction mixture was centrifuged and 20 μ l was injected onto HPLC column Nucleosil C18 ec (125 $\times$ 3 mm, 5 μ m) (Macherey–Nagel, Düren, Germany) at 40 °C using isocratic mobile phase composed of 0.1% of formic acid in 50% acetonitrile (v/v), flow rate 400 μ l/min.

The HPLC system was connected to the mass spectrometer QTRAP 4000 (Sciex, Prague, Czech Republic). MDA and MDA-D2 DNPH derivatives (MDA-DNPH and MDA-D2-DNPH) were detected in positive multiple reaction monitoring (MRM) mode. MDA-DNPH was monitored at m/z 235 $\rightarrow$ 189 and MDA-D2-DNPH at m/z 237 $\rightarrow$ 191. MDA and MDA-D2 DNPH derivatives eluted at 3.00 min. Analyst v.1.6 from SCIEX was used for the acquisition and analysis of data.

Real-time quantitative PCR. Liver samples were dissected and immediately frozen in liquid nitrogen. Total RNA was extracted using the Qiagen Mini RNeasy isolation kit (Qiagen, Hilden, Germany). A DNAase step was included to avoid possible DNA contamination. The integrity was detected on the fragment analyzer Automated CE System (Advanced Analytical Technologies, Inc., Ames, USA). The concentration of total RNA

was measured on Qubit (ThermoFisher Scientific, USA). A standard amount of total RNA (700 ng) was used to synthesize the first-strand cDNA with the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). The RT-PCR amplification mixture (20 μ l) contained 1 μ l template cDNA, 5xHOT FIREPol Eva Green qPCR SuperMix (Solis BioDyne, Tartu, Estonia) and 400 nM (10 pmol/reaction) sense and antisense primer. The reaction was run on the ViiA 7 Real-Time PCR System (ThermoFisher Scientific, USA). Results were analyzed using ViiA software v 1.1 (ThermoFisher Scientific, USA). The expression of genes of interest was normalized to the housekeeper gene (B2M and Cyc1) and calculated using the $\Delta\Delta C_t$ method.

Primer design. Primers were based upon known rat sequences available from the GeneBank Graphics database <https://www.ncbi.nlm.nih.gov>. List of primers is shown in Supplementary Table 7.

Lipidomics analysis. The liver extracts were prepared by a previously described method relying on MTBE:MeOH (methyl tert-butyl ether: methanol) extraction⁵⁶ with the modification of resuspending the dried extract in a mixture of isopropyl alcohol:MeOH:H₂O (65:30:5,v/v/v), the full preparation is described in Supplementary Methods. For the lipidomic analysis, U-HPLC (Infinity 1290, Agilent) coupled to a high-resolution mass spectrometer with a hyphenated quadrupole time-of-flight mass analyzer (6560 Ion Mobility Q-TOF LC/MS; Agilent) with the Agilent Jet Stream (AJS) electrospray (ESI) source were employed. Mass spectrometer acquired data in the m/z range of 100–1200 in MS1 mode for all the samples and a repeatedly (every 10 samples) injected QC sample. Fragmentation experiments for lipid identification were carried out on the QC sample using ddMS2 acquisition mode. The collision energy was set to 10, 20 and 40 eV. Chromatographic separation was based on 150 mm BEH C18 reverse-phase column (details in supplemental) with a mobile phase gradient of A: ACN:H₂O 60:40) and B: 2-propanol/ACN (90:10) using ammonium formate and formic acid (ESI+) or ammonium acetate and acetic acid (ESI-) as additives.

Lipid identification and statistical analyses. The mass spectrometry data was processed LipidMatch suite⁵⁷ which uses MZmine 2 for feature extraction and an R script for lipid identification based on in-silico fragmentation databases. At least a class-specific fragment was required for lipid identification. One item, FAHFA (18:2/18:2)_FAHFA(16:1/20:3), was not identified unequivocally. Due to the number of isomers the separation technique does not allow to separate some of them properly, the fragmentation spectrum contains fatty acyl ions confirming both lipid identities.

Lipidomics data processing was performed in both web-based and R based MetaboAnalyst (metaboanalyst.ca) packages followed by SIMCA (Umetrics) and subjected to two-dimensional hierarchical cluster analysis (HCA). A custom-built R script based on the MetaboAnalystR package was used to filter out features based on their univariate statistics. In MetaboAnalyst, sum normalization, log transformation, and Pareto scaling were used for signal processing. Statistically insignificant compounds were filtered out if they did not meet the criteria of a t-test or ANOVA p-value < 0.01 (FDR adjusted). These filtered data matrices were the first sum normalized in MS Excel and then loaded to SIMCA, where statistical models were built. When OPLS-DA (Orthogonal PLS-DA) models in SIMCA logarithmic transformation and Pareto scaling was used. Fragmentation spectra of the VIP score based significant compounds were double-checked both against the in-silico fragmentation library and against METLIN and LIPIDMAPS databases for their identities to be confirmed. Profiles of oxidized forms of free fatty acids were obtained by feeding a database with their respective molecular formulas to MassHunter Profinder, which scans the LC-HRMS data for respective features, which satisfy molecular formula requirements. These requirements were set as m/z value of 5 ppm, high confidence of isotopic pattern distribution and isotope spacing within 10 ppm. The features with a score of less than 80 were discarded.

Proteomic analysis. The tissue was lysed in the detergent-containing buffer, cysteines were reduced, blocked and samples were digested with trypsin⁵⁸. The samples were injected on nanoLC coupled with Orbitrap Fusion™ Tribrid™ (Thermo Scientific, USA, San Jose) mass spectrometer. Data were collected during a two-hour gradient. For protein identification and quantification were used MaxQuant software. The search was performed with the Rattus Norvegicus UniProt proteome database from 11/2018 (29965 entries). Gene names are listed according to HGNC guidelines (<https://www.genenames.org>). The detailed workflow is present in Supplementary Methods.

Bile acid analysis. The BAs were determined in methanol extract of lyophilized liver tissue. Liquid chromatography separation was performed using 1290 Infinity LC (Agilent Technologies) followed by mass spectrometry using 6550 iFunnel LCQ- TOF-MS (Agilent Technologies) equipped with a Dual AJS ESI probe in negative-ion mode. System control and data acquisition were performed by Agilent MassHunter Quadrupole Time of Flight Acquisition Software (B.06) with Qualitative Analysis (B.07 SP2) Software. The detailed workflow is present in Supplementary Methods.

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Competing interests

The authors declare no competing interests.

Additional information

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PŘÍLOHA XII

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SHORT COMMUNICATION

MicroRNAs as Potential Markers of Parenteral Nutrition-Associated Liver Disease in Adult Patients

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Summary

Parenteral nutrition-associated liver disease (PNALD) is a severe complication in patients completely dependent on parenteral nutrition (PN). The gold diagnostic standard, liver biopsy, is associated with significant health risk and therefore its use is limited. MicroRNAs (miRNAs) are small non-coding regulatory RNA molecules with highly tissue-specific expression and the secreted miRNAs may serve as non-invasive diagnostic biomarkers. The aim of this study was to evaluate the expression of a panel of specific miRNAs associated with liver diseases of different origin in PN-dependent adult patients in order to design miRNA panel enabling to precise monitoring of PNALD progression. Twelve PN-dependent patients with short bowel syndrome (SBS) were monitored on three/four-month basis for up to 24 months. Forty-five age- and sex-matched subjects without any known liver pathology served as controls. Specific miRNAs expression was determined by RT-qPCR using TaqMan probes (ThermoFisher). Liver function test parameters were determined in certified clinical laboratories. Six of the tested miRNAs exhibited significantly altered expression compared with healthy controls, three of them (MIR122, MIR1273g, and MIR500a) were upregulated while three were down-regulated (MIR505, MIR199a, MIR139). MIR122 positively correlated with serum AST and ALT activities while MIR1273g positively correlated with serum CRP concentration and GGT activity. MIR505, MIR199a, and MIR139 negatively correlated with serum GGT activity. Fluctuation of these parameters well paralleled serum miRNA concentrations in all patients throughout the whole

observation period. We identified six miRNAs whose serum concentrations are significantly altered in PN-dependent patients with PNALD and correlate with markers of inflammation, cholestasis or hepatic injury. Their reliability as markers of PNALD progression needs to be further evaluated.

Key words

miRNA • Parenteral nutrition-associated liver disease • Biomarker

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Long-term administration of total parenteral nutrition (PN) is often associated with the development of parenteral nutrition-associated liver disease (PNALD). The origin of this pathologic condition is multifactorial with numerous contributing factors, such as sepsis, intestinal inflammation, cholangitis, cholelithiasis, bacterial translocation, short bowel syndrome, the disturbance of hepato-biliary circulation, the lack of enteral nutrition, etc. PNALD clinical manifestations – which range from steatosis, cholestasis, gallbladder sludge/stones, fibrosis, and cirrhosis – can occur separately or in combination (Drongowski *et al.* 2009, Luman *et al.* 2002). The history of PNALD in adult patients is characterized by elevated liver enzymes in

association with steatosis lasting for years, followed by steatohepatitis, cholestatic hepatitis as well as fibrosis and cirrhosis (Cahova *et al.* 2017). The exact staging of the disease progression is necessary for the determination of the right prognosis and efficient treatment, including the indication for the intestine transplantation. It was repeatedly shown that liver tests alone are not sensitive enough for the diagnosis (Klek *et al.* 2016). Therefore, a liver biopsy remains the gold diagnostic procedure. Nevertheless, liver biopsy is associated with significant health risk and therefore its use is limited. There is an urgent need for seeking novel diagnostic tools for PNALD. These would help to optimize existing PN administration regimen/composition in order to delay or even prevent the development of PNALD. The aim of our study was to determine serum concentrations of selected miRNAs associated with liver pathologies of different origin in a cohort of adult PN-dependent patients in order to design miRNA panel enabling to precise monitoring of PNALD progression.

miRNAs are small endogenous RNA molecules that post-transcriptionally regulate gene expression by preferentially targeting the 3'-untranslated region of specific mRNA (Marin *et al.* 2014). The specific miRNA/mRNA interaction typically results in negative regulation of the expression of the protein encoded by target mRNA (Grimson *et al.* 2007). The occurrence of miRNAs is not restricted into intracellular space, in contrast, they are found in extracellular body fluids like blood, milk, urine, cerebral spinal fluid, semen, saliva and bile (Shigehara *et al.* 2011). Extracellular miRNAs are quite stable (Gori *et al.* 2014). Many of the circulating miRNAs are highly tissue-specific (Ninomiya *et al.* 2013) and emerging evidence shows that they can serve as non-invasive diagnostic biomarkers for various diseases, including non-alcoholic fatty liver disease (Yamada *et al.* 2013), steatohepatitis (Jin *et al.* 2012), biliary diseases (Munoz-Garrido *et al.* 2012) or hepatocellular cancer (Gailhouste *et al.* 2013).

We performed an extensive computer-based search of published articles in PubMed to identify relevant studies on the usefulness of serum miRNAs as non-invasive biomarkers for the detection of liver pathologies. The used Medical Subject Headings terms and keywords were “miRNA”, “biomarker”, „liver disease”, “PNALD”, “cholestasis” and “NASH”. We found 52 miRNAs proposed as putative biomarkers of liver injury (Table 1) that were further analyzed in a cohort of adult patients with chronic intestinal failure.

The discovery cohort consisted of 12 subjects with short bowel syndrome of different etiologies who were repeatedly monitored on three/four-month basis for up to 24 months. Underlying cause of SBS were mesenteric ischemia (n=4), Crohn disease (n=1), ulcerative colitis (n=1), Gardner syndrome (n=1), post radiation enteritis (n=3), postsurgical adhesion (n=1) and trauma (n=1). Control cohort included 45 apparently healthy age- and sex-matched subjects without any known liver pathology. Blood sample with no additives was taken between 7-8 a.m. in a fasting state and it was left at room temperature for 30 min. Then it was centrifuged twice for 3000 g, 15 min, 4 °C, serum removed to the new tube and centrifuged again 3000g, 10 min, 4 °C in order to remove any blood elements. The serum was aliquoted and stored at -80 °C until analysis. miRNA extraction was performed using miRCURY RNA isolation kit – biofluids (Exiqon) with RNA Carrier MS2 10 ng/μl (Roche). miRNA detection system included specific Taqman MicroRNA Reverse Transcription kit and TaqMan microRNA assays (Thermofisher Scientific). The PCR reaction was performed on ViiA7 Real-Time PCR system (Thermofisher Scientific). The specific miRNA expression was normalized to Stock Serum/Plasma spike-in control *Caenorhabditis elegans* MIR39 (cel miR-39-3p), 2 x 10⁶ molecules per sample (Qiagen). The data are expressed as 2^{ΔCt} (ΔCt = Ct_{miRNA} – Ct_{cel miR-39}) and presented as a median and interquartile range. Statistical analysis was performed using the Kruskal-Wallis test. Differences were considered statistically significant at the level of p<0.05. Spearman's rank correlation coefficient was used to assess the correlation between the studied variables.

SBS patients represent a highly diverse cohort with respect to the primary diagnosis, duration of PN-dependence or age. Most of the patients (11 out of 12) exhibited chronically abnormal liver function tests (Table 2). Among all miRNAs tested, six exhibited significantly altered expression compared with healthy controls. Three of them (MIR122, MIR1273g, and MIR500a) were upregulated while three were down-regulated (MIR505, MIR199a, MIR139) in SBS patients. MIR122 positively correlated with s-AST and s-ALT activities while MIR1273g positively correlated with s-CRP concentration and with s-GGT activity. MIR505, MIR199a, and MIR139 negatively correlated with s-GGT activity (Table 3). Fluctuation of these parameters well paralleled serum miRNA concentrations in all patients throughout the whole observation period.

Table 1. miRNAs identified as potential biomarkers of liver injury.

HGNC ID		TaqMan assay ID		reference
31476	MIRLET7A	000377	liver fibrosis	10.1371/journal.pone.004836
31479	MIRLET7b-5p	002619	APAP-induced liver injury	10.1093/toxsci/kfy200
	MIR16	000391	NAFLD, NASH	10.1371/journal.pone.0023937
31575	MIR19b	002425	liver fibrosis	10.1371/journal.pone.0048366
31586	MIR21	000397	APAP-induced liver injury liver inflammation	10.1093/toxsci/kfy200 10.1371/journal.pone.0023937
31599	MIR22	002301	liver inflammation	10.1371/journal.pone.0048366
	MIR24	000402	liver fibrosis	10.1371/journal.pone.0048366
31616	MIR29A	002112	lower circulating levels in	10.1002/hep.23922
31619	MIR29B1	000413	patients with liver fibrosis	
31621	MIR29C	000587		
31625	MIR30B	000602	primary biliary cirrhosis NAFLD	10.1371/journal.pone.0066086 10.1016/j.hep.2018.08.008
31634	MIR33a	002135	primary biliary cirrhosis	10.1371/journal.pone.0066086
32791	MIR33b	002085	NAFLD	10.1016/j.hep.2018.08.008
31635	MIR34a	000426	APAP-induced liver injury liver inflammation NAFLD	10.1002/jat.3722 10.1371/journal.pone.0048366 10.1016/j.hep.2018.08.008
31648	MIR96	000186	apoptosis, necrosis	10.1080/1354750X.2018.1528631
31650	MIR99A	000435	NASH	10.4254/wjh.v6.i8.613
31495	MIR106b	000442	liver fibrosis	10.1371/journal.pone.0048366
31501	MIR122	002245	drug-induced liver injury apoptosis, necrosis oxidative stress NASH NAFLD	10.1093/toxsci/kfy200 10.1080/1354750X.2018.1528631 10.3164/jcbrn.17-123 10.1016/j.cca.2013.05.021 10.1371/journal.pone.0153497
31505	MIR125	002198	NAFLD	10.1136/gutjnl-2014-306996
31514	MIR130a	000454	apoptosis, necrosis liver inflammation	10.1080/1354750X.2018.1528631 10.1371/journal.pone.0048366
31526	MIR139	001096	primary biliary cirrhosis NAFLD, NASH	10.1371/journal.pone.0066086 10.1038/ijo.2017.21
31530	MIR143-3p	002249	cholestasis	10.1093/toxsci/kfy200
32079	MIR146B	001097	NAFLD, NASH	10.1136/gutjnl-2015-309456 10.4254/wjh.v6.i8.613
31537	MIR150	002637	NAFLD, NASH	10.1136/gutjnl-2015-309456 10.1016/j.bbrc.2017.10.149
31762	MIR151a	002642	APAP-induced liver injury	10.1002/jat.3722

Table 1., continued.

31549	MIR181a	000480	NAFLD progression liver cirrhosis	10.1016/j.taap.2012.04.018 10.1016/j.bbrc.2012.03.025
31554	MIR183	002269	apoptosis, necrosis	10.1080/1354750X.2018.1528631
31560	MIR190	000489	cholestasis	10.1097/MOG.0000000000000051
31562	MIR192-5p	000491	drug-induced liver injury oxidative stress NAFLD, NASH	10.1093/toxsci/kfy200 10.3164/jcbn.17-123 10.1016/j.hep.2018.08.008
31563	MIR193a	002281	APAP-induced liver injury liver inflammation	10.1002/jat.3722 10.1371/journal.pone.0048366
	MIR194	000493	APAP-induced liver injury	10.1002/jat.3722
31567	MIR196	241070_mat	apoptosis, necrosis	10.1080/1354750X.2018.1528631
31569	MIR197	474626_mat	primary biliary cirrhosis liver inflammation	10.1371/journal.pone.0066086 10.1371/journal.pone.0048366
31571	MIR199a	000498	alcoholic liver disease liver fibrosis	10.3390/ijms17030280 10.1038/nrgastro.2013.87
31579	MIR200B	002274	liver inflammation steatosis	10.1016/S0168-8278(15)31170-3 10.18632/oncotarget.9183
	MIR218a-5p	000521	cholestasis	10.1093/toxsci/kfy200
31601	MIR221	002096	liver fibrosis hepatocellular carcinoma	10.1038/nrgastro.2013.87 10.1073/pnas.0907904107
31771	MIR320-3p	002230	steatosis	10.1093/toxsci/kfy200
31868	MIR375	000564	NASH	10.1136/gutjnl-2014-306996
32053	MIR451	001141	NAFLD	10.1016/j.cca.2013.05.021
32134	MIR500a	002428	primary biliary cirrhosis	10.1371/journal.pone.0066086
32140	MIR505	002087	primary biliary cirrhosis	10.1371/journal.pone.0066086
32827	MIR571		correlates with disease stages during alcoholic or HCV- induced liver cirrhosis	10.1371/journal.pone.0032999
32828	MIR572	001614	NASH	10.3748/wjg.v18.i37.5188
32831	MIR575	001617	NASH	10.3748/wjg.v18.i37.5188
32894	MIR638	001582	NASH	10.3748/wjg.v18.i37.5188
32915	MIR659	001514	liver inflammation	10.1371/journal.pone.0048366
37316	MIR711	241090_mat	liver inflammation	10.1371/journal.pone.0048366
33658	MIR744	002324	NASH	10.3748/wjg.v18.i37.5188
33923	MIR-1224-5p	002752	oxidative stress	10.3164/jcbn.17-123
	MIR1273g	462577_mat	primary biliary cirrhosis	10.1371/journal.pone.0066086
	MIR1274B	002884	liver inflammation	10.1371/journal.pone.0048366

MIR122 is highly enriched in the liver but absent in other tissues (Lagos-Quintana *et al.* 2002). MIR122 participates on the regulation of the expression of enzymes involved in crucial metabolic pathways in the liver including glycolysis and gluconeogenesis, carbohydrate digestion and absorption, glucagon signaling pathway, starch and sucrose metabolism, cholesterol synthesis or iron homeostasis (Joppling 2012). Several lines of evidence indicate that it functions as a tumor suppressor (Bai *et al.* 2009). Roderburg *et al.* (2015) showed that serum MIR122 concentrations were strongly elevated in mice after hepatic ischemia/reperfusion injury, as well as in the cellular supernatants in an *in vitro* model of hepatocyte injury,

supporting the hypothesis that the passive release of MIR122 represents a surrogate for hepatocyte death in liver injury. This finding corresponds with our observation that serum MIR122 levels correlate with ALT and AST concentrations. Taken together, MIR122 levels may serve as an independent marker of ongoing liver injury and hepatic cell death.

Serum content of all other miRNAs deregulated in PN-dependent patients significantly correlated with GGT serum activity, which is a marker of cholestasis. To our knowledge, this association has not been described yet. Three of these miRNAs (MIR199a, MIR505, and MIR139) were described as tumor suppressors and their down-regulation is associated with disease progression.

Table 2. Clinical characteristics of patients.

patient	no. of assayed samples	sex	Age (yr)	SBS type	diagnosis	remnant small bowel (cm)	time on PN (month)	bilirubin total ($\mu\text{mol.l}^{-1}$)	bilirubin conjugated ($\mu\text{mol.l}^{-1}$)	AST ($\mu\text{kat.l}^{-1}$)	ALT ($\mu\text{kat.l}^{-1}$)	ALP ($\mu\text{kat.l}^{-1}$)	GGT ($\mu\text{kat.l}^{-1}$)	CRP (mg.l^{-1})
1	6	M	36	I	1	50	8	60* (37)	32.8* (22)	1.4* (0.7)	2.6* (1.3)	4.1* (1.4)	2.4* (1.1)	7.7* (54.9)
2	4	F	71	I	2	100	87	28* (15)	12* (3)	0.5 (0.08)	0.5 (0.16)	2.2* (0.2)	1.9* (0.2)	0.6 (0.2)
3	1	F	52	I	3	200	84	9.4	n.d.	0.4	0.5	1.8*	0.6*	5.3
4	3	F	64	I	5	40	96	19.5* (6.3)	11.0* (2.4)	0.7* (0.2)	0.6* (0.2)	3.6* (2.3)	1.3* (0.3)	75.8* (39.8)
5	1	M	64	I	7	?	13	19.9	11.0	0.6*	0.7*	5.1*	2.7*	2.3
6	4	F	53	I	5	120	16	7 (2.6)	3.7 (1.5)	0.7* (0.3)	0.5* (0.3)	6.5* (1.7)	1.6* (0.6)	31* (41)
7	5	F	38	II	4	15	27	8.7 (3.4)	3.4 (0.9)	0.5 (0.3)	0.7* (0.62)	1.8* (0.7)	0.3 (0.9)	3.7 (4.3)
8	4	F	68	II	1	40	88	14.6 (3.9)	7.6 (1.7)	0.5* (0.2)	0.7* (0.41)	2.2* (3.3)	2.4* (1.7)	11.4* (19.4)
9	5	F	40	II	1	30	61	27* (18.8)	10.3* (4.5)	0.4 (0.2)	0.6* (0.19)	3.0* (1.4)	0.3 (0.2)	0.2 (0.1)
10	3	F	37	II	1	30	52	6.1 (1.9)	2.9 (0.4)	0.3 (0.02)	0.53 (0.08)	1.6 (0.1)	0.5 (0.1)	0.5 (0.4)
11	1	M	21	II	6	80	18	6.7	2.7	0.7*	0.9*	0.7	0.3	2.5
12	1	F	50	II	5	?	84	6.2	3.1	1.8*	1.1*	2.3*	0.4	13.2*
controls		29F/1 6M	40 (25)	N/A	N/A	N/A	N/A	7.8 (1.9)	2.8 (0.7)	0.3 (0.4)	0.4 (0.6)	0.9 (0.5)	0.2 (0.2)	1.8 (2.1)

SBS type I: end-ostomy, SBS type II: bowel in continuity. Diagnoses: 1 mesenteric ischemia, 2 Crohn disease, 3 ulcerative colitis, 4 Gardner syndrome, 5 post-radiation enteritis, 6 post-surgical adhesion, 7 trauma. AST aspartate transaminase; ALT alanine transaminase; ALP alkaline phosphatase; GGT gamma-glutamyl transpeptidase; CRP C-reactive protein. When applicable, data are given as a median and interquartile range. Values marked with * were above the normal range in more than half samples during the observation period.

Table 3. Content of selected miRNA in the serum of PN-dependent patients and healthy controls.

symbol	HGNC ID	fold change	p-value	Spearman's correlation coefficient				
				AST	ALT	ALP	GGT	CRP
<i>MIR122</i>	31501	1.7 (1.8)	0.024	0.685*	0.873*	0.305	0.254	0.008
<i>MIR1273G</i>	-	3.7 (6.9)	0.003	-0.048	-0.131	0.316	0.761*	0.531*
<i>MIR500A</i>	32134	2.1 (1.9)	0.049	-0.157	-0.126	-0.038	-0.463*	-0.021
<i>MIR199A1</i>	31571	0.3 (0.5)	2.7×10^{-5}	-0.070	-0.085	-0.246	-0.510*	-0.325
<i>MIR505</i>	32140	0.1 (0.1)	2.4×10^{-12}	0.336	0.250	-0.323	0.697*	0.056
<i>MIR139</i>	31526	0.5 (0.3)	0.025	-0.195	-0.254	-0.044	-0.599*	-0.338

Fold change is calculated as the ratio of normalized Ct values (patients vs a median of control cohort) and expressed as a median and interquartile range (IQR). p-value shows the significance of the difference between control and patient cohorts (Kruskal-Wallis test with Bonferroni correction). Spearman's correlation coefficient: values marked by * are statistically significant at the level $p < 0.05$.

MIR505 is down-regulated in the serum of pancreatic cancer patients (Schultz *et al.* 2014) and patients with hepatocellular carcinoma (HCC) (Li *et al.* 2015) as well as in serum of patients with primary biliary cirrhosis (Ninomiya *et al.* 2013). In hepatoma cell lines down-regulation of MIR505 promoted proliferation, invasion and epithelial-mesenchymal transition (Lu *et al.* 2016). Serum means values of MIR199a were significantly decreased among HCC patients (Kamel *et al.* 2016, Yin *et al.* 2015) and served as a predictor of hepatitis B- or hepatitis C-related HCC (Fiorino *et al.* 2016). MIR139 suppresses tumor growth and metastasis in HCC and its decreased serum levels may serve as biomarker of this pathology (Zou *et al.* 2018). MIR500a promotes the progression of hepatocellular carcinoma and enhances HCC (Bao *et al.* 2018, Jiang *et al.* 2017, Zhao *et al.* 2017). The biological function of MIR1273g has not been described yet but the increased MIR1273g content was observed in mice pancreatic cancer tissue (Rachagani *et al.* 2015) and in human colorectal carcinoma tissue (Vishnubalaji *et al.* 2015). Interestingly, the expression pattern of all these five miRNAs in patients' cohorts (down MIR199a, MIR505, MIR139; up MIR500a,

MIR1273g) follows the signature characteristic of hepatocellular or pancreatic cancer. In our cohort of patients, there was only one case with diagnosed GIT-related cancer (Gardner syndrome) but our data suggest the increased risk of increased proliferation and possible malignant transformation in the liver of PN-dependent patients.

In conclusion, we identified a panel of six miRNAs differently expressed in sera of PN-dependent patients with abnormal liver function tests compared with healthy controls. These miRNAs correlated with liver injury and hepatic cell death (MIR122), cholestasis (MIR505, MIR199a, MIR139, MIR500a, MIR1273g) or inflammation (MIR1273g). This study suggests that specific miRNAs profile in serum has potential as a diagnostic biomarker of PNALD progression.

Conflict of Interest

There is no conflict of interest.

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PŘÍLOHA XIII

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Hepatic Gene Expression Profiles Differentiate Steatotic and Non-steatotic Grafts in Liver Transplant Recipients

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Background: Liver transplantation leads to non-alcoholic fatty liver disease or non-alcoholic steatohepatitis in up to 40% of graft recipients. The aim of our study was to assess transcriptomic profiles of liver grafts and to contrast the hepatic gene expression between the patients after transplantation with vs. without graft steatosis.

Methods: Total RNA was isolated from liver graft biopsies of 91 recipients. Clinical characteristics were compared between steatotic ($n = 48$) and control ($n = 43$) samples. Their transcriptomic profiles were assessed using Affymetrix HuGene 2.1 ST Array Strips processed in Affymetrix GeneAtlas. Data were analyzed using Partek Genomics Suite 6.6 and Ingenuity Pathway Analysis.

Results: The individuals with hepatic steatosis showed higher indices of obesity including weight, waist circumference or BMI but the two groups were comparable in measures of insulin sensitivity and cholesterol concentrations. We have identified 747 transcripts (326 upregulated and 421 downregulated in steatotic samples compared to controls) significantly differentially expressed between grafts with vs. those without steatosis. Among the most downregulated genes in steatotic samples were *P4HA1*, *IGF1*, or fetuin B while the most upregulated were *PLIN1* and *ME1*. Most influential upstream regulators included *HNF1A*, *RXRA*, and *FXR*. The metabolic pathways dysregulated in steatotic liver grafts comprised blood coagulation, bile acid synthesis and transport, cell redox homeostasis, lipid and cholesterol metabolism, epithelial adherence junction signaling, amino acid metabolism, AMPK and glucagon signaling, transmethylation reactions, and inflammation-related pathways. The derived mechanistic network underlying major transcriptome differences between steatotic samples and controls featured *PPARA* and *SERPINE1* as main nodes.

Conclusions: While there is a certain overlap between the results of the current study and published transcriptomic profiles of non-transplanted livers with steatosis, we have identified discrete characteristics of the non-alcoholic fatty liver disease in liver grafts potentially utilizable for the establishment of predictive signature.

Keywords: microarray, non-alcoholic fatty liver disease (NAFLD), transcriptomics profile, predictive signature, liver transplant

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in industrialized countries, its prevalence being estimated at 19–31.3% (1). It encompasses a range of conditions that are thought to arise from fatty liver (simple steatosis) through non-alcoholic steatohepatitis (NASH), which refers to findings on liver biopsy reflecting typical metabolic and inflammatory changes (fat-related inflammation) with or without fibrosis in the absence of significant alcohol consumption (2, 3). NASH may develop into liver cirrhosis and hepatocellular carcinoma. Although NAFLD itself may be rather benign in the majority of patients, it is associated with increased specific (liver-related) mortality (4) and represents a substantial risk particularly for both fatal and non-fatal adverse cardiovascular events (5, 6) and chronic kidney disease (7).

The pathogenesis of NAFLD is multifactorial and multiple genetic and behavioral factors were identified to contribute to hepatic mishandling of fatty acids and carbohydrates as energy sources, as recently reviewed in detail. The overload of fatty acids in hepatocytes is deemed to result in the formation of lipotoxic species, paving the way toward NASH and fibrosis (5). As no targeted pharmacological therapy for NASH is currently in general clinical use, the modification of lifestyle is of particular importance in the prevention and therapy of this condition. Physical exercise, diet, and their combination belong to major modifiable aspects of non-pharmacological management of NAFLD. Reduction in caloric intake and macronutrients such as fat and carbohydrate have been utilized, sometimes with supplementation of natural antioxidants, with the aim to treat NAFLD (8–10). Although results of the studies have been so far somewhat conflicting, consistent effects leading to alleviation of NAFLD were repeatedly shown for weight loss and physical exercise (11).

It has been previously reported that among liver transplant recipients, post-transplant NAFLD affects 18–40% of subjects (12–14) and even 39–70% (15, 16) of those transplanted for NAFLD-related cirrhosis. At our Center, we performed a study based on repeated liver graft biopsies up to 15 years from liver transplantation and diagnosed graft steatosis in 56.4% patients, a proportion significantly exceeding the NAFLD prevalence in general population (17). In that study, liver graft steatosis was associated with a trend for diminished survival (mostly due to cardiovascular and oncologic reasons), and graft steatosis is considered a risk factor for poor outcome (18).

In clinical practice, simple steatosis is a frequent finding on ultrasonography, computer tomography or magnetic resonance examination. Nevertheless, the disease progression and impact on patient survival over time is the result of complex and dynamic interactions among many processes and the pathophysiological mechanisms leading to the development of a progressive and deleterious form of damage to the liver graft post-transplant as well as to the liver in general population are incompletely understood. The possibility to identify markers indicating the pro-steatotic condition of the graft prior to clinical manifestation of NAFLD may allow to start the specific therapy early and to reverse the negative trend. Moreover, we assume

that the situation in liver graft recipients could serve as a valuable model for the study of NAFLD applicable to the general population. Liver transplantation offers a unique opportunity to follow graft recipients since engraftment, and unlike in general population, most factors putatively involved in pathogenesis could be extensively studied during the comprehensive follow-up including the repeated graft biopsies.

While the gene expression and other “-omic” signatures have been recently reported mostly for distinct oncological conditions including liver cancer (19), the systems biology-level analyses indicate that similar types of analyses applied to NAFLD might produce clinically-relevant results (20–22). Therefore, the aim of the presented study was to identify and contrast the gene expression profiles in liver graft biopsies in a cohort of patients either with or without histologically proven steatosis who successfully underwent liver transplantation.

MATERIALS AND METHODS

Cohort

The cohort consisted of unselected adult liver transplant recipients who received cadaveric whole or partial liver graft in between 1995 and 2013 at the Institute for Clinical and Experimental Medicine (IKEM) Prague, scheduled for regular surveillance biopsy, and consented to participate in the study. Recipients transplanted for HCV-related cirrhosis were excluded unless they achieved sustained virologic response after the antiviral treatment. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Joint Ethics Committee of the Institute for Clinical and Experimental Medicine and Thomayer Hospital. All patients signed informed consent. Patients received different immunosuppression protocols based either on cyclosporin or tacrolimus depending on transplantation era. All relevant clinical data are provided in **Supplementary Table S1**.

Clinical Assessment

All patients were followed by transplant physicians at the Center at regular intervals. At the time of biopsy (± 1 month), clinical assessment (blood pressure, body weight, and abdominal circumference measurement), as well as laboratory evaluations of bilirubin, ALT, AST, GGT, ALP, total cholesterol, LDL- and HDL-cholesterol, and triglycerides were performed. Patients further underwent examination focused on insulin sensitivity: fasting glucose, HbA1c concentration, C-peptide, and fasting insulin. Based on these data, HOMA (homeostatic model assessment) and QUICKI (quantitative insulin sensitivity check) indexes were calculated. All biochemical analyses were performed by the in-house routine biochemistry laboratory according to the approved protocols. Total Bilirubin, ALT, AST, GGT, ALP, total cholesterol, HDL-cholesterol, triglycerides and glucose were determined on automatic Architect c16000 Analyzer (Abbott Diagnostics) using the following kits: Bilirubin Total: 6L45-21, ALT: 8L92-41, AST: 8L91-41, GGT: 7D65-21, ALP: 7D55-22, Cholesterol: 7D62-21, HDL-Cholesterol: 3K33-21, Triglycerides: 7D74-21, and Glucose: 3L82-41). LDL-cholesterol was calculated using the Friedwald equation. HbA1c was measured by HPLC on Tosoh

Automated Glycohemoglobin Analyzer HLC 723 G8 (Tosoh Corp., Japan). C-peptide was determined by immunochemistry (ECLIA method) on Cobas e801 Analyzer (Roche Diagnostics). Insulin was measured using an immunoradiometric (IRMA) kit (Beckman Coulter, Prague, CR).

Histological Assessment

Liver graft biopsies were obtained either by Menghini technique (1.6 G needle) or by true cut biopsy device. Formalin-fixed, paraffin-embedded liver graft tissue was routinely processed and 4 μ m thick serial sections were stained according to the standard protocol of our laboratory with hematoxylin and eosin, elastic-van Gieson and Shikata's orcein method, Perls' Prussian Blue reaction, and periodic acid-Schiff technique after diastase digestion. For the purpose of the study, all biopsies were evaluated by two experienced pathologists. All biopsies were classified according to the Kleiner's histological scoring system for NAFLD (23). NAS (NAFLD activity score) was calculated as the sum of the scores for the hepatocellular steatosis (0–3), lobular inflammation (0–3), and ballooning (0–2). Additionally, the extent of the liver graft fibrosis stage was classified as proposed by Kleiner et al. (23), i.e., (stage 1A—mild perisinusoidal fibrosis in zone 3; stage 1B—moderate perisinusoidal fibrosis in zone 3; stage 1C—portal/periportal fibrosis; stage 2—perisinusoidal and portal/periportal fibrosis; stage 3—perisinusoidal and bridging fibrosis; stage 4—cirrhosis). Liver graft steatosis was defined as the presence of liver fat in $\geq 5\%$ hepatocytes on hematoxylin-eosin staining.

RNA Isolation, Transcriptomics, qPCR Validation

Liver tissue (20–30 mg) was placed into RNAlater (ThermoFisher Scientific, Waltham, MA, USA) immediately after the biopsy and then stored in -80°C until further processed. Total RNA was isolated using TRIzol Reagent (ThermoFisher Scientific, Waltham, MA, USA) and RNeasy Mini Kit (Hilden, Germany). The quality and integrity of the total RNA were evaluated on the Agilent 2100 Bioanalyzer system (Agilent, Palo Alto, CA, USA). Only samples surpassing the minimal quality threshold (RIN > 8.0) were used in the subsequent transcriptomic assessment. Microarray experiments were performed using the GeneChip™ Human Gene 2.1 ST Array Strip (Thermo Fisher Scientific, Waltham, MA, USA) on Affymetrix GeneAtlas™ system (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. The quality control of the chips was performed using Affymetrix Expression Console (Thermo Fisher Scientific, Waltham, MA, USA); Partek Genomics Suite 7.0 (Partek Inc., St. Louis, Missouri, USA) was used for subsequent data analysis. Transcriptomic data were then processed by standardized sequence of analyses (hierarchical clustering and principal component analysis, gene ontology, gene set enrichment, "Upstream Regulator Analysis," "Mechanistic Networks," "Causal Network Analysis," and "Downstream Effects Analysis") using Ingenuity Pathway Analysis (IPA hereafter, Qiagen Redwood City, Inc., Redwood City, CA, USA). For qPCR validation, the expressions of 19 selected transcripts were verified in 47 randomly chosen samples (19 control, 28 steatotic).

The complete procedure involving reverse transcription (TATAA GrandScript cDNA Supermix; TATAA Biocenter AB, Göteborg, Sweden), pre-amplification (iQ Supermix, Bio-Rad Laboratories, Inc., Hercules, CA, USA + Applied Biosystems TaqMan Assays; Thermo Fisher Scientific, Waltham, MA, USA) and High-throughput qPCR System BioMark (Fluidigm Corporation, South San Francisco, CA, USA) analysis using 48.48 Gene Expression Dynamic Chip (Fluidigm Corporation, South San Francisco, CA, USA). The primary analysis of the data was performed by Fluidigm Real-Time PCR Analysis 4.1.2, the comparisons were assessed using the Livak analysis method (24) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference gene (**Supplementary Figure S1**).

Statistics

After applying quality filters and data normalization by Robust Multichip Average algorithm, the set of obtained differentially expressed probesets [analysis of variance with hepatocellular steatosis (0 vs. 1, 2, and 3) as fixed effect] was subsequently filtered by false discovery rate (FDR) method implemented in PARTEK Genomics Suite 7.0 (Partek Inc., St. Louis, Missouri, USA). For further analysis, only genes showing statistically significant (FDR < 0.05) differences in expression between patients with vs. without steatosis were included in subsequent analyses. Selected genes and patients were subject to two-dimensional hierarchical cluster analysis with a Pearson correlation coefficient being a measure of dissimilarity. Outcomes of the cluster analysis were displayed in a heatmap. Moreover, selected covariates (time from transplantation, fibrosis, ballooning, inflammation, NAS score) were analyzed with respect to steatosis using a Spearman correlation coefficient. The results were also displayed in heatmaps. Analyses were performed using a statistical package R version 3.4.4 (25). *P*-values $< 5\%$ were considered statistically significant.

RESULTS

Cohort Comparison

Samples from 91 liver graft recipients (45 men, 46 women) passed all the quality controls and were included in the subsequent analyses. The relevant clinical characteristics are shown in **Table 1**. The individuals with hepatic steatosis showed higher indices of obesity including waist circumference and BMI. Except for the latter two parameters, the subjects with mild steatosis (grade 1) were comparable in remaining measures with controls. The individuals with more severe steatosis (grade 2+3) in their liver grafts had higher levels of ALT, total cholesterol and triglycerides compared to controls, yet the values of these parameters were still in the normal range. As expected, the parameters associated with insulin sensitivity, i.e., fasting glucose, HbA1c, fasting insulin, C-peptide, and HOMA index were higher in patients with grade 2+3 steatosis compared to controls and for fasting insulin, C-peptide and HOMA index even when compared to subjects with grade 1 steatosis.

Immunosuppressive medication of patients in both cohorts was comparable (**Supplementary Table S2**). All participants were treated with calcineurin inhibitor tacrolimus, only a

TABLE 1 | Clinical characteristics of patients without or with graft steatosis.

	Non-steatosis (n = 43)	Steatosis grade 1 (n = 37)	Steatosis grade 2, 3 (n = 11)
Sex (women/men)	25/18	16/21	4/7
Age (years)	51.6 (49.0; 59.7)	61.7* (54.6; 66.6)	58.4 (47.1; 64.5)
Time from Tx (days)	906 (443; 3756)	899 (433; 1922)	751 (482; 1880)
BMI (kg/m ²)	24.0 (21.5; 28.4)	26.2* (24.4; 31.0)	28.4* (26.8; 32.2)
Waist circumference (cm)	91.0 (17.5; 99.5)	103.0* (89.0; 111.0)	108.0* (102.5; 113.5)
LIVER FUNCTION TESTS			
Total bilirubin (μmol/l)	12.1 (9.3; 17.8)	12.5 (9.9; 18.9)	13.5 (8.35; 16.4)
AST (μkat/l)	0.38 (0.32; 0.44)	0.41 (0.33; 0.47)	0.49 (0.38; 0.58)
ALT (μkat/l)	0.4 (0.34; 0.48)	0.45 (0.35; 0.61)	0.59* (0.58; 0.72)
ALP (μkat/l)	1.32 (1.08; 1.16)	1.31 (1.07; 1.69)	1.54 (1.21; 2.18)
GGT (μkat/l)	0.36 (0.28; 0.54)	0.38 (0.28; 0.73)	0.93 [#] (0.53; 1.44)
LIPID METABOLISM			
TAG (mmol/l)	1.0 (0.8; 1.3)	1.1 (0.9; 1.4)	1.8* [#] (1.3; 2.6)
Total cholesterol (mmol/l)	4.5 (3.8; 5.0)	4.4 (3.7; 4.9)	4.8* [#] (4.2; 5.4)
LDL-cholesterol (mmol/l)	2.5 (2.0; 3.0)	2.5 (1.9; 3.0)	3.2 (1.9; 3.4)
HDL-cholesterol (mmol/l)	1.3 (1.1; 1.5)	1.2 (1.0; 1.4)	1.1 (0.8; 1.2)
GLUCOSE METABOLISM			
Fasting glucose (mmol/l)	5.2 (4.9; 5.6)	5.2 (4.9; 6.0)	5.8* (5.6; 6.6)
HbA1c (mmol/mol)	37 (32; 40)	37 (32; 43)	41 (36; 47)
C-peptide (nmol/l)	0.7 (0.6; 0.9)	0.8 (0.7; 0.9)	1.2* [#] (0.9; 1.5)
Fasting insulin (μIU/ml)	7.1 (4.35; 8.8)	7.0 (5.2; 9.6)	10.1* [#] (6.9; 18.1)
HOMA-IR	1.554 (0.982; 2.182)	1.647 (1.139; 2.559)	2.646* [#] (1.518; 4.361)
QUICKI	0.337 (0.339; 0.3845)	0.354 (0.332; 0.375)	0.330 (0.308; 0.360)

Data are given as a median (1st quartile; 3rd quartile). Statistical significance between both groups was calculated by Student's *t*-test with Bonferroni correction. **p* < 0.05 compared to non-steatosis, [#]*p* < 0.05 compared to steatosis grade 1. ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; GGT, gamma-glutamyl transferase; HDL, high-density lipoprotein; HbA1c, glycated hemoglobin; HOMA-IR, homeostatic model assessment for insulin resistance; LDL, low-density lipoprotein; QUICKI, quantitative insulin sensitivity check index; TAG, triacylglycerol; Tx, transplantation.

few were receiving cyclosporine. In addition, most of the patients received mycophenolate mofetil, while azathioprine, sirolimus or everolimus were prescribed only to the minor portion of patients. Eleven patients without steatosis and twelve patients with steatosis received antilymphocyte induction with either antithymocyte globulin or anti-CD25 monoclonal antibody (daclizumab or basiliximab). All received a high dose of methylprednisolone during anhepatic phase (immunosuppressive therapy is detailed in **Supplementary Table S1**). In the subcohorts of patients with steatosis, statin, insulin and PAD treatment was more frequent compared to the patients without steatosis.

In the whole cohort, the prevailing primary diseases leading to liver transplantation were alcoholic cirrhosis and biliary cirrhosis. In patients with graft steatosis, the alcoholic cirrhosis was more frequent (29.2 vs. 11.6% in steatosis group vs. controls, respectively), while in controls, the biliary cirrhosis dominated (18.8 vs. 41.9%). Other diagnoses represented in both groups included hepatitis B (10.4 vs. 2.3% in steatosis group vs. controls, respectively), hepatitis C (all of them genotype 1) (4.2 vs. 7%), autoimmune disorders (8.3 vs. 7%) and cryptogenic cirrhosis (8.3 vs. 2.3%). Less frequent diagnoses distinct from the above comprised 20.8 and 23.3% of steatotic cases and controls, respectively. These diagnoses included polycystic liver disease

(*n* = 5), epithelioid hemangioendothelioma (*n* = 3), acute liver failure (*n* = 3), hepatic adenoma (*n* = 2), cholangiocarcinoma (*n* = 2), Wilson's disease (*n* = 2), alpha-1 antitrypsin deficiency (*n* = 1), neuroendocrine carcinoma (*n* = 1), and Rendu-Osler disease (*n* = 1).

Transcriptome Comparison

In order to find genes significantly associated with steatosis in transplanted liver grafts, we adopted a strategy based on testing the difference of transcriptome profiles between patient's steatotic and non-steatotic cohorts. All subjects with the liver fat content higher than 5% were included in the steatotic group while those with <5% of liver fat were considered as non-steatotic. After correction for multiple testing (FDR < 0.05), we identified 747 significantly differentially expressed transcripts (326 upregulated and 421 downregulated in steatotic samples compared to controls) out of 53,617. The top differentially expressed genes are shown in **Table 2**, the complete set is provided in **Supplementary Table S3**.

We further performed hierarchical clustering of the genes selected in the previous step. Most of the graft recipients tended to form separate clusters according to the level of steatosis (**Figure 1A**). This analysis, expectedly, revealed two distinct clusters of transcripts based on their expression

TABLE 2 | Top differentially expressed transcripts.

Symbol	Gene name	Fold change	p
<i>P4HA1</i>	Prolyl 4-hydroxylase subunit alpha 1	−2.26	1.03E-07
<i>CYP1A1</i>	Cytochrome P450 family 1 subfamily A member 1	−1.98	7.75E-05
<i>IGF1</i>	Insulin-like growth factor 1	−1.73	3.27E-06
<i>SHBG</i>	Sex hormone binding globulin	−1.72	3.10E-07
<i>SLC2A12</i>	Solute carrier family 2 member 12	−1.66	2.71E-06
<i>ENST00000560967</i>	Predicted non-coding transcript	−1.59	3.46E-07
<i>IGFBP2</i>	Insulin-like growth factor binding protein 2	−1.58	7.75E-08
<i>PROZ</i>	Protein Z, vitamin K dependent plasma glycoprotein	−1.50	6.29E-04
<i>APOA4</i>	Apolipoprotein A4	1.55	1.95E-04
<i>ME1</i>	Malic enzyme 1	1.55	4.30E-05
<i>SERPINE1</i>	Serpin family E member 1	1.56	5.73E-04
<i>PLIN1</i>	Perilipin 1	1.65	5.33E-05
<i>SCUBE1</i>	Signal peptide, CUB domain and EGF like domain containing 1	1.66	3.42E-07
<i>MAMDC4</i>	Mam domain containing 4	1.67	2.31E-07
<i>DOPEY2</i>	Dopey family member 2	1.69	3.43E-04
<i>LOC101928635</i>	Uncharacterized LOC101928635	1.70	2.73E-04

Only the transcripts showing >50% difference in fold change of gene expression in steatotic vs. non-steatotic grafts ratio are shown. Unadjusted p-values are indicated (FDR < 0.05 cut-off value is 8.29 E-04).

patterns. We further wanted to know whether the genes differentially expressed in steatotic grafts show association with other features of liver histology typical for NAFLD/NASH and therefore we displayed inflammation, ballooning, fibrosis level, and NAS score, in the clustering heatmaps as well. We found no association between steatosis and fibrosis grade ($p = 0.8877$) (**Supplementary Figure S2**) or the time interval from transplantation ($p = 0.2873$) (**Supplementary Figure S3**) respective. On the other hand, the graft recipients showed significant associations between steatosis and the NAS score ($p < 0.0001$), ballooning ($p < 0.0001$), and inflammation ($p < 0.0001$) (**Figures 1B–D**).

Identification of Deregulated Metabolic Pathways

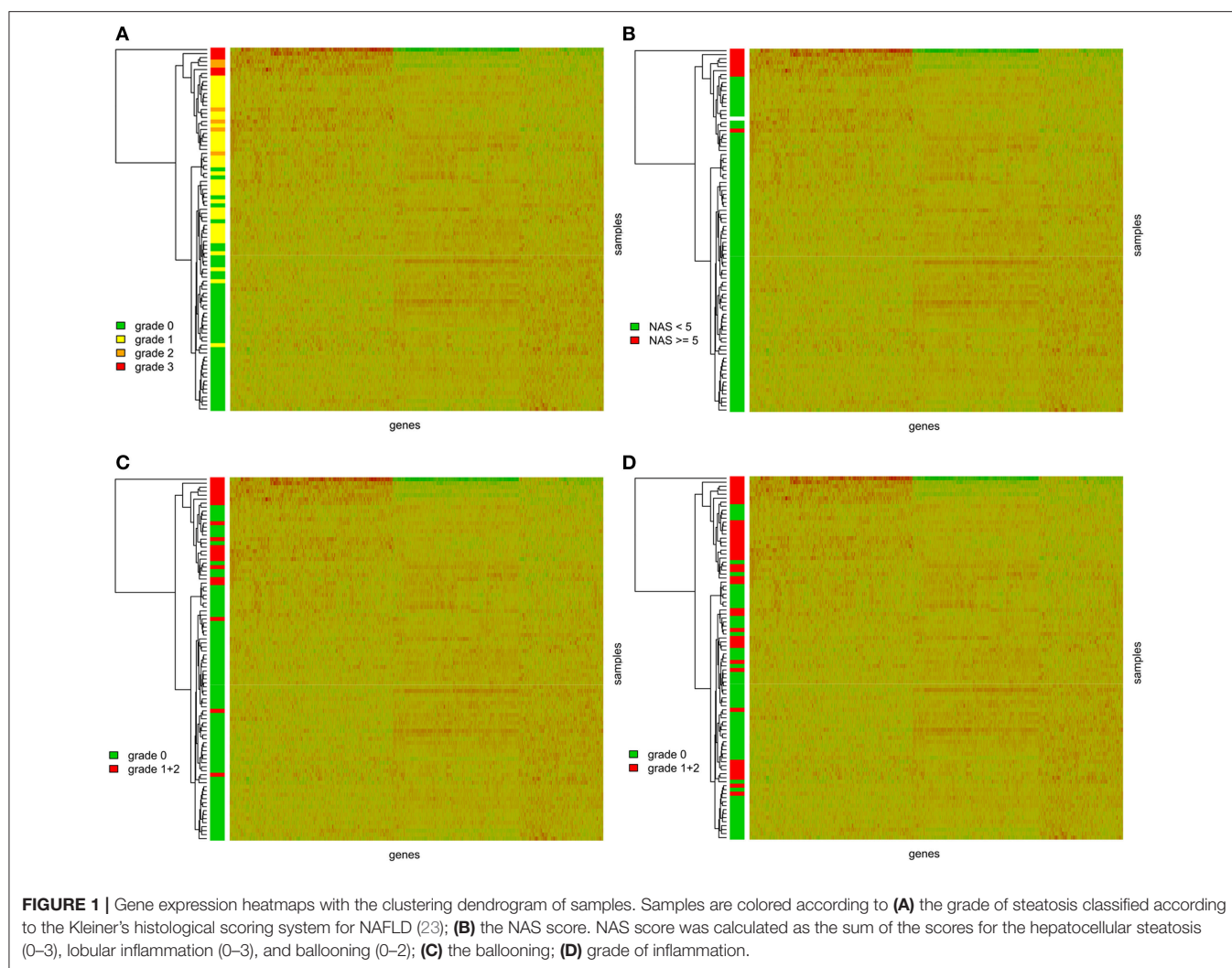
In order to identify the metabolic processes and functions deregulated in steatotic grafts we subjected the set of 747 differentially expressed genes to systematic set of gene

enrichment, clustering and network analyses using several dedicated tools and databases—IPA, KEGG (Kyoto Encyclopedia for Genes and Genomes) and DAVID (Database for Annotation, Visualization and Integrated Discovery).

We identified following significantly enriched biological processes: blood coagulation, bile acid synthesis, and transport, cell redox homeostasis, lipid and cholesterol metabolism, epithelial adherence junction signaling, amino acid metabolism, AMPK and glucagon signaling, transmethylation reactions, and inflammation-related pathways. The list of all significantly deregulated pathways and involved genes is shown in **Table 3**. Employing IPA, we predicted the potential upstream regulators that may modulate the gene expression in steatotic grafts, including *FXR-ligand-FXR-retinoic acid-RXR α* , *NR1H4*, *RXR α* , *HNF1 α* , *HNF4 α* , and *INSR*. Furthermore, using the Regulator effect module, we identified the downstream processes most likely to be perturbed in steatotic grafts, including the transport of specific substances and cellular export machinery (**Figure 2**). Then we proceeded to generate mechanistic networks based on the observed significant expression shifts. The network reaching the highest arbitrary score in IPA is shown in **Figure 3**. Several distinct major nodes are apparent including *PPARA* downregulated in steatotic grafts and *SERPINE1* upregulated in steatosis compared to controls. These results combined show systematic shifts of gene expression that distinguish liver grafts with vs. those without signs of steatosis development.

DISCUSSION

In this study, we identified a set of 747 genes associated with the development of graft steatosis in patients after liver transplantation. Previously, Ryaboshapkina (26) published systematic meta-analysis of available human gene expression studies on liver biopsies and bariatric surgery samples from NAFLD patients. Using regression models, they identified a set of 280 genes showing a consistent association with NAFLD in at least three independent studies. Thirty genes from this list overlap with those identified by us including genes involved in cellular stress (annexins, *DNAJC12*), mitochondrial metabolism (*PPARGC1*, *ATPIA1*), lipid and cholesterol metabolism (*PPARA*, *LPIN1*, ABC transporters, *APOF*), regulation of gene expression (*H2AFY*) or extracellular matrix metabolism (*LAMA 2,3/LAMA5*, *PCOLCE2*). The possible explanation for the difference between our and others' studies may arise from several reasons. First, the cohort represents a rather unique subset in the “garden variety” NAFLD patients given the fact that our study was performed on liver transplant recipients. Therefore, while presumably there are common mechanisms underlying most NAFLD cases, specific changes may be at play in its pathogenesis in the transplanted graft. Also, we cannot exclude the effect of administered immunosuppressive and concomitant therapy (ursodeoxycholic acid, statins). Second, all of the above-mentioned studies were focused on markers of NAFLD progression toward NASH and fibrosis; however, an only minor fraction of our patients exhibited these pathologies.



Traditionally NAFLD is one of the hallmarks of insulin resistance. In our cohort of patients with graft steatosis, we saw clear markers of deregulated glucose metabolism in individuals with steatosis grade 2+3 but not in those with the only mild form of the disease (grade 1). Because of the limited cohort size, we had to combine all steatotic samples into one set in order to perform a transcriptomic analysis. When we performed hierarchical clustering on the selected set of NAFLD-associated genes we observed clear grouping of individuals with grade 3 steatosis but subjects with grade 2 steatosis did not form a separate cluster and were mixed with both grade 1 and grade 3 samples in a diffused manner. This result indicates that the expression of NAFLD-associated genes is altered even in the early stages of NAFLD prior to the onset of insulin resistance.

Within our subset of deregulated genes, we identified metabolic pathways associated predominantly with cholesterol and bile acid metabolism, inflammation, lipid metabolism, blood coagulation, and oxidative stress. Interestingly, similar pathways were unveiled by meta-analysis performed by Wruck et al. (27) on NAFLD datasets published by Ahrens (28), du Plessis (29), Horvath (30), and Wruck (31). Several deregulated

pathways are associated with cholesterol metabolism. There is a growing body of evidence that poor NAFLD outcome, i.e., progression toward NASH and cirrhosis, is not associated with triglyceride accumulation *per se* but rather with altered cholesterol homeostasis and free cholesterol accumulation (32). In our cohort of patients, several pathways profoundly involved in cholesterol metabolism (FXR/RXR activation, LXR/RXR activation, bile acid biosynthesis, bile acid excretion, ABC transporters) were significantly downregulated in steatotic grafts. Consequently, this implicates that cholesterol conversion to bile acids, cholesterol efflux to the bile as well as cholesterol transport to apo-A1 and HDL-C formation were reduced.

Farnesoid X receptor (FXR) pathway downregulation in grafts that developed steatosis corroborates the data on this major bile acid sensor and metabolism regulator (33) involved in the gut-liver axis homeostasis. Observations showing that activation of FXR directly leads to decrease in liver lipogenesis and amelioration of insulin sensitivity served as the rationale for the development of FXR agonists (e.g., obeticholic acid) as potential therapeutic agents for NAFLD (34, 35). Taken together, all these data suggest that alteration of cholesterol

TABLE 3 | Metabolic pathways deregulated in steatotic liver.

Pathway ID	Database	Description	p-value	Involved genes
GO:0002576	DAVID	Platelet degranulation	0.000	<i>A2M, F5, FGA, IGF1, SERPINE1</i>
hsa04610	KEGG	Complement and coagulation cascades	0.000	<i>A2M, CPB2, F5, C2, C9, FGA, KLKB1, MBL2, SERPINC1, SERPINE1</i>
GO:0007596	DAVID	Blood coagulation	0.000	<i>GNAQ, CPB2, F5, FGA, RAC1, SERPINC1</i>
GO:0042730	DAVID	Fibrinolysis	0.008	<i>CPB2, FGA, KLKB1, SERPINE1</i>
	IPA	Coagulation system	0.008	<i>A2M, F5, FGA, KLKB1, SERPINC1, SERPINE1</i>
	IPA	Intrinsic prothrombin activation pathway	0.045	<i>COL5A3, F5, FGA, KLKB1, SERPINC1</i>
GO:0006699	DAVID	Bile acid biosynthetic process	0.000	<i>ABCB11, ACOX2, CYP27A1, CYP7B1, SLC27A5</i>
hsa00120	KEGG	Primary bile acid biosynthesis	0.030	<i>ACOX2, CYP27A1, CYP7B1, SLC27A5</i>
hsa04976	KEGG	Bile secretion	0.010	<i>ABCC2, ATP1B1, SLC27A5, ABCG2, AQP8, ABCB11, ATP1A1</i>
hsa02010	KEGG	ABC transporters	0.042	<i>ABCA6, ABCB11, ABCC2, ABCG1, ABCG2</i>
GO:0045454	DAVID	Cell redox homeostasis	0.022	<i>ADI1, ALDH1A1, ALDH6A1, AOX1, CBS, CYP1A1, CYP27A1, CYP7B1, LIPF, ME1, MTHFD1, MAOB, NOS1, P4HA1, SESN3</i>
hsa01230	KEGG	Biosynthesis of amino acids	0.003	<i>ACO1, ASS1, CTH, CBS, MAT1A, MAT2B, OTC, PSAT1</i>
	IPA	Cysteine biosynthesis/homocysteine degradation	0.027	<i>CBS/ CBSL, CTH</i>
	IPA	S-adenosyl-L-methionine biosynthesis	0.045	<i>MAT1A, MAT2B</i>
hsa03320	KEGG	PPAR signaling pathway	0.036	<i>ACOX2, CYP27A1, PLIN1, PPARα, PCK2, SLC27A5</i>
	IPA	FXR/RXR activation	0.000	<i>ABCB11, ABCC2, AKT1, APOA4, APOF, C9, CYP27A1, FETUB, FGA, GC, ITIH4, ORM1, PCK2, PON3, PPARα, PPARGC1α, SLC27A5</i>
	IPA	LXR/RXR activation	0.013	<i>ABCG1, APOA4, APOF, C9, FGA, GC, IL18RAP, ITIH4, ORM1, PON3</i>
	IPA	AMPK signaling	0.045	<i>AKT1, EP300, INSR, KAT2B, MAPK13, PCK2, PIK3R4, PPARGC1α, PPM1D, RAB11A, RAB27A, SIRT1</i>
hsa04922	KEGG	Glucagon signaling pathway	0.004	<i>AKT1, EP300, GNAQ, CAMK2D, ITPR2, PPARα, PCK2, PPP4R3B, SIRT1</i>
	IPA	LPS/IL-1 mediated inhibition of RXR function	0.013	<i>ABCB11, ABCC2, ABCG1, ACOX2, ALDH1A1, ALDH6A1, IL18RAP, MAOB, MGST2, PPARα, PPARGC1α, SLC27A5, SULT1A2, SULT1C3</i>
	IPA	Acute phase response signaling	0.036	<i>A2M, AKT1, C2, C9, FGA, ITIH4, KLKB1, MAPK13, MBL2, ORM1, SERPINE1</i>
	IPA	Epithelial adherence junction signaling	0.041	<i>ACVRC1, AKT1, APC, ARPC1A, CDH2, CTNNA1, MET, PARD2, RAC1, RAP1A</i>

homeostasis, cholesterol accumulation within hepatocytes, and down-regulation of bile acid synthesis are characteristic features of graft steatosis and may play a role in NAFLD progression.

As expected, we identified deregulation of lipid metabolism-related pathways, i.e., the down-regulation of PPAR signaling and AMPK signaling. This metabolic milieu setting promotes the triglyceride accumulation and weakens their oxidation what further establish the pro-steatotic feedback loop. Our previous study demonstrated that high serum triglyceride level was an independent risk for graft steatosis (17).

Oxidative stress (36) and inflammation (37) belong to the well-recognized components of NAFLD/NASH pathophysiology. In our cohort of patients, we observed significant down-regulation of 15 genes involved in the maintenance of redox homeostasis what implicates at least an increased susceptibility to the oxidative stress. The patients in our cohort did not manifest histological markers of severe inflammation and therefore it is not surprising that we did not find an increased activation of pro-inflammatory cytokines and other related genes that

were reported in transcriptomic studies focused on NAFLD progression toward NASH or fibrosis (26, 38). Furthermore, all liver graft recipients are subjected to immunosuppressive treatment. Nevertheless, we detected the up-regulation of LPS/IL-1 mediated inhibition of RXR function suggesting the presence of subclinical inflammation or infection. RXR inactivation results in attenuation of the expression of hepatic transport and biosynthesis enzymes (ABC, CYP) what, together with other factors, may contribute to the described metabolic alterations in the liver.

We observed down-regulation of S-adenosyl-L-methionine biosynthetic pathway, particularly S-adenosylmethionine synthetases *MAT1A* and *MAT2B*, in steatotic grafts. Their product S-adenosylmethionine is a cofactor involved in methyl group transfers. It is essential for numerous biological processes like methylation of phospholipids that affects membrane fluidity (39), or epigenetic silencing via methylation of gene promoter regions (40). Its role in NAFLD/NASH development was unraveled in experimental animal models (41) and confirmed in

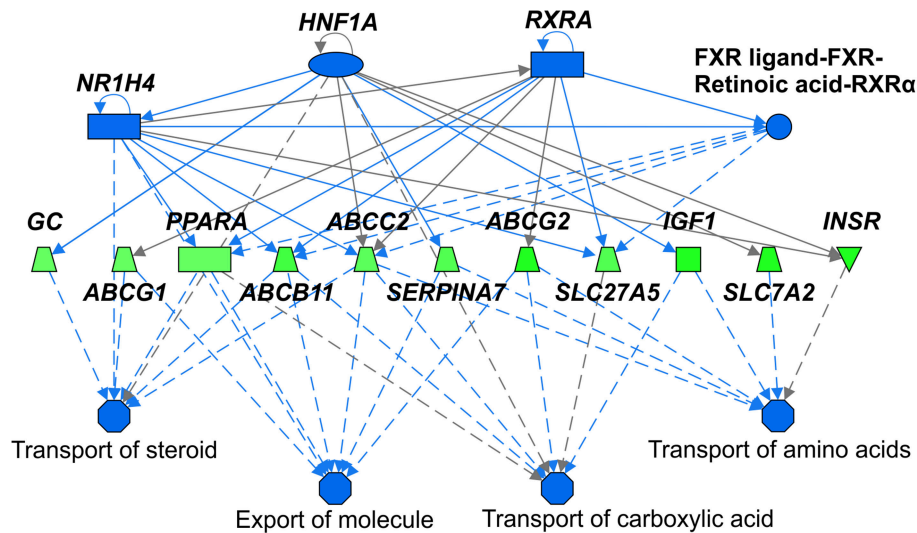


FIGURE 2 | Upstream regulators and downstream processes most affected in steatotic liver grafts. Mechanistic network summarizing main differences in activation (not present) or inhibition (shades of blue) of upstream regulators (top part) and downstream processes (bottom part) in steatotic liver grafts as compared with the non-steatotic transplanted controls. Gene expression comparison between the two groups is shown according to the level of its difference in shades of green (downregulation in steatotic samples) or red (upregulation, not present). Lines depict direct (full lines) or indirect (dashed lines) known interactions. Derivation of the network was performed using Ingenuity Pathways Analysis. The gene symbols are used in accordance with the names approved by the HUGO Gene Nomenclature Committee.

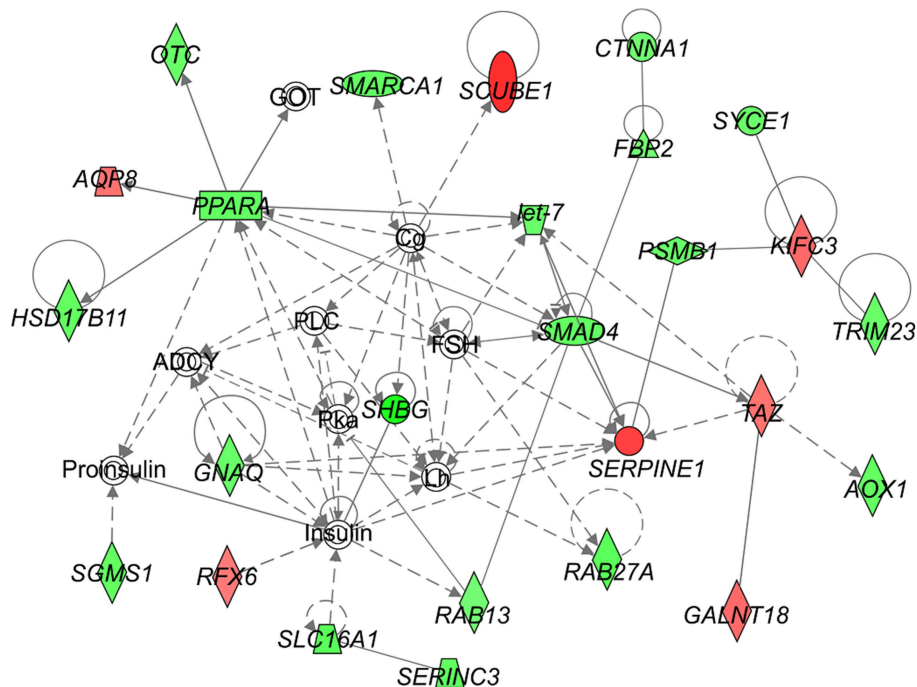


FIGURE 3 | Network analysis of steatosis in transplanted liver grafts. The figure represents the network with the highest score derived using the set of transcripts showing significant difference in expression between steatotic liver grafts and the non-steatotic transplanted controls. The level of difference in gene expression between the two groups is shown in shades of green (downregulation in steatotic samples) or red (upregulation in steatotic samples). Empty symbols indicate entities not found in the experimental dataset of differentially expressed genes. Lines depict direct (full lines) or indirect (dashed lines) known interactions. Derivation of the network was performed using Ingenuity Pathways Analysis. The gene symbols are used in accordance with the names approved by the HUGO Gene Nomenclature Committee.

human studies (42–44). Importantly, *MAT1A* gene expression in the liver can distinguish between patients with mild vs. advanced NAFLD (38, 45). In our cohort with rather mild steatosis, we found modest but significant downregulation of *MAT1A* and *MAT2B*. Together with other findings, our data indicate that the expression of these genes may serve as one of the indicators of NAFLD progression.

In our study, several pathways associated with blood coagulation (intrinsic prothrombin pathway, platelet degranulation, fibrinolysis and complement/coagulation cascade) were significantly deregulated. Rather surprisingly, their activation pattern was incoherent. In accordance with previously published observations that consider NAFLD to be the pro-thrombotic state (46), we confirmed significantly increased expression of *SERPINE1* (*PAI-1*). In contrast, all other genes involved in blood coagulation pathways were down-regulated. We could speculate that this downregulation may either represent a counter-regulatory mechanism compensating for the increased risk of thrombosis or reflect the decreased general proteosynthesis in the transplanted liver.

In an attempt to identify the major drivers of the observed gene expression changes, we identified *NRIH4*, *HNF1 α* , *HNF4 α* , *RXR α* , and *FXR-RXR* among top upstream regulators that govern the expression of the number of downstream targets differentially expressed in steatotic grafts. These transcription factors belong to the family of liver-enriched transcription factors that regulate hepatocyte-specific gene transcription (47). They control numerous functions including glucose and fatty acid metabolism, synthesis of blood coagulation factors, detoxification (CYP450 activity) and biliary metabolism. In our cohort of patients, we did not observe the downregulation of HNF transcription factors itself but we observed deregulation of many of their downstream targets. However, the activity of HNF transcription factors is regulated on several posttranscriptional levels and our findings suggest that deregulation of these transcription factors may represent an early event in the deterioration of overall hepatic function.

Not surprisingly, hierarchical clustering analysis confirmed the association between the selected set of differentially expressed genes and groups of graft recipients based on the level of steatosis. More interestingly, we found that based on the expression patterns of these “pro-steatotic” genes the graft recipients tend to cluster also according to the other histological markers of NAFLD activity score (NAS), inflammation or ballooning (a histological marker of mitochondrial dysfunction). This finding supports the hypothesis that our set of genes may be indicative for the negative prognosis of further NAFLD development in the transplanted liver.

We have found no association between gene expression pattern and graft fibrosis, despite fibrosis is considered an important feature of NAFLD progression. The explanation could be the low number of patients with high NAS score (with advanced NAFLD), which comprised <10% in our study with extensive follow up of our recipients (17). Other reason for this finding could arise from the nature of population studied, where many other factors besides NAFLD could contribute to graft fibrosis, namely subclinical graft rejection, disease recurrence,

and transfer from the donor, which could not be excluded. Importantly we have found no association of gene expression pattern and time elapsed between engraftment and liver biopsy.

Despite its merits, our study has limitations that are important to acknowledge. First, prior further considerations, validation of the ascertained expression profiles and networks must be performed on an independent cohort. Second, the cross-sectional design of the study precludes the use of discrete data points to reflect NAFLD development. The fact that a particular gene or pathway is differentially expressed in NAFLD patients may identify it as a marker but it does not imply its causative role in NAFLD development. Further mechanistic studies are necessary to verify or disprove the role of identified genes in the disease progression.

CONCLUSIONS

In summary, we present transcriptomic profile and pathway deregulation patterns distinguishing steatosis-prone from steatosis-free transplanted liver grafts. While some parts of the identified molecular signature are shared with those found in NAFLD in non-transplanted individuals, the unique revealed characteristics may, upon validation in independent studies, lay the groundwork for the establishment of predictive assessment of NAFLD risk in liver grafts.

ETHICS STATEMENT

The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Joint Ethics Committee of the Institute for Clinical and Experimental Medicine and Thomayer Hospital. All patients signed informed consent. Patients received different immunosuppression protocols based either on cyclosporin or tacrolimus depending on transplantation era. All relevant clinical data are provided in **Supplementary Table S1**.

AUTHOR CONTRIBUTIONS

OŠ, MC, and PT designed the study, analyzed the data, discussed the manuscript, coordinated and directed the project, developed the hypothesis, and wrote the manuscript. IM, DE, and PT recruited the patients. HD, MH, MB, ND, and BC performed the analyses. VČ and LŠ analyzed the data and performed the statistical evaluation. All authors read and approved the final manuscript and joined the analysis and interpretation of data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2019.00270/full#supplementary-material>

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PŘÍLOHA XIV

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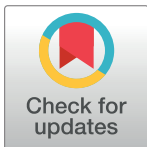
RESEARCH ARTICLE

Serum miR-33a is associated with steatosis and inflammation in patients with non-alcoholic fatty liver disease after liver transplantation

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Abstract

Background & aims

MiR-33a has emerged as a critical regulator of lipid homeostasis in the liver. Genetic deficiency of miR-33a aggravates liver steatosis in a preclinical model of non-alcoholic fatty liver disease (NAFLD), and relative expression of miR-33a is increased in the livers of patients with non-alcoholic steatohepatitis (NASH). It was unknown whether miR-33a is detectable in the serum of patients with NAFLD. We sought to determine whether circulating miR-33a is associated with histological hepatic steatosis, inflammation, ballooning or fibrosis, and whether it could be used as a serum marker in patients with NAFLD/NASH.

Methods

We analysed circulating miR-33a using quantitative PCR in 116 liver transplant recipients who underwent post-transplant protocol liver biopsy. Regression analysis was used to determine association of serum miR-33a with hepatic steatosis, inflammation, ballooning and fibrosis in liver biopsy.

Results

Liver graft steatosis and inflammation, but not ballooning or fibrosis, were significantly associated with serum miR-33a, dyslipidemia and insulin resistance markers on univariate analysis. Multivariate analysis showed that steatosis was independently associated with serum miR-33a, ALT, glycaemia and waist circumference, whereas inflammation was independently associated with miR-33a, HbA1 and serum triglyceride levels. Receiver operating characteristic analysis showed that exclusion of serum miR-33a from multivariate analysis

Abbreviations: AIH, autoimmune hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUROC, area under the receiver operating characteristic curve; BMI, body mass index; Hb1Ac, haemoglobin A1c; HBV, hepatitis B virus; HCV, hepatitis C virus; HDL-cholesterol, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; LDL-cholesterol, low-density lipoprotein cholesterol; LTx, liver transplant; MDRD-GFR, modification of diet in renal disease—glomerular filtration rate; miRNA, micro ribonucleic acid; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PCR, polymerase chain reaction; QUICKI, quantitative insulin sensitivity check index; RNA, ribonucleic acid; ROC, receiver operating characteristics; SPSS, statistical package for the social sciences; SREBP, sterol regulatory element-binding protein; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

resulted in non-significant reduction of prediction model accuracy of liver steatosis or inflammation.

Conclusions

Our data indicate that circulating miR-33a is an independent predictor of liver steatosis and inflammation in patients after liver transplantation. Although statistically significant, its contribution to the accuracy of prediction model employing readily available clinical and biochemical variables was limited in our cohort.

Introduction

NASH has become a major cause of cirrhosis and hepatocellular carcinoma, and represents one of the most common indications for liver transplant in the United States [1]. NASH develops in the context of hepatic steatosis, a process during which hepatocytes accumulate excessive amount of lipids via mechanisms that have only recently been characterized [2] [3]. Liver steatosis develops in up to 50% of liver transplant recipients and shares many etiopathogenetic factors with steatosis in NAFLD patients in general population [4–7].

Recent reports have highlighted the critical role of microRNAs in regulation of hepatic steatosis. MicroRNAs negatively regulate expression of a wide variety of proteins involved in lipid metabolism, and thus post-transcriptionally modify lipolysis, lipogenesis and lipoprotein turnover [8]. In addition, microRNAs are exported from liver cells and their profile in the serum correlates with the underlying mechanism of liver pathology in preclinical models of liver disease [9].

Numerous studies have shown that miR-33 is a critical microRNA regulating metabolism of fatty acids and cholesterol by cooperating with transcription factors SREBP-1 and -2 (sterol-regulatory element-binding protein-1 and -2), respectively, and by regulating expression of genes of lipid and cholesterol synthesis [10] [11]. Mice deficient in miR-33a exposed to high-fat diet develop severe fatty liver disease, compared to wild-type littermates [12]. In a recently published clinical trial, increased expression of miR-33a in the liver was associated with steatohepatitis in morbidly obese humans [13].

Considering the crucial role of miR-33a in hepatic lipid metabolism and its increased presence in steatotic livers in humans, we hypothesized that miR-33a will be increased in the serum of patients with NAFLD and could be used as non-invasive diagnostic marker. We evaluated this hypothesis in a cohort of patients after liver transplant and used this cohort because of its well-defined demography, clinical data, meticulous follow-up and availability of protocol liver biopsy. Here we show that circulating miR-33a is significantly increased in serum of patients with fatty liver disease after liver transplantation, and that circulating miR-33a is independently associated with steatosis and lobular inflammation in liver biopsy.

Patients and methods

Patients

One hundred and sixteen liver transplant recipients undergoing protocol liver biopsy during standard post-transplant follow up between May 2015 and May 2017 were enrolled in this prospective study. We excluded patients with known or suspected alcohol abuse after liver transplantation (LTx), with HCV infection of the graft or with corticosteroids administration

higher than 5 mg of prednisone per day. The most common indications for LTx in our cohort were biliary disorders (PBC, PSC, overlap PSC/AIH; 33 patients, 28.5%), alcoholic liver disease (28 patients, 24.1%) and HBV, autoimmune hepatitis and cryptogenic cirrhosis (8 patients, 7% each, [S1 Fig](#)). Only one patient in our cohort was indicated for LTx because of NASH (0.9%). All patients included in the study signed informed consent for participation in the study. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Joint Ethics Committee of the Institute for Clinical and Experimental Medicine and Thomayer Hospital.

Clinical data and laboratory testing

Blood samples were collected in fasted state in the morning prior to liver biopsy. We measured serum glucose, HbA1c, C-peptide, insulin, triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol, bilirubin, ALT, AST, blood count, and creatinine. All these analyses were performed in an accredited biochemistry laboratory according to the standard manufacturer's protocols. Homeostatic model assessment index (HOMA-IR), the quantitative insulin-sensitivity check index (QUICKI) and the glomerular filtration rate (MDRD-GFR) were calculated using standard formulas. We also analysed clinical and anthropometric data, including age, body mass index (BMI), waist circumference, time from liver transplantation (LTx), presence of comorbidities such as hypertension, diabetes, and prescription medications (especially immunosuppressive drugs). Next, we analysed clinical and anthropometric data of liver graft donors.

Circulating miRNAs relative expressions analysis

Blood samples for miRNA relative expressions analysis were collected at the same time as blood samples for biochemical testing on same day when liver biopsy was performed. Serum was separated by centrifugation and stored at -80°C. MiRNAs were isolated from serum using miRCURY RNA isolation kit for biofluids (EXIQON). Before isolation, serum samples were spiked with control miRNA cel-miR-39-3p (QIAGEN), which served as control of quality of isolation and as reference gene. RNACarrier—bacteriophage MS2 (Roche) was added for better yield.

Liver biopsy

Liver biopsies were performed using the Menghini technique. Histologic sections of formalin-fixed, paraffin-embedded liver tissue were routinely stained according to the standard protocols. All samples were reviewed by an experienced histopathologist who was blinded to miRNA analysis. Liver biopsies were graded according to the scale published by Kleiner, where four morphological features related to NAFLD were semi-quantitatively appraised on light microscopy: steatosis, lobular inflammation, hepatocellular ballooning and fibrosis [[14](#)].

Statistical analysis

One-way ANOVA or Mann-Whitney test, when appropriate, were used for comparison of continuous variables, and chi-square or Fisher's exact test were used for comparison of proportions. To assess the role of miRNAs, clinical and demographic variables in clinical outcomes, stepwise logistic regression was used. Predicted probability values for clinical outcomes calculated in regression models were used to construct receiver operating characteristic curves. Goodness-of-fit statistics were assessed for all regression models. The level of significance was set at P less than 0.05. All P values were two sided. Statistical calculations were performed using SPSS Statistics Version 25 (IBM Corporation).

Results

Cohort characteristics

Demographic and clinical data of all patients enrolled in the study are shown in Table 1 and S1 Table. In total, 116 patients (median BMI was 25.3 kg/m²) in stable clinical condition participated in the study (60 men and 56 women). Median time from LTx was 2.2 years (minimum: 1 year, maximum: 20.5 years). Thirty-one patients had diabetes and 70 patients had hypertension. On average, plasma levels of aminotransferases were within normal range (Table 1).

Donor characteristics and immunosuppression regimens are described in S1 Table.

Liver biopsy findings are shown in Fig 1. Steatosis, lobular inflammation, ballooning of any grade, and fibrosis of any stage were present in 53%, 40%, 18% and 100% patients, respectively (Fig 1). Advanced degree of steatosis (≥ 2), lobular inflammation (≥ 2), ballooning (≥ 2) or fibrosis (≥ 3) was present in 13%, 8%, 2% and 18% patients, respectively (Fig 1).

Table 1. Demographic and clinical characteristics of enrolled patients. Data are given as N (%) or median (1st - 3rd quartile).

	N = 116	
Male gender	60 (52%)	
Age [years]	56.8 (42.0–64.8)	
Time from LTx [days]	805 (446–2381)	
BMI [kg/m²]	25.3 (22.4–29.5)	
Waist circumference [cm]	96 (85–107)	
Hypertension	70 (60.3%)	
Diabetes	31 (27%)	
Statins	19 (16.4%)	
Laboratory values:		Reference range:
Bilirubin [μmol/L]	12.2 (9.2–18)	3.4–20
AST [μkat/L]	0.40 (0.33–0.47)	0.17–0.75
ALT [μkat/L]	0.45 (0.37–0.59)	0.17–1.17
Glycaemia [mmol/L]	5.3 (4.9–6.0)	3.6–5.59
HbA1c [%]	37.0 (32.8–41.3)	20–42
C-peptide [nmol/L]	0.8 (0.6–1.1)	0.26–1.03
Insulinemia [mIU/mL]	7.6 (4.8–10.0)	2.1–22
HOMA-IR	1.8 (1.1–2.6)	0.5–1.4
QUICKI	0.35 (0.33–0.38)	0.45–0.339
Triglycerides [mmol/L]	1.1 (0.8–1.6)	0.5–1.69
Total cholesterol [mmol/L]	4.5 (3.8–5.0)	2.9–5
LDL-cholesterol [mmol/L]	2.6 (2.0–3.1)	1.2–3
HDL-cholesterol [mmol/L]	1.2 (1.0–1.4)	1–2.1
Creatinine [μmol/L]	92 (79–115)	49–90
MDRD-GFR [ml/min/1.73²]	68 (53–80)	> 80
WBC [x10⁹/L]	6.1 (4.9–7.3)	4–10
Erythrocytes [x10¹²/L]	4.7 (4.3–5.1)	3.8–5.2
Haemoglobin [g/L]	136 (125–150)	120–160
Thrombocytes [x10³/L]	174 (143–219)	150–400
CRP [mg/L]*	2.4 (1–4.5)	0–5

* CRP was measured only in 59 patients from the cohort

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Distribution of liver histology findings

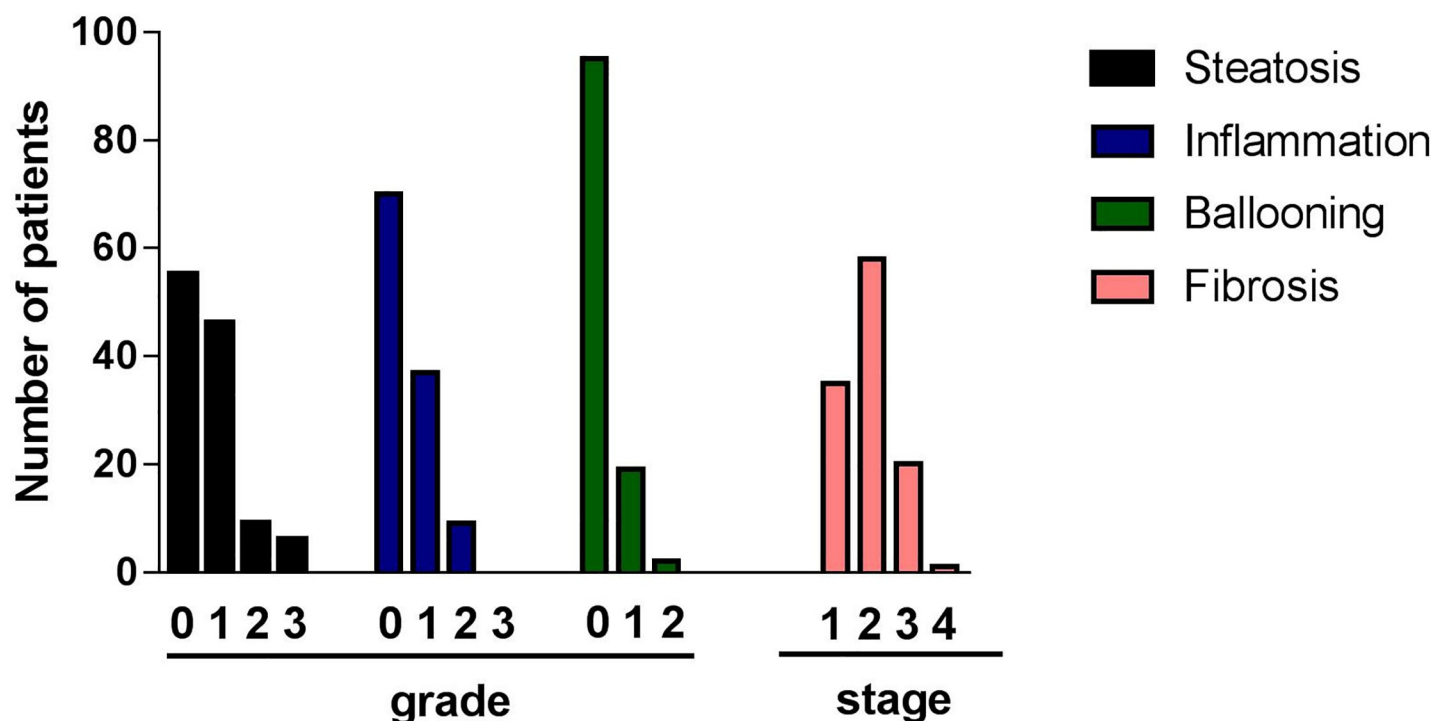


Fig 1. Distribution of liver histology findings in our cohort. Steatosis, lobular inflammation, ballooning of any grade, and fibrosis of any stage were present in 53%, 40%, 18% and 100% patients, respectively. Advanced degree of steatosis (≥ 2), lobular inflammation (≥ 2), ballooning (≥ 2) or fibrosis (≥ 3) was present in 13%, 8%, 2% and 18% patients, respectively.

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Circulating miR-33a is increased in patients with liver graft steatosis and lobular inflammation

Given its crucial role in lipid metabolism regulation, we compared serum levels of miR-33a in patients with no steatosis and patients with liver graft steatosis (Table 2). In order to validate this analysis, we also evaluated serum levels of two miRNAs with known association with NASH (miR-34a and miR-122, positive validation) and one miRNA not previously associated with NASH (miR-106b, negative validation). Next, we compared serum of those four miRNAs in patients with and without lobular inflammation, with and without ballooning and with and without fibrosis.

Table 2. Associations of miRNAs with liver biopsy findings. Top row shows fold changes and 95% confidence intervals. Bottom row shows p-values derived from 1-way ANOVA. Statistically significant results are printed in bold.

	Steatosis	Inflammation	Ballooning	Fibrosis
miR-33a	1.17 (1.04–1.30) 0.034	1.25 (1.08–1.42) 0.003	1.10 (0.86–1.35) 0.30	1.02 (0.93–1.11) 0.79
miR-34a	1.42 (0.74–2.10) 0.28	1.96 (0.88–3.04) 0.030	2.23 (0.27–4.20) 0.86	1.38 (0.81–1.96) 0.39
miR-122	1.19 (0.92–1.46) 0.29	1.46 (1.06–1.87) 0.031	1.57 (1.02–2.12) 0.023	0.76 (0.60–0.92) 0.13
miR-106b	1.06 (0.73–1.38) 0.62	1.04 (0.74–1.34) 0.83	1.18 (0.57–1.80) 0.36	1.05 (0.79–1.36) 0.82

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We found that serum miR-33a was associated with liver graft steatosis and lobular inflammation. Specifically, patients with steatosis on liver biopsy had 17% (95% CI 1.04–1.30) increase of serum miR-33a, compared to subjects without steatosis ($p = 0.034$, Table 2), and patients with lobular inflammation on liver biopsy had 25% (95% CI 1.08–1.42) increase of serum miR-33a in comparison to subjects without inflammation ($p = 0.003$, Table 2). There was no association between miR-33a and ballooning or fibrosis (Table 2). Consistent with published reports, miR-34a and miR-122, used for positive validation, but not miR-106, used for negative validation, showed a significant increase in patients with liver inflammation [15].

Circulating miR-33a is an independent predictor of graft steatosis in context of clinical and laboratory characteristics

The increased serum levels of miR-33a in patients with steatosis prompted us to investigate whether miR-33a has a potential role as non-invasive biomarker of graft steatosis. We also postulated that if such role exists, miR-33a should be associated with graft steatosis independently of demographic, clinical, and biochemical variables.

To answer this question, we first used univariate analysis to identify demographic, clinical and biochemical variables associated with graft steatosis. To do so we divided all patients into group without steatosis and group with steatosis (Table 3). This analysis showed that in addition to serum miR-33a, steatosis was also associated with laboratory or demographic variables including age, BMI, waist circumference, diabetes, treatment with statins, ALT, glycaemia, HbA1c, C-peptide, HOMA-IR and triglycerides. Multivariate regression analysis showed that miR-33a was independent predictor of graft steatosis. For each fold-change of miR-33a serum level, the odds ratio for graft steatosis increased 2.9-fold (Table 4). In addition, glycaemia, waist circumference and ALT were found to be independent predictors of graft steatosis as well, consistently with previous reports [4].

Circulating miR-33a is an independent predictor of liver graft inflammation in context of clinical and laboratory characteristics

Next, we asked whether miR-33a is independently associated with liver graft inflammation. Hence, we divided all patients into two groups. The first one included patients without lobular inflammation in their liver biopsy and the second one included patients with present lobular inflammation. Based on the results of univariate analysis, we analysed miR-33a along with age, BMI, waist circumference, diabetes, glycaemia, HbA1c, C-peptide, HOMA-IR, triglycerides, total cholesterol plasma concentration, glomerular filtration rate (MDRD-GFR) and C-reactive protein (CRP) (Table 5). In addition, we found that liver graft inflammation was also associated with miR-34a and miR-122 in univariate analysis (Table 2 and Table 5). Multivariate regression analysis showed that miR-33a, but not miR-34a or miR-122 was an independent predictor of lobular inflammation. For each fold-change of miR-33a serum level, the odds ratio for lobular inflammation was increased 4-fold (Table 6). In addition, HbA1c and triglycerides were found to be independent predictors of lobular inflammation as well.

Clinical utility of miR-33a in non-invasive diagnosis of liver graft steatosis or inflammation

Multivariate regression showed that miR-33a, glycaemia, waist circumference and ALT represent independent predictors of hepatic steatosis. Based on the Wald's statistics, which assesses the relative contribution of each variable to the outcome in regression model, we hypothesized that the relative significance of miR-33a (Wald's coefficient 4.3) in prediction of liver graft

Table 3. Steatosis. Univariate analysis of the effect of clinical and laboratory findings on developing graft steatosis. Data are given as N (%) or median (1st - 3rd quartile). Significant results are printed in bold. Normal ranges of biochemical values are mentioned in Table 1. Non-steatosis group includes subjects without histologically proven graft steatosis ($\leq 5\%$ of hepatocytes); steatosis group comprises all patients with steatosis grade 1–3.

	Non-steatosis N = 55 (47.4%)	Steatosis N = 61 (52.6%)	p-value
Male gender	23 (41.8%)	37 (60.7%)	0.06
Age [years]	48.7 (37.5–59.2)	60.1 (50.8–65.9)	0.001
Time from LTx [days]	798 (471–3711)	894 (445–1916)	0.51
BMI [kg/m²]	23.6 (21.5–27.4)	27.3 (24.5–31.1)	< 0.001
Waist circumference [cm]	88 (81.5–96)	103 (92–111)	< 0.001
Hypertension	29 (52.7%)	41 (67.2%)	0.13
Diabetes	8 (14.5%)	23 (37.7%)	0.006
Statins	5 (9.1%)	14 (23%)	0.049
Liver function tests:			
Bilirubin [μmol/L]	12.1 (9.4–19.2)	12.4 (9.1–17.1)	0.67
AST [μkat/L]	0.39 (0.32–0.45)	0.40 (0.34–0.50)	0.12
ALT [μkat/L]	0.41 (0.34–0.49)	0.52 (0.40–0.69)	0.021
Glucose metabolism:			
Glycaemia [mmol/L]	5.2 (4.9–5.6)	5.5 (4.9–6.5)	0.003
HbA1c [%]	36 (32–39)	38 (33–45)	0.010
C-peptide [nmol/L]	0.7 (0.6–0.9)	0.8 (0.6–1.2)	0.030
Insulinemia [mIU/mL]	7.2 (4.6–9.1)	7.6 (5.0–11.2)	0.06
HOMA-IR	1.7 (1.0–2.2)	2.1 (1.1–3.2)	0.01
QUICKI	0.35 (0.34–0.38)	0.34 (0.32–0.38)	0.19
Lipid metabolism:			
Triglycerides [mmol/L]	1.0 (0.8–1.3)	1.2 (0.9–1.9)	0.004
Total cholesterol [mmol/L]	4.4 (3.8–4.9)	4.5 (3.8–5.2)	0.27
LDL-cholesterol [mmol/L]	2.5 (2.1–3.0)	2.6 (1.9–3.2)	0.42
HDL-cholesterol [mmol/L]	1.3 (1.0–1.5)	1.1 (0.9–1.4)	0.07
Renal function:			
Creatinine [μmol/L]	91 (76–101)	99 (82–121)	0.08
MDRD-GFR [ml/min/1.73²]	73 (54–82)	63 (51–77)	0.11
Blood count:			
WBC [x10⁹/L]	5.9 (4.8–7.3)	6.2 (5.1–7.5)	0.86
Erythrocytes [x10¹²/L]	4.6 (4.3–5.0)	4.8 (4.3–5.1)	0.39
Haemoglobin [g/L]	136 (122–148)	136 (128–152)	0.39
Thrombocytes [x10³/L]	184 (142–238)	172 (145–216)	0.34
CRP [mg/L]*	1.8 (0.8–3.1)	2.9 (1.3–5.7)	0.06
miRNAs:			
miR-33a	1.19 (0.96–1.48)	1.29 (0.89–1.76)	0.034
miR-34a	1.37 (0.91–2.65)	1.76 (1.15–3.02)	0.28
miR-106b	1.62 (1.06–2.18)	1.37 (1.03–2.12)	0.62
miR-122	3.31 (1.44–5.95)	3.99 (2.05–8.44)	0.29

* CRP was measured only in 59 patients from the cohort.

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steatosis will be, at most, at par with clinical and laboratory parameters (waist circumference (Wald's coefficient 13.6), glycaemia (Wald's coefficient 4.5), ALT (Wald's coefficient 4.93)) (Table 4). To confirm or reject this hypothesis, we constructed a receiver operating

Table 4. Steatosis. Multivariate logistic regression involving all significant variables from univariate analysis (including miR-33a). ALT underwent logarithmic transformation.

	p-value	Odds ratio	95% CI	Wald
miR-33a	0.039	2.86	1.06–7.75	4.27
waist circumference	< 0.001	1.07	1.03–1.11	13.63
glycemia	0.034	1.51	1.03–2.21	4.48
ALT	0.026	3.71	1.17–11.79	4.93

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characteristics (ROC) curve, which showed that inclusion of all four variables rendered 80.2% accuracy of the regression model for steatosis (Fig 2). Exclusion of miR-33a from the model decreased the accuracy of the regression model by 0.7% to 79.5% (Fig 2).

Utilizing a similar approach for assessment of relative contribution of miR-33a to lobular inflammation in regression model, we found that the area under the curve (AUROC) for all three independent predictors (serum miR-33a, triglycerides, HbA1c) was 75.7%, whereas exclusion of miR-33a decreased accuracy to 74.7% (Fig 3). Taken together, although serum miR-33a was an independent predictor of liver graft steatosis or lobular inflammation, its contribution to the predictive model was limited.

Discussion

Our results have shown that serum levels of miR-33a are significantly increased in liver transplant recipients with graft steatosis or lobular inflammation. Using multivariate regression analysis we showed that miR-33a is an independent predictor of liver graft steatosis or lobular inflammation in the context of clinical and biochemical variables. To the best of our knowledge this is the first study that described increased circulating miR-33a in patients with NAFLD, and these novel findings are consistent with our current understanding of the role of miR-33a in lipid metabolism. We believe, that as the transplant recipients with graft steatosis share most of risk factors associated with NAFLD in general population, particularly high prevalence of risk alleles of *PNPLA3* rs738409 and *TM6SF2* rs58542926 gene polymorphism in corresponding donors, higher BMI, higher triglycerides plasma concentration and diabetes mellitus [4,16,17], our finding could probably, with caution, also apply to regular NAFLD/NASH patients.

Our finding of increased serum level of miR-33a in patients with liver graft steatosis is consistent with the known role of miR-33a in lipid metabolism. First, miR-33 is encoded by an intronic sequence within genetic loci encoding SREBP-1 and SREBP-2, two transcription factors critically involved in regulation of fatty acid and cholesterol homeostasis [10] [11]. Second, expression of miR-33, along with expression of SREBP-1 and -2, is upregulated by insulin resistance, which has a causal role in pathogenesis of NAFLD [18]. Third, suppression of miR-33a by genetic approaches or by therapeutic RNA in preclinical models of NAFLD [12] [19] resulted in the development of liver steatosis or in major changes of plasma lipoprotein profile. Although our findings suggest that the increased serum levels of miR-33 may reflect increased expression of SREBP-1 and -2 driving an increased lipid and cholesterol synthesis, we cannot completely rule out the possibility that increased miR-33 reflects insulin resistance rather than increased lipogenesis. Similarly, we cannot attribute the increased levels of miR-33 in the serum solely to its release from liver cells as miR-33 (and SREBP-1) are expressed in all tissues metabolizing lipids or cholesterol, albeit to a lesser degree compared to hepatocytes [11].

Although we identified miR-33a as independent predictor of liver graft steatosis and inflammation, it needs to be emphasized that statistical significance does not always imply clinical relevance. Using ROC analysis we showed only limited contribution of miR-33a to prediction modelling of steatosis and lobular inflammation in the context of other independent

clinical or demographic predictors. This finding of limited clinical relevance is consistent with previously published reports, that showed that AUROC of microRNA (namely miR-34a miR-122) in the diagnostics of lipid accumulation or liver inflammation was between 0.6–0.8 [20].

Table 5. Lobular inflammation. Univariate analysis of the effect of clinical and laboratory findings on developing liver graft inflammation. Data are given as N (%) or median (1st - 3rd quartile). Significant results are printed in bold. Normal ranges of biochemical values are mentioned in Table 1. Non-lobular inflammation group includes subjects without histologically proven lobular inflammation; lobular inflammation group comprises all patients with lobular inflammation grade 1–3.

	Non- inflammation N = 70 (60.3%)	Lobular inflammation N = 46 (39.7%)	p-value
Male gender	35 (50%)	25 (54.3%)	0.71
Age [years]	52.5 (40.9–62.4)	60.1 (50.8–65.3)	0.038
Time from LTx [days]	898 (482–3680)	771 (433–1901)	0.39
BMI [kg/m²]	24.2 (21.9–28.2)	27.7 (24.5–30.9)	0.002
Waist circumference [cm]	91 (83–103)	103 (93–113)	< 0.001
Hypertension	40 (57.1%)	30 (65.2%)	0.44
Diabetes	11 (15.7%)	20 (43.5%)	0.001
Statins	10 (14.3%)	9 (19.6%)	0.46
Liver function tests:			
Bilirubin [μmol/L]	12.4 (9.4–19.3)	12 (9.1–16.3)	0.42
AST [μkat/L]	0.40 (0.32–0.45)	0.39 (0.34–0.54)	0.15
ALT [μkat/L]	0.44 (0.36–0.52)	0.53 (0.39–0.70)	0.09
Glucose metabolism:			
Glycaemia [mmol/L]	5.2 (4.9–5.7)	5.6 (5.0–6.8)	0.003
HbA1c [%]	36 (32–40)	39 (34–47)	< 0.001
C-peptide [nmol/L]	0.7 (0.5–0.9)	0.8 (0.7–1.1)	0.032
Insulinemia [mIU/mL]	7.1 (5.0–9.5)	8.1 (4.3–11.4)	0.13
HOMA-IR	1.6 (1.1–2.5)	2.2 (1.0–3.2)	0.037
QUICKI	0.36 (0.33–0.38)	0.34 (0.32–0.38)	0.21
Lipid metabolism:			
Triglycerides [mmol/L]	1.0 (0.8–1.3)	1.3 (1.0–2.1)	< 0.001
Total cholesterol [mmol/L]	4.2 (3.7–4.9)	4.7 (4.1–5.3)	0.019
LDL-cholesterol [mmol/L]	2.4 (1.9–3.0)	2.7 (2.3–3.2)	0.06
HDL-cholesterol [mmol/L]	1.3 (1.0–1.5)	1.1 (0.9–1.4)	0.08
Renal function:			
Creatinine [μmol/L]	91 (77–104)	100 (83–123)	0.07
MDRD-GFR [ml/min/1.73²]	73 (54–83)	63 (50–76)	0.032
Blood count:			
WBC [x10⁹/L]	5.8 (4.8–7.2)	6.6 (5.5–7.9)	0.08
Erythrocytes [x10¹²/L]	4.7 (4.4–5.0)	4.8 (4.3–5.2)	0.40
Haemoglobin [g/L]	135 (125–148)	142 (125–151)	0.56
Thrombocytes [x10³/L]	173 (140–218)	176 (146–223)	0.60
CRP [mg/L]*	1.8 (0.75–3.4)	3.1 (1.4–5.2)	0.027
miRNAs:			
miR-33a	1.18 (0.92–1.50)	1.35 (0.93–1.92)	0.003
miR-34a	1.37 (0.94–2.39)	2.09 (1.16–3.22)	0.03
miR-106b	1.41 (1.06–2.06)	1.38 (1.01–2.41)	0.83
miR-122	3.34 (1.46–5.61)	4.97 (1.98–10.14)	0.031

* CRP was measured only in 59 patients from the cohort.

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Table 6. Lobular inflammation. Multivariate logistic regression involving all significant variables from univariate analysis (including miR-33a, miR-34a, miR-122).

	p-value	Odds ratio	95% CI	Wald
miR-33a	0.006	3.95	1.49–10.46	7.62
HbA1c	0.049	1.07	1.00–1.15	3.87
Triglycerides	0.053	2.40	0.99–5.82	3.74

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Not only miRNAs, that failed to act as useful biomarker of NAFLD/NASH but most of other recently suggested noninvasive biomarkers are lacking sufficient discriminatory power, or poses other shortcomings preventing them from use in routine clinical practice [21].

The inherent drawback of using miRNAs as non-invasive markers of liver disease relies in their mechanism of activation, which is usually dependent upon gene or metabolic pathways they are part of. If, as it is often in the case of NAFLD, those genes are involved in glucose or lipid regulation, then readily available laboratory markers of insulin resistance or dyslipidemia will provide similar diagnostic information and therefore it would be of no surprise that adding the corresponding miRNA does not further contribute to the diagnostic model accuracy.

We used a cohort of liver transplant recipients for our study because we believe that these represent appropriate in vivo model of liver steatosis and steatohepatitis demonstrating most of the epidemiologic and genetic risks described in general population [5,16,17]. We are not

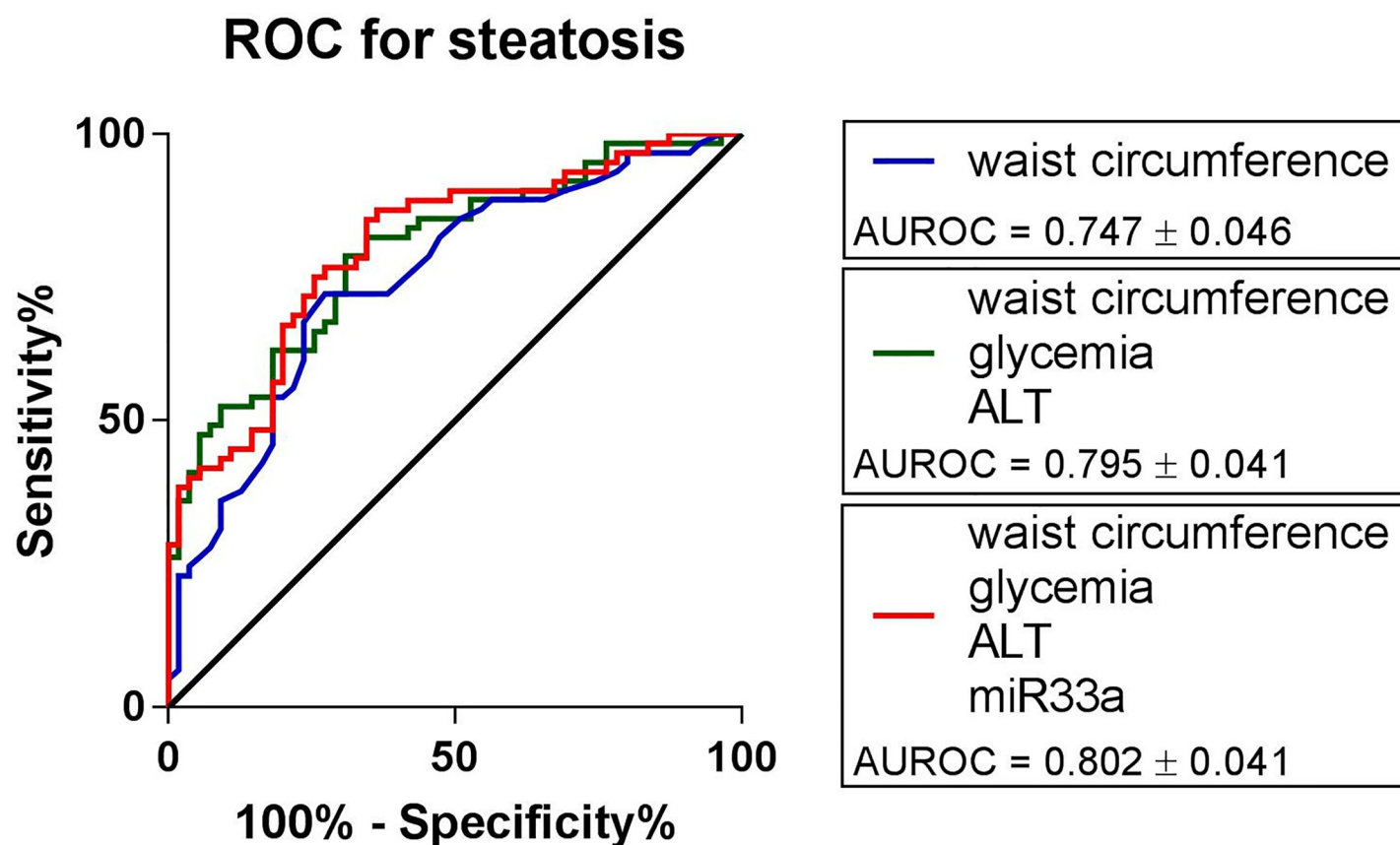


Fig 2. ROC curve for liver graft steatosis. ROC curve for steatosis shows that exclusion of miR-33a from the model decreased the accuracy of the regression model by 0.7%.

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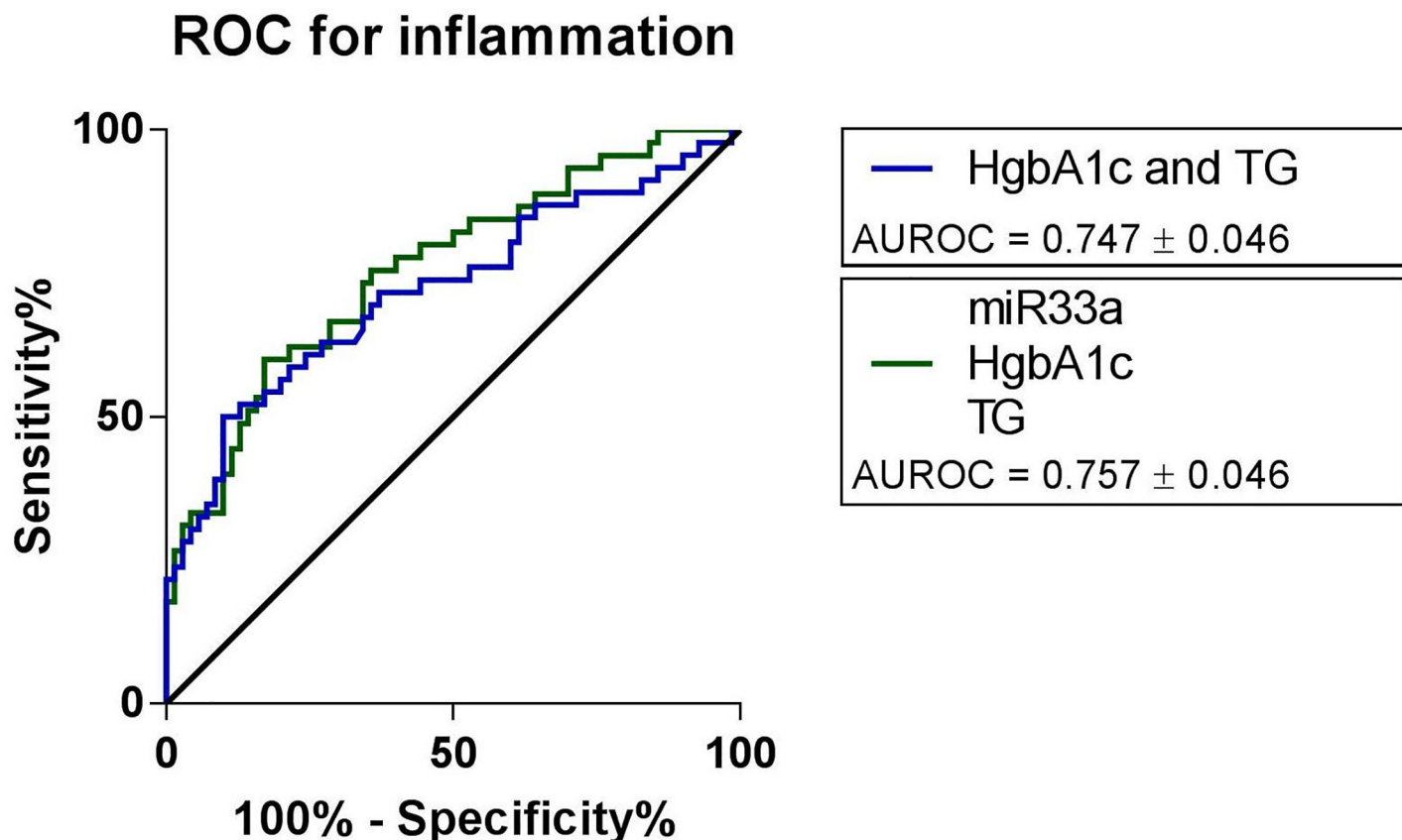


Fig 3. ROC curve for lobular inflammation. ROC curve for liver graft inflammation shows that exclusion of miR-33a from the model decreased the accuracy of the regression model by 1%.

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aware of any difference between pathogenetic mechanisms employed in NAFLD in general population and in liver transplant recipients. It was also described previously that graft steatosis is not transferred from the donor as it rather resolves shortly after transplantation [22–24]. Despite we have found no association between immunosuppressive treatment and development of graft steatosis after liver transplantation in cohort of 268 liver transplant recipients, this influence cannot be easily ruled out [17]. The advantage of this cohort is that graft steatosis after liver transplant develops with high prevalence (20–40% on average) and faster than in general population [5] [6]. In addition, liver transplant recipients have close follow-ups including protocol liver biopsy, which is still the gold standard in diagnosis of NAFLD. We also used the two most investigated miRNAs (miR-34a and miR-122) in non-transplanted patients with NAFLD as positive validation of our results and they were also upregulated in case of liver transplant recipients. Next, we are aware that most of our patients had mild NAFLD phenotype, and we believe that including patients with more advanced NAFLD could unravel greater contribution of serum miR-33a to the liver phenotypes investigated in this study.

Nevertheless it is necessary to keep in mind that transplanted patients are specific cohort, due to immunosuppression including steroids, (described in detail in S1, S2 and S3 Tables), and host-graft interactions which are nowadays not completely understood. Taken all together, patients with NAFLD after liver transplantation has many similarities with the general NAFLD population and probably serve as valuable model, but the findings should apply to general population with considerable caution.

In conclusion, we have shown that circulating miR-33a is associated with steatosis and inflammation in patients with non-alcoholic fatty liver disease after liver transplantation. If validated in more robust cohorts of patients with more advanced stages of NAFLD/NASH, preferably from general population, miR-33a could potentially be used as a useful biomarker.

Supporting information

S1 Table. Donor characteristics and immunosupresion regimens—All patients enrolled in study. Data are given as N (%) or median (1st - 3rd quartile).

(DOCX)

S2 Table. Donor characteristics and immunosupresion regimens—Steatosis. Data are given as N (%) or median (1st - 3rd quartile).

(DOCX)

S3 Table. Donor characteristics and immunosupresion regimens—Lobular inflammation. Data are given as N (%) or median (1st - 3rd quartile).

(DOCX)

S1 Fig. Graph of indications for liver transplant in our cohort. Biliary cirrhosis was indication for LTx in 33 patients (28.5%), alcoholic liver disease in 28 (24.1%), HBV, autoimmune and cryptogenic in 8 patients each (7%), HCV in 4 patients (3.4%), NASH in 1 patient (0.9%) and other diagnoses in 26 patients (22.4%).

(JPG)

S1 Data. Underlying data of all patients enrolled in the study.

(XLSX)

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