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Efekt nutričních intervencí na vlastnosti tukové tkáně u obézních

Effect of nutritional intervention on characteristics of adipose tissue in obese

Školitel: prof. MUDr. Vladimír Štich, Ph.D.

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Eva Krauzová

SEZNAM ZKRATEK:

- AMPK – 5´AMP aktivovaná proteinkináza
- ATGL – adipose triglyceride lipase
- BMI – body mass index
- DAG – diacylglycerol
- DM – diabetes mellitus
- ER – endoplasmatické retikulum
- HFM – high fat meal
- HOMA-IR – homeostatis model assessment of insulin resistence
- ICAM – intracelulární adhesní molekula
- IL – interleukin
- IR- inzulinová rezistence
- LCD – nízkokalorická dieta (low calorie diet)
- MK - mastné kyseliny
- MCP-1/CCL2 – chemoatraktant monocytů (monocyte chamoattractant protein 1)
- TAG – triacylglycerol
- TLR – toll like receptor
- TNF α – tumor necrosis factor alfa
- TT – tuková tkáň
- VCAM – vascular cells adhesion molecule
- VEGF α – vaskulární endoteliální růstový faktor

- VLCD – velmi nízkokalorická dieta (very low calorie diet)
- WHO – světová zdravotnická organizace
- FGF 21 – růstový faktor fibroblastů 21

SHRNUTÍ

Obezita je celosvětově nejrozšířenější metabolické onemocnění a její trvalý vzestup je alarmující. Závažnost obezity spočívá v tom, že je spojena s výrazným zvýšením rizika metabolických chorob jako je diabetes mellitus (DM) 2. typu, hyperlipidémie, kardiovaskulární a některá nádorová onemocnění. Obezita se tak podílí významně na zvyšování nákladů celého zdravotnického systému. Zásadní pro snížení této zátěže je prevence vzniku výše zmíněných chorob. Tato spočívá jednak v prevenci obezity samotné, jednak v poznání a následném ovlivnění mechanismů vzniku metabolických poruch s obezitou spojených. Podkladem vzniku těchto poruch jsou změny v klíčových metabolických orgánech: tukové tkáni, játrech, kosterním svalu a slinivce břišní. Předkládaná práce se soustředila na obezitou vyvolané poruchy tukové tkáně na úrovni metabolické, endokrinní a imunitní, jednotně prezentované jako dysfunkční tuková tkáň. Pozornost byla specificky věnována škodlivému účinku zvýšených hladin základních makronutrientů (sacharidů a lipidů) a na straně druhé příznivému působení nízkokalorické diety na molekulární charakteristiky tukové tkáně. Získané poznatky osvětlují mechanismy působení výživových podnětů na dysfunkci tukové tkáně na molekulární a buněčné úrovni a poskytují tak důležitý podklad k prevenci obezity a s ní spojených metabolických poruch. Spojitost mezi rizikovými faktory výživy a etiologií vzniku dysfunkční tukové tkáně tak poskytuje vědecky podložená fakta nezbytná k úpravě výživových doporučení. Tento přístup bychom mohli nazvat „Molekulární preventivní lékařství“.

Předkládaná dizertační práce je zpracována formou souboru publikací zabývajících se účinkem intervencí vedoucích k elevaci cirkulujících hladin nutrientů – hyperlipidémie, hyperglykémie na straně jedné a kalorickou restrikcí na straně druhé - na charakteristiky podkožní tukové tkáně. Zahrnuto je celkem 6 publikací, které vznikly na Ústavu pro studium obezity a diabetu 3. LF UK nyní nově přejmenovaném Ústavu patofyziologie 3. LF UK. Ústav se ve své výzkumné aktivitě dlouhodobě zabývá výzkumem tukové tkáně, obezitou, metabolickými komplikacemi obezity a jejich prevencí. Práce je tématicky rozdělena do dvou částí. V první části je společným tématem vliv krátkodobých intervencí na prozánětlivý profil jak na úrovni tukové tkáně, tak v cirkulaci. V publikaci č. 1 a 2 je zhodnocen efekt krátkodobé hyperlipidémie a hyperglykémie na imunitní profil tukové tkáně u obézních žen. Publikace č. 3 popisuje efekt jednorázově podaného vysokotukového pokrmu na zánětlivé změny v cirkulaci u mladých zdravých mužů. Publikace č. 4 sleduje regulaci mobilizace mastných kyselin z tukové tkáně během tělesné zátěže při podání metforminu. Druhá část práce zahrnující publikace č. 5 a 6 je tématicky věnována efektu

hypokalorické diety a jejích různých fází na modulaci metabolických, endokrinních a imunitních funkcí tukové tkáně u obézních žen.
Studie sdružené v této dizertační práci poskytují příspěvky k poznání regulace vzniku prozánětlivého stavu organizmu a úlohy energetického příjmu i hladiny základních makronutrientů v tomto procesu. Tyto střípky vědění mohou přispět k pochopení funkčních změn na úrovni tukové tkáně a vést tak k účinnému rozvoji preventivních a léčebných strategií.

Klíčová slova: obezita, tuková tkáň, hyperlipidémie, hyperglykémie, dietní intervence.

SUMMARY

Obesity is the world's most widespread metabolic disease and its continous rise in prevalence is alarming. The severity of obesity is that it is associated with a significant increase in the risk of metabolic diseases such as type 2 diabetes mellitus, hyperlipidemia, cardiovascular disease and some types of cancer. Obesity thus contributes significantly to increasing the cost of healthcare system. Preventing the aforementioned diseases is essential to reduce this burden. This is based both on the prevention of obesity itself, on the other hand, on the knowledge and consequent influence on the mechanisms of metabolic disorders associated with obesity. These disorders are based on changes in key metabolic organs: adipose tissue, liver, skeletal muscle and pancreas. The present thesis is focused on obesity-induced disorders of adipose tissue at the metabolic, endocrine and immune levels, presented as dysfunctional adipose tissue. Special attention was paid to the detrimental effect of elevated levels of basic macronutrients (carbohydrates and lipids) and, on the other hand, to the beneficial effects of low calorie diets on molecular characteristics of adipose tissue. The acquired knowledge illuminates the mechanisms of action of nutritional stimuli on adipose tissue dysfunction at the molecular and cellular level and thus provides an important basis for the prevention of obesity and related metabolic disorders. Thus, the link between nutritional risk factors and the etiology of dysfunctional adipose tissue provides scientifically-based facts necessary to adjust nutritional recommendations. This approach could be called "Molecular Preventive Medicine".
This thesis is elaborated in the form of a set of publications dealing with the effect of interventions leading to the elevation of circulating levels of nutrients - hyperlipidemia, hyperglycemia on the one

side, and calorie restriction on the other - on subcutaneous adipose tissue characteristics. A total of 6 publications that were created at the Department for the Study of Obesity and Diabetes, newly renamed as Department of Pathophysiology on the Third Faculty of Medicine of Charles University are included. This Department has been engaged in the research of adipose tissue, obesity, metabolic complications of obesity and their prevention for a long time. The thesis is thematically divided into two parts. In the first part, the common theme is the influence of short-term interventions on the pro-inflammatory profile both at the level of adipose tissue and in the circulation. In publication No. 1 and 2 is evaluated the effect of short-term hyperlipidemia and hyperglycemia on the adipose tissue immune profile of obese women. Publication No. 3 describes the effect of a single-dose high-fat meal on inflammatory changes in circulation in young healthy men. Publication No. 4 is focused on the regulation of fatty acid mobilization from adipose tissue during exercise influenced by antidiabetic medication - metformin. The second part of the thesis, which includes publications No. 5 and 6, focuses on the effect of hypocalorie diet and its various phases on the modulation of metabolic, endocrine and immune functions of adipose tissue in obese women.

The studies in this thesis provide contributions to the knowledge of the regulation of pro-inflammatory state of the organism and the role of energy intake and the level of basic macronutrients in this process. These fragments of knowledge can help to understand the functional changes at the level of the adipose tissue and thus lead to the effective development of preventive and treatment strategies.

Key words: obesity, adipose tissue, hyperlipidemia, hyperglycemia, diet intervention.

OBSAH

Předmluva a poděkování 4
SEZNAM ZKRATEK: 5
SHRNUTÍ 7
SUMMARY 8
OBSAH 10
1. ÚVOD 12
1.1. Epidemiologie obezity 12
1.2. Etiopatogeneze obezity 14
1.3. Prevence a léčba obezity 14
1.4. Metabolické komplikace obezity 19
2. TUKOVÁ TKÁŇ 23
2.1. Složení tukové tkáně 23
2.2. Distribuce tukové tkáně 26
2.2. Lipolýza tukové tkáně a její regulace 28
2.3. Endokrinní funkce tukové tkáně 29
2.4. Imunitní buňky v tukové tkáni 34
2.5. Dysfunkce tukové tkáně 36
2.6. Úloha hyperlipidémie a hyperglykémie v patogenezi dysfunkce tukové tkáně a metabolického syndromu. 38
2.7. Vliv redukční diety na tukovou tkáň u obézních 40
3. POPIS VYBRANÝCH METODIK 42
3.1. Jehlová biopsie podkožní tukové tkáně 42
3.2. Euglykemicky a Hyperglykemický clamp 42
3.3. Mikrodialýza 43
3.4. Průtoková cytometrie 45
3.5. Analýza metabolitů a cytokinů v plasmě 45
3.6. Statistická analýza 46
4. CÍLE PRÁCE A HYPOTÉZY 47
5. PŘEHLED PUBLIKACÍ 49
ČÁST A: Krátkodobé intervence 50
5.1. Publikace č. 1: 50

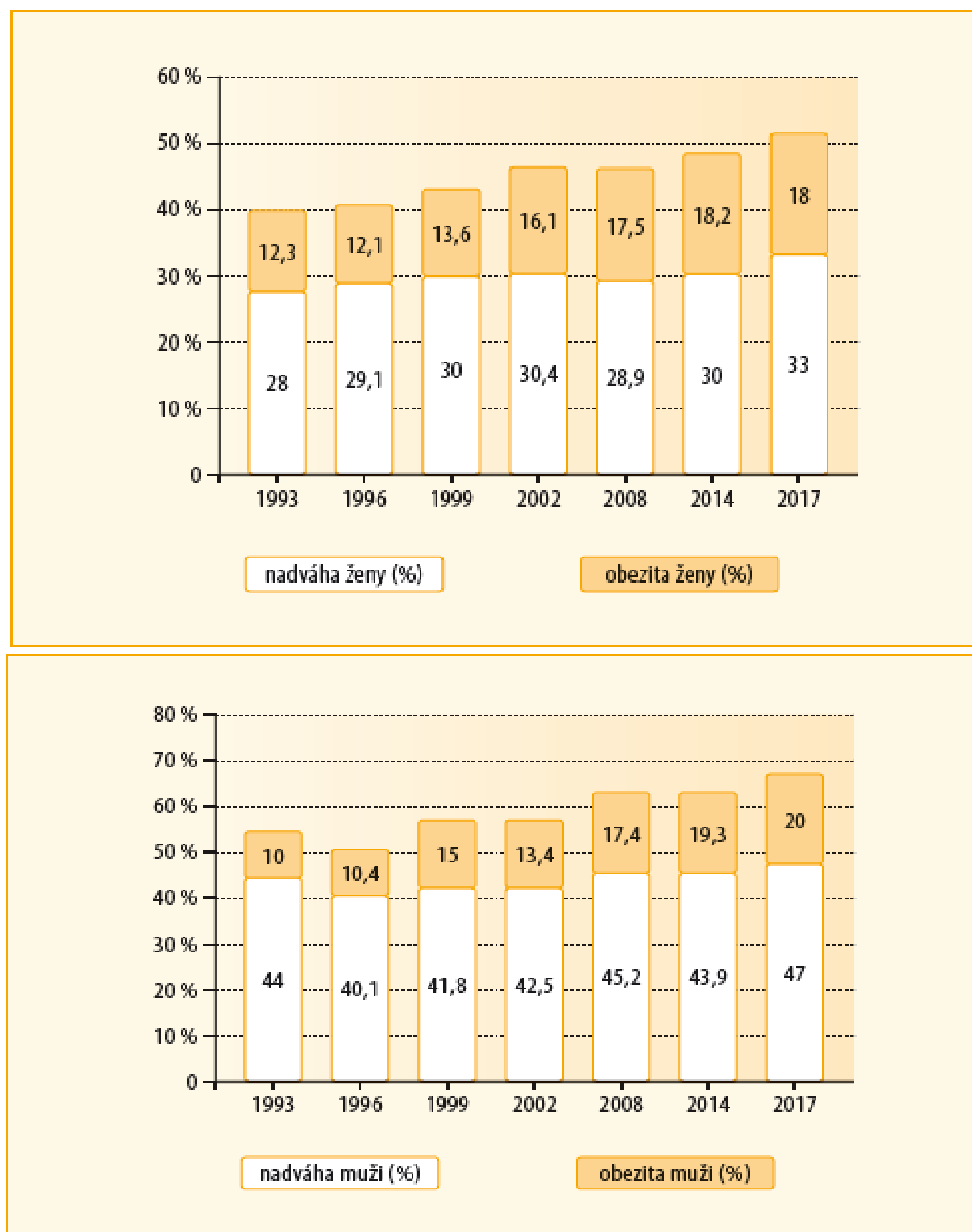
5.2. Publikace č. 2: ... 52
5.3. Publikace č.3: ... 54
5.4. Publikace č. 4: ... 56
ČÁST B: Dietní intervence ... 58
5.5. Publikace č. 5: ... 58
5.6. Publikace č. 6: ... 60
6. ZÁVĚRY A DISKUZE ... 63
7. OSTATNÍ PUBLIKAČNÍ AKTIVITA ... 68
8. LITERÁRNÍ ZDROJE ... 70
PŘÍLOHA 1 ... 82
PŘÍLOHA 2 ... 90
PŘÍLOHA 3 ... 104
PŘÍLOHA 4 ... 115
PŘÍLOHA 5 ... 123
PŘÍLOHA 6 ... 133

1. ÚVOD

1.1. Epidemiologie obezity

Obezita je charakterizována nadměrnou akumulací tukové tkáně v organismu. Její příčinou je nerovnováha mezi energetickým příjmem a výdejem. Obezitou se rozumí stav s hmotnostním indexem BMI ≥ 30 kg/m^2. Dle Světové zdravotnické organizace je obezita považována za epidemii 21. století nyní přerůstající do pandemických rozměrů. V roce 2016 se 39 % dospělé populace ve světě nacházelo v pásmu nadváhy (BMI 25,1 -29,9 kg/m^2) a 13% v pásmu obezity. 11 % bylo mužů a 15 % žen (http://www.who.int/mediacentre/factsheets/fs311/en/ 2018). V české populaci dle kontinuálního průzkumu společnosti STEM/MARK z roku 2013 se s nadváhou potýká 35 % a s obezitou 23 % dospělé populace (https://www.slideshare.net/stemmark/obezita-2013-stemmark-vzp 2013). Obézních je 20 % mužů a 18 % žen dle dat Českého statistického úřadu z roku 2018 (viz obrázek č.1). Ve většině evropských zemích se prevalence obézních dospělých pohybuje mezi 10 - 20 % , v ČR je to 23 %, což je srovnatelné s okolními zeměmi jako je Německo, Litva, Belgie i Francie. Některé části Evropy jsou typické prevalencí obesity nad 30%, např. jižní Itálie či Malta. V rámci Evropské unie se ČR pohybuje na 13. místě z 28 zemí (Svačina 2013). Oproti výzkumu v roce 2011 nedošlo v roce 2018 v ČR k významnému nárůstu prevalence osob s nadváhou či obezitou. Vysvětlení mohou být mnohá. Jedním z nich je, že naše populace již dosahuje maxima vzhledem k dané genetické výbavě, případně také informovanost populace o preventivních opatřeních a rizicích obezity mohou vést k zastavení vzestupu (Matoulek, Svacina, and Lajka 2010).

Obrázek č. 1: Vývoj prevalence nadváhy a obezity u mužů a žen v ČR od roku 1993 – 2017 (zdroj ČSÚ)



1.2. Etiopatogeneze obezity

Nejvýznamnější etiopatogenetický faktor vzniku obezity je nerovnováha mezi energetickým příjmem a výdejem. Za posledních 25 let došlo k významným změnám jídelníčku: např. ke snížení spotřeby hovězího a vepřového masa a k nárůstu spotřeby masa drůbežího. Více než o pětinu stoupla spotřeba ovoce a zeleniny. Přesto je v ČR stále vysoká konzumace tučných jídel a sekundárně zpracovaného masa. V mladší populaci se zvýšila spotřeba sladkých nápojů. Významnější změny jsou patrné v energetickém výdeji, kdy došlo k dramatickému nárůstu sedavého způsobu života (Matoulek, Svacina, and Lajka 2010).

Ve většině případů je obezita polygenní onemocnění, vzácná je monogenní obezita např. s mutací ob genu kodujícího produkci leptinu, mutací genu pro leptinový receptor, receptor pro melanocortin 4 nebo proopiomelanocortin. Z dalších příčin lze zmínit endokrinní faktory jakoje např. porušená diurnální sekrece kortikoidů, Cushingův syndrom, těžká hypotyreóza a taktéž psychologické faktory podílející se na vzniku poruch příjmu potravy jako jsou bulimie, syndrom nočního přejídání nebo binge eating syndrom.

1.3. Prevence a léčba obezity

Obezita představuje významný rizikový faktor vzniku diabetu 2.typu, hyperlipidémie, kardiovaskulárních, nádorových a neurodegenerativních onemocnění (Lengyel et al. 2018; Pistollato et al. 2018). V důsledku těchto komorbidit dochází k významnému vzestupu morbidity a mortality (Miller et al. 2013). Proto je kladen důraz především na preventivní a léčebná opatření obezity a metabolického syndromu. Primárně preventivní intervence mají příznivější efektivitu vložených nákladů ve srovnání s léčbou již vzniklé nemoci. Strategie léčby obezity zahrnuje nejen

redukci hmotnosti, ale především snížení metabolických rizik. Modifikace životního stylu zahrnuje tři základní komponenty: dietu, pohybovou aktivitu a behaviorální terapii. Komplexní intervence zahrnuje redukci hmotnosti o 0,5 – 1 kg/ týdně během prvních 12 týdnů a dále udržení hmotnosti po 6-9 měsíců. Klinický benefit je dosažen už při mírné hmotnostní redukci (o 5 -10 % tělesné hmotnosti). Důležité je věnovat pozornost nejen samotné redukci hmotnosti, ale změně tělesného složení s cílem snížení tukové hmoty a navýšení svalové hmoty. Součástí léčebné strategie je zvládnutí komorbidit – kontrola dyslipidémie, glykémie, udržení normálního krevního tlaku, léčba syndromu spánkové apnoe a psychosociálních omezení (Yumuk et al. 2015).

Součástí léčby obezity jsou (Wilding 2007):

- Dietní intervence
- Pohybová aktivita
- Behaviorální terapie
- Farmakoterapie
- Baritrická chirurgie

1.3.1. Dietní intervence

Evropská Obezitologická společnost doporučuje snížení energetického příjmu o 600 kcal/den k dosažení redukce tělesné hmotnosti o 0,5 kg/ týdně. Dieta obsahující ≥ 1200kcal/den,je nazývána „**nízkokalorická vyvážená dieta“.** Dieta s energetickým příjmem nižším tj. 800-1200 kcal/den, nazývaná **„nízkokalorická dieta“,** může vést k deficitu mikronutrietů a je vhodné užívat suplementaci minerálů a stopových prvků. Nízkokalorická dieta je doporučována pouze jako

krátkodobá intervence 2-3 měsíce vedoucí k signifikantní redukci hmotnosti. Neukázal se benefit nízkokalorických diet s různými poměry makronutrientů (nízkosacharidová, nízkotuková nebo vysokoproteinová dieta) oproti vyvážené hypokalorické dietě (Yumuk et al. 2015; Shai et al. 2008).Vyvážené nízkokalorické diety mají pozitivní efekt na redukci rizikových faktorů pro kardiovaskulární onemocnění a diabetes mellitus 2.typu. Tento typ diety je nejvhodnější volbou pro déletrvající úspěch léčebné strategie obezity (Yumuk et al. 2015).

Velmi nízkokalorická dieta obsahuje 400-800 kcal/den a je obvykle preskribována na kratší časový úsek 4-8 týdnů, kdy nejprudší pokles hmotnosti bývá dosažen během prvního týdne (2-8 kg) a následně dochází k redukci maximálně 2 kg/ týden (Heiner 2004). Tyto diety jsou využívány nejčastěji pro pacienty s BMI ≥35kg/m2 nebo BMI ≥30kg/m2 s komplikacemi při nutnosti rychlé redukce váhy nebo rychlého ovlivnění insulinové resistence. Je dostupné velké množství komerčních přípravků VLCD k přípravě tetkuté diety (např. Ultra Fit and Slim, Redita, Optifast, Modifast aj.). K udržení hmotnosti po absolvování redukční diety je důležitá fáze stabilizace hmotnosti vedoucí k adaptaci na nově nastavenou energetickou bilanci.

Tabulka č. 2: Typy dietních intervencí (upraveno dle (Tsigos et al. 2008))

Typ dietní intervence	**Denní energetický příjem**
Vyvážená nízkokalorická dieta	≥1200 kcal/den
Nízkokalorická dieta (LCD)	800 – 1200 kcal/day
Velmi nízkokalorická dieta (VLCD)	≤800 kcal/day
Multifázové kombinace výše uvedených diet	

1.3.2. Pohybová aktivita

Pohybová aktivita je nedílnou součástí režimových opatření vedoucích společně s dietou k redukci hmotnosti a tukové hmoty. Je nesporné, že kombinace aerobního a rezistentního tréninku má zdravotně prospěšné účinky a vede k zachování svalové hmoty u obézních na rozdíl od diety samotné (Willis et al. 2012). Odborné společnosti (jako je Evropská obezitologická a Česká obezitologická společnost) doporučují 150 minut týdně formou aerobní aktivity mírné intenzity (např. rychlá chůze) v kombinaci s rezistentním tréninkem 1-3x týdně pro zlepšení svalové síly (Willis et al. 2012). Pravidelná aerobní pohybová aktivita bez dietní intervence vede k významnější redukci hmotnosti až při velkých objemech aktivity, nicméně i při nepřítomnosti výrazné redukce váhy má příznivý vliv na metabolickcý profil (Slentz et al. 2004). K dosažení metabolických cílů postačí např. aerobní aktivita 30 minut 5x týdně mírné intenzity (Jensen et al. 2014). Pohybová aktivita vede ke zlepšení lipidových parametrů, poklesu krevního tlaku, zlepšení glukózové tolerance a zvýšení inzulinové citlivosti, redukci množství viscerálního tuku a zvýšení svalové hmoty, čímž se podílí na zmírnění negativního poklesu klidového energetického výdeje indukovaného redukcí hmotnosti při dietě. Pohybová aktivita zvyšuje spolupráci pacientů k dodržování dietního režimu a má pozitivní efekt v udržení hmotnosti, zároveň má pozitivní vliv na psychosomatické aspekty, snižuje anxiózní a depresivní stavy (Ross et al. 2004; Lee et al. 2005). Pohybová aktivita je také důležitý článek v prevenci sarkopenie u seniorů (Palus et al. 2017).

1.3.3. Kognitivně – behaviorální terapie

Obezitu je třeba pojímat i z hlediska psychologického jako odlišnost v kognitivním a emočním nastavení. Jedním z nejefektivnějších psychoterapeutických přístupů k obézním pacientům je kognitivně behaviorální terapie (KBT) vycházející z teorie učení. Cílem terapie je

navodit žádoucí změny v nevhodném chování, myšlení a emocích, změnit životní styl a v konečném důsledku zvýšit kvalitu života obézního (Málková 2006). Základním pilířem KBT je self-monitoring jídelního chování, záznamy kalorického příjmu, pohybové aktivity a vývoje hmotnosti (Webb and Wadden 2017).

1.3.4. *Farmakoterapie*

Farmakoterapie může pomoci pacientům k prohloubení efektu redukční diety, k zajištění dlouhodobého poklesu tělesné hmotnosti a ke stabilizaci úbytku hmotnosti. Dle novelizace Doporučených postupů v ČR (Svačina Štěpán et al. 2018) je indikována u pacientů s BMI ≥ 30 kg/m2 a u pacientů s BMI nad 27 kg/m2 s komplikacemi (DM 2.typ, dyslipidémie). V současnosti lze užívat buď přípravky navozující centrálně pocit sytosti nebo látky blokující vstřebávání tuku z trávícího traktu. Z prvé skupiny je nově dostupný kombinovaný preparát naltrexon –bupropion, který vykazuje efekt na snížení hmotnosti i na složky metabolického syndromu. Antiobezitikem, které může být podáváno dlouhodobě, je blokátor střevní lipázy orlistat, který vede ke snížené resorpci tuků v trávicím traktu a navozuje tak negativní energetickou bilanci. K jeho účinkům patří i to, že vede k obtížím pacienta po požití tučných jídel. U diabetiků lze využít antidiabetika, která vedou k redukci hmotnosti- jako inkretinová analoga (exenatid, liraglutid, lixisenatid, albiglutid, dulaglutid) a blokátory vstřebávání glukózy v ledvině – SGLT2 inhibitory tzv. glifloziny. Inkretinový analog, který je od roku 2018 dostupný i pro léčbu obézních bez diabetu, je liraglutid (Victoza v diabetologii a Saxenda v obezitologii). Jeho efekt je jak centrální inhibice příjmu potravy, tak i pomalejší vyprazdňování žaludku i efekt metabolický (Svačina Štěpán et al. 2018).

1.3.5. *Chirurgická léčba obezity*

Bariatrická chirurgie je nejúčinnějším způsobem léčby pacientů s BMI > 35 nebo BMI > 30 s přítomností komorbidit typu DM 2. typu, hypertenze, syndromu spánkové apnoe a dalších. Proto se užívá i název metabolická chirurgie a pacienti s DM 2.typu mohou být operování již od BMI 30 kg/m2 (Kasalický Mojmír 2018). Současné dlouhodobé studie ukazují, že po bariatrickém výkonu dochází k podstatnému snížení úmrtnosti, k remisi DM 2. typu, k poklesu rizika vzniku chorob souvisejících s obezitou a snížení nákladů na zdravotní péči.Největší efekt na vyléčení DM 2.typu mají malabsorpční operace, což dokládá Buchwald et.al ve své metaanalýze. (Buchwald et al. 2007; Buchwald et al. 2009). Rozsáhlá 20-ti letá studie Swedish Obese Subjects ukazuje diferencovaný efekt bariatrie na metabolická onemocnění s maximem ovlivnění DM 2.typu, méně pak hypertenze a dyslipidémie (Sjostrom 2013).

Typy bariatrických výkonů dělíme na restriktivní (bandáž žaludku, tubulizace žaludku, plikace žaludku) nebo kombinované, kde převažuje navození malabsorpce živin (žaludeční bypass, biliopankretická diverze). První typ výkonů vede k remisi diabetu 2. typu v 50–80 %, druhý typ až v 90 % (Kasalický Mojmír 2018). U kombinovaných výkonů je však pacient ohrožen rozvojem proteinové malnutrice a karencí vitamínů, proto je nutné dlouhodobé sledování.

1.4. Metabolické komplikace obezity

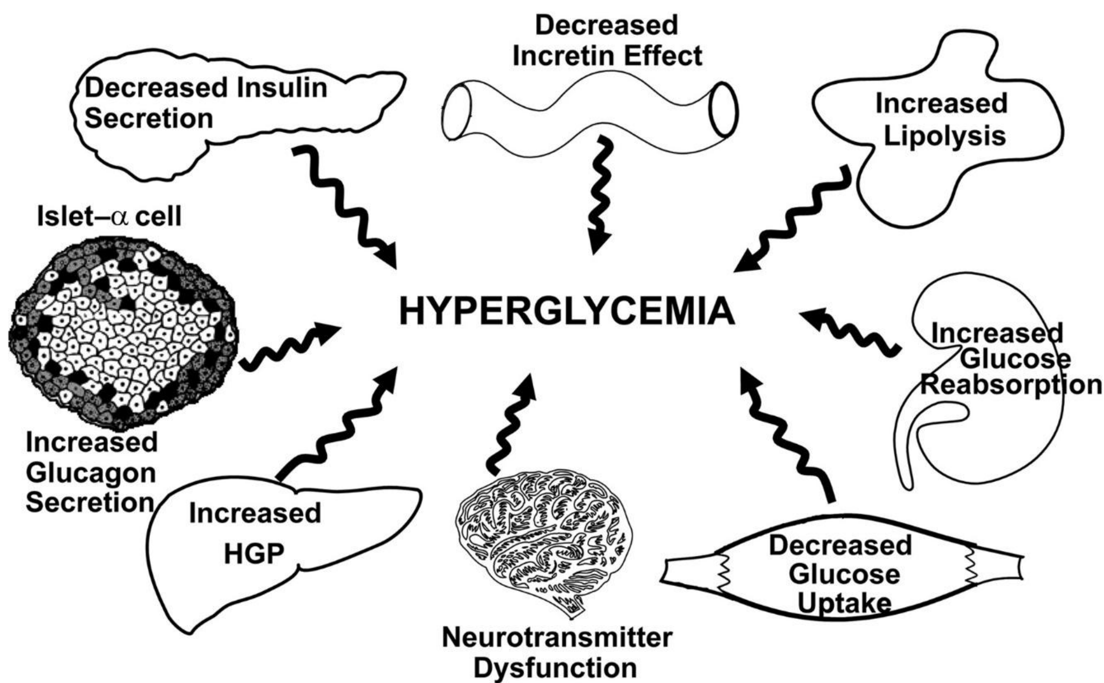
1.4.1. *Inzulinová rezistence*

Inzulinová rezistence (IR) je definována jako stav, kdy normální hladina inzulinu nevyvolává adekvátní odpověď v cílových tkáních a k vyvolání normální odpovědi je potřebné větší množství inzulínu (Kahn 1978). Manifestuje se jako porucha na inzulinu závislém odsunu glukózy, ale také

porucha suprese hepatální produkce glukózy (Reaven 1995). Postprandiální hyperglykémie stimuluje sekreci inzulinu, který se naváže na receptor s tyrozin-kinázovou aktivitou, tím dojde k fosforylaci receptoru a spuštění intracelulární kaskády kinázových reakcí vedoucích k samotnému biologickému efektu inzulinu. Jedním z nich je exprese glukózového přenašeče GLUT-4, který je exprimován v inzulin-senzitivních tkáních jako je tuková tkáň a kosterní sval (Pessin and Saltiel 2000). Současně inzulin stimuluje glykolýzu, syntézu glykogenu a inhibuje glykogenolýzu a glukoneogenezi v játrech. Tyto procesy pak zajišťují udržení normoglykémie v organismu. Inzulin také reguluje metabolismus lipidů – inhibuje lipolýzu v TT a oxidaci MK v játrech a svalech, stimuluje syntézu triglyceridů (TG) a jejich vychytávání v TT a v kosterním svalu (Dimitriadis et al. 2011). Jsou popsány mutace mnoha genů inzulinové signalizace a jejich polymorfismy jsou spojeny s prokazatelně vyšším rizikem rozvoje IR a DM 2.typu, což indikuje genetickou predispozici k jejich rozvoji (Pedersen 1999; Brown and Walker 2016). Předpokládá se, že rozhodující jsou však faktory environmentální a nutriční, zejména nedostatek fyzické aktivity a nevhodná životospráva, které zhoršují citlivost k inzulinu. Pokud nedojde k intervenci snižující inzulinovou rezistenci – jako je nízkokalorická dieta a fyzická aktivita, β-buňky vyčerpají svou sekreční schopnost a dojde tak k relativnímu nedostatku inzulinu a k manifestaci poruchy metabolismu sacharidů s rozvojem postprandiálních hyperglykémií následovaných rozvojem lačných hyperglykémií a konečně DM 2.typu (Jallut et al. 1990). Obézní subjekt s poruchou glukózové tolerance má sníženou inzulinovou citlivost a manifestace diabetu je dána poklesem sekreční schopnosti β-buňky (Jallut et al. 1990). Defronzo postuloval nejprve tzv. triumvirát (DeFronzo 1988) a následně oktet etiopatologických mechanismů podílejících se na vzniku IR a DM 2.typu (Defronzo 2009). Nejprve popsal roli kosterního svalu, jater a beta-buněk pankreatu v mechanismech vzniku IR a následně roli tukové tkáně (porucha lipolýzy), gastrointestinálního

traktu (deficit inkretinů), alfa-buněk pankreatu (hyperglukagonémie), ledvin (zvýšená reabsorpce glukózy) a mozku (inzulinová rezistence). Tento oktet poukazuje také na vhodně zvolený typ léčby dle patofyziologických mechanismů.

Obrázek č. 7: Defronzův oktet etiopatogeneze diabetu mellitu 2.typu (Defronzo 2009)



V tukové tkáni vede inzulinová rezistence ke zvýšenému uvolňování volných MK z adipocytů a překročí-li se zásobní kapacita adipocytů, dochází k „přetékání“ MK do cirkulace (Iozzo 2009) a k akumulaci TG v ektopických tukových depech a ukládání MK ve formě toxických ceramidů a sfingolipidů ve svalech, játrech, pankreatu a endotelu, kde způsobují klinické známky inzulinové rezistence. Tato tzv. lipotoxicita vede k akceleraci hepatální produkce glukózy, ke snížení odsunu glukózy ve svalech a k selhání β-buňky pankreatu (Boden et al. 1994; Roden et al. 1996; Unger and Zhou 2001).

1.4.2. Metabolický syndrom

Metabolický syndrom je koncept, který představuje možnost identifikovat jedince, kteří se vyznačují inzulinovou rezistencí a metabolickými abnormitami asociovanými s viscerální obezitou – tedy mají vysoké riziko vzniku diabetu mellitu 2.typu a kardiovaskulárních onemocnění. V literatuře je známo více možných kritérií metabolického syndromu. Já zde uvádím kritéria dle IDF (International Diabetes Federation), kdy metablický syndrom je diagnostikován, pokud splňuje tři z následujících pěti kritérií uvedených v tabulceč č. 1.

Tabulka č. 1: Kritéria metabolického syndromu dle International Diabetes Federation (Alberti, Zimmet, and Shaw 2005)

Kritéria metabolického syndromu	**Muži**	**Ženy**
Obvod pasu (cm)	>94	>80
Koncentrace HDL (mmol/l)	< 1,0	< 1,3
Triacylglyceroly (mmol/l)	> 1,7	> 1,7
Krevní tlak (mm Hg)	> 130 / 85	> 130 / 85
Glykémie (mmol/l)	> 5,6	> 5,6

Z dalších komplikací obezity můžeme zmínit plicní abnormality jako např. syndrom spánkové apnoe, gastrointestinální obtíže jako je např. cholecystolithiáza či gastro-ezofageální reflux. Taktéž reprodukční komplikace spojeny s polycystickými ovárii a anovulačními cykly. Obézní pacienti mají vyšší prevalenci psychosociálních obtíží. Studie dokládají také vyšší prevalenci nádorových onemocnění u obézních (Segula 2014).

2. TUKOVÁ TKÁŇ

2.1. Složení tukové tkáně

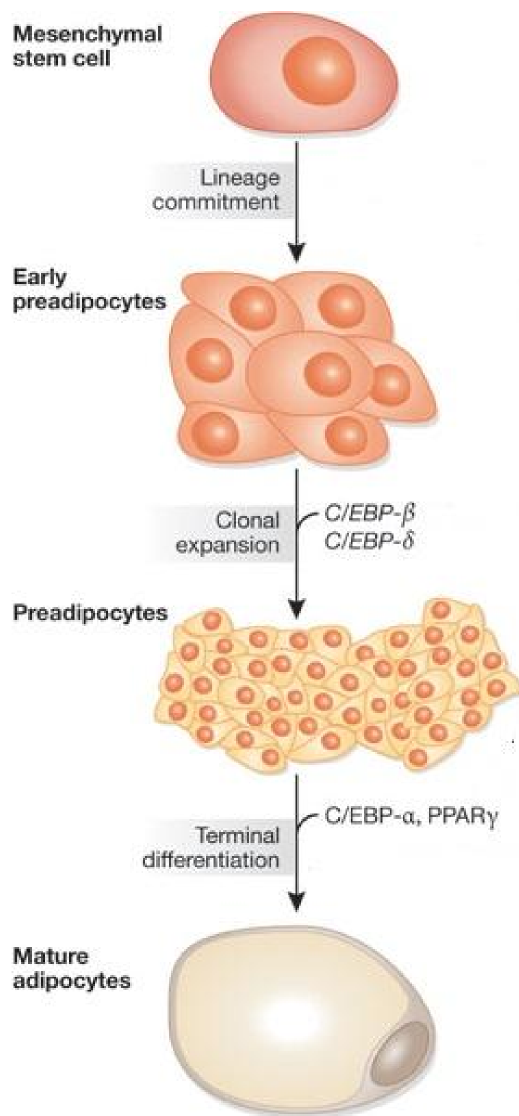
Tuková tkáň (TT) je složena z tukových buněk - adipocytů a stromovaskulární frakce (SVF), která zahrnuje mesenchymální kmenové buňky, preadipocyty, imunitní buňky, endotelie a extracelulární matrix. Adipocyt má oproti jiným buňkám proměnlivou velikost v závislosti

na fyziologických okolnostech. Průměr buňky je přibližně od 10 do 180 μm.

Při nadměrném příjmu energie dochází k ukládání tukových zásob v organismu ve formě triglyceridů (Mingrone and Castagneto-Gissey) a tuková tkáň může takto zvětšovat svůj objem dvojím mechanismem - hypertrofií (zmnožením intracelulárních lipidů a zvětšením adipocytů) nebo hyperplazií (zvýšením počtu adipocytů). Menší adipocyty jsou pozorovány u štíhlých jedinců, zatímco obézní jedinci mají objem adipocytů větší v důsledku vysokého obsahu lipidů. Velikost adipocytů a obsah lipidů významně ovlivňuje jejich metabolické vlastnosti včetně citlivosti na inzulin (snížená u velkých adipocytů) a endokrinní produkce (velké adipocyty obézních produkují více prozánětlivých a méně protizánětlivých faktorů než u jedinců štíhlých) (Ravussin and Smith 2002). Zrání adipocytů představuje proces adipogeneze (Obrázek č. 2), kdy zásadní roli zde hrají transkripční faktory PPARγ (proliferator activated receptor gamma) a C/EBP (CCAAT/enhancer binding protein), které spustí kaskádu změn v morfologii a genové expresi vedoucí k maturaci adipocytu (Gregoire, Smas, and Sul 1998). Adipogenní kapacita preadipocytu je negativně spojena s velikostí zralého adipocytu, tedy velké hypertrofické adipocyty zhoršují adipogenní kapacitu preadipocytů (Gustafson and Smith 2012) a může vést k akumulaci viscerální a ektopické TT (Alligier et al. 2013; Bluher 2010). Ve studii naší skupiny bylo prokázáno, že dietní intervence u obézních žen zlepšila adipogenní potenciál preadipocytů cestou snížení exprese osteogenního

faktoru RUNX2 interferujícího s aktivitou PPARγ (Rossmeislova, Malisova, Kracmerova, Tencerova, et al. 2013). Lidská tuková tkáň zahrnuje dva typy adipocytů: bílé adipocyty (bílá TT) a hnědé adipocyty (hnědá TT). Liší se svým buněčným složením i funkcí. Zatímco bílé adipocyty reprezentují hlavní funkci tukové tkáně v akumulaci lipidů a lipidová kapénka v nich zaujímá většinu objemu, tak hnědé adipocyty obsahují několik malých lipidových kapének a mají vyšší denzitu mitochondrií, proto hnědé adipocyty sehrávají kruciální roli v netřesové termogenezi. Hnědé adipocyty jsou přítomny u novorozenců a hibernujících zvířat, ale publikace dokazují jejich přítomnost i u dospělé populace (Nascimento and van Marken Lichtenbelt 2018). V současnosti se popisuje i „ třetí“ typ adipocytů tzv. „béžové adipocyty“, které vycházejí z bílé TT, ale mají velké množství lipidových kapének a vysokou mitochondriální aktivitu (Sepa-Kishi and Ceddia 2018).

Obrázek 2: Schéma diferenciace adipocytů (upraveno dle (Ricoult and Manning 2013).

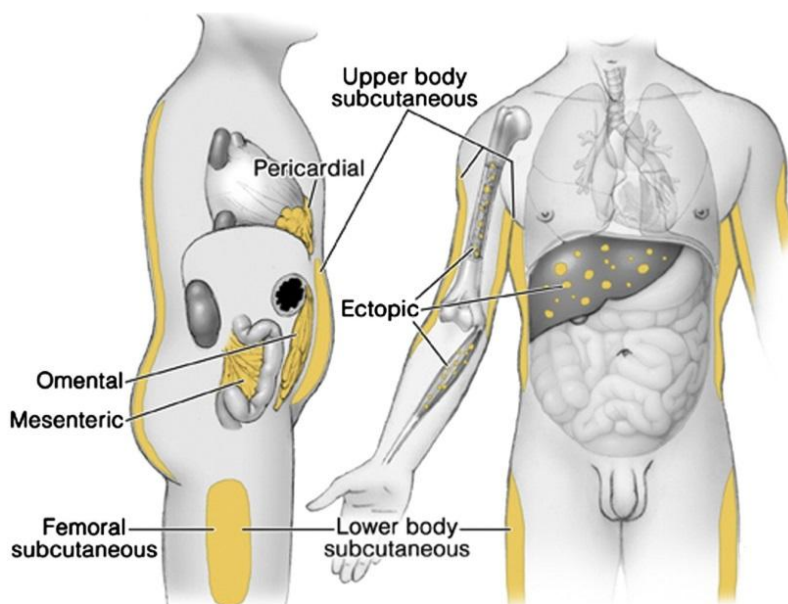


2.2. Distribuce tukové tkáně

Tuková tkáň je lokalizována primárně ve třech oblastech (Obrázek č. 3):

1) Podkožní TT - abdominální, gluteální a femorální depo
2) Intraabdominální TT - mesenterické, omentální a retroperitoneální depo)
3) Ektopická TT - epikardiální, intramuskulární, intrahepatální a perivasální tuk

Obrázek č.3: Distribuce tukové tkáně (upraveno dle Tchkonia, (Tchkonia et al. 2013))



Distribuce TT má vliv na metabolický profil daného jedince (Tchkonia et al. 2013). Větší množství podkožní a viscerální TT v oblasti břicha je spojeno s inzulinovou rezistencí, progresí aterosklerózy, rozvojem arteriální hypertenze a diabetu mellitu 2.typu. (Wajchenberg 2000; Iozzo 2009). Tento typ obezity je označován jako “androidní obezita “ a z hlediska kardiovaskulárních komplikací je rizikovější než “gynoidní obezita”, která je spojena s nahromaděním podkožní TT v

oblasti boků a hýždí (Ebbert and Jensen 2013; Despres 2001). Existují ještě další uložiště TT s odlišnými vlastnostmi jako je např. epikardiální TT, která naléhá na srdeční sval a jeho obaly. Studie ukazují, že větší množství této TT je spojeno s vyšším kardiovaskulárním a metabolickým rizikem (Kim et al. 2016) a produkuje více prozánětlivých faktorů než viscerální TT u pacientů s manifestní aterosklerózou (Kremen et al. 2006).

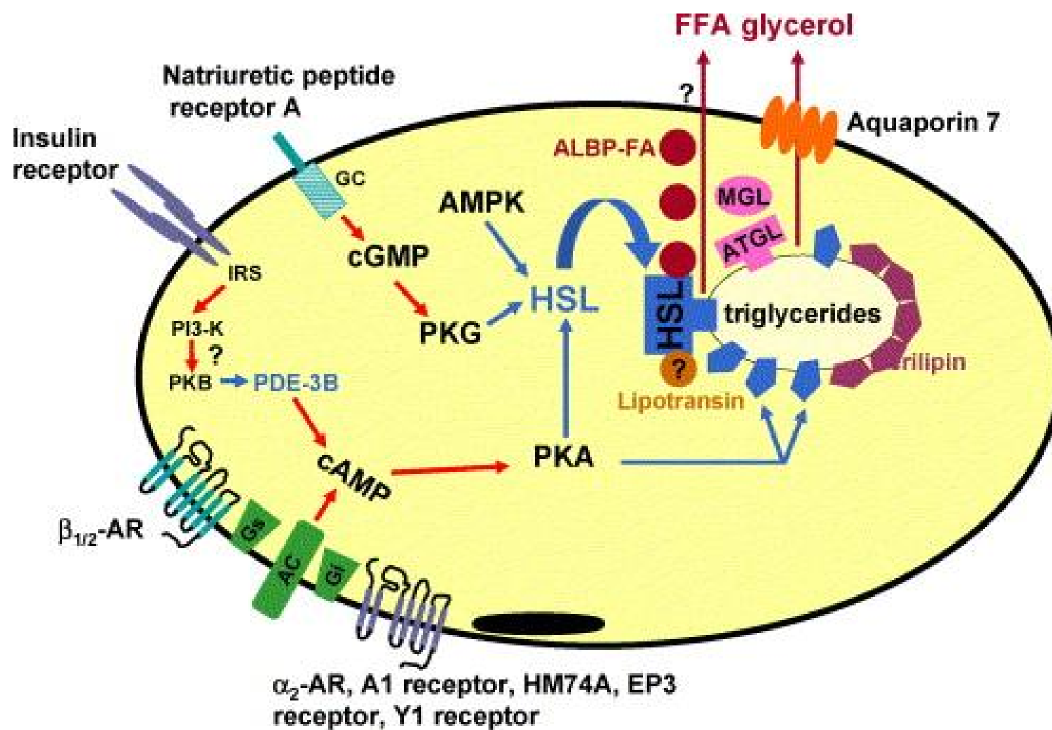
Rozložení tukové tkáně se liší v závislosti na pohlaví – pro ženy je typická gynoidní distribuce s akumulací TT v gluteofemorální oblasti a u mužů je typičtější akumulace abdominální TT. Rozdílné anatomické rozložení TT vykazuje diverzitu v odpovědích na externí signály (např. inzulin, lipolytické signály), rozdílný sekreční profil a zastoupení imunokompetentních buněk. Některé práce uvádějí, že viscerální TT je v porovnání s podkožní TT více infiltrována imunitními buňkami a produkuje vice prozánětlivých působků ve srovnání s podkožní TT (Dolinkova et al. 2008). Rozdíl mezi těmito dvěma depy je dán rozdílnou prozánětlivou charakteristikou TT, kdy viscerální TT je asociována s vyšším rizikem rozvoje metabolických komplikací (Klimcakova, Roussel, Kovacova, et al. 2011). Dalším vysvětlením rozdílného metabolického profilu těchto dvou dep může být výšší hladina volných MK pocházející z lipolýzy z abdominální TT, která je drénována přímo do portálního řečiště, což je spojováno s rozvojem inzulinové rezistence (Karpe, Dickmann, and Frayn 2011). Na druhé straně gluteofemorální TT představuje protektivní funkci a je spojena s nižším metabolickým rizikem a nižší kardiovaskulární morbiditou a mortalitou ve srovnání s abdominální obezitou (Snijder et al. 2004; Pischon et al. 2008). Studie naší skupiny neprokázala rozdílnou genovou expresi prozánětlivých cytokinů ani markerů makrofágů v podkožní abdominální TT a v gluteální TT v bazálním stavu ani při dietní intervenci (Malisova et al. 2014).

Několik studií dokládá vyšší uvolňování MK (Berman et al. 1998; Berman et al. 2004) a aktivitu LPL (Arner et al. 1991; Ferrara et al. 2002) v podkožní abdominální TT ve srovnání s gluteální, což předpokládá větší kapacitu gluteální TT k akumulaci lipidů.

2.2. Lipolýza tukové tkáně a její regulace

Lipolýza je proces mobilizace tuků z tukové tkáně, kdy se hydrolyzují triglyceridy (TG) (Mingrone and Castagneto-Gissey) a dojde k uvolnění volných MK a glycerolu do intersticia a následně do cirkulace (Stich and Berlan 2004). TG jsou hydrolyzovány 3 lipázami: adipotriglycerid lipáza (ATGL), která hydrolyzuje triacylglycerol na diacylglycerol (DAC) a volné MK, dále hormon-senzitivní lipáza (HSL) hydrolyzuje DAG na monoacylglycerol (MAG), a monoacylglycerol lipáza (MGL), která hydrolyzuje MAG na glycerol a MK. Lipolytický účinek má řada hormonů a cytokinů, které se podílejí na regulaci lipolýzy. Katecholaminy a Atriální natriuretický peptid (ANP) jsou hlavními stimulátory lipolýzy (Langin, Lucas, and Lafontan 2000). Katecholaminycestou β1 a β2 adrenoreceptorů stimulují a cestou α2 receptorů inhibují lipolýzu, inzulin působí také inhibičně, zatímco atriální natriuretický peptid a růstový hormon jsou faktory stimulační. Byla popsána také řada endogenních působků s lipolytickým působením – např. TNF a IL-6 (shrnuto v review (Rossmeislova, Malisova, Kracmerova, and Stich 2013). U obézních je popsána porucha regulace lipolýzy v podkožní tukové tkáni vyjádřena jako rezistence ke katecholaminy-stimulované lipolýze. Tato rezistence může být dána vlivem snížení exprese β2 adrenoreceptorů a zvýšení exprese α2 adrenoreceptorů (Mauriege et al. 1991). Dalším z defektů u obézních je snížená exprese HSL v podkožní tukové tkáni (Large et al. 1999), tento defekt byl popsán i u prvostupňpvých příbuzných obézních pacientů (Hellstrom et al. 1996).

Obrázek č. 4: Lipolýza (Langin 2006)



2.3. Endokrinní funkce tukové tkáně

Tuková tkáň je považována za aktivní endokrinní orgán, který uvolňuje do cirkulace cytokiny podílející se na regulaci systémového metabolismu a ovlivňující funkci imunitních buněk i adipocytů samotných (Hotamisligil, Shargill, and Spiegelman 1993). Dle současných poznatků je známo více než 600 působků produkovaných tukovou tkání (Bluher 2013). Tyto působky se obecně nazývají adipokiny či adipocytokiny, jsou produkovány nejen adipocyty, ale také imunokompetentními buňkami, endoteliálními buňkami, fibroblasty a dalšími buňkami v tukové tkáni. Jsou popsány stovky adipokinů zahrnující protizánětlivé (např. adiponectin) i prozánětlivé

působky (tumor necrosis factor α: TNFα, monocyte chemoattractant protein 1: MCP1, interleukin 6: IL6). Přehled některých adipokinů shrnuje Tabulka č. 3.

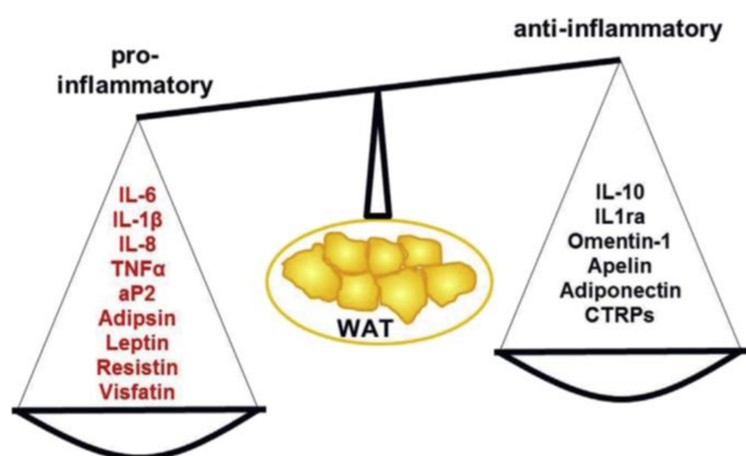
Tabulka č. 3: Přehled adipokinů (upraveno dle (Ouchi et al. 2011)

Název hormonu	**Zdroj**	**Funkce**
Leptin	Adipocyty	Kontrola chuti k jídlu a energetické homeostázy, imunitní funkce
Adiponectin	Adipocyty	Protizánětlivý, inzulinový senzitizér, antilipolytický
Resistin	Monocyty/makrofágy	Zvyšuje sekreci prozánětlivých cytokinů z makrofágů, spojen s inzulinovou rezistencí
Monocytární chemotaktický protein (MCP-1 = CCL-2)	Adipocyty, SVF buňky	Recruitment monocytů
Interleukin 6	Adipocyty, SVF buňky, hematocyty, svalové buňky	Liší se dle tkáně
Interleukin 18	SVF buňky	prozánětlivý

Tumor necrosis factorα	Adipocyty, SVF buňky	Prozánětlivý, antagonista inzulinové signalizace
Retinol binding protein 4	Hepatocyty, adipocyty, makrofágy	inzulinová rezistence
Visfatin	Adipocyty	Inzulin –mimetický efekt
Fatty acids binding protein 4	Adipocyty	Intracelulární transport MK, ovlivnění inzulinové rezistence

Exprese prozánětlivých a protizánětlivých adipokinů je ve zdravé tukové tkáni v rovnováze. Nicméně u obézních převažují prozánětlivé adipokiny, které vedou k rozvoji inzulinové rezistence.

Obrázek č. 5: Převaha prozánětlivých adipokinů u obézních (Aguilar-Valles et al. 2015)



Leptin

Leptin je proteinový hormon kódovaný *ob* genem, který byl objeven v roce 1994 metodou pozičního klonování u morbidně obézních *ob/ob*myší (Zhang et al. 1994). Ob/ob myš se vyznačuje mutací genu pro leptin a trpí hyperfágií, morbidní obezitou, inzulinovou rezistencí a diabetem mellitem 2.typu (Friedman and Halaas 1998). Podáním rekombinantního leptinu došlo ke zvratu těchto odchylek. Nicméně, u obézních pacientů nepřineslo podávání leptinu v léčbě obezity žádné výsledky. Hladiny leptinu jsou u obézních zvýšeny, což dokládá existenci leptinové rezistence (Cui, Lopez, and Rahmouni 2017). Tento adipokin se podílí na centrální regulaci jídelního chování aktivací anorexigenní osy s následnou redukcí energetického příjmu a zvýšením energetického výdeje (Vong et al. 2011). Hladiny leptinu pozitivně korelují s množstvím tukové tkáně. Nejpravděpodobnější úlohou leptinu je jeho regulační vliv u malnutričních stavů. Navíc má další mnohočetné role v imunitním systému, kdy se považuje za převážně prozánětlivou molekulu schopnou aktivovat adaptivní i vrozenou imunitu (Naylor and Petri 2016).

Adiponektin

Rok po objevu leptinu byl popsán adiponektin, protein produkován *adipo*O genem a sekretován ze zralých adipocytů do cirkulace, kde dosahuje násobně vyšších hladin než ostatní cytokiny (3-30 µg/ml) (Ouchi et al. 2003). Adiponektin je ve vysoké míře exprimován funkčními, inzulin-citlivými adipocyty a jeho exprese je snížena u dysfunkčních hypertrofovaných adipocytů vyskytujících se převážně u obézních (Lihn, Pedersen, and Richelsen 2005). Hladiny adiponektinu jsou snížené při obezitě, inzulinové rezistenci a diabetu mellitu 2.typu (Arita et al. 1999; Abbasi et al. 2004). Vyskytuje se v několika multimerních izoformách – jako trimer o nízké molekulové hmotnosti, hexamer o střední molekulové hmotnosti a multimer o vysoké molekulární hmotnosti.

Všechny tyto izoformy jsou detekovatelné v krvi. Jednotlivé izoformy vykazují různou aktivitu a afinitu v různých tkáních a stav polymerizace adiponektinu ovlivňuje jeho biologickou aktivitu (Waki et al. 2003). Ve studii naší skupiny bylo prokázáno, že hladina adiponektinu se nemění po redukci hmotnosti, což indikuje, že adiponektin zřejmě není hlavní determinanta inzulinové citlivosti navozené redukcí hmotnosti (Polak et al. 2008). V experimentu na myším modelu však podávání adiponektinu upravilo parametry inzulinové rezistence. Podání perorálního antidiabetika thiazolidindionu (glitazony) zvýšilo hladinu adiponektinu, což může částečně vysvětlovat mechanismus účinku tohoto léku (Maeda et al. 2001).

Interleukin 6

IL-6 patří do skupiny prozánětlivých cytokinů a tuková tkáň produkuje zhruba 1/3 cirkulujícího množství IL-6 (Ziccardi et al. 2002). Exprese IL-6 v TT i koncentrace cirkulujícího IL-6 pozitivně koreluje s obezitou, poruchou glukozové tolerance a inzulinovou rezistencí (Fernandez-Real and Ricart 2003). V práci naší skupiny bylo ukázáno, že se hladina i exprese IL-6 v tukové tkáni snižuje při redukci hmotnosti (Siklova-Vitkova et al. 2012). IL-6 také inhibuje adipogenezi a snižuje sekreci adiponektinu (Fernandez-Real and Ricart 2003). Role IL-6 je však velmi komplexní a rozdílné efekty na energetickou homeostázu vykazují účinky IL-6 v periferii a v CNS (Kershaw and Flier 2004).

MCP-1

Obezita je asociována se zvýšenou infiltrací TT makrofágy, které svou sekreční aktivitou přispívají k inzulinové rezistenci (např. IL-6 a TNF- α). MCP-1 je chemokin, který je exprimován v tukové tkáni adipocyty i buňkami SVF a působí jako chemoatraktant makrofágů do TT (Weisberg et al. 2003). Infiltrace TT makrofágy pak může přispívat k metabolickým odchylkám u obezity a

inzulinové rezsitence (Sartipy and Loskutoff 2003). Toto dokládá in vitro studie, kdy adipocyty inkubované s MCP-1 vykazovaly snížené vychytávání glukózy a rezistenci k inzulinu (Gerhardt et al. 2001). Studie naší skupiny ukazují vyšší sekreci MCP-1 z TT při akutní 3-hodinové hyperglykémii (Siklova et al. 2015).

TNF–α

Tento cytokin je jeden z hlavních prozánětlivých mediátorů, který je zvýšeně exprimován adipocyty a buňkami SVF v tukové tkáni u obezity a inzulinové rezistence (Hotamisligil, Shargill, and Spiegelman 1993). Hladiny cirkulujícího TNF-α jsou zvýšené u obézních diabetiků 2.typu na rozdíl od neobézních diabetiků (Miyazaki et al. 2003). Tento mediátor ovlivňuje metabolismus lipidů v tukové tkáni. *In vitro* bylo prokázáno na izolovaných adipocytech, že TNF-α suprimuje aktivitu genů regulujících vychytávání MK v TT. Zároveň TNF-α aktivuje lipolýzu v TT a dochází tak ke zvýšení hladin volných MK, což zřejmě přispívá k rozvoji inzulinové rezitence (Akash, Rehman, and Liaqat 2018).

2.4. Imunitní buňky v tukové tkáni

Obezita je asociována s chronickým mírným prozánětlivým stavem organismu spojeným s produkcí a uvolňováním prozánětlivých molekul do cirkulace a do vlastní tukové tkáně a se zvýšenou infiltrací TT imunitními buňkami (Rosen and Spiegelman 2014). Poruchy funkce TT jsou spojeny s metabolickými odchylkami na celotělové úrovni, jak dokládají práce, kdy inzulinová rezistence koreluje s infiltrací podkožní TT makrofágy a s expresí a sekrecí prozánětlivých cytokinů (Klimcakova, Roussel, Kovacova, et al. 2011; Klimcakova, Roussel, Marquez-Quinones, et al.

2011). Fenotyp imunitních buněk je ovlivňován cytokiny produkovanými přímo adipocyty nebo buňkami SVF. Např. leptin stimuluje aktivaci makrofágů a indukuje proliferaci TH1 lymfocytů (Bruun et al. 2002), dále spouští uvolnění prozánětlivých cytokinů (TNFα, IL1β, IL6) z imunitních buněk (Zarkesh-Esfahani et al. 2004). Na druhé straně adiponektin indukuje sekreci protizánětlivých cytokinů z makrofágů (Wolf et al. 2004).

Imunitní buňky v tukové tkáni štíhlých jedinců vyjadřují protizánětlivý fenotyp v zastoupení T- helper (T_H) a T-reg lymfocyty a M2 typ makrofágů (Zeyda et al. 2011). U obézních jedinců převažuje prozánětlivý fenotyp imunitních buněk - T_H1 lymfocyty, cytotocické T-lymfocyty (T_C) a M1 typ makrofágů (Osborn and Olefsky 2012). Je nutno brát v úvahu, že tyto dobře odlišitelné skupiny makrofágů M1 a M2 byly popsány ve zvířecím experimentu (Lumeng, Bodzin, and Saltiel 2007), ale v lidské tukové tkáni zřejmě není toto fenotypické rozdělení na prozánětlivé a protizánětlivé makrofágy jednoznačně prokázáno, spíše se předpokládá celé kontinuum fenotypu od M1 k M2 makrofágům. Jak bylo ukázáno ve studii skupiny Anne Boulomié - makrofágy v lidské tukové tkáni vykazují fenotyp s prozánězliváými i protizánětlivými charakteristikami (Curat et al. 2004).

Ačkoli se za hlavní faktor v „osídlení" TT imunitními buňkami dlouhou dobu považovaly právě makrofágy, které svými působky přitahovaly další imunitní buňky do tukové tkáně, při dalším studiu se ukázalo, že akumulaci makrofágů předchází infiltrace T- lymfocyty, které pravděpodobně reagují na metabolické stimuly jako je vysokotuková dieta dříve než makrofágy (Feuerer et al. 2009). Jiné studie poukazují na to, že dalším možným stimulem infiltrace tukové tkáně imunitními buňkami mohou být volné mastné kyseliny, které se uvolňují z hypertrofických adipocytů a vedou k expresi chemoatraktantů (MCP -1, IL-8) (Graham et al. 2006). Důležitými stimuly ovlivňujícími vstup imunokompetentních buněk do TT jsou také: lipotoxicita (Unger and Scherer 2010), zvýšená

apoptóza hypertrofických adipocytů v důsledku hypoxie (Halberg et al. 2009) a stres endoplazmatického retikula (Ozcan et al. 2004). Vliv nutriční a dietní intervence na infiltraci tukové tkáně imunitními buňkami je jedním z cílů této práce.

2.5. Dysfunkce tukové tkáně

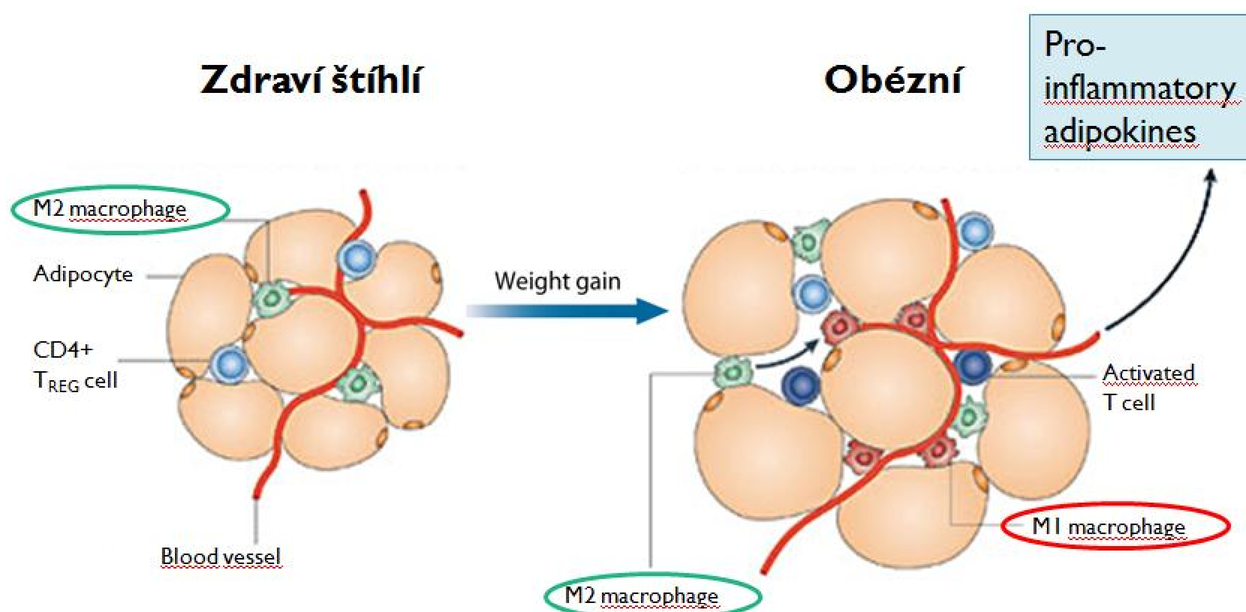
Z pohledu metabolických komplikací je zcela zásadní schopnost tukové tkáně ukládat lipidy a jejich metabolity, aby nedocházelo k jejich ektopickému ukládání mimo tukovou tkáň (Ravussin and Smith 2002). Jsou dva základní principy expanze tukové tkáně, jak již bylo zmíněno výše.

Hyperplastický typ je charakterizován tvorbou nových (pre)adipocytů s vysokou schopností ukládat tukové kapénky. Tento typ expanze je spojen s nižším rizikem metabolických komplikací obezity a představuje jedno z vysvětlení tzv. metabolicky zdravých obézních (Tan and Vidal-Puig 2008).

Hypertrofický typ je častější cesta ukládání nadměrného množství energie založena na zvětšování adipocytů. Tyto zvětšené adipocyty podléhají stresovým podmínkám jako je mechanický stres, lokální hypoxie v důsledku nedostatečné vaskularizace (Trayhurn, Wang, and Wood 2008) a stres endoplazmatického retikula. Dochází tak k aktivaci kaskád, které svými působky přispívají k prozánětlivému stavu organismu (Hotamisligil 2010). Alterovaná exprese prozánětlivých a protizánětlivých faktorů produkovaných imunitními buňkami působí zpětně na adipocyty a podílí se na vzniku dysfunkce tukové tkáně. Navíc hypertrofický adipocyt vykazuje poruchu inzulinem stimulované suprese lipolýzy a poruchu katecholaminy a ANP (atriální natriuretický paptid) stimulované lipolýzy v TT (Ryden et al. 2016; Nielsen et al. 2014). Zvláště pak postprandiálně dochází k poruše skladování lipidů a to vede k přetížení kapacity adipocytů a k ektopickému ukládání tuků v periferních tkáních jako je kosterní sval, játra, pankreas, srdce a ledviny, což přispívá k rozvoji systémové inzulinové rezistence (Verboven et al. 2018). Schopnost expanze

podkožní tukové tkáně při nadměrném kalorickém příjmu tak může hrát zásadní roli v rozvoji metabolických komplikací u obézních pacientů. Studie naznačují, že redukce tělesné hmotnosti navozená dietním režimem pozitivně ovlivňuje schopnost expanze a adipogenní potenciál tukové tkáně (Rossmeislova, Malisova, Kracmerova, Tencerova, et al. 2013).

Obrázek č. 6: Dysfunkční tuková tkáň u obézních - infiltrace imunokompetentními buňkami a sekrece prozánětlivých adipocytokinů (upraveno dle (Gleeson et al. 2011).



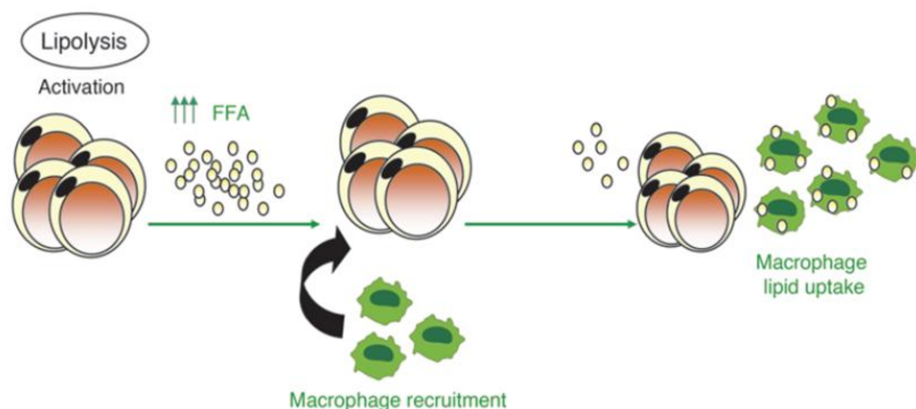
2.6. Úloha hyperlipidémie a hyperglykémie v patogenezi dysfunkce tukové tkáně a metabolického syndromu.

Hyperlipidémie

Jak již bylo v této práci zmíněno, obezita je spojena se zvýšenou aktivací imunitních buněk a jejich infiltrací do tukové tkáně, což vede ke změnám v její metabolické i endokrinní funkci. Metabolické faktory, které u člověka regulují zvýšenou infiltraci a akumulaci makrofágů a dalších imunitních buněk v tukové tkáni, nejsou stále známy. Jedním z faktorů hrajících zásadní roli v tomto procesu jsou zřejmě volné mastné kyseliny (Klimcakova, Roussel, Marquez-Quinones, et al. ; Kosteli et al. 2010). Práce Kosteliho a kol. na myším modelu prokázala, že zastoupení makrofágů v tukové tkáni korelovalo s koncentrací cirkulujících MK a s jejich zvýšeným uvolňováním při farmakologické stimulaci či hladovění. V *in vitro* studiích samotné saturované MK způsobují zvýšení mRNA exprese i sekrece prozánětlivých adipokinů a chemokinů (MCP-1, IL-6, IL-8) u adipocytů i makrofágů (Haversen et al. 2009a; Takahashi et al. 2008): mechanismem působení může být stres endoplasmatického retikula a aktivace TLR2/4 receptorů, které hrají v imunitní odpovědi významnou roli. Zvýšené hladiny MK způsobují zvýšení mRNA exprese CD-36 na povrchu monocytů, což vede k akumulaci lipidů v těchto buňkách a je předpokladem rozvoje IR a aterosklerózy (Kashyap et al. 2009). Ve shodě s tímto bylo opět na myším modelu prokázáno, že vlivem vysokotukové stravy může dojít ke změně fenotypu/polarizaci rezidentních makrofágů ve směru aktivace makrofágů produkujících prozánětlivé látky (Lumeng, Bodzin, and Saltiel 2007). Tato změna je spojena i s vyšší akumulací lipidů přímo v makrofázích (Prieur et al. 2011). Tyto poznatky naznačují, že makrofágy zachycují přebytek lipidů, který není adipocyty efektivně ukládán a zvýšená akumulace lipidů v makrofágu následně ovlivňuje jeho fenotyp ve směru prozánětlivé aktivace. V několika studiích na zdravých neobézních i obézních subjektech byl

prokázán postprandiální nárůst prozánětlivých cytokinů (IL-6, PAI-1) v plasmě po HFM (Blackburn et al. 2006; Poppitt et al. 2008). Jedním z důležitých zdrojů cirkulujících cytokinů je tuková tkáň, nicméně změny ukazatelů prozánětlivého stavu TT v podmínkách zvýšené hladiny lipidů, respektive mastných kyselin, jsou u člověka prozkoumány jen málo.

Obrázek č. 7: Zvýšená hladina volných MK atrahuje makrofágy do tukové tkáně (Kosteli et al. 2010).



Hyperglykémie

Porucha glukózového metabolismu u obézních může představovat jeden z faktorů podílejících se na deterioraci funkce TT. Lu a kol. exponovali adipocyty hyperglykemickým podmínkám a prokázali snížení inzulinové citlivosti a zvýšení reaktivních forem kyslíku in vitro na krysích adipocytech (Lu et al. 2001). Podobné závěry byly prokázány i na humánních adipocytech, kterépři hyperglykemických podmínkách in vivo vykazovali snížení insulinem stimulovaného odsunu glukózy (Lin et al. 2005). Navíc bylo prokázáno, že fluktuace hladin glykémie je více škodlivá než chronická expozice hyperglykémii (Monnier et al. 2006). Škodlivý efekt hyperglykémie může být

zprostředkován cestou indukce oxidačního stresu (cestou konečných produktů glykace a aktivace protein kinázy C) a aktivací prozánětlivých kaskád vedoucí ke zvýšení sekrece prozánětlivých cytokinů (Marfella et al. 2001; Chen 2006). Je popsán efekt hyperglykémie na zvýšení hladin leptinu a snížení hladin adiponektinu, což přispívá k metabolické dysfunkci u diabetiků 2.typu (Faraj et al. 2008). Je také popsán efekt hyperglykémie na zvýšení mRNA a proteinové exprese TLR-2 a TLR-4 na monocytech, což zřejmě přispívá k prozánětlivému stavu organismu (Dasu et al. 2010). V této dizertační práci v ČÁSTI A se zabývám právě vlivem hyperlipidémie a hyperglykémie na systémový prozánětlivý stav a imunitní odpověď v tukové tkáni.

2.7. Vliv redukční diety na tukovou tkáň u obézních

Klíčovou složkou prevence a léčby obezity je redukční dieta, která vede nejen ke snížení tukové hmoty, ale i metabolických poruch s obezitou spojených. Nízkokalorická dieta vyvolává funkční i morfologické změny v TT a lze předpokládat, že takto napomáhá k „úpravě“ dysfunkce TT. Studie porovnávající efekt přísné nízkokalorické diety (VLCD) a bariatrické chirurgie ukazují téměř stejný efekt na zlepšení inzulinové citlivosti, funkce β-buněk a lipidových parametrů, pokud je dosaženo srovnatelné redukce tukové hmoty (Jackness et al. 2013). Nicméně pozitivní efekt kalorické restrikce je pozorován již v době, kdy není dosaženo úbytku tukové hmoty. Inzulinová citlivost vyjádřena indexem HOMA-IR (homeostasis model assessment for insulin resistence) se zvýšila již po 2 dnech VLCD (Jazet et al. 2005). Stejně tak efekt bariatrické chirurgie na parametry glukózového metabolismu byl pozorován u diabetiků 2. typu již v časné pooperační fázi bez dosažené redukce tukové hmoty (Mingrone and Castagneto-Gissey 2009). Mechanismus časného efektu bariatrické chirurgie je vysvětlován vyloučením proximální části tenkého střeva, tím dojde k supresi anti-inkretinových působků, zvýšení cirkulují hladiny glukagon-like peptidu 1

(GLP-1) a stimulaci sekrece inzulinu (Laferrere et al. 2008; Laferrère 2016). Mechanismus efektu samotné kalorické restrikce není dosud zcela prozkoumán. Podílet se může modifikace charakteristik TT a zastoupení imunitních buněk, přestože se nemění množství TT. Odpověď prozánětlivých cytokinů na kalorickou restrikci (VLCD) byla popsána ve více studiích shrnutých v následujících přehledech (Rossmeislova, Malisova, Kracmerova, and Stich 2013), (Klimcakova et al. 2010). Obecně lze shrnout, že redukce hmotnosti 5-10% moduluje sekreci adipokinů a vede ke zlepšení metabolického profilu jedince. Ve studiích naší skupiny bylo ukázáno, že modulace adipokinů při kalorické restrikci závisí na době trvání a míře restrikce (Siklova-Vitkova et al. 2012, Siklova et al. 2014). V dalších studiích byl prokázán pokles genové exprese markerů makrofágů (Capel et al. 2009a) či přímý pokles makrofágů v TT měřený průtokovou cytometrií v odpovědi na kalorickou restrikci. (Kovacikova et al. 2011). Dietní intervence také ovlivňují regulaci lipolýzy. Krátkodobé hladovění stimuluje lipolýzu u neobézních subjektů, u obézních k této změně nedochází (Horowitz et al. 1999).Ve studii naší skupiny byla u obézních jedinců zjištěna aktivace lipolýzy po 2 dnech velmi přísné kalorické restrikce (VLCD) bez redukce hmotnosti: bylo zjištěno zvýšení exprese HSL v podkožní TT a zvýšené uvolňování glycerolu z TT (Siklova et al. 2014). Aktivovaná lipolýza přetrvává i po měsíci přísné kalorické restrikce, kdy studie prokázaly vyšší expresi HSL (Stich et al. 1997) a také vyšší citlivost ke smimulaci lipolýzy katecholaminy (Barbe et al. 1997) nebo ANP (Sengenes et al. 2002). Regulace lipolýzy a uvolňování volných MK z tukové tkáně do cirkulace se mění v jednotlivých fázích dietního režimu, což může být podkladem dynamiky odpovědi inzulinové rezistence, imunitního stavu a dalších metabolických změn na nutriční intervenci. Je zřejmé, že změny vyvolané kalorickou restrikcí vedou k úpravě poruchy regulace lipolýzy u obézních (Stich 2016). V této dizertační práci v ČÁSTI B se zabývám efektem kalorické restrikce na ovlivnění metabolických a imunitních charakteristik v tukové tkáni.

3. POPIS VYBRANÝCH METODIK

Jednotlivé metody jsou popsány v Přehledu publikací v Kapitole 5 a podrobně dále v přiložených originálních publikacích, proto jsou zde popsány pouze některé klíčové metody zejména s klinickým zaměřením.

3.1. Jehlová biopsie podkožní tukové tkáně

Tato metoda spočívá v odběru malého množství (1-2g) podkožního tuku z oblasti břicha. V cca 10-15 cm laterálně od umbiliku za sterilních kautel po znecitlivění lokální anestezií (1% Mesocain Zentiva, Praha) zavádíme punkční jehlu s 20 ml stříkačkou, která nám vytvoří podtlak. Takto několik minut odsáváme podkožní tuk, který je následně pročištěn a rozdělen pro následné analýzy.

3.2. Euglykemicky a Hyperglykemický clamp

Euglykemický hyperinzulinový clamp (zámek) je „zlatý standart" ke zjištění periferní inzulinové rezistence organismu a jeho protokol detailně popsal v roce 1979 DeFronzo (DeFronzo, Tobin, and Andres 1979). Principem je zhodnotit inzulinovou citlivost na základě rychlosti odsunu glukózy při intravenózním podání předem definovaného množství inzulinu (1 mIU.kg-1 .min-1). Tato koncentrace inzulinu v plazmě (hyperinzulinémie) je dostačující k inhibici endogenní produkce glukózy a sekrece inzulinu. Pacient se dostaví po 12-ti hodinách lačnění a jsou mu zavedeny periferní žilní kanyly v kubitální jamce pro podávání roztoku inzulinu (Humulin R, Eli Lilly, ČR) a zároveň je podáván nitrožilně roztok 15% glukózy s 30ml 7,45% KCl tak, aby byla udržována euglykémie. Odběry krevních vzorků ke stanovení glykémie (Precission PCx , Abbott Laboratories, Německo) probíhají po 5-ti minutách z žíly na dorsu ruky, která je v průběhu

vyšetřování ohřívána k dosažení arterializace žilní krve. Rychlost infuze glukózy potřebná k udržení euglykémie odpovídá odsunu glukózy z extracelulárního prostředí do buněk a je měřítkem účinku inzulinu. Tato hodnota je vyjádřena jako tzv. M-value (metabolizovaná glukóza). M-hodnota je vypočtena z rychlosti průtoku roztoku glukózy v rovnovážné fázi v posledních 30-ti minutách clampu vydělením střední rychlosti infuze glukózy za určitou periodu clampu tělesnou hmotností (mg/kg/min) (Pelikánová and Zadák 2011).

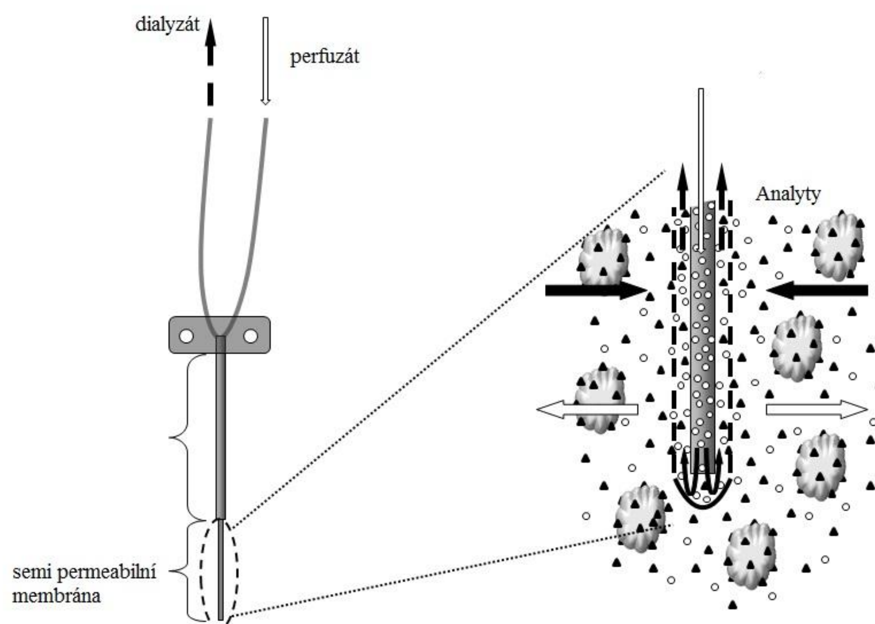
Hyperglykemický clamp, který se standartně využívá k testování sekrece inzulinu, jsme použili ve studii č. 2 k navození experimentální hyperglykémie. Pacient se dostaví po 12-ti hodinách lačnění a jsou mu zavedeny periferní žilní kanyly v kubitální jamce. 5 minut před zahájením bolusu glukózy je pacientovi podána infuze octreotidu (Sandosatin, Novartis) k zablokování endogenní produkce inzulinu. Iniciálně podáváme 25 µg intravenózně a následně infuzi rychlostí 30ng/min/kg hmotnosti pacienta. Po bolusové dávce 0,33 g/kg glukózy jsme intravenózně podali roztok 20% glukózy k dosažené stabilních hodnot glykémie v rozmezí 15 – 18 mmol/l po dobu třech hodin. Odběry krevních vzorků ke stanovení aktuální glykémie (Precission PCx , Abbott Laboratories, Německo) byly prováděny po 5-ti minutách.

3.3. Mikrodialýza

Mikrodialyzační technika je mini-invazivní metoda umožňující monitorování metabolitů v extracelulární tekutině *in vivo*. V našich studiích využíváme tuto metiodiku především ke sledování změn v podkožní tukové tkáni během různých intervencí. Mikrodialyzační sonda je vybavena semi-permeabilní membránou, která umožňuje průchod látek po směru osmotického gradientu, tedy z tkáně do dialyzátu, ve kterém jsou posléze tyto látky analyzovány. Nejčastěji jsou

stanovovány nízkomolekulární metabolity, cytokiny apod. Ve studii č. 4 jsme analyzovali hladinu metforminu, laktátu a glycerolu v tukové tkáni během akutní tělesné zátěže. Mikrodialzyční sondu (20 × 0.5 mm; 20 kDa cut-off; Carnegie Medicine, Stockholm, Sweden) zavádíme do podkožní tukové tkáně v oblasti břicha cca 10-15 cm od umbiliku v lokálním zneciltlivění (1% Mesocain, Zentiva, Praha) za sterilních kautel. Sonda je napojena na mikrodialyzační pumpu (Harvard apparatus, France) a promývána Ringerovým roztokem (139 mM Na^{+}, 2.7 mM K^{+}, 0.9 mM Ca^{2+}, 140.5 mM Cl^{-}, 2.4 mM HCO_3^{-}, Baxter, Czech Republic) rychlostí 2.5 µL/min. Vzorky dialyzátu jsou sbírány po 60 minutách ekvilibrace v 15-30 minutových intervalech, dále zmraženy v -80 °C a následně analyzovány pomocí enzymatických kolorimetrických metod (Randox Laboratories Ltd., UK) nebo pomocí kapilární elektroforézy (Tuma, Sommerova, and Siklova 2019).

Obrázek č. 8: Princip mikrodialýzy



3.4. Průtoková cytometrie

Průtoková cytometrie je laboratorní metoda umožňující analýzu různých buněčných subpopulací pomocí specifických markerů. Typizace jednotlivých buněčných populací využívá reakce antigen-protilátka, kdy reakce je vizualizována označením protilátky fluorochromem a odečtení této fluorescence při průchodu laserovým paprskem. Výstupem měření jsou pak výsledky v grafické podobě histogramů a v procentuálním zastoupení buněk nesoucích daný antigen. K detekci povrchových antigenů jsme použili fluresceněně značené protilátky CD4, CD14, CD16 and CD36, CD3, CD11c, CD14, Toll-like receptor TLR2 a TLR4, CD8 and CD56 (BD Biosciences), které byly přidány ke vzorku plné krve nebo stromo-vaskulární frakce z tukové tkáně a ponechány 30 minut v pokojové teplotě. Poté bylo přidáno lyzační činidlo k lýze erytrocytů na 15 minut a po promytí fosfátovým pufrem byl vzorek analyzován průtokovým cytometrem (FACS-Calibur a CellQuest Pro Software, BD Bio-sciences). Metodickou originalitou naší laboratoře bylo použití průtokové cytometrie ke stanovení zastoupení imunitních buněk v malém vzorku podkožní tukové tkáně (500mg). Tato metodika nebyla v této aplikaci v době vzniku studie používána.

3.5. Analýza metabolitů a cytokinů v plasmě

Vzorky plasmy a séra byly připravovány z nesrážlivé periferní krve a následně centrifugovány a rozděleny do alikvotů. Základní biochemické parametry byly stanovovány v certifikovaných laboratořích (FNKV, Spadia Lab). K analýze plasmatických hladin cytokinů jako IL-6, IL-8, IL-10, TNF-α MCP-1, FGF-21, IL-1β , leptin, VEGF-A , sICAM , sVCAM byla využita imunoanalýza pomocí metody ELISA (**E**nzyme-**L**inked **I**mmuno**S**orbent **A**ssay) za použití komerčních kitů (eBioscience; RaD; MyBiosource, USA) nebo multiplexová imunoanalýza (High Sensitivity

Human Cytokine Milliplex panel; Merck.Millipore, USA) na přístroji Luminex 200. Pro stanovení hladiny glycerolu a neesterifikovaných mastných kyselin v séru byly použity komerční analytické soupravy na principu kolorimetrie „RX Monza“ (Randox Laboratories Ltd., UK).

3.6. Statistická analýza

Analýza a grafické zpracování výsledků byly provedeny v programu GraphPad Prism 5.0 (GraphPad Software, USA). Výsledky jsou uvedeny jako průměr ± standardní chyba průměru (SEM). Hladina statistické významnosti byla stanovena na $p < 0,05$. Normalita dat byla posuzována Shapiro-Wilkovým testem. Data byla vyhodnocena dvouvýběrovým Studentovým T-testem nebo neparametrickým Mann-Whitney testem, dle normálního či nenormálního rozložení dat. Efekty intervence byly testovány jednocestnou nebo dvoucestnou ANOVA (analýza rozptylu) metodou s Bonferroni post hoc analýzou. Korelace byly provedeny pomocí Personova nebo Spearmanova korelačního testu.

4. CÍLE PRÁCE A HYPOTÉZY

Porucha metabolických, endokrinních a imunitních funkcí TT se podílí na rozvoji inzulinové rezistence u obézních jedinců a následném zvýšeném riziku rozvoje metabolických a kardiovaskulárních chorob. Předmětem intenzivního výzkumu řady pracovišť je identifikovat molekuly a mechanismy zprostředkovávající regulaci výše zmíněných funkcí TT tak, aby se mohly stát potenciálním cílem preventivního i léčebného ovlivnění metabolických důsledků obezity.

Cílem tohoto projektu bylo přispět k poznání regulace výše zmíněných funkcí TT různými nutričními podněty: od restrikce energetického příjmu až k navození hyperlipidémie či hyperglykemie. Byly sledovány základní charakteristiky prozánětlivého stavu v TT tj. a) zastoupení a/nebo aktivace makrofágů a dalších buněčných populací v tukové tkáni, b) produkce prozánětlivých cytokinů buňkami tukové tkáně.

Poznatky získané uvedenými studiemi poukazují na mechanismy vzniku nutricí vyvolaných metabolických změn v tukové tkáni na molekulární a buněčné úrovni a otevírají cestu k účinnější prevenci a léčbě obezity.

Specifické cíle:

ČÁST A)

- Objasnit některé z mechanismů lipotoxicity resp. glukotoxicity sledováním účinku krátkodobých parenterálních intervencí vedoucích k hyperlipidémii a hyperglykémii na imunitní stav TT, prozánětlivý stav organismu a vztah k ateroskleróze u obézních žen (Publikace č. 1 a 2)

  Hypotéza: Hyperlipidémie a hyperglykémie imitují obezitogenní prostředí a vedou k rozvoji prozánětlivého stavu na úrovni organismu i tukové tkáně.

- analyzovat prozánětlivou reakci v cirkulaci vyvolanou jednorázovým vysokotukovým pokrmem u mladých zdravých mužů (Publikace č. 3)

  Hypotéza: Vysokotukový pokrm vede k rozvoji prozánětlivého stavu, na kterém se podílí aktivace stresu endoplasmatického retikula.

- sledovat regulaci mobilizace MK z TT během tělesné zátěže při podání insulin-sensibilizujícího a antilipolytického agens - metforminu- u mladých zdravých mužů (publikace č. 4)

  Hypotéza: Metformin inhibuje lipolýzu aktivovanou fyzickou aktivitou u zdravých mužů.

ČÁST B)

- sledovat účinek několikafázové kalorické restrikce na regulaci lipolýzy v TT na úrovni exprese regulačních genů v TT u obézních žen (Publikace č. 5)

  Hypotéza: Jednotlivé fáze lišící se dobou trvání a mírou kalorické restrikce jsou charakterizovány rozdílnou regulací lipolýzy v TT tj. rozdílnou expresí genů regulujících metabolismus a lipolýzu.

- Porovnat efekt 2-denní a 28-denní velmi přísné kalorické restrikce na metabolické a prozánětlivé charakteristiky v cirkulaci a v podkožní tukové tkání u obézních žen (Publikace č. 6)

  Hypotéza: a) Již po dvou dnech trvání přísné nízkokalorické diety dochází ke změnám na úrovni systémového metabolického stavu organismu, které mohou být podmíněny změnami na úrovni molekulárních charakteristik tukové tkáně. b) Odpověď tukové tkáně na 2-denní a 28-denní kalorickou restrikci se liší jak v metabolických, tak v imunitních parametrech.

5. PŘEHLED PUBLIKACÍ

ČÁST A: Krátkodobé intervence

1) **Krauzová E**, Kračmerová J, Rossmeislová L, Mališová L, Tencerová M, Koc M, Štich V, Šiklová M. Acute hyperlipidemia initiates proinflammatory and proatherogenic changes in circulation and adipose tissue in obese women. Atherosclerosis. 2016 Jul;250:151-7.

2) Tencerová M, Kračmerová J, **Krauzová E**, Mališová L, Kováčová Z, Wedellová Z, Šiklová M, Štich V, Rossmeislová L. Experimental hyperglycemia induces an increase of monocyte and T-lymphocyte content in adipose tissue of healthy obese women. PLoS One. 2015 Apr 20;10(3).

3) Kračmerová J, **Czudková E,** Koc M, Mališová L, Siklová M, Stich V, Rossmeislová L. Postprandial inflammation is not associated with endoplasmic reticulum stress in peripheral blood mononuclear cells from healthy lean men. Br J Nutr. 2014 May 28:1-10.

4) **Krauzová E**, Tůma P, de Glisezinski I, Štich V, Šiklová M. Metformin Does Not Inhibit Exercise-Induced Lipolysis in Adipose Tissue in Young Healthy Lean Men. Front Physiol. 2018 May 23; 9:604.

ČÁST B: Dietní intervence

5) Koppo K, Valle C, Šiklová-Vítková M, **Czudková E**, de Glisezinski I, van de Voorde J, Langin D, Štich V. Expression of lipolytic genes in adipose tissue is differentially regulated during multiple phases of dietary intervention in obese women. Physiol Res. 2013;62:527-35.

6) Šrámková V, Rossmeislová L, **Krauzová E**, Kračmerová J, Koc M, Langin D, Štich V, Šiklová M. Comparison of Early (2 Days) and Later (28 Days) Response of Adipose Tissue to Very Low-Calorie Diet in Obese Women. J Clin Endocrinol Metab. 2016 Dec;101(12):5021-5029.

ČÁST A: Krátkodobé intervence

5.1. Publikace č. 1:

Originální publikace s obrázky a grafy je přiložena na str. 82.

Akutní hyperlipidémie vede k prozánětlivým a proaterogenním změnám v cirkulaci a v tukové tkáni u obézních žen.

Úvod: V této studii jsme se zaměřili na efekt parenterálního podání exogenních lipidů na zánětlivé a imunitní charakteristiky tukové tkáně. Za spouštěč prozánětlivého prosesu jsou považovány volné mastné kyseliny (Klimcakova, Roussel, Marquez-Quinones, et al.) a triglyceridy (Mingrone and Castagneto-Gissey), zejména saturované MK aktivují klasickou imunitní odpověď a regulují sekreci cytokinů v různých typech buněk (Peairs, Rankin, and Lee 2011; Boni-Schnetzler et al. 2009; Haversen et al. 2009b). Schopnost lipidů aktivovat prozánětlivou odpověď byla porkázána v další publikaci č. 3 po podání vysokotukového pokrmu, v jiné práci byla tato odpověď více vyjádřena u obézních pacientů (Marques-Lopes et al. 2001). Efekt lipidů na imunitní procesy v TT nejsou příliš probádány. Saturované MK mohou indukovat zvýšenou expresi prozánětlicýh cytokinů v adipocytech. Navíc jsou MK považovány za důležitý článek pro akumulaci makrofágů v TT u myší (Kosteli et al. 2010). Studie dokazují, že zvýšená hladina cirkulujících MK u lidských subjektů je asociována se zvýšenou expresí CD36 na monocytech, což vede k větší akumulaci lipidů v těchto buňkách a k rozvoji aterosklerózy (Kashyap et al. 2009; den Hartigh et al. 2010). Ateroskleróza je, stejně jako obezita, spojována s chronickým prozánětlivým stavem, endoteliální dysfunkcí s následnou aktivací cirkulujících leukocytů na místa poškození endotelu (Jaipersad et al. 2014

). Cílem studie bylo sledovat efekty zvýšených MK v tukové tkáni i na systémové úrovni.

Metody: Tato studie byla provedena u 15 obézních žen (BMI 31.4 ± 2.7 kg/m^2), u nichž byla nitrožilně podána infuze 20% Intralipidu (20% sojový olej,1.2% fosfolipidy vaječného žloutku, 2.25% glycerol, voda) po dobu 7h rychlostí 60ml/hod. Zastoupení jednotlivých MK v Intralipidu je: 11.3% palmitová, 4.9% stearová, 39.7% olejová, 46% linolová a 8,1% linolenová kyselina. V kontrolním experimentu byla u 10 žen (BMI 31.6 ± 3.4 kg/m^2) podána infuze fyziologického roztoku s 2.25% glycerolu. Před započetím a po ukončení infuze byla provedena biopsie podkožní tukové tkáně a odběry krve k provedení průtokové cytometrie a dalších analýz. Pomocí ELISA a multiplexové imunoanalýzy na přístroji Luminex byly stanovovány plasmatické hladiny cytokinů (IL-6, IL-8, TNF-α a MCP-1). Dále byly sledovány aterogenní markery spojené s adhezí imunitních buněk (sICAM, sVCAM) a angiogenesí (VEGF-A). Cca 1g tukové tkáně byl použit k analýze imunitních buněk v krvi a tukové tkáni pomocí průtokové cytometrie. Dále byla analyzována mRNA exprese prozánětlivých markerů a markerů imunitních buněk v tukové tkáni (CD3, CD25, RORC, FOXP3, CD14, CD68, CD206, CD163, CD36, TLR4, IL-6, IL-8, IL-10, MCP-1, TNF-α aj.)

Výsledky: Akutně zvýšená hladina MK vyvolaná infuzí Intralipidu způsobila zvýšení relativního zastoupení T-lymfocytů, a to především populace Th lymfocytů (CD45+/3+/4+) v cirkulaci. Ačkoli v tukové tkáni nedošlo k významným změnám v populacích T-lymfocytů, došlo zde ke zvýšení exprese markeru RORC (Th17 prozánětlivé lymfocyty). Relativní zastoupení monocytů CD45+/14+ a CD45+/14+/16+ v krvi bylo vlivem zvýšených hladin lipidů sníženo. Zároveň došlo ke zvýšení plasmatických hladin některých cytokinů (IL-6, MCP-1), adhesivních molekul (sICAM, sVCAM) a angiogenního markeru VEGF-A. V tukové tkáni došlo k nárůstu populace CD14+/206- a populace CD14/TLR4+ monocytů/makrofágů, a ke zvýšené expresi cytokinů IL-6 a MCP-1.

Diskuze: Akutní zvýšení hladin MK vyvolává zvýšení některých ukazatelů prozánětlivého a proaterogenního stavu jak v podkožní tukové tkáni, tak na systémové úrovni. Zjištěné změny naznačují, že zvýšení hladin lipidů v cirkulaci navozuje adhezi cirkulujících monocytárních buněk na povrch endotelií a indukci prozánětlivého stavu, jenž je asociován s aterogenezí. V tukové tkáni pravděpodobně dochází ke změně polarizace makrofágů z fenotypu M2 na prozánětlivý M1 fenotyp. Expozice akutní hyperlipidémii tak přispívá k rozvoji aterosklerózy a zhoršuje prozánětlivý stav obézních žen.

5.2. Publikace č. 2:

Experimentální hyperglykémie vede ke zvýšení obsahu monocytů a T-lymfocytů v tukové tkáni u obézních žen.

Originální publikace s obrázky a grafy je přiložena na str. 90.

Úvod: Metabolické komplikace obezity jsou spojovány s mírným prozánětlivým stavem organismu (zvýšení hladin cirkulujících cytokinů např. IL-6, TNF-α, CCL2, CCL5) a se zvýšenou akumulací imunitních buněk v tukové tkáni. Bylo prokázáno, že se významně zvýšila exprese markerů makrofágů u obézních s metabolickým syndromem ve srovnání s obézními bez metabolického syndromu (Klimcakova, Roussel, Kovacova, et al. 2011). Nicméně příčina vyšší zánětlivé infiltrace tukové tkáně u obézních s metabolickým sydnromem není zcela osvětlena. Jedním z mechanismů může být právě hyperglykémie, která může indukovat oxidační stres a spouštět prozánětlivé buněčné pochody (Esposito et al. 2002). Jedna z možností, jak experimentálně sledovat vliv jednotlivých nutrientůje např. vyvolání akutní hyperglykémie. Cílem studie bylo sledovat efekty hyperglykémie v tukové tkáni a na systémové úrovni.

Metody: Studie byla provedena na 30 obézních ženách bez poruchy glukózové tolerance či hyperlipidémie. 10 žen podstoupilo hyperglykemický clamp (endogenní produkce inzulinu byla blokována infuzí octreotidu), jako kontrolní skupina podstoupilo 10 žen infuzi octreotidu a 10 žen infuzi fyziologického roztoku po dobu tří hodin. Před započetím a po ukončení infuze byla provedena biopsie podkožní tukové tkáně a odběry krve. Pomocí průtokové cytometrie byly sledovány změny v buněčných populacích imunitních buněk a jejich aktivace v krvi a v tukové tkáni. Dále byla analyzována mRNA exprese pomocí qRT-PCR prozánětlivých markerů (CCL2, CCL5, CXCL12, IL1β, IL8, TNFα), markerů makrofágů (CD14, CD206, TLR 2, TLR4) and T lymphocytů (CD3g, CD4, TBX21/TH1, GATA3/TH2, RORC/TH17, FoxP3/TREG).

Výsledky: Akutní hyperglykémie vyvolala zvýšení monocytů/makrofágů charakterizovaných expresí CD45+/14+ v tukové tkáni. Tyto změny byly nezávislé na obsahu granulocytů v TT (17.8 ± 2.3% před a 17.4 ± 1.9% po infuzi). Rezidenční makrofágy exprimující manosový receptor CD206 v TT nebyly ovlivněny hyperglykémií. V cirkulaci nedošlo ke změnám v obsahu monocytů/makrofágů. Populace T-lymfocytů (CD45+/3+ cells; T helper subpopulace CD45+/3+/4+; T cytotoxické lymfocyty- CD45+/3+/8+) se v cirkulaci nezměnily, ale došlo k nárůstu obsahu těchto buněk v TT. Současně došlo ke zvýšené mRNA exprese prozánětlivých cytokinů (CCL2, TLR4, TNFα) a lymfocytárních markerů (CD3g, CD4, CD8a, TBX21, GATA3, FoxP3) v TT ($p<0.05$). Kontrolní infuze octreotidu ani fyziologického roztoku nevyvolala žádné z výše uvedených změn v cirkulaci ani v TT.

Diskuze: Hyperglykémie vyvolala prozánětlivou imunitní reakci vyjádřenou zvýšením populace makrofágů CD45+/14+ v tukové tkáni. Z literatury je známo, že diabetici 2.typu exprimují na monocytech více receptorů TLR2 a TLR4, jakožto spouštěčů prozánětlivé kaskády, ve srovnání se zdravou populací (Dasu et al. 2010). Proto jsme tyto receptory analyzovali jak v TT, tak

v periferní krvi. Populace monocytů CD45+/14+/TLR4+ se při hyperglykémii zvýšila v TT, nepozorovali jsme změny v obsahu monocytlů CD45+/14+/TLR2+. Tento selektivní efekt hyperglykémie na monocyty TLR4+ vypovídá o možném efektu glukózy na monocyty v TT. Potvrdili jsme i zvýšenou expresi mRNA TLR4 společně s TNFα v TT při hyperglykémii. Studie dokládají, že sekrece prozánětlivých cytokinů se liší na základě aktivace TLR4 nebo TLR2 makrofágů (Jones et al. 2001). A právě tato cytokinová regulace může hrát důležitou roli v migraci a aktivaci monocytů. Zároveň došlo ke zvýšení populace T-helper a T-cytotoxických lymfocytů a zvýšení exprese prozánětlivých cytokinů a markerů T- lymfocytů v TT. Výsledky potvrzují, že krátkodobá hyperglykémie – charakteristický rys nedobře kompensovaného diabetika - indukuje zvýšení obsahu monocytů a T- lymfocytů v tukové tkáni a může tak přispívat ke zhoršení prozánětlivého stavu tukové tkáně u obézních žen.

5.3. Publikace č.3:

Postprandiální zánětlivá reakce není asociována se stresem endoplazmatického retikula v periferních monocytech u zdravých štíhlých mužů.

Originální publikaces obrázky a grafy je přiložena na str. 104

Úvod : Nadměrný příjem lipidů a cukrů je asociován s nadměrnou hladinou glykémie a lipidů a protrahovaná elevace těchto metabolitů vyvolává postprandiální prozánětlivý stav (O'Keefe, Gheewala, and O'Keefe 2008). Bylo prokázáno, že buněčná expozice saturovaným lipidům a vysokým hladinám glykémie vyvolává stres endoplasmatického retikula (ER stres), který může regulovat postprandiální zánětlivou odpověď (Zhang and Kaufman 2008). Snížení ER stresu může potencovat ursodeoxycholová kyselina (UDCA), která se běžně užívá v terapii cholestázy.

Ve studii *in vitro* na buněčných kulturách byl prokázán její efekt na snížení ER stresu (Berger and Haller 2011). V naší práci jsme sledovali efekt postprandiálního zvýšení MK na zánětlivé parametry v cirkulaci a jejich vztah se stresem endoplasmatického retikula u 10 zdravých štíhlých mužů.

Metody: Subjektům byl podán vysokotučný vysokokalorický pokrm (high fat meal- HFM) - McDonalds, 6151 kJ (1469 kcal), 32.8% sacharidy, 47.4% tuky, 11.3% bílkoviny. Před jeho konzumací byly mužům podány 2 dávky placeba nebo Ursosanu (UDCA – chemický chaperon účinný v blokaci ER stresu). Před započetím experimentu a 4 hodiny po konzumaci HFM byly odebrány vzorky krve pro provedení analýzy. Pomocí ELISA a multiplexové imunoanalýzy na přístroji Luminex byly stanoveny plasmatické hladiny cytokinů (IL-6, IL-8, TNF-α a MCP-1). Pomocí průtokové cytometrie byly sledovány změny v buněčných populacích imunitních buněk a jejich aktivace v krvi, dále byla sledována mRNA exprese zánětlivých markerů a markerů ER stresu v izolovaných CD14+ a CD14- mononukleárních periferních buňkách (PBMC).

Výsledky: HFM indukoval zvýšení všech populací leukocytů v krvi - granulocytů, lymfocytů i monocytů. Z monocytárních populací došlo ke zvýšení exprese především CD11c+ na povrchu monocytů. Současně došlo v naší práci ke zvýšené mRNA expresi prozánětlivých cytokinů IL-6, IL-1β, IL-8, TNF-α a MCP-1. HFM neovlivnil expresi markerů ER stresu na PBMC. Podání ursodeoxycholové kyseliny před konzumací HFM neovlivnilo změny v mRNA expresi zánětlivých markerů ani markerů ER stresu.

Diskuze: Ve shodě s jinými pracemi jsme zde potvrdili zvýšenou mRNA expresi IL-6 i jeho zvýšenou hladinu v plazmě po HFM. Vlivem HFM byla indukována prozánětlivá imunitní reakce vyjádřená vzestupem exprese prozánětlivých cytokinů a zvýšením populací monocytů exprimujících toll-like receptory (TLR2, TLR4) a CD11c. Je známo, že aktivace těchto receptorů vede k signalizaci zánětlivé odpovědi a produkci cytokinů. Marker CD11c na monocytech je

považován za marker aktivující adhezi monocytů k endoteliím a jejich potenciální migraci do tkání. Tento fenotyp je považován za pro-zánětlivý M1 fenotyp, je ve zvýšené míře exprimován na monocytech u obézních subjektů a pozitivně koreluje s insulinovou rezistencí vyjádřenou pomocí indexu HOMA-IR (Wu et al. 2010). Po HFM byla zvýšená exprese CD11c na monocytech ve shodě s literaturou (Gower et al. 2011). Můžeme se domnívat, že vysokotuková strava vyvolává zvýšení exprese tohoto markeru stejně jako u obezity spojené s nadměrným kalorickým příjmem. Absence změn ukazatelů stresu endoplasmatického retikula (ER stres) po HFM a absence efektu ursodeoxycholové kyseliny na prozánětlivou odpověď po HFM nasvědčují tomu, že tato prozánětlivá odpověď není zprostředkována ER stresem.

5.4. Publikace č. 4:

Metformin neinhibuje lipolýzu v tukové tkáni během cvičení u mladých zdravých mužů.

Originální publikace s obrázky a grafy je přiložena na str. 115.

Úvod: Metformin je efektivní antiadiabetikum první volby a je široce podávaný u prediabetu a diabetu mellitu 2.typu. Ovšem některé studie na lidských adipocytech ukazují, že metformin může mít anti-lipolytický efekt cestou aktivace AMP-aktivované protein kinasy (AMPK) (Bourron et al. 2010; Zhang et al. 2009). Proto jsme se rozhodli ověřit, zda jedním z negativních vedlejších účinků metforminu může být inhibice cvičením indukované lipolýzy v tukové tkáni. Cílem této studie bylo tedy sledovat anti-lipolytický efekt metforminu během cvičení za standartních fyziologických podmínek u zdravých štíhlých mužů. Dále také sledovat farmakokinetiku metforminu v tukové tkáni po p.o. podání.

Metody: Studii se podrobilo 10 zdravých mladých mužů (věk 27.2 ± 0.4 let; BMI 23.6 ± 0.5 kg/m^2), kteří absolvovali 60 minut jízdy na bicyklovém ergometru. První den v kombinaci s perorálně podaným metforminem, druhý den s lokálně podaným metforminem do podkožní tukové tkáně mikrodialyzačním katetrem. Mikrodialýza je metoda, kterou jsme schopni změřit míru lipolýzy v podkožní tukové tkáni, dále jsme v dialyzátu meřili hladiny laktátu a metforminu pomocí enzymových kolorimetrických kitů a kapilární elektroforézy.
Výsledky: Hladina metforminu v plazmě se kontinuálně zvyšovala během 3 hodin po podání 2550 mg p.o.metforminu a maximální hladiny bylo dosaženo po 3,5 hodinách (koncentrace 4ug/ml). Podobnou dynamiku dosahovaly hladiny metforminu v dialyzátu z tukové tkáně s maximální koncentrací 1,3 ug/ml. Míra lipolýzy v tukové tkáni byla měřena jako množství glycerolu v dialyzátu. Během cvičení se zvýšila lipolýza oproti bazálnímu stavu (4.3 násobně ± 0.5 vs. basal; p = 0.002) a nedošlo k supresi po podání p.o. ani lokálně podaného metforminu. Hladina laktátu rostla během cvičení v plazmě i v dialyzátu po 30-ti a 60-ti minutách cvičení (3.6 násobně vs. basal; p = 0.015; 2.75 násobně vs. basal; p = 0.002). Metformin neměl efekt na hladinu laktátu během cvičení.

Diskuze: V této studii jsme demonstrovali, že metformin je distribuován v podkožní tukové tkáni po perorálním podání 2550mg a nevykazuje inhibiční efekt na lipolýzu aktivovanou jednorázovým tělesným cvičením u zdravých mužů. Jiné publikace prokazují, že metformin inhibuje katecholaminy-stimulovanou a ANP-stimulovanou lipolýzu cestou aktivace AMPK. Nicméně tyto efekty byly studovány *in vitro* a koncentrace metforminu byla cca 200x vyšší, než jakou jsme detekovali v dialyzátu z tukové tkáně v této studii. *In vivo* jsme použili koncentraci metforminu v perfuzátu podobně jako v publikaci Flechtner-Mors (Flechtner-Mors et al. 1999). Nicméně v naší práci nebyla prokázaná inhibice lipolýzy metforminem během cvičení. Náš protokol představuje

fyziologickou a více komplexní cestu stimulace lipolýzy, navíc je proveden u štíhlých mužů, kdežto ve studii Flechter-Mors (Flechtner-Mors et al. 1999) byli zavzaty morbidně obézní ženy. Použili jsme intenzitu cvičení optimální v modelu stimulace lipolýzy (Moro et al. 2007), obdobnou intenzitu cvičení jsme volili i v předchozích studiích zaměřených na regulaci lipolýzy během cvičení (Stich, de Glisezinski, Berlan, et al. 2000), (Stich, De Glisezinski, Crampes, et al. 2000).V regulaci lipolýzy během cvičení může hrát roli velká škála regulátorů a signálních drah jako jsou např. cytokiny IL-6, IL-15 (Ajuwon and Spurlock 2004), insulin nebo FGF21 (Hotta et al. 2009). Metformin některé z těchto drah ovlivňuje, je schopen inhibovat IL-6 a inzulinovou signalizaci (Li et al. 2014; Kisfalvi et al. 2009). Role AMPK během cvičením stimulované lipolýzy není jednoznačná (Gaidhu and Ceddia 2011), a proto je potřeba další studium detailních efektů metforminu nejen na AMPK, ale i jiné signalizační dráhy během fyziologických podmínek jako je cvičení. Z naší publikace vyplývá, že metformin nehraje zásadní roli v mobilizaci lipidů během cvičení.

ČÁST B: Dietní intervence

5.5. Publikace č. 5:

Exprese lipolytických genů v tukové tkáni je rozdílně regulována během jednotlivých fází dietní intervence u obézních žen.

Originální publikace s obrázky a grafy je přiložena na str. 123

Úvod: V této studii jsme sledovali efekt jednotlivých fází 6-ti měsíční dietní intervence na expresi genů regulujících lipolýzu a vývoj inzulinové rezistence. Porucha regulace lipolýzy je jedním

z mechanismů podílejících se na rozvoji inzulinové rezistence (Reynisdottir et al. 1994). S obezitou je spojena jak zvýšená míra bazální lipolýzy (Ryden and Arner 2017), tak porucha regulace katecholaminy-indukované lipolýzy v TT (Reynisdottir et al. 1995).

Metody: Studii absolvovalo 15 obézních žen (BMI 34.7 ± 1.0 kg/m^2), které podstoupily 6-ti měsíční dietní intervenci skládající se z 1 měsíce velmi přísné nízkokalorické diety (VLCD), následované 2-mi měsíci nízkokalorické diety (LCD) a dále 3-mi měsíci udržení hmotnosti. Před začátkem intervence a po skončení jednotlicých fází byla provedena jehlová biopsie podkožní tukové tkáně z oblasti břicha a proveden hyperinsulinemický euglykemický clamp ke zhodnocení inzulinové citlivosti. Ze vzorků tukové tkáně byly analyzovány mRNA exprese genů regulujících lipolýzu – adrenergní β2 receptor (ADRB2), adrenergní α2A receptor (ADRA2A), adipose-triglycerid lipáza (ATGL), hormon-senzitivní lipáza (HSL), phosphodiesterasa-3B (PDE3B) a insulinový receptor (INSR).

Výsledky: Dietní intervence vedla ke snížení hmotnosti o 9,8%, které bylo dáno především úbytkem tukové hmoty. Na konci dietní intervence došlo ke snížení plasmatických hladin triglyceridů, snížení hladiny bazálního inzulinu a ke zlepšení inzulinové citlivosti měřené metodou clampu. Exprese mRNA adrenergního β2-receptoru v TT signifikantně vzrostla na konci VLCD a navrátila se k bazálním hodnotám během LCD a ve fázi udržení hmotnosti. Exprese adrenergního α2 receptoru se signifikantně snížila na konci VLCD a LCD a k původním hodnotám se vrátila během fáze udržení hmotnosti. Exprese HSL a ATGL byla ovlivněna podobně: došlo k signifikantnímu snížení na konci LCD a k původním hodnotám se vrátila během fáze udržení hmotnosti. Na konci VLCD byla nižší exprese ATGL, ale rozdíl nebyl statisticky významný (p=0.076). Exprese fosfodiesterázy – 3B a insulinového receptoru se během jednotlivých fází dietní intervence nezměnila.

Diskuze: Výsledky této studie demonstrují, že multi-fázová dietní intervence modifikuje expresi genů regulujících lipolýzu v TT. Tyto poznatky se shodují s předchozími studiemi, které dokazují, že exprese genů regulujících metabolismus a imunitní funkce jsou závislé na fázi dietní intervence (Vitkova et al. 2007), (Kovacikova et al. 2011). Hlavní roli v regulaci lipolýzy v TT hrají katecholaminy. Prokázali jsme zvýšení mRNA exprese β2-adrenergního receptoru na konci VLCD, v souladu s dřívější prací prokazující zvýšení lipolytické odpovědi po β2-adrenergní stimulaci za 4 týdny VLCD (Stich et al. 1997). Exprese lipolytického genu β2-adrenergního receptoru byla up-regulována na konci VLCD, zatímco exprese α_2-adrenergního receptoru (zprostředkovávajícího anti-lipolytický efekt) byla down-regulována na konci VLCD a LCD. Ve fázi udržení hmotnosti se exprese mRNA adrenoreceptorů vrátily zpět k bazálním hodnotám. Výsledky jsou ve shodě s naší dřívější studií, v níž bylo metodou mikrodialýzy TT zjištěno snížené α_2-adrenergní anti-lipolytické působení na konci VLCD a LCD (Koppo et al. 2012). Lze shrnout, že exprese genů regulujících lipolýzu je ovlivněna jednotlivými fázemi dietní intervence, lišícími se trváním a energetickou bilancí: tyto faktory jsou tedy důležitými determinantami této metabolické regulace.

5.6. Publikace č. 6:

Porovnání časné (2 dny) a pozdní (28 dnů) odpovědi tukové tkáně na velmi nízkokalorickou dietu u obézních žen.

Originální publikace s obrázky a grafy je přiložena na str. 133.

Úvod: V této studii byl sledován vliv 2-denní a 28-mi denní velmi nízkokalorické diety (VLCD) na prozánětlivý stav organismu a tukové tkáně. Dvoudenní přísná kalorická restrikce byla v této studii

modelem intervence, která vede ke zvýšení hladin cirkulujících MK (v důsledku stimulace lipolýzy) bez poklesu tělesného tuku. Případné změny v tukové tkáni mohou tak být pokládány za důsledek zvýšení hladin volných MK bez interferujícího vlivu změny množství tělesného tuku, resp. změny rozměru adipocytů. Dvoudenní efekty byly srovnány s dlouhodobější (28 denní) intervencí, při níž dochází k již významné redukci tělesného tuku.

Metody: Studie se zúčastnilo 17 obézních žen (BMI 32.7 ± 0.9 kg/m^2), které po dobu 28 dní dodržovaly přísnou nízko-kalorickou dietu (VLCD; 500kcal/ den). Před započetím diety, po 2 dnech a po 28 dnech VLCD byla provedena jehlová biopsie podkožní tukové tkáně z oblasti břicha a byla analyzována sekrece cytokinů/adipokinů z explantů tukové tkáně a jejich cirkulující hladiny (IL-6, IL-8, IL-10, MCP-1, TNF-α, leptin). Dále byla ve vzorcích tukové tkáně analyzována genová exprese markerů lipidového metabolismu (SCD1, FASN, DGAT, PPARγ, HSL, ATGL, CD36), a cytokinů/adipokinů (IL-6, IL-8, IL-10, MCP-1, TNF-α, leptin).

Výsledky: Dle očekávání došlo po 2 dnech VLCD ke zvýšení plasmatických hladin MK, které přetrvávalo až do 28. dne diety. Insulinová resistence hodnocená HOMA-IR indexem byla, ve srovnání s basálním stavem, snížena již po 2 dnech intervence a snížena zůstala i po 28 dnech. Po 2 dnech VLCD bylo pozorováno zvýšení exprese lipolytických enzymů (HSL, ATGL), po 28 dnech došlo k jejich poklesu na bazální hodnoty. Po 2 dnech VLCD se zvýšily hladiny cirkulujících cytokinů (IL-6, MCP-1), po 28 dnech se hodnoty vrátily k basálnímu stavu před dietou. Sekrece a mRNA exprese cytokinů v tukové tkáni nebyla po 2 dnech změněna, vzestup sekrece a exprese cytokinů (IL-6, MCP-1, TNF-α) v tukové tkáni byl pozorován po 28 dnech VLCD.

Diskuze: Výsledky nasvědčují tomu, že krátkodobá 2-denní kalorická restrikce a současná mobilizace MK v tukové tkáni nevede k indukci prozánětlivého stavu v tukové tkáni. Naproti tomu,

po 28 dnech diety – tedy již za podmínek redukce množství tělesného tuku - byl prozánětlivý stav tukové tkáně zvýšen. Kromě prozánětlivých cytokinů byly zvýšeny i cytokiny protizánětlivé. Po delší VLCD tedy dochází v tukové tkáni k indukci zánětlivých změn, které pravděpodobně souvisí s remodelací tkáně samotné (jejíž součástí je zmenšení objemu adipocytů, změny v infiltraci/polarizaci makrofágů, změny stupně fibrosy, stupně prokrvení aj.). Výsledky jsou zajímavé v kontextu současných diskusí o mechanismech zlepšení metabolického stavu pozorovaného u jedinců v časných fázích po bariatrickém výkonu. Dále výsledky přinesly zajímavé poznatky o regulaci lipolýzy a lipogeneze: změny lipolytických i lipogenních genů a jejich regulátorů PPARγ po 2 dnech i po 28 dnech VLCD korelovaly se změnami exprese a sekrece leptinu v tukové tkáni; změny lipogenních a lipolytických genů během 28 dnů VLCD korelovaly negativně se změnou plasmatických hladin FGF21. Tento faktor produkovaný játry je uvolňován zejména při dlouhodobějším hladovění (Emanuelli et al. 2014). Korelační výsledky nasvědčují významné roli leptinu a FGF-21 v regulaci změn toků lipidů během přísné kalorické restrikce. Role FGF21 v tukové tkáni je pravděpodobně v potlačení lipolýzy a lipogenese při nedostatečném přísunu živin a tak šetření substrátů při dlouhodobějším hladovění.

6. ZÁVĚRY A DISKUZE

Obezita je charakterizovaná zvýšenou akumulací tukové tkáně a je spojena se zvýšeným rizikem vzniku metabolických poruch vedoucích k inzulinové rezistenci a následně diabetu mellitu 2.typu, kardiovaskulárním a nádorovým onemocněním (Lengyel et al. 2018; Pistollato et al. 2018). Mechanismy zprostředující vztah mezi obezitou a jejími komplikacemi nejsou stále zcela zřejmé, nicméně jejich poznání je zásadní pro prevenci a léčbu obezity a následně také prevenci jejích komplikací.

Možným pojítkem mezi obezitou a chorobami s ní spojenými je dysfunkce tukové tkáně projevující se jak na úrovni metabolismu tukových buněk (lipolýza a lipogeneze), tak na úrovni endokrinní či imunitní. Dysfunkce na úrovni imunitní je vyjádřena prozánětlivým stavem tukové tkáně, který se podílí na vzniku prozánětlivého stavu na úrovni celého organismu. Mezi poruchami na výše zmíněných úrovních existuje úzká souvislost: např. metabolická porucha spočívající ve zvýšeném uvolňování mastných kyselin v procesu lipolýzy v adipocytech vyvolává změnu v endokrinní funkci samotných adipocytů a v atrakci imunitních buněk z cirkulace do TT. Následně se zvyšuje produkce prozánětlivých působků jak v migrujících imunitních buňkách, tak v adipocytech samotných (Kosteli et al. 2010). Soubor hypertrofický adipocyt + zvýšená přítomnost imunitních buněk + jejich prozánětlivý fenotyp + fibrosa tukové tkáně tvoří základní charakteristiky dysfunkční tukové tkáně u obézního jedince.

Cílem této dizertační práce je přispět k pochopení mechanismů a regulačních pochodů, které zprostředkovávají spojení mezi zvýšenými hladinami nutrientů (glukóza a mastné kyseliny) a prozánětlivým stavem organismu u obézních jedinců. K dosažení vyšší hladiny nutrientů jsme zvolili buď podání exogenní (lipidy, glukóza či vysokotukový pokrm) nebo indukci endogenní při dietní intervenci či fyzické zátěži. Studie si kladly za cíl objasnit některé z mechanismů, jimiž

nutriční podněty vyvolávají metabolické poruchy u obézních jedinců a přispět tak k poznání rizikových faktorů výživy a tím i k účinnější prevenci a léčbě obezity v budoucnu.

V první části této práce byla sledována akutní reakce imunitních buněk na experimentálně zvýšené hladiny nutrientů. **V publikaci č. 1** jsme se věnovali vlivu hyperlipidémie a lipotoxicity na zánětlivé a proaterogenní charakteristiky v tukové tkáni i cirkulaci. Zvoleným modelem bylo vyvolání hyperlipidémie exogenním podáním lipidů infuzí 20% Intralipidu. Ukázali jsme, že parenterální podání lipidů spojené se zvýšením hladiny cirkulujících MK a TG, typické pro obézního jedince s metabolickým syndromem, vyvolává aktivaci proaterogenních změn v cirkulaci, kdy monocyty zřejmě adherují k endotelu cévní stěny, což je v souladu s dosavadními poznatky (den Hartigh et al. 2014; Motojima et al. 2008). Současně dochází ke zvýšení hladin adhezních molekul v cirkulaci (sICAM, sVCAM). Výsledky mohou nasvědčovat tomu, že hyperlipidémie vyvolává oxidační stres a endoteliální dysfunkci, v souladu s prací Gowera at al. (Gower et al. 2011). Prozánětlivé změny byly patrné i v tukové tkáni. Osvětlen byl tak jeden z mechanismů lipotoxicity: expozice akutní hyperlipidémii působí u obezních žen proaterogenní změny na úrovni cirkulace a prozánětlivé změny v tukové tkáni a na systemové úrovni.

V publikaci č. 2 jsme jako model možného toxického působení glukózy - tj. glukotoxicity - zvolili krátkodobý hyperglykemický klemp tj. hyperglykémii navozenou parenterálním podáním exogenní glukózy. Hyperglykémie vyvolala prozánětlivou imunitní reakci vyjádřenou zvýšením populace makrofágů v TT. Zároveň došlo ke zvýšení populace T-helper a T-cytotoxických lymfocytů a zvýšení mRNA exprese markerů T-lymfocytů v TT. Výsledky potvrzují, že krátkodobá hyperglykémie indukuje prozánětlivou reakci v tukové tkáni a může tak přispívat ke zhoršení prozánětlivého stavu u populací jak s vyšší lačnou, tak s postprandiálně zvýšenou glykémií např.

u obézních jedinců s prediabetem. Výsledky jsou dalším elementem potvrzujícím fenomén glukotoxicity a následně i důležitost dostatečné kompenzace hladin glykémie u pacientů s prediabetem a diabetem mellitem 2.typu.

V publikaci č. 3 jsme studovali vliv hyperlipidémie v kontextu vzniku zánětlivého stavu tukové tkáně. Tentokrát bylo zvýšené hladiny lipidů dosaženo podáním vysokotukového vysokokalorického pokrmu (HFM). Tento pokrm vyvolal postprandiální leukocytózu a prozánětlivou reakci detekovanou v krevních monocytech. Hypotéza o tom, že podkladem prozánětlivé reakce je stres endoplasmatického retikula (ER stres) však potvrzena nebyla. Ukazatelé ER stresu nebyly po podání HFM změněny, ani podání inhibitoru ER stresu neprokázalo žádný efekt na sledované parametry. Tato studie dokládá lipotoxické působení vysokotukového pokrmu a potvrzuje důležitost dietních doporučení a osvěty jako významného faktoru prevence metabolických onemocnění.

V další **publikaci č.4** jsme k dosažení zvýšené hladiny volných MK zvolili model aktivace lipolýzy krátkodobou pohybovou aktivitou. Metformin je široce používaný lék první volby u prediabetu a diebetu 2.typu a bylo prokázáno, že vykazuje anti-lipolytický efekt (Bourron et al. 2010; Zhang et al. 2009). Znalost jeho působení na TT během cvičení je zásadní pro preskripci pohybové aktivity u prediabetiků a diabetiků jako součást prevence i léčby. Proto jsme testovali hypotézu, zda metformin může inhibovat cvičením indukovanou lipolýzu v tukové tkáni. Originálním metodickým přínosem práce bylo sledování distribuce metforminu v podkožní TT metodou mikrodialýzy. Hypotézu o inhibici lipolýzy během cvičení jsme nepotvrdili. Metformin tedy nehraje zásadní roli v mobilizaci lipidů během cvičení a pozitivní vliv cvičení na redukci tukové hmoty není metforminem negativně ovlivněn. Výsledek je tak přínosem pro preskripci pohybové aktivity jako součást prevence a léčby prediabetu a diabetu mellitu 2. typu.

Ve druhé části této dizertační práce bylo snahou prohloubit znalosti o pozitivních efektech redukce hmotnosti jakožto základním preventivním i léčebném opatřením obezity a jejich komplikací. **V publikaci č. 5** jsme prokázali, že míra a regulace lipolýzy během redukčního režimu je závislá na míře a době trvání kalorické restrikce. Ukázali jsme, že ve změnách lipolýzy během kalorické restrikce hraje pravděpodobně roli rozdílná exprese genů spojených s adrenergní signalizací a následnou regulací lipolýzy. Výsledky jsou ve shodě s dřívější studií, kde byla demonstrována změna v adrenergní regulaci lipolýzy během dietní intervence pomocí *in vivo* mikrodialýzy tukové tkáně (Koppo et al. 2012). V souladu s klinickou zkušeností tato regulace pravděpodobně souvisí s výraznou redukcí tukové hmoty v prvním měsíci podávání nízkoenergetických diet a adaptací na nízkoenergetický režim v dalších fázích, dále také souvisí se závislostí redukce tukové hmoty na míře kalorické restrikce.

V publikaci č. 6 jsme sledovali změny širšího spektra metabolických a endokrinních charakteristik TT při velmi krátkém – dvoudenním - působení výrazné kalorické restrikce. Tato situace je vhodným modelem ke sledování působení kalorické restrikce *per se* tj. bez průvodní redukce tukové tkáně. Z publikovaných studií i z klinických zkušeností je známo, že k metabolickým změnám po bariatrických intervencích dochází již v prvních dnech po zákroku (Bojsen-Moller et al. 2014), ovšem podklad těchto změn není stále zřejmý. V naší studii byly změny v TT po dvou dnech kalorické restrikce srovnány se změnami po 28 denní intervenci, při níž dochází k již významné redukci tělesného tuku. V našem souboru došlo již po dvou dnech kalorické restrikce ke snížení inzulinové rezistence hodnocené indexem HOMA-IR. Na úrovni TT výsledky ukázaly, že již po 2 dnech byla zvýšena exprese genů podílejících se na regulaci lipoýzy v TT, na druhé straně po dvou dnech diety nedošlo k indukci prozánětlivého stavu v TT, zatímco po 28 dnech diety byl prozánětlivý stav TT spíše zvýšen. Tyto výsledky naznačují, že v dynamické fázi výrazné kalorické

restrikce mohou být charakteristiky zánětlivého stavu zčásti determinovány remodelací tukové tkáně: zmenšením adipocytů, změnami v infiltraci či polarizaci makrofágů, stupněm fibrózy a prokrvením TT. Naše předchozí studie (Capel et al. 2009b; Vitkova et al. 2007) ukázaly, že ke zlepšení prozánětlivého stavu TT v souvislosti s redukcí tukové hmoty je třeba režimů trvajících alespoň 3 měsíce, obsahující nejlépe i fázi uržení hmotnosti. V těchto studiích bylo též ukázáno, že změny v zánětu tukové tkáně nejsou jedinou determinantou zlepšení metabolického stavu obezního jedince při dietních intervencích.

Lze shrnout, že na základě analýz účinků krátkodobých intervencí byla potvrzena hypotéza o toxickém prozánětlivém působení jak zvýšených hladin glukózy (např. u prediabetiků a diabetiků), tak zvýšených hladin volných MK (charakteristické pro obézní jedince) na úrovni TT i celého organismu. Nízkoenergetická dieta zlepšuje metabolický stav organismu obézního jedince již po několika dnech trvání, nicméně toto metabolické zlepšení není asociováno s odpovídajícími změnami v ukazatelích prozánětlivého stavu tukové tkáně. Pro toto zlepšení je třeba intervencí dlouhodobějších. Kalorická restrikce ovlivnila významě mechanismy regulace lipolýzy TT, což může být podkladem pozitivních změn spojených s redukcí hmotnosti.

Výsledky studií zahrnutých v této práci přispěly k pochopení regulace vzniku prozánětlivého stavu organismu a úlohy energetického příjmu i hladiny základních makronutrientů v tomto procesu. Další výzkum funkčních změn na úrovni tukové tkáně je nadále nezbytný k pochopení mechanismů a další implikace pro preventivní a léčebná doporučení.

7. OSTATNÍ PUBLIKAČNÍ AKTIVITA

SEZNAM NEZAHRNUTÝCH PUBLIKACÍ S IF

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PREZENTACE VÝSLEDKŮ NA KONFENRENCÍCH:

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8. TRAINING ON INSULIN RESISTANCE AND ADIPOSE TISSUE MACROPHAGES IN ELDERLY WOMEN. EASD 12.-15.9.2017. Lisabon. Poster.

9. ROLE TUKOVÉ TKÁNĚ V ROZVOJI INSULINOVÉ REZISTENCE U ZDRAVÝCH NEOBÉZNÍCH MUŽŮ S GENETICKOU PREDISPOZICÍ K T2DM. 54. Diabetologické dny Luhačovice. 2018. Ústní sdělení.

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PŘÍLOHA 1

Acute hyperlipidemia initiates proinflammatory and proatherogenic changes in circulation and adipose tissue in obese women.

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Acute hyperlipidemia initiates proinflammatory and proatherogenic changes in circulation and adipose tissue in obese women

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ABSTRACT

Background: Obesity represents a high risk factor for the development of atherosclerosis and is associated with a low-grade inflammation and activation of immune cells.
Aims: The aim of our study was to investigate the effect of a short-term lipid infusion on immune cells in blood and subcutaneous abdominal adipose tissue (SAAT) in obese women.
Methods: Seven-hour intravenous lipid/control infusions were performed in two groups of women ($n = 15$, $n = 10$, respectively). Before and at the end of the infusion, SAAT and blood samples were obtained and relative content and phenotype of immune cells were analyzed using flow cytometry. Analysis of immune cell markers, inflammation and angiogenesis markers was performed in SAAT by RT-PCR and in plasma by immunoassays.
Results: Relative content of CD45+/14+ and CD45+/14+/16+ populations of monocytes was reduced in circulation by 21% ($p = 0.004$) and by 46% ($p = 0.0002$), respectively, in response to hyperlipidemia, which suggested the increased adhesion of these cells to endothelium. In line with this, the levels of sICAM and sVCAM in plasma were increased by 9.4% ($p = 0.016$), 11.8% ($p = 0.008$), respectively. In SAAT, the relative content of M2 monocyte/macrophages subpopulation CD45+/14+/206+/16+ decreased by 27% ($p = 0.012$) and subpopulations CD14+/CD206− and CD14/+TLR4+ cells increased ($p = 0.026$; $p = 0.049$, respectively). Intralipid infusion promoted an increase of mRNA levels in SAAT: RORC (marker of proinflammatory Th17 lymphocytes) by 43% ($p = 0.048$), MCP-1 (78%, $p = 0.028$) and VEGF (68.5%, $p = 0.0001$).
Conclusions: Acute hyperlipidemia induces a proinflammatory and proatherogenic response associated with altered relative content of immune cells in blood and SAAT in obese women.
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1. Introduction

Obesity represents a high risk factor for the development of cardiovascular diseases and atherosclerosis. The common feature of these complications is a low-grade inflammation and activation of immune cells. It was proposed that one of the triggers of these proinflammatory processes are circulating free fatty acids (FFA) and triglycerides (TG) [1,2]. Lipids, namely saturated FFA, were found to activate classical inflammatory responses in immune cells and to regulate secretion of proinflammatory cytokines in several types of cells [3,4]. The ability of lipids to activate proinflammatory responses was documented also in vivo in humans upon the postprandial increase of lipid metabolites: the consumption of high-fat meal was accompanied with an increase of proinflammatory cytokine plasma levels [5,6] and increased circulating leukocyte counts [7,8]. Importantly, the postprandial increase of circulating lipids (TG, FFA) as well as the signs of systemic postprandial inflammatory response were more pronounced in obese [9,10].

Effects of FFA on cells can be mediated through binding to the receptors/sensors, such as toll like receptor 4 (TLR4) and fatty acid translocase (CD36), that control inflammatory signaling pathways

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[2,11]. Indeed, in humans, increased circulating levels of FFA were associated with increased expression of CD36 on monocytes, which led to lipid accumulation in these cells [12]. Such a lipid overload caused monocytes to form foam cells that are implicated in the development of atherosclerosis [13]. Atherosclerosis is now, similarly as obesity, widely accepted as a low-grade chronic inflammatory disease. It is initiated by the dysfunction of the vascular endothelium and followed by the activation and recruitment of circulating leukocytes to sites of endothelial damage [14,15].

However, the effects of lipids on immune processes within the adipose tissue (AT) are known only partially. Saturated FFA may induce an increased expression of proinflammatory cytokines in adipocytes similarly as was shown in immune cells [16]. In vivo in rats, the postprandial increase of mRNA expression of IL-6 and NF-kB activation in AT was observed after high-fat meal [17]. Moreover, FFA appeared as an important driver of macrophage accumulation in AT in mice [18]. In humans, it was documented that postprandial triglyceridemia increased levels of soluble cell adhesion molecules (sICAM, sVCAM), which may regulate the infiltration of monocytes to the endothelium [19,20] and potentially recruitment to AT [17,18].

Based on these studies, we hypothesize that acute elevation of systemic lipid levels may modify proinflammatory characteristics of AT, and so worsen the AT dysfunction and contribute to proatherogenic changes in metabolically healthy obese women. Thus, the aim of the current study was to investigate whether the acute experimentally-induced hyperlipidemia modifies the relative content and phenotype of immune cells, and other immunity-related features in subcutaneous adipose tissue (SAAT) and in circulation in obese women.

2. Materials and methods

2.1. Subjects

Seventeen obese healthy premenopausal women were sequentially recruited by referral from obesity consultations at the *University Hospital Královské Vinohrady* and by local obesity-management organizations (STOB). Subjects were randomly divided into two groups: the intervention group with Intralipid infusion (n = 15, age 43 ± 7 year, BMI 31.4 ± 2.7 kg/m^2) and the control group with infusion of glycerol (n = 10, age 44 ± 6 years, BMI 31.6 ± 3.4 kg/m^2). Exclusion criteria were weight changes of more than 3 kg within the 3 months before the study, hypertension, impaired fasting glucose, diabetes, hyperlipidemia, drug-treated obesity, smoking, drug or alcohol abuse, irregular menstrual cycle, pregnancy or participation in other studies. All individuals in the two groups of subjects showed the “metabolically healthy obese” phenotype [21,22], i.e. they did not meet the criteria of metabolic syndrome as defined by International Diabetic Federation. The two groups were homogenous from this point of view. All the subjects were non-smokers, sedentary, did not take any medications and did not suffer from any disease except for obesity. All subjects were fully informed about the aim and the protocol of the study and signed an informed consent approved by the Ethical committee of the Third Faculty of Medicine (Charles University in Prague, Czech Republic).

2.2. Experimental protocol

The subjects entered the laboratory at 7.00 a.m. after an overnight fast. A complete clinical investigation was performed, anthropometric parameters were measured and body composition was determined with multifrequency bioimpedance (Bodystat QuadScan 4000; Bodystat Ltd., Isle of Man, British Isles). Subsequently, the subjects were placed in a semi-recumbent position and a catheter was placed in the antecubital vein. To increase plasma FFA and TG concentration intravenous infusion of lipid emulsion (Intralipid 20%) was applied. Intralipid 20% (Fresenius Kabi, Bad Hamburg, Germany) consists of soya-bean oil (20%) stabilized with egg yolk phospholipids (1.2%) and glycerol (2.5%). The fatty acid composition was as follows: palmitic acid 11.3%, stearic acid 4.9%, oleic acid 29.7%, linoleic acid 46.0% and linolenic acid 8.1%. The infusion of Intralipid 20% was administered through cannula at a rate 60 ml/h for one hour and then it was continued at constant rate 90 ml/h for following six hours.

In the control group, saline infusion with 2.5% glycerol was administered at the same rate for seven hours.

Before the start of infusions and every 60 min during infusions venous blood was collected into 50 μl of an anticoagulant and antioxidant cocktail (Immunotech SA, Marseille, France) and immediately centrifuged (1300 rpm, 4 °C). The plasma samples were stored at −80 °C until analyses.

The needle biopsies and 2 ml of uncoagulated blood samples were taken 30 min before the start of the experimental infusions and 15 min before the end of infusions. Needle biopsies of SAAT were obtained approximately 10–15 cm laterally to the umbilicus under local anesthesia (1% Mesocain, Zentiva, Prague, Czech Republic), as previously described [23]. Approximately 1 g of SAAT was used for isolation of stroma-vascular fraction (SVF). SVF and 2 ml of venous blood were used for flow cytometry analyses. An aliquot of SAAT (approx. 0.2 g) was immediately frozen in liquid nitrogen and stored at −80 °C until gene expression analysis.

2.3. Determination of plasma levels of biochemical parameters

Homeostasis model assessment of the insulin resistance index (HOMA-IR) was calculated as follows: ((fasting insulin in mU/l) × (fasting glucose in mmol/l)/22.5). Plasma levels of FFA and TG were measured using enzymatic colorimetric kits (Randox, Crumlin, UK). The concentrations of VEGF-A, and MCP-1 in plasma were measured by ELISAs (eBioscience, San Diego, USA; R&D Systems, Minneapolis, USA). The concentrations of sVCAM, sICAM, IL-8, IL-6, and TNFα were measured by multiplex immunoassays (Milliplex Cardiovascular disease panel HCVD2MAG and High sensitivity T-Cell panel HSTCMAG, Merck-Millipore, USA). The intra-assay coefficients of variation for individual immunoassays obtained in our laboratory were: VEGF-A 7.5%, MCP-1 7.1% IL-8 3.6%, IL-6 5.3%, and TNFα 3.3%, sVCAM 2.7%, sICAM 5.8%. Plasma levels of other parameters were determined using standard clinical biochemical methods.

2.4. Isolation of SVF cells

SAAT was washed with saline, minced into small pieces and digested with type I collagenase (SERVA, Heidelberg, Germany) for 1 h in 37 °C shaking water bath and subsequently centrifuged at 200 g for 10 min and filtered through 100- and 40-μm sieves to isolate SVF cells.

2.5. Flow cytometry analyses

The whole blood and isolated SVF cells were analyzed immediately after isolation for flow cytometry analyses as described before [7]. Briefly, SVF cells were resuspended to final concentration 10^6 cells/ml in PBS solution containing 0.5% BSA and 2 mmol/l EDTA and 100 μl of this suspension was incubated with fluorescence-labelled monoclonal antibodies (FITC-conjugated antibody CD14, CD16, CD4; PE-conjugated antibody CD14, TLR4, CD3, CD36; PerCP-conjugated CD45 antibody and APC-conjugated

antibodies CD206 and CD8) or the appropriate isotype controls (BD Bioscience, Bedford, MA, USA; Exbio, Prague, Czech Republic) for 30 min at 4 °C.

The whole blood samples were stained with the same set of fluorescence-labelled monoclonal antibodies as used for SVF cells for 30 min at room temperature. After cell staining, erythrocytes were lysed by erythrocyte lysis buffer for 15 min at room temperature. Cells were washed with PBS and analyzed on FACS Calibur flow cytometer and CellQuest Pro Software (BD Biosciences, Bedford, MA, USA). The number of immune cell populations was expressed as percentage of gated events. Background was set up to 5% of positive cells of isotype control.

2.6. Gene expression analysis

Total RNA extraction and reverse transcription were performed as previously described [7]. Before reverse transcription, genomic DNA was eliminated by DNase I (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR (RT-qPCR) was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers and TaqMan probes were obtained from Applied Biosystems. Results are presented as fold change values calculated by the ΔΔ Ct method normalized to endogenous control GUSB.

2.7. Statistical analyses

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, California, USA). Differences of baseline clinical data between the two groups of patients were assessed using nonparametric Mann-Whitney rank test for unpaired observations. The effect of Intralipid infusion or control infusion on biochemical, gene expression and flow cytometry-derived variables was assessed using a nonparametric Wilcoxon matched-pairs signed rank test. The relative changes of variables (fold change) during Intralipid infusion compared to control experiment were analyzed using Mann-Whitney rank test. Correlations between anthropometric data and flow cytometry variables were analyzed using Spearman's correlation. Data are presented as mean ± SD. Differences at the level of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Subject characteristics and levels of free fatty acids and triglycerides during the experimental infusions

The anthropometric and biochemical data of subjects participating in the interventions are shown in Table 1. There were no significant differences in anthropometric and metabolic indices (including fasting blood glucose, plasma insulin levels, HOMA-IR, cholesterol, FFA and TG) between Intralipid and control group. Lipid infusion resulted in a continuous significant increase of plasma levels of FFA and TG ($p < 0.001$). In control intervention, no changes in FFA and TG levels compared to basal levels were observed (Fig. 1).

3.2. Effect of lipid infusion on monocyte/macrophage content in blood and SAAT of obese women

Monocyte/macrophage content was identified by CD45+ (common leukocyte antigen) and CD14+ (co-receptor of Toll like receptor 4, TLR-4) markers. Two populations of monocytes/macrophages were distinguished by CD16 expression (Supplemental Fig. 1). In blood, lipid infusion reduced the relative content of whole monocyte population and CD16+ monocytes by 21% ($p = 0.004$) and by 46% ($p = 0.0002$), respectively (Fig. 2A). In SAAT, the relative content of total and CD16+ monocyte/macrophage population was not significantly changed (Fig. 2B). The relative content of M2 resident macrophages-CD206+ and CD206+/CD16+ populations - were reduced by 29% and 27% ($p = 0.02$; $p = 0.012$), respectively (Fig. 2B), while the subset of non-resident CD206− cells was increased by 44% ($p = 0.026$) (Fig. 2B). Moreover, the subset of monocytes/macrophages expressing TLR4+ was increased by 27% ($p = 0.049$) in SAAT. Control infusion did not exert any effect on monocyte/macrophage content in SAAT (data not shown).

3.3. Effect of lipid infusion on T-lymphocyte content in peripheral blood and SAAT of obese women

T-lymphocytes were identified by the combination of general leukocyte and T-cells marker CD45+/CD3+ (Supplemental Fig. 1). The total T-cells population in blood was increased in response to lipid infusion ($p = 0.03$) (Fig. 2A). This increase was associated with a rise of T-helper (T_H) CD4+ cells sub-population by 24% ($p = 0.007$) (Fig. 2A), while no change in T-cytotoxic CD8+ cells population was observed. In SAAT, the increase of T-lymphocytes populations in response to lipid infusion was not consistent (Fig. 2B). The control infusion did not exert any effect on any T-cells content either in blood or in SAAT (data not shown).

3.4. Effect of lipid infusion on plasma levels of cytokines, VEGF-A and adhesion molecules

The observed decrease of monocyte populations in blood led us to the hypothesis that, in response to hyperlipidemia, these cells adhere to endothelium of vascular wall. Therefore, we analyzed the plasma levels of molecules related to adhesion of immune cells to endothelial surface (sICAM, sVCAM) and to angiogenesis (VEGF-A). In response to lipid infusion the plasma levels of sICAM, sVCAM and VEGF-A markedly increased by 9.4% ($p = 0.016$), 11.8% ($p = 0.008$) and 31.7% ($p = 0.03$), while no change was observed during control infusion (Fig. 3). Moreover, plasma levels of IL-6 and MCP-1 were increased in response to lipid infusion by 131%, and 42% ($p = 0.033$, $p = 0.017$), respectively (Fig. 3), the increase of IL-6 and MCP-1 was significantly higher than in control infusion ($p = 0.038$, $p = 0.017$).

3.5. Effect of lipid infusion on expression of genes related to immune response in SAAT

mRNA expression of markers of monocytes/macrophages (CD14, CD206), and markers of T_H lymphocytes subtypes (T_H1: TBX21, T_H2: GATA3, T_{REG}: FOXP3, T_H17: RORC) were determined in SAAT. Among these markers, the expression of RORC was increased by 43% ($p = 0.048$) after lipid infusion (Fig. 4). Further, mRNA levels of chemokines/cytokines (MCP-1, IL-8, IL-6, TNFα), angiogenic marker (VEGF-A), fatty acid translocase (CD36) and toll like receptor 4 (TLR4) were analyzed. The mRNA levels of MCP-1 (78%, $p = 0.028$) and VEGF (68.5%, $p = 0.0001$) were increased in response to lipid infusion (Fig. 4), compared to control experiment.

4. Discussion

In this study, we have shown that the lipid infusion, increasing levels of circulating FFA and TG, induced modifications of relative content of particular sub-populations of monocytes/macrophages and T-lymphocytes in blood and SAAT in obese women, and modified expression of several proinflammatory genes in SAAT along with the increased levels of cytokines and adhesion

Table 1
Anthropometric and biochemical characteristics of two experimental groups of obese women.

	Intralipid (n = 15)	Glycerol controls (n = 10)	P
Age (years)	43 ± 7	44 ± 6	NS
Weight (kg)	89.1 ± 8.0	89.0 ± 6.6	NS
BMI (kg/m^2)	31.4 ± 2.7	31.6 ± 3.4	NS
Waist circumference (cm)	96.6 ± 7.0	98.5 ± 6.8	NS
Waist-to-hip ratio	0.8 ± 0.1	0.8 ± 0.1	NS
Fat mass (%)	38.5 ± 5.0	39.5 ± 4.3	NS
Fat-free mass (%)	61.0 ± 5.8	60.5 ± 4.3	NS
BP–systolic (mmHg)	125 ± 9	124 ± 10	NS
BP–diastolic (mmHg)	78 ± 6	80 ± 7	NS
Total Cholesterol (mmol/L)	5.4 ± 0.8	4.8 ± 0.9	NS
HDL-C (mmol/L)	1.4 ± 0.3	1.4 ± 0.3	NS
Triglycerides (mmol/L)	1.2 ± 0.5	1.1 ± 0.3	NS
Glucose (mmol/L)	5.3 ± 0.3	4.9 ± 0.3	NS
Insulin (mU/L)	7.3 ± 4.3	6.6 ± 2.2	NS
Ureic acid (μmol/L)	276 ± 53	244 ± 63	NS
HOMA-IR	1.8 ± 0.8	1.3 ± 0.6	NS

Data are presented as mean ± SD; NS: non-significant ($p > 0.05$).
BMI: body mass index; BP: blood pressure; HOMA-IR: homeostasis model assessment of the insulin resistance index; HDL-C: HDL Cholesterol.

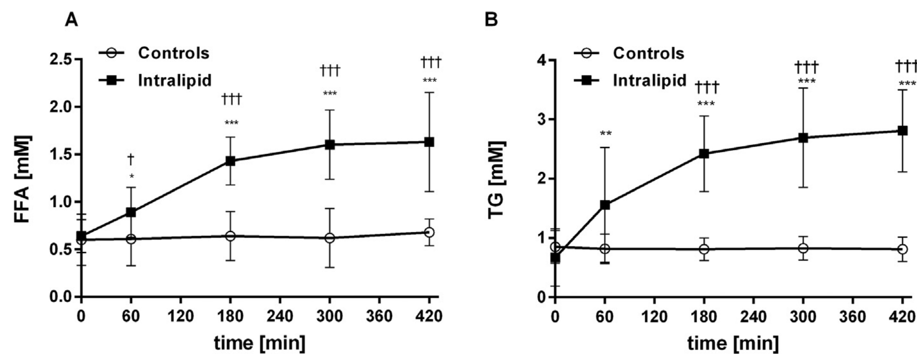

Fig. 1. Plasma levels of FFA (A) and TG (B) during experimental infusion of Intralipid and glycerol-control. Values are presented as means ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$: significant difference during infusion compared to basal state (Wilcoxon matched-pairs signed rank test); †$p < 0.05$, ††$p < 0.01$, †††$p < 0.001$: significantly different value during infusion when compared to control infusion in the same time-point (Man-Whitney rank test).

molecules in circulation in obese women.

Monocyte/macrophages and T-lymphocytes are among the most abundant immune cells invading both adipose tissue and atherosclerotic lesions and appear to be essential for the initiation and/or progression of the metabolic disturbances in obese [24,25]. The present study provides evidence that acute systemic elevation of lipids induced by Intralipid infusion decreased the relative content of monocytes in circulation. This result led us to hypothesis that these cells adhere to the endothelium of vascular wall. This is in agreement, and further extends, the previous findings showing that monocytes in circulation increasingly adhered to the vascular wall in response to repeated postprandial hypertriglyceridemia in rats [26]. Similarly, it was shown in vitro that treatment of endothelial cells with TG-rich lipoproteins increased adherence of human monocytes to these cells [13,27]. Moreover, the increased expression of surface adhesion molecules (CD11b, CD62L etc.) in immune cells was demonstrated postprandially in lean and obese men [1,8,19]. From the two analyzed subpopulations of monocytes, we observed a decrease specifically in CD16+ subpopulation. Nevertheless, it should be mentioned that in humans not only the two monocyte subpopulation are present, in vivo cells are exposed to highly complex and constantly changing mixture of cytokines, which stimulates their differentiation into several intermediate phenotypes [28]. The CD16+ monocytes might be identified as "non-classical" activated cells (M2), i.e. exhibiting antiinflammatory properties [29]. Auffray et al. postulated that these "non-classical" activated monocytes patrol healthy tissue through crawling along the endothelium [30]. It was suggested that CD16+ monocytes/macrophages are present also as reparative mechanism in reaction to the damaged vessel in the early stage atherosclerotic lesions [24]. Thus, we may speculate that CD16+ monocytes represent the first cells that react to an acute hyperlipidemia and that adhere to endothelium damaged by high concentrations of lipids in order to protect it. Indeed, the dysfunctional endothelium was suggested as one of the first steps in atherosclerosis development [24,31,32]. It has been shown previously, that the exposure to high concentration of FFAs and TG induces oxidative stress in endothelium leading to an impairment of its function [33–35] and to elevated expression and secretion of chemokines and adhesion molecules (ICAM, VCAM) [26,27]. Similarly, in this study we found an increase of plasma levels of soluble adhesion molecules (sICAM, sVCAM), chemokines (MCP-1) and also angiogenic factor VEGF-A in response to lipid infusion in obese subjects. The increased expression of angiogenic marker VEGF-A and chemokine MCP-1 was detected also in SAAT. Moreover, the tendency to increase of expression of ICAM was found on blood monocytes (Suppl. Fig 2). These findings support the above mentioned hypothesis of monocytes adhesion to endothelia. It should be noted that a slight increase of IL-6 was observed also during control glycerol infusion: this might correspond to a non-specific IL-6 stimulation by the infusion itself, similarly as in the study of Keller et al. [36]. Importantly, the increase of IL-6 induced by Intralipid infusion was

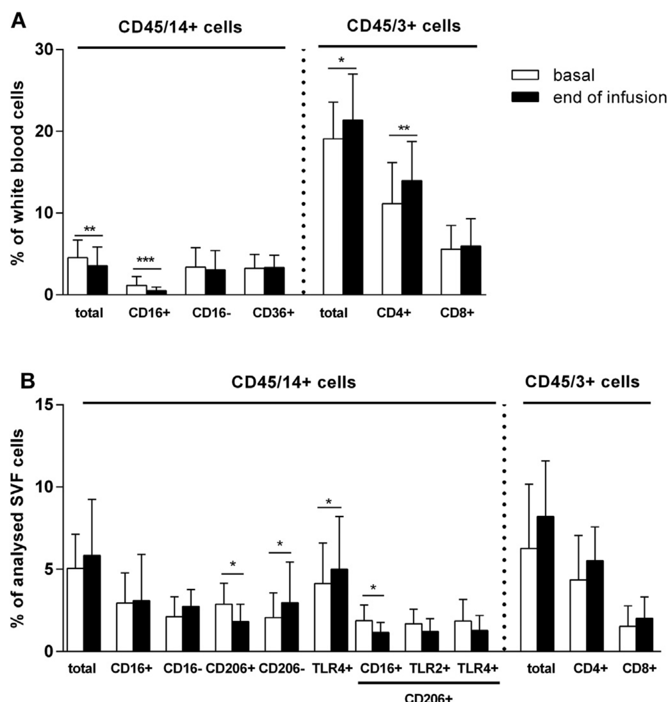

Fig. 2. Relative content of monocyte/macrophage and T-lymphocyte populations in blood (A) and subcutaneous adipose tissue (B) in obese women. Data are presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001: value after infusion significantly different from that of basal state (Wilcoxon matched-pairs signed rank test).

M2, macrophages [37,38], similarly as CD16. Therefore the decrease in these CD206+ and CD206+/CD16+ populations may suggest a switch from M2 to "classical" M1 activated proinflammatory phenotype of macrophages in response to lipid infusion. The switch to M1 proinflammatory phenotype in SAAT could be indeed supported by the observed upregulation of MCP-1 expression in SAAT. In addition, the subpopulation of CD206− monocytes increased in response to lipid infusion. Such a population of CD206− monocytic cells was described by Wentworth et al. [39] and was shown to be elevated in human obesity. It is plausible that these monocytes represent "the newest arrivals" into AT and later can mature into proinflammatory macrophages.

The effects of FFA on leukocytes were shown to be mediated by several receptors, i.e. TLR4 or CD36 [12,40]. In this study, we observed increased relative content of TLR4+ monocytes/macrophages in SAAT in response to Intralipid infusion. TLR4 signaling induced by free fatty acids was shown as important mediator of chronic inflammation in patients with metabolic syndrome and atherosclerosis [41]. The increased content of monocytic cells expressing TLR4 could represent the switch of these cells to the proinflammatory phenotype.

In our study, we found an increase of T_H cell content and total T lymphocyte content in circulation in response to lipid infusion. This finding is in line with previous studies, in which lymphocyte counts were shown to be increased postprandially in healthy as well as in hyperlipidemic subjects with coronary artery disease [1,42]. Moreover, intra-venous administration of CD4+ cells enhanced atherosclerosis in immunodeficient ApoE knockout mice [43] and the development of atherosclerosis was significantly reduced in

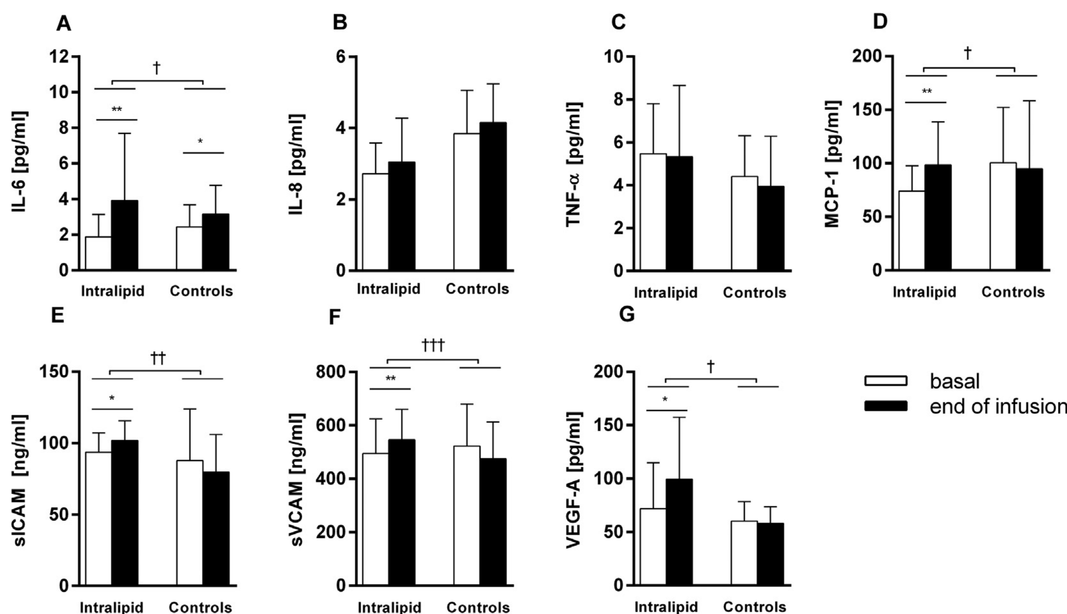

Fig. 3. Plasma levels of cytokines IL-6 (A), IL-8 (B), TNFα (C), MCP-1 (D), sICAM (E), sVCAM (F) and VEGF-A (G) during experimental infusion of Intralipid and glycerol-control. Values are presented as means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001: value after infusion (black bars) significantly different from that of basal state (white bars) (Wilcoxon matched-pairs signed rank test). †p < 0.05, ††p < 0.01, †††p < 0.001: relative change during Intralipid infusion significantly different from the change during control infusion (Man-Whitney rank test).

significantly higher when compared with the effect of control infusion.

In SAAT, the relative content of CD16+ monocytes remained unchanged, but the subset of CD206+/CD16+ macrophages was decreased. CD206 is considered not only as a marker of AT resident macrophages but also a marker of "non-classically" activated, i.e.

CD4/ApoE deficient mice [44]. Thus, our data support the possible involvement of CD4+ cells in the proinflammatory and proatherogenic changes in the presence of hyperlipidemia in obese.

In SVF of SAAT, no changes of T-lymphocyte relative counts in response to Intralipid were detected. Similarly mRNA levels of the T-lymphocyte markers that were used in FACS analysis were not

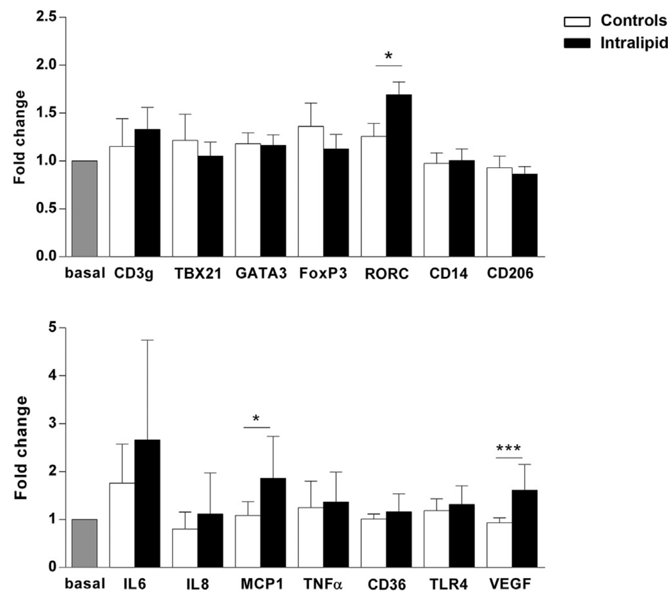

Fig. 4. Effect of Intralipid on gene expression of macrophage, T lymphocyte and selected inflammatory markers in subcutaneous adipose tissue of obese women. Data are presented as mean fold change ± SD. Relative mRNA levels are normalized to housekeeping gene GUSB. *p < 0.05, **p < 0.01, ***p < 0.001: fold change during Intralipid infusion significantly different from that of control infusion (Man-Whitney rank test).

altered. However, mRNA analysis of SAAT revealed an increase in the expression of RORC, a T_H17 lymphocyte marker. T_H17 cells represent a subtype of CD4+ T-lymphocytes with a proinflammatory phenotype. Their numbers were found to be elevated in adipose tissue of metabolically unhealthy obese subjects or in adipose tissue of mice with diet-induced obesity [45,46]. Therefore the increase of expression of T_H17 marker (RORC) might suggest a selective activation/recruitment of T_H17 lymphocytes, which may be interpreted as a proinflammatory change in obese SAAT in response to high lipid levels.

It should be mentioned that the Intralipid infusion consists mostly from poly-unsaturated fatty acids (PUFA)-linoleic and α-linolenic acid. These FFA contribute over 95%, and perhaps as much as 98% of dietary PUFA intake in most Western diets [2]. Although the detrimental effects in respect to inflammation were reported mainly for saturated FFA, the role of linoleic acid as the precursor of arachidonic acid, which is, in turn, the substrate for the synthesis of proinflammatory eicosanoids and leukotrienes, should be considered [2].

Several limitations of this study might be taken into account. These include the limited number of subjects that is, nevertheless, common in interventional dynamic studies with Intralipid infusion [47]. Also, the alterations in SAAT might be different during oral fat load when compared with intravenous infusion. Nevertheless, the Intralipid infusion creates a condition that corresponds exactly to the aim of this study: i.e. to investigate the response of SAAT to a steady exposure of increased circulating levels of lipids. Several studies (including ours) showed that the elevation of triglycerides and fatty acids is lower in response to oral fat load when compared with lipid infusion [7,48]. In addition, the response to oral fat load involves alterations in gastrointestinal hormones and other variables that might show quite higher inter-individual variability compared with intravenous administration. Moreover, the response to the oral lipid load was shown to be genotype-dependent [49]: the genotype dependency cannot be excluded also in respect to Intralipid infusion. As this study was performed in metabolically healthy obese women, the gender and metabolic status specificity should be taken into account when extending the results to other populations. Concerning methodological aspects of the study, ELISA analysis might be influenced by lipemia of samples at the end of Intralipid infusion. Nevertheless, our experiments (not reported here) showed that lipemic plasma generate negative bias, which does not exceed 10%. The negative bias should not change the main findings of the study.

In conclusion, the acute hyperlipidemia induced by Intralipid infusion was associated with proinflammatory and proatherogenic changes in monocyte and lymphocyte populations in SAAT and blood as well as in soluble proinflammatory mediators in circulation in obese women. The proinflammatory changes in SAAT were represented by a decrease of M2 macrophages content and increased expression of several proinflammatory cytokines and of the marker of T_H17 cells. Together, these results point at the processes that could contribute to the development of atherosclerosis in obese exposed to higher chronically, as well as acutely (e.g. postprandially), increased levels of FFA and TG. These processes might play a role in the development of detrimental proinflammatory changes in obese humans and, thus, reduction of these peak lipid levels through nutritional and life-style recommendations should become a part of preventive strategies in obese and/or metabolic syndrome subjects.

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Contribution of authors

E.K. performed the experiments, researched data, and wrote the manuscript. J.K. performed the experiments, researched data, and edited manuscript. M.T. designed the study, performed the experiments, and researched data. L.M., Z.K. and M.K. performed the experiments. L.R. researched data, and reviewed/edited manuscript. V.S. designed the study, and reviewed/edited the manuscript. M.S. researched data and wrote the manuscript.

M.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of interest

Authors have nothing to disclose.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2016.04.021.

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PŘÍLOHA 2

Experimental hyperglycemia induces an increase of monocyte and T-lymphocyte content in adipose tissue of healthy obese women.

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RESEARCH ARTICLE

Experimental Hyperglycemia Induces an Increase of Monocyte and T-Lymphocyte Content in Adipose Tissue of Healthy Obese Women

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Abstract

Background/Objectives

Hyperglycemia represents one of possible mediators for activation of immune system and may contribute to worsening of inflammatory state associated with obesity. The aim of our study was to investigate the effect of a short-term hyperglycemia (HG) on the phenotype and relative content of immune cells in circulation and subcutaneous abdominal adipose tissue (SAAT) in obese women without metabolic complications.

Subjects/Methods

Three hour HG clamp with infusion of octreotide and control investigations with infusion of octreotide or saline were performed in three groups of obese women (Group1: HG, Group 2: Octreotide, Group 3: Saline, n=10 per group). Before and at the end of the interventions, samples of SAAT and blood were obtained. The relative content of immune cells in blood and SAAT was determined by flow cytometry. Gene expression analysis of immunity-related markers in SAAT was performed by quantitative real-time PCR.

Results

In blood, no changes in analysed immune cell population were observed in response to HG. In SAAT, HG induced an increase in the content of CD206 negative monocytes/ macrophages ($p<0.05$) and T lymphocytes (both T helper and T cytotoxic lymphocytes, $p<0.01$). Further, HG promoted an increase of mRNA levels of immune response markers (CCL2, TLR4, TNFα) and lymphocyte markers (CD3g, CD4, CD8a, TBX21, GATA3,

Competing Interests: The authors have declared that no competing interests exist.

FoxP3) in SAAT ($p<0.05$ and 0.01). Under both control infusions, none of these changes were observed.

Conclusions

Acute HG significantly increased the content of monocytes and lymphocytes in SAAT of healthy obese women. This result suggests that the short-term HG can modulate an immune status of AT in obese subjects.

Introduction

Obesity represents a high risk factor for the development of various metabolic and cardiovascular diseases such as insulin resistance, type 2 diabetes, liver steatosis or atherosclerosis. The common feature of these complications is a low-grade inflammation characterized by increased circulating levels of pro-inflammatory cytokines and chemokines (e.g. IL-6, TNF-α, CCL2, CCL5) and enhanced accumulation of immune cells (macrophages, lymphocytes) in adipose tissue (AT) [1–3].

In a previous study focused on subcutaneous abdominal AT (SAAT), we found a progressive increase in the mRNA expression of macrophage markers from obese towards obese with metabolic syndrome (MS) individuals [4]. Similar findings based on a comparison of insulin-resistant with insulin-sensitive subjects were presented by other laboratories [5–7]. However, the cause of higher AT inflammation in obese subjects with metabolic syndrome compared to metabolically healthy obese remains only partly elucidated. The altered control of glycaemia on the obese background might be one factor that plays a role in the further deterioration of AT functions. Indeed, it was suggested that the deterioration of postprandial glucose control precedes long-term elevation of fasting glucose concentration [8,9]. Moreover, it was shown that fluctuations in glucose levels are more harmful than chronic hyperglycemia (HG) *per se* [10]. Detrimental effects of acute HG might be mediated through induction of oxidative stress (via production of glycosylation end product and activation of protein kinase C) and through the activation of inflammatory pathways in various cells resulting in increased secretion of pro-inflammatory cytokines [10–13]. Still, only a few reports addressed responses of cells of adaptive and innate immunity to this metabolic stimulus *in vivo* in obese individuals [14,15].

Therefore, the objective of this study was to investigate whether acute experimental HG has an impact on phenotype and relative content of monocytes/macrophages and lymphocytes in circulation and the SAAT of healthy obese women.

Subjects and Methods

Subjects

The co-author and the head of the Department of Sport Medicine, Vladimir Stich, MD, PhD, recruited subjects for this study among the subjects consulting at the Obesity unit of the University Hospital Kralovske Vinohrady. 30 healthy obese premenopausal women were recruited and divided into 3 groups (n = 10) matched for BMI and age (group 1- HG clamp with octreotide infusion, group 2—octreotide infusion, group 3- saline infusion study). The subjects were matched for BMI and age (range 27–32 kg/m^2 and 40–44 years, respectively) and then they were assigned to one of the three experimental procedures without systematic randomization. All women were drug-free and without signs of metabolic syndrome [16], except for obesity.

To exclude subjects with metabolic syndrome we followed NCEP-ATP III guidelines (https://www.nhlbi.nih.gov/files/docs/guidelines/atglance.pdf), i.e. only women exerting less than 3 out 5 risk factors (waist circumference > 88cm, TAG > 1.7mmol/l, HDL-cholesterol <1.3mmol/l, blood pressure > 130/85mmHg, fasting glucose ≥ 5.6mmol/l) were admitted to the study. Their body weight had been stable for 3 months prior to the examination. Participants signed a written informed consent before the study. The study was performed according to the Declaration of Helsinki and approved by the Ethical Committee of the Third Faculty of Medicine (Charles University in Prague, Czech Republic).

Design of clinical investigation

Clinical investigation was performed before intervention in the fasting state and at the end of the 3-hour HG clamp, or octreotide, or saline infusion. Anthropometric measurements and blood processing were performed as previously reported [17,18]. Body composition was assessed using multi-frequency bioimpedance (Bodystat, Quad scan 4000, Isle of Man, British Isles). 1–2 ml of un-coagulated blood samples was used for flow cytometry analysis. SAAT was obtained by needle biopsy carried out in the abdominal region (10 cm laterally from umbilicus) under local anesthesia (1% Xylocain) as previously described [17]. Biopsies were performed 30 min before the start of the experimental infusions and within the last 15 min of infusions on the contralateral side of abdomen. 1–2 g of SAAT was used for isolation of stromal vascular fraction (SVF) cells to perform flow cytometry analyses. In a subgroup of 6 women in HG and in 9 women from the two remaining experimental groups, 0.1 g of SAAT was immediately frozen in liquid nitrogen and stored at -80°C until RNA isolation.

Hyperglycemic clamp

A bolus injection of 0.33 g/kg glucose followed by a varying 20% glucose infusion was used to achieve steady-state plasma glucose concentrations of 15 mmol/l for 180 minutes. Continuous infusion dose was adjusted every 5 to 10 minutes according to the measured plasma glucose. 5 minutes before the priming glucose, octreotide (Sandostatin, Novartis) infusion was started in order to block the release of endogenous insulin. The initial 25 μg IV bolus administered over 1 min was followed by an infusion at the rate 30 ng/min/kg body weight. To prevent hypokalemia, 0.26 mmol/l KCl was added to the glucose infusion.

To exclude any direct effect of infusion itself or infusion of octreotide on circulating cells and on SAAT characteristics, 2 groups of subjects as control groups (n = 10 per each group) different from those participating in the HG clamp received infusion of saline or octreotide alone (i.e. in the absence of the glucose infusion) at the duration, resp. dose identical to the hyperglycemic condition.

Isolation of SVF cells

SAAT was washed with saline, further minced and digested with type I collagenase 300 U/ml in PBS/ 2%BSA (SERVA, Heidelberg, Germany) for 1h in 37°C shaking water bath. Digested tissue was subsequently centrifuged at 200 g for 10 minutes and filtered through 100- and 40-μm sieves to isolate SVF cells.

Flow cytometry analysis

The whole blood and freshly isolated SVF cells were used for immediate flow cytometry analyses. SVF cells were resuspended in 100 μl PBS solution containing 0.5% BSA and 2 mM EDTA and incubated with fluorescence-labeled monoclonal antibodies (FITC-conjugated antibody

CD14, CD4; PE-conjugated antibody CD14, TLR2, TLR4, CD3; PerCP-conjugated antibodies CD45 and APC-conjugated antibodies CD206 and CD8) or the appropriate isotype controls (BD Bioscience, Bedford, MA) for 30 min at 4°C according to protocol of Curat et al. [19]. The whole blood samples were stained with the same set of fluorescence-labelled monoclonal antibodies as used for SVF cells (except for CD206) for 30 min at room temperature. After staining, erythrocytes were lysed by erythrocyte lysis buffer for 15 min at room temperature. Cells were washed with PBS and analysed on FACS Calibur flow cytometer with CellQuest Pro Software (BD Biosciences, NJ, USA). The number of immune cells belonging to specified populations was expressed as percentage of gated events.

Quantitative real time PCR (RT-qPCR)

Total RNA extraction and reverse transcription (RT-PCR) were performed as previously described [17]. Before reverse transcription, genomic DNA was eliminated by DNase I (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR (RT-qPCR) was performed using an ABI PRISM 7000 and 7500 instrument (Applied Biosystems, Foster City, CA, USA). Primers and TaqMan probes were obtained from Applied Biosystems. Results are presented as fold change values calculated by $\Delta\Delta$ Ct method normalized to geometric mean of two endogenous controls (18S rRNA and GUSB).

Determination of plasma levels of biochemical parameters

Plasma glucose and insulin were determined using the glucose-oxidase technique (Beckman Instruments, Fullerton, CA) and an Immunotech Insulin Irma kit, resp. (Immunotech, Prague, Czech Republic). Homeostasis model assessment of the insulin resistance index (HOMA-IR) was calculated as follows: ((fasting insulin in mU/l) x (fasting glucose in mmol/l) / 22.5). Circulating levels of selected bioactive molecules were measured by commercial ELISA kits: RANTES/CCL5 (Duoset, R&D Systems, Minneapolis, MN, USA) and MCP-1 (Ready-SET-Go, eBioscience, San Diego, CA, USA). Plasma levels of other parameters were determined using standard biochemical methods.

Statistical analyses

Statistical analysis was performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, Inc., San Diego, California, USA). The data were log-transformed for the analyses. The effect of HG clamp or octreotide/saline infusion was tested using parametric t-test. Differences of baseline clinical data between the three groups of patients were analysed by one-way ANOVA with Tukey multiple comparison tests. To compare the effect of HG vs. control infusions, the data were analysed by two-way ANOVA with repeated measures. Data are presented as mean ± SEM. Differences at the level of $p < 0.05$ were considered statistically significant.

Results

Clinical characteristics of obese subjects

The clinical data of subjects participating in three short-term interventions are shown in Table 1. There were no significant differences in anthropometric and laboratory parameters (including fasting blood glucose, plasma insulin levels, and HOMA-IR) between HG and octreotide group of subjects. Fasting glucose levels were lower in the saline group (vs. HG group) but no other differences were found between the two groups.

Table 1. Characteristics of obese subjects in experimental groups.

Characteristics	Hyperglycemia (HG)	Octreotide	Saline
N	10	10	10
Age (years)	42 ± 1	42 ± 2	44 ± 2
Weight (kg)	86.9 ± 2.7	87.5 ± 4.4	89.0 ± 2.2
BMI (kg/m^2)	30.8 ± 0.8	31.9 ± 1.5	31.6 ± 1.1
Fat (kg)	33.2 ± 1.6	34.5 ± 3.0	35.8 ± 2.1
Waist circumference (cm)	95.2 ± 2.6	100.8 ± 3.4	98.5 ± 2.3
Systolic blood pressure (mm Hg)	117. ± 4.6	124.3 ± 3.3	124.4 ± 3.5
Diastolic blood pressure (mm Hg)	76 ± 3.2	77.2 ± 1.5	79.5 ± 2.3
Glucose (mmol/L)	5.4 ± 0.1	5.2 ± 0.1	5.0 ± 0.1 [a]
Insulin (mU/L)	6.5 ± 0.9	7.3 ± 0.8	6.6 ± 0.8
C-peptide (mU/L)	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
HOMA-IR	1.6 ± 0.2	1.7 ± 0.2	1.4 ± 0.2
Cholesterol (mmol/L)	4.7 ± 0.2	4.4 ± 0.2	4.8 ± 0.3
Triglycerides (mmol/L)	0.9 ± 0.1	1.2 ± 0.2	1.1 ± 0.1
HDL-C (mmol/L)	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.1

Data are presented as mean ± SEM

[a] $p < 0.05$

hyperglycemia vs saline; BMI: body mass index; HOMA-IR: homeostasis model assessment of the insulin resistance index; HDL-C: HDL cholesterol

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Plasma insulin, C peptide and glucose levels during hyperglycemic clamp, octreotide and saline infusions

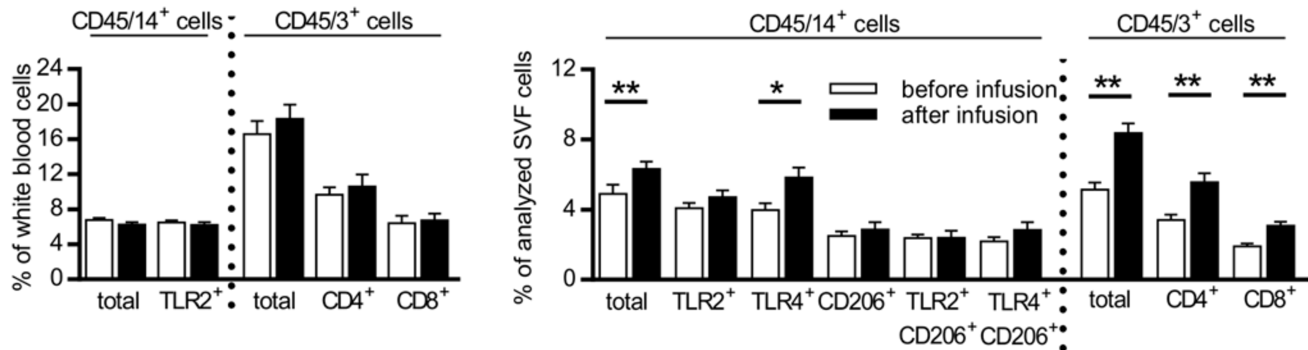
Throughout the HG clamp plasma glucose was maintained at 15 mmol/l (coefficient of variation 7.2 ± 0.7%), being approximately three times higher compared with baseline values. The addition of octreotide prevented hyperglycemia-stimulated endogenous production of insulin except at the end of the 3-hours hyperglycemia when plasma insulin and C-peptide concentrations were modestly increased (insulin 6.45 ± 0.86 mU/l at baseline vs. 10.76 ± 2.1, $p < 0.05$, C-peptide 0.72 ± 0.06 mU/l at baseline vs. 1.08 ± 0.18, $p < 0.05$).

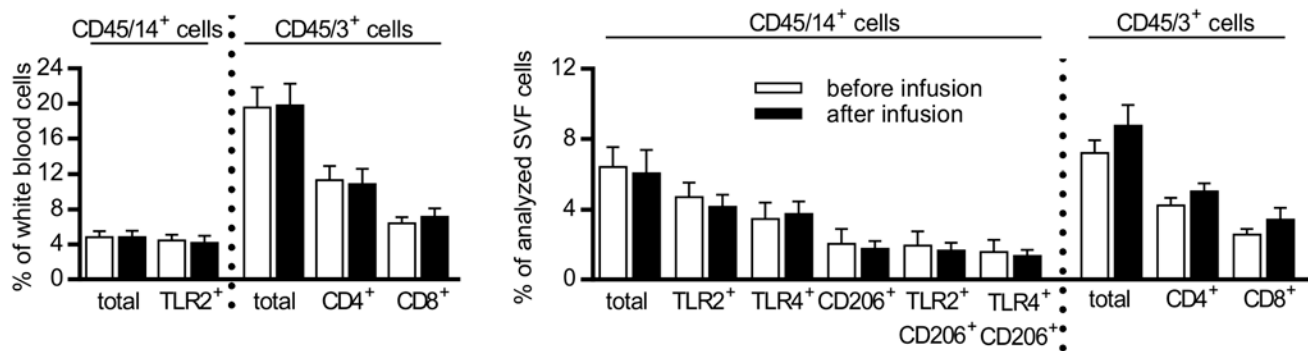
The infusion of octreotide alone decreased plasma insulin and C-peptide below basal levels (insulin, 7.34 ± 0.84 mU/l at baseline vs. 2.42 ± 0.39 mU/l at the end of infusion, $p < 0.001$, C-peptide 0.76 ± 0.84 mU/l at baseline vs. 0.27 ± 0.04 mU/l at the end of infusion, $p < 0.001$) and this was accompanied with a slight elevation of glucose levels (baseline 5.23 ± 0.11 mmol/l, end of infusion 5.95 ± 0.27 mmol/l, $p < 0.01$).

Glucose, insulin and C-peptide levels remained stable during the saline infusion (data not shown).

Monocyte/macrophage and T lymphocyte content in peripheral blood and SAAT of obese women in response to hyperglycemic clamp, octreotide and saline infusion

The content of monocytes/macrophages characterized by expression of CD45+/14+ did not change in response to HG in blood but significantly increased in SAAT (Fig 1A). Similarly, no changes in relative content of monocytes/macrophages expressing Toll-like receptor (TLR) 2 and 4 were induced by HG in blood, while there was a significant HG-induced increase in relative content of CD45+/14+/TLR4+ population in SAAT (Fig 1A). These changes were independent of the content CD45+ cells with high granularity (granulocytes), in SAAT biopsy samples,





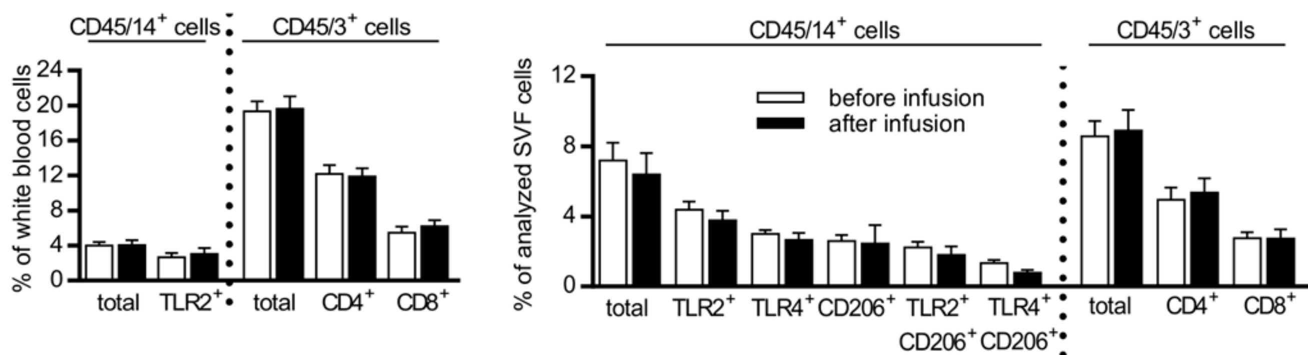


Fig 1. Effect of hyperglycemic clamp (A), octreotide infusion (B), and saline infusion (C) on relative content of monocyte/macrophage and T-lymphocyte populations in peripheral blood and stromal vascular fraction (SVF) of subcutaneous abdominal adipose tissue of obese women. A population of TLR4+ monocytes in blood is not shown due to a low frequency. White bars- before infusion, black bars- after infusion. Data are presented as mean ± SEM, each investigated group n = 10, *p < 0.05; ** p < 0.01: before vs after clamp.

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because their content was not different before or at the end of the HG clamp or other experimental infusions (17.8 ± 2.3% before and 17.4 ± 1.9% after infusion, n = 30). Resident AT macrophage populations were identified by the expression of mannose receptor CD206 on CD45+/CD14+ cells (i.e. CD45+/14+/206+, CD45+/14+/206+/TLR2+ and CD45+/14+/206+/TLR4+ cells) in SAAT (Fig 1A) and they were not affected by HG.

Populations of T lymphocytes (CD45+/3+ cells; T helper subpopulation-CD45+/3+/4+; T cytotoxic subpopulation- CD45+/3+/8+) remained unchanged in response to HG in blood but

significantly increased in SAAT (Fig 1A). The ratio between subpopulations of T helper and T cytotoxic lymphocytes (CD4+/CD8+) in SAAT did not change during HG clamp (data not shown). Importantly, neither octreotide nor saline infusion had a significant effect on relative content of monocyte/macrophage and T lymphocyte populations in blood and/or SAAT (Fig 1B and 1C). Of note, HG-specific increases in total T cell and T helper cell content in SAAT was confirmed by two-way ANOVA.

SAAT mRNA levels of macrophage, lymphocyte and inflammatory markers in response to hyperglycemic clamp, octreotide and saline infusion

To extend the results of flow cytometry, mRNA levels of chemokines/cytokines (CCL2/MCP1, CCL5/RANTES, CXCL12/SDF-1α, IL8, IL1β, TNFα), markers of macrophages (CD14, CD206), Toll like receptors (TLR2, TLR4), lymphocyte markers (CD3g, CD4, CD8a) and remodeling marker (MMP9) were analysed in SAAT. Levels of CCL2 and CCL5 chemokines were also evaluated in plasma. The mRNA levels of CCL2, TLR4, TNFα and all measured T lymphocyte markers (CD3g, CD4, CD8a) including Th1 (TBX21), Th2 (GATA3) and T regs (FoxP3) markers significantly increased in response to HG (Fig 2A) but not after octreotide or saline infusion (Fig 2B and 2C, confirmed also by two-way ANOVA).

Plasma levels of chemokines in response to hyperglycemic clamp, octreotide and saline infusion

Circulating levels of two chemokines involved in attraction of monocytes and lymphocytes, i.e. CCL2 and CCL5, were not changed in response to either conditions (data not shown).

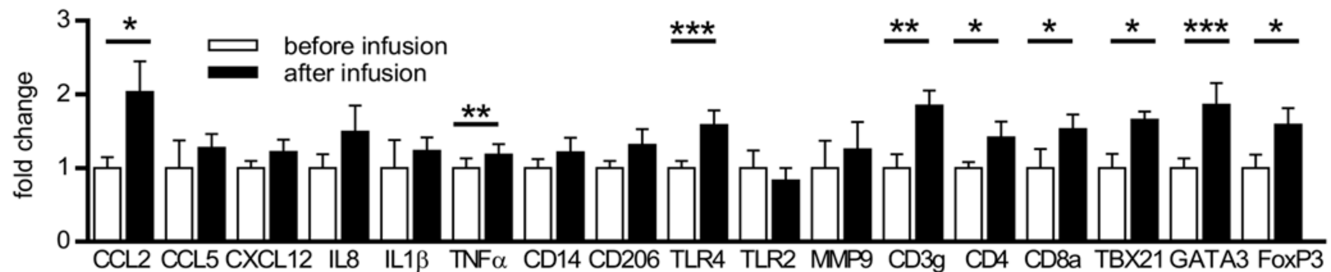
Discussion

Obesity-related inflammation has been considered one of the major risk factors for the development of metabolic and cardiovascular diseases. Short-term HG represents one of the possible triggers to aberrant activation of the immune system [20]. This could contribute to the further worsening of the inflammatory state in obese subjects resulting in metabolic syndrome or type 2 diabetes. Thus, we investigated the effect of HG on immune cell phenotype and content in circulation and SAAT. The present study was carried out in healthy obese women representing an optimal model for studying the processes contributing to the deterioration of metabolic status of obese subjects.

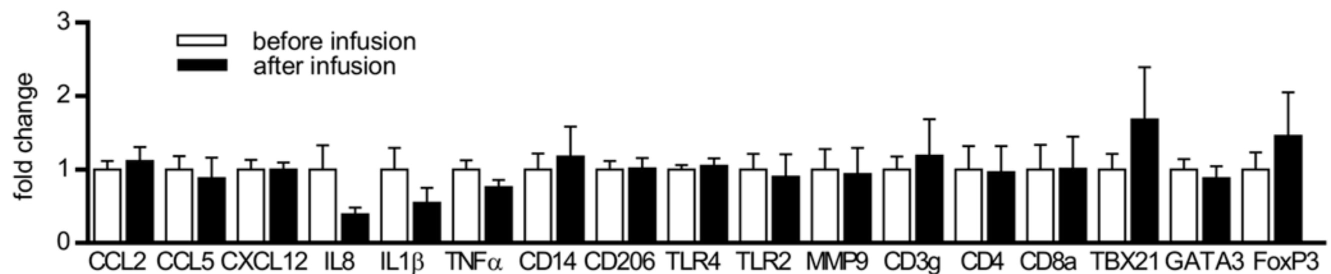
We documented that HG induced an increase in CD45+/14+ monocyte/macrophage population in SAAT. Upon the octreotide or saline infusion, no changes in monocyte/macrophage population in SAAT were detected; therefore the above-mentioned increased numbers of monocytes/macrophage in SAAT cannot be attributed to octreotide or infusion *per se.*

Since it was shown previously that HG treatment of monocytes *in vitro* increases expression of Toll-like receptors [21] and also monocytes from patients with type 2 diabetes show a higher expression of TLR2 and TLR4 compared to healthy subjects [15], we investigated the expression of these two receptors in circulating blood cells and SAAT in obese women. While the relative content of activated monocyte/macrophage population defined as a triple positive population CD45+/14+/TLR4+ was increased in response to HG, no significant changes in CD45+/14+/TLR2+ population were observed in SAAT. Thus, the selective effect of HG on TLR4+ monocyte/macrophage population could point to a specific physiological function of this subtype of monocytes/macrophages in HG-affected SAAT. Indeed, recent findings suggest that TLR4 and TLR2 activation in macrophages results in the differential expression and

A) **Hyperglycemic clamp**



B) **Octreotide infusion**



C) **Saline infusion**

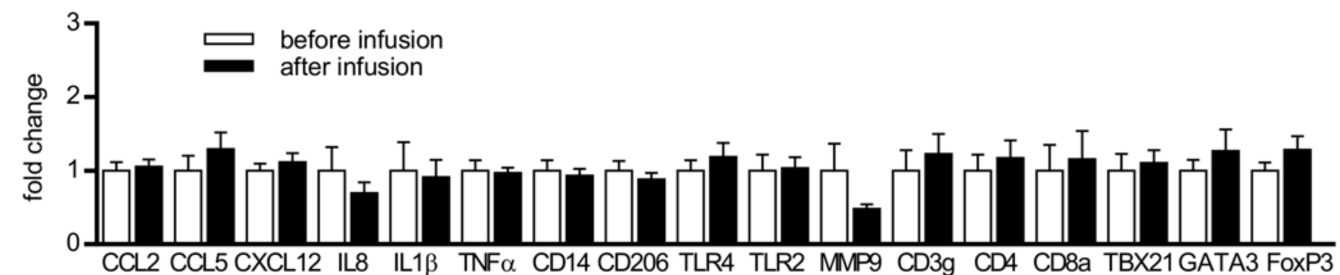


Fig 2. Effect of hyperglycemic clamp (A), octreotide infusion (B), and saline infusion (C) on mRNA levels of selected immunity-related genes in subcutaneous abdominal adipose tissue of obese women. White bars- before infusion, black bars- after infusion. Data are presented as mean fold change ± SEM. Relative mRNA levels are normalized to geometrical mean of 2 housekeeping genes 18S and GUSB, n = 6 (hyperglycemic clamp), n = 9 (octreotide and saline infusion), *p < 0.05; ** p < 0.01; *** p < 0.001: before vs after clamp.

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secretion of pro-inflammatory cytokines [22,23]. We found increased mRNA levels of TLR4 along with TNF in the AT of obese women after HG clamp, which has been shown to be up-regulated after TLR4 but not TLR2 stimulation in macrophages [23]. This mechanism of cytokine regulation is considered to be important for the control of migration and subsequent activation of inflammatory monocytic cells. Notably, we observed that the surface expression of both TLR2 and TLR4 was detectable in the majority of monocytes present in SAAT despite low expression of TLR4 in circulating monocytes (low expression of TLR4 on circulating

monocytes was also documented by Kashiwagi et al [24]). Thus, we speculate that the expression of TLR4 on monocytes could either be stimulated by the SAAT microenvironment or, alternatively, only those monocytes expressing TLR4 could reach the SAAT. However, further studies will be needed to clarify this hypothesis.

Contrary to monocyte population, a population of resident AT macrophages did not show any changes in response to HG in terms of relative content and TLRs expression (i.e. content of CD45+/14+/206+/TLR2+ and TLR4+). Therefore, it seems that SAAT microenvironment, changed by HG, activated only monocytic cells that are not fully differentiated into macrophages. Such a population of CD206- monocytic cells was described by Wentworth et al. [25] and was shown to be elevated in human obesity. It is plausible that these monocytes represent "the newest arrivals" into AT but then later can mature into CD206+ macrophages. Nevertheless, CD206 marker used to identify resident AT macrophages was previously suggested to be preferentially expressed by M2 macrophages [26], and thus it is also possible that observed increase in CD45+/14+/206- population could be attributed to M1 macrophages. This hypothesis however could not be tested as M1 macrophage marker CD40 [26] is expressed also on 60% of circulating monocytes (not shown).

The pro-inflammatory state associated with metabolic complications represents a bridging of innate and adaptive immune systems in AT physiology. Previous studies investigating the dynamics of immune cell infiltration of AT during the onset of obesity suggested that lymphocytes are the first players of immunity which infiltrate the AT [27–29]. In our study, we found an increased content of total T lymphocytes and both major subpopulations of T lymphocytes, i.e. T helper CD4+ and T cytotoxic CD8+ in SAAT of obese women in response to short-term HG. Importantly, the role of CD4+ and CD8+ T cells in modulating AT inflammation and overall metabolic status has been documented previously in both animal models and human. According to animal studies [28], CD8+ T cells direct macrophage infiltration into AT. CD4+ T cells have both anti- and pro-inflammatory roles based on their further specialization [30] and the balance between these individual CD4+ subpopulations is responsible for the control of metabolic inflammation [31]. Notably, at least two of the CD4+ subpopulations, i.e. Th1 and Th17 cells, are pro-inflammatory and their numbers are significantly elevated in AT of metabolically unhealthy obese subjects or in diet-induced obesity in mice [31,32]. Thus, we could speculate that the increase of CD4+ cells upon HG could be attributed to these two subpopulations (Th1 and Th17 cells) however this hypothesis has to be proven in further study.

In blood, short-term HG caused no alteration in relative content of immune cell populations or their phenotype, along with no change in circulating levels of chemokines, i.e. CCL2 and CCL5, involved in chemo-attraction of monocytes and lymphocytes. Thus, a short metabolic stimulus of 3-hour HG is probably insufficient to alter relative content of various leukocyte populations in circulation but it has a significant effect on immune response in SAAT of obese healthy women. In fact, relative content of immune cell populations in SAAT was analysed in the context of other cell types (i.e preadipocytes, endothelial cells) whose numbers in AT are presumably insensitive to short-term metabolic insults, which may facilitate a detection of even small changes in numbers of immune cells.

It was shown that HG modulates expression of genes related to immune response in SAAT of lean subjects [14,33]. We observed that mRNA levels of TLR4 (also expressed on adipocytes and endothelial cells [34]; [35]), CD3g, CD4 and CD8a increased in the experimental condition of HG in obese women, which nicely supports the flow cytometry results. Unlike circulating levels of CCL2, mRNA levels of CCL2 in SAAT were increased after a short-term HG. One could hypothesize that these local changes of immune response genes in the AT could affect monocyte/macrophage population. Indeed, recent paper of Amano et al. [36], suggested that CCL2 promotes proliferation of resident macrophages in AT in obesity. Likewise, other clinical

studies have reported an increased expression of activation markers on monocytes and neutrophils in type 2 diabetic patients [15,37,38].

Unlike other studies analysing T cell subpopulations in mice [39], we did not analyse among T cell subtypes by flow cytometry due to the limited numbers of SVF cells derived from needle biopsy samples. However, we found the up-regulation of TBX21, GATA3 and FoxP3 mRNA levels (representing major differentiation factors of Th1, Th2 and Tregs subtypes) in SAAT after HG condition in obese women. It has been shown that Th1, Tregs are increased and Th2 subpopulation is decreased with obesity [40,41]. Based on the combination of our results from flow cytometry and mRNA analysis, one can hypothesize that HG enhanced infiltration of both pro- and anti-inflammatory T cells in order to maintain immune homeostasis in AT. However, to determine a comprehensive picture of the sequence of the immune cells activation in circulation, accumulation in AT, and their role in the induction of the AT pro-inflammatory state further analyses need to be performed. In fact, the role of immune cell infiltration in AT is not unequivocal: it still remains unknown whether it reflects the dysfunction of AT metabolism or prevents this event. The study of Duffaul et al. [42] documented that early T cells infiltration into AT has protective role since it inhibits pro-inflammatory reaction of innate cells. Similar finding by Sultan et al. [43] showed that adaptive cells alone are not responsible for the impairment of insulin sensitivity in obesity.

For characterization of particular immune cell populations in blood and SAAT, we used flow cytometry. This method enables simultaneous detection of several surface markers and provides results superior over immunohistochemistry or gene expression analysis alone. However, the flow cytometry analysis of needle biopsy-derived samples may raise concerns of a possible contamination of SAAT sample by blood cells. Similar to our previous study [18], where this possible limitation was already discussed, the content of granulocytes, i.e. CD45+ cells with high granularity, in SAAT samples, was not different before or at the end of the HG clamp or other experimental infusions used in this study. This suggests that blood contamination does not affect the outcome of the flow cytometric data in SAAT. Another possible limitation of this study was a slight increase of plasma insulin levels at the end of the HG clamp. Noteworthy, this final concentration of insulin remained within the range of normal fasting levels and was negligible when compared with the usual postprandial concentrations. In addition, the reports showing an acute effect of insulin on the circulating levels of pro-inflammatory cytokines [44,45] were based on the exposure to 4 fold higher levels of insulin than those detected in the present study. Moreover, circulating resting T lymphocytes are devoid of insulin receptor [38]. Thus even though we cannot completely rule out the possibility that the slight increase of plasma insulin may contribute to the observed effect of HG on immune cells, it seems rather unlikely.

In summary, our results show that the short-term HG induces an increase in the content of monocytes and T lymphocytes in SAAT of healthy obese women and thus suggest that the oscillations in glycaemia levels may modulate an immune status of AT in obese individuals.

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Author Contributions

Conceived and designed the experiments: MT VS LR. Performed the experiments: MT JK EK LM ZK ZW MS VS LR. Analyzed the data: MT JK LM ZK MS LR. Contributed reagents/materials/analysis tools: MT JK LM ZK LR. Wrote the paper: MT LR VS.

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PŘÍLOHA 3

Postprandial inflammation is not associated with endoplasmic reticulum stress in peripheral blood mononuclear cells from healthy lean men.

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Postprandial inflammation is not associated with endoplasmic reticulum stress in peripheral blood mononuclear cells from healthy lean men

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Abstract

The consumption of lipids and simple sugars induces an inflammatory response whose exact molecular trigger remains elusive. The aims of the present study were to investigate (1) whether inflammation induced by a single high-energy, high-fat meal (HFM) is associated with endoplasmic reticulum stress (ERS) in peripheral blood mononuclear cells (PBMC) and (2) whether these inflammatory and ERS responses could be prevented by the chemical chaperone ursodeoxycholic acid (UDCA). A total of ten healthy lean men were recruited to a randomised, blind, cross-over trial. Subjects were given two doses of placebo (lactose) or UDCA before the consumption of a HFM (6151 kJ; 47·4 % lipids). Blood was collected at baseline and 4 h after the HFM challenge. Cell populations and their activation were analysed using flow cytometry, and plasma levels of inflammatory cytokines were assessed by ELISA and Luminex technology. Gene expression levels of inflammatory and ERS markers were analysed in $CD14^+$ and $CD14^-$ PBMC using quantitative RT-PCR. The HFM induced an increase in the mRNA expression levels of pro-inflammatory cytokines (*IL-1β*, 2·1-fold; *IL-8*, 2·4-fold; *TNF-α*, 1·4-fold; monocyte chemoattractant protein 1, 2·1-fold) and a decrease in the expression levels of *miR181* (0·8-fold) in $CD14^+$ monocytes. The HFM challenge did not up-regulate the expression of ERS markers (*XBP1*, *HSPA5*, *EDEM1*, *DNAJC3* and *ATF4*) in either $CD14^+$ or $CD14^-$ cell populations, except for *ATF3* (2·3-fold). The administration of UDCA before the consumption of the HFM did not alter the HFM-induced change in the expression levels of ERS or inflammatory markers. In conclusion, HFM-induced inflammation detectable on the level of gene expression in PBMC was not associated with the concomitant increase in the expression levels of ERS markers and could not be prevented by UDCA.

Key words: Peripheral blood mononuclear cells: Ursodeoxycholic acid: Postprandial inflammation: Endoplasmic reticulum stress

The pandemic of obesity in the Western world has been attributed to the lack of physical activity and availability of highly palatable, easily digestible and energy-dense food. Palatability is based on a high content of lipids and simple sugars. However, the overconsumption of lipids and simple sugars is associated with the exaggeration of postprandial blood glucose and lipid levels[1]. The protracted elevations of blood metabolites are the signs of postprandial dysmetabolism associated with so-called postprandial inflammation[1–3]. Postprandial inflammation is manifested by increased plasma levels of inflammatory cytokines and leucocyte activation[4,5], although the precise contribution of blood monocytes and lymphocytes to these pro-inflammatory changes remains unknown. While in healthy people, postprandial inflammation is transient, it is prolonged in obese people and in subjects with type 2 diabetes[2,3,6]. Thus, prolonged postprandial inflammation has been suggested to promote insulin resistance and atherosclerosis. The exact molecular trigger of postprandial inflammation is not fully elucidated yet. Nevertheless, it has been shown previously that exposure of cells to saturated lipids and a high concentration of glucose may cause endoplasmic reticulum stress (ERS), as documented by the increased mRNA levels of several ERS markers or by the increased activity of an ERS-responsive LacZ reporter

Abbreviations: ATF, activating transcription factor; DNAJC3, DnaJ (Hsp40) homolog, subfamily C, member 3; EDEM1, ER degradation enhancer, mannosidase alpha-like 1; ERS, endoplasmic reticulum stress; HFM, high-fat meal; HSPA5, heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa); MCP1, monocyte chemoattractant protein 1; miRNA, microRNA; PBMC, peripheral blood mononuclear cell; RANTES, regulated on activation, normal T-cell expressed and secreted; TLR, Toll-like receptor; UDCA, ursodeoxycholic acid; UPR, unfolded protein response; XBP1, X-box binding protein 1; XBP1s, X-box binding protein 1 spliced.

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system[7–9]. ERS leads to the activation of pathways that primarily decrease the burden of endoplasmic reticulum or eliminate the affected cell. Meanwhile, however, it leads to the stimulation of classic inflammatory regulatory molecules such as NF-κB and Jun N-terminal kinase[10]. Thus, postprandial inflammation could be triggered by ERS. Notably, ERS-induced inflammation may be alleviated by chemical chaperones such as bile acids[11]. One such chemical chaperone, ursodeoxycholic acid (UDCA), currently used therapeutically for the treatment of cholestasis, has been shown to prevent chemically induced ERS *in vitro*[12,13]. Given these facts, we analysed inflammation induced by a single high-fat meal (HFM) in two subpopulations of peripheral blood mononuclear cells (PBMC) representing cells of innate and adaptive immunity, and tested whether this HFM-induced inflammation is associated with ERS. Furthermore, we investigated whether the inflammatory or ERS response may be modified or prevented by the non-toxic chemical chaperone UDCA.

Experimental methods

Subjects and study design

A total of ten healthy lean male subjects were recruited to a randomised, blind, cross-over trial consisting of two 1 d studies, separated by at least 1 week (when the subjects followed their habitual diet and level of exercise). Exclusion criteria were as follows: weight changes of >3 kg within the 3 months before the start of the study; participation in other trials; hyperbilirubinaemia; smoking; alcohol or drug abuse. The characteristics of the subjects are provided in Table 1. Subjects were given 10 mg/kg of placebo (lactose) or UDCA (Ursosan; PRO.MED.CS) in gelatin capsules with the last evening meal (20.00 hours) before the experimental day. Upon admission (08.00 hours), a catheter was placed in the antecubital vein. After baseline blood sampling, subjects were given 15 mg/kg of placebo or Ursosan. Within 15 min, they consumed a high-energy, HFM consisting of a breakfast sandwich with pork meat and egg omelette, French fries, ketchup, Nutella spread, croissant, ice tea (McDonalds; 6151 kJ; 32·8 % carbohydrates, 47·4 % lipids and 11·3 % proteins). After the meal was consumed, blood was drawn each hour up to the 4th hour. During the intervention, subjects had free access to drinking-water. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the ethical committee of the Third Faculty of Medicine of Charles University in Prague, Czech Republic. Written informed consent was obtained from all subjects before the study.

Table 1. Characteristics of the subjects
(Mean values with their standard errors, *n* 10)

	Mean	SEM
Age (years)	26·3	1·04
BMI (kg/m^2)	23·11	0·59
Weight (kg)	77·51	2·48
Waist circumference (cm)	81·5	1·96
Fat mass (%)	13·46	1·06
Glucose (mmol/l)	4·77	0·11
Insulin (mU/l)	5·4	0·66
HOMA-IR	1·16	0·15
TAG (mmol/l)	0·82	0·13
HDL-cholesterol (mmol/l)	1·59	0·15
Total cholesterol (mmol/l)	4·81	0·28

HOMA-IR, homeostasis model assessment of the insulin resistance index.

Determination of plasma levels of biochemical parameters

Plasma glucose levels were determined using the glucose oxidase technique (Beckman Instruments, Inc.). Plasma insulin level was measured using an Immunotech Insulin Irma kit (Immunotech). Homeostasis model assessment of the insulin resistance (HOMA-IR) index was calculated as follows:

$$\text{HOMA-IR} = (\text{fasting insulin (mU/l)} \times \text{fasting glucose (mmol/l)})/22{\cdot}5.$$

Plasma levels of glycerol, NEFA and TAG were measured by colorimetric enzymatic assays using kits from Randox.

Flow cytometry analysis

To determine the absolute numbers of cells in the blood, TruCOUNT tubes containing defined numbers of beads detectable by flow cytometry were used according to the manufacturer's protocol (BD Biosciences). Subpopulations of blood cells representing lymphocytes, granulocytes and monocytes were analysed according to their size and granularity. To detect specific surface antigens, whole-blood samples were stained with fluorescence-labelled monoclonal antibodies (fluorescein isocyanate-conjugated antibodies: CD4, CD14, CD16 and CD36; phycoerythrin-conjugated antibodies: CD3, CD11c, CD14, Toll-like receptor (TLR)2 and TLR4; allophycocyanin-conjugated antibodies: CD8 and CD56) or the appropriate isotype controls (BD Biosciences) for 30 min at room temperature. After cell staining, erythrocytes were lysed by erythrocyte lysis buffer for 15 min at room temperature. The cells were then washed with PBS and analysed on a FACSCalibur flow cytometer and CellQuest Pro Software (BD Biosciences). The number of immune cells in the analysed populations was expressed as a percentage of gated events or the absolute numbers calculated from data obtained by TruCOUNT analysis. Background was set up to 5 % of positive cells of the isotype control.

Isolation of peripheral blood mononuclear cells and CD14$^+$ cells

PBMC were isolated by gradient centrifugation. Briefly, 9 ml of uncoagulated blood were diluted in PBS to 16 ml and applied onto Leucosep tubes (Greiner Bio-One) filled with 3 ml of Histopaque-1077 separation medium (Sigma-Aldrich). After centrifugation for 15 min at 800 ***g***, plasma was discarded and PBMC located above the frit were transferred to a tube containing endothelial cell basal medium (PromoCell). The cells

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were washed three times, diluted in isolation buffer (PBS supplemented with 0·1 % bovine serum albumin and 2 mM-EDTA, pH 7·4) and counted. Up to 10 million cells were mixed with 25 μl CD14 Dynabeads (Invitrogen) and incubated on a rotator for 20 min at 4°C, and then $CD14^{+}$ PBMC were separated with a magnet and lysed in RLT (Qiagen). $CD14^{-}$ PBMC were collected by centrifugation and lysed in RLT. Both fractions of PBMC were then used for RNA isolation. Separation efficiency was confirmed by both fluorescence-activated cell sorting and quantitative RT-PCR analysis (data not shown).

Gene expression analysis

Total RNA was isolated using a miRNeasy Mini Kit (Qiagen). Genomic DNA was removed by DNase I treatment (Invitrogen). Complementary DNA was obtained by reverse transcription (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems) of 300 or 600 ng of total RNA. Complementary DNA equivalent to 5 ng of RNA was used for real-time PCR analysis using the Gene Expression Master Mix and Gene Expression Assay for heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa) (*HSPA5*) (Hs99999174_m1), activating transcription factor 4 (*ATF4*) (Hs00909569_g1), *ATF3* (Hs00231069_m1), ER degradation enhancer, mannosidase alpha-like 1 (*EDEM1*) (Hs00976004_m1), DnaJ (Hsp40) homolog, subfamily C, member 3 (*DNAJC3*) (Hs00534483_m1), regulated on activation, normal T-cell expressed and secreted (*RANTES*; Hs00174575_m1), *IL-1β* (Hs01555410_m1), *IL-8* (Hs00174103_m1), monocyte chemoattractant protein 1 (*MCP1*; Hs00234140_m1), *PPARα* (Hs00947539_m1), *PPARγ* (Hs01115513_m1), *TLR2* (Hs00152932_m1) and *TLR4* (Hs010 60206_m1) (Applied Biosystems). *TNF-α*, X-box binding protein 1 (*XBP1*) total and *XBP1* spliced (*XBP1s*) were detected by specific primers (TNF-α: forward 5′-TCTCGAACCCCGAGTGACA-3′ and reverse 5′-GGCCCGGCGGTTCA-3′; *XBP1* total: forward 5′-CGCTGAGGAGGAAACTGAA-3′ and reverse 5′-CACTTGCTGTTCCAGCTCACTCAT-3′; *XBP1s*: forward 5′-GAGTCCGCAGCAGGTGCA-3′ and reverse 5′-ACTGGGTCCAAGTTGTCCAG-3′) using a SYBR Green technology (Power SYBR® Green Master Mix; Applied Biosystems). The microRNA (miRNA) were transcribed by a miScript II RT kit (Qiagen) without prior DNase I treatment. Complementary DNA equivalent to 1 ng of RNA was used for real-time PCR analysis using the miScript SYBR Green PCR Kit and miScript Primer Assay for *miR146a* and *miR181a* (Hs_miR-146a*_1 and Hs_miR-181a*_1; Qiagen). All samples were run in duplicate on a 7500 Fast ABI PRISM instrument (Applied Biosystems). Gene expression of target genes was normalised to the expression of ribosomal protein S13 (*RPS13*) (mRNA, Hs01011487_g1) or RNA, U6 small nuclear 2 (*RNU6-2*) (miRNA, Hs_RNU6-2_1) (Qiagen), and expressed as fold changes calculated using the $\Delta\Delta C_t$ method.

Plasma cytokine analysis

Plasma levels of leptin and adiponectin were measured by ELISA (DuoSet; R&D Systems), with a limit of detection of 62·5 pg/ml. Plasma TNF-α, IL-6, IL-1β and IL-8 levels were measured by the MILLIPLEX MAP Human High Sensitivity Cytokine Panel (Merck), with a limit of detection of 0·13 pg/ml.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 6 and SPSS 12.0 for Windows (SPSS, Inc.). Data of plasma metabolites, gene expression (ΔC_t) and flow cytometry-derived variables were log transformed, and normality of the data was assessed by the Shapiro–Wilk normality test. The effects of the HFM in the placebo and UDCA treatments were tested using the one-way and two-way ANOVA with Bonferroni *post hoc* analysis. Correlations among the relative mRNA levels were analysed using Spearman's correlation. Data are presented as means with their standard errors. Differences at the level of $P < 0·05$ were considered to be statistically significant.

Results

Postprandial changes in plasma metabolites

Evolution of postprandial plasma levels of glycerol, NEFA, TAG, glucose and insulin in response to the HFM challenge is shown in Fig. 1. NEFA levels declined after the consumption of the HFM and then gradually increased during the time course of the experiment but not above the fasting levels (Fig. 1(a)). Glycerol and TAG concentrations reached peak values 3 h after ingestion of the HFM (Fig. 1(b) and (c)). Glucose levels did not alter significantly during the whole intervention (Fig. 1(d)), whereas insulin levels increased 1 h after ingestion of the HFM and remained elevated above the fasting levels (Fig. 1(e)). Baseline plasma levels of NEFA and glycerol were lower in the UDCA treatment, though this difference did not reach a significant level. Thus, no differences in baseline or postprandial plasma levels of the tested metabolites between the placebo and UDCA treatments were detected.

Postprandial changes in blood cell populations

At the fasting state, numbers of leucocytes per μl of blood were not different between the placebo and UDCA treatments (placebo: 9821 (SE 704) cells/μl; UDCA: 9380 (SE 763) cells/μl). The HFM challenge significantly increased the absolute numbers of monocytes, lymphocytes and granulocytes and the total numbers of leucocytes (Fig. 2(a) and (b)). This increase was similar in the presence of UDCA. In addition, the relative distribution of two main leucocyte populations, namely lymphocytes and granulocytes, in the blood changed postprandially, i.e. the relative proportion of lymphocytes decreased, while that of granulocytes decreased reciprocally in response to the test meal in the placebo treatment (data not shown). The relative proportion of monocytes within the whole leucocyte population remained unaltered in response to the HFM challenge. Given that both the relative distribution of the leucocyte population and the absolute counts of cells were affected by the consumption of the test meal, the numbers of events representing gated cells were normalised by

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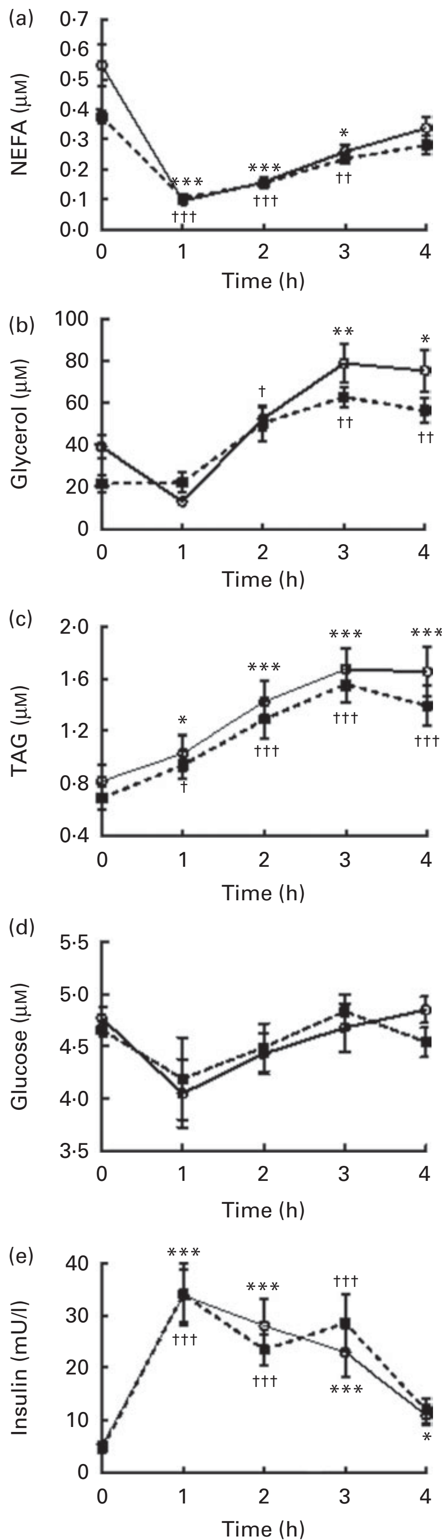

Fig. 1. Evolution of plasma levels of (a) NEFA, (b) glycerol, (c) TAG, (d) glucose and (e) insulin following a high-fat meal challenge. Values are means, with their standard errors represented by vertical bars. Mean value was significantly different from that of baseline levels in the placebo (-○-) treatment: * $P<0·05$, ** $P<0·01$, *** $P<0·001$. Mean value was significantly different from that of baseline levels in the ursodeoxycholic acid (-■-) treatment: † $P<0·05$, †† $P<0·01$, ††† $P<0·001$.

TruCOUNT data (the percentage of positive cells multiplied with the absolute number of events in either the monocyte, lymphocyte or granulocyte gate).

The HFM increased the counts of $CD14^+/CD11c^+$ and $CD14^+/TLR2^+$ monocytes in both placebo and UDCA treatments. The counts of $CD14^+/TLR4^+$ monocytes were increased after ingestion of the test meal in the placebo treatment only. However, only in the UDCA treatment, the HFM challenge increased the counts of $CD4^+$ and $CD8^+$ lymphocytes (Fig. 2(c) and (d)).

The evaluation of the expression levels of individual surface markers (expressed as geometric mean fluorescence intensity) revealed that the HFM enhanced the expression levels of the activation marker CD11c in monocytes. This increase was significant in both placebo and UDCA treatments (Fig. 2(e)).

Postprandial changes in plasma adipokines and inflammatory cytokines

Plasma levels of leptin, adiponectin, IL-8 and TNF-α did not alter during the HFM intervention in either the placebo or UDCA treatment (data not shown). Plasma IL-6 levels increased gradually over the 4 h period in both placebo and UDCA treatments (Fig. 3). However, in most samples, plasma levels of IL-1β were under the detection limit.

Postprandial changes in the gene expression levels of cytokines in peripheral blood mononuclear cells

At baseline levels, $CD14^+$ cells expressed substantially higher mRNA levels of *IL-1β*, *IL-8*, *MCP1* and *TNF-α* and lower mRNA levels of *RANTES* compared with the $CD14^-$ cell population (Fig. 4(a)). Therefore, the effect of the HFM on the expression levels of *IL-1β*, *IL-8*, *MCP1* and *TNF-α* was analysed in $CD14^+$ cells, and of *RANTES* in $CD14^-$ cells.

In $CD14^+$ cells, gene expression levels of all the measured cytokines were increased in response to the HFM challenge (Fig. 4(b)–(e)). This increase was similar in both treatments except for TNF-α that was not altered in response to the HFM challenge in the UDCA treatment. Subsequently, the expression levels of two miRNA (*miR181a* and *miR146a*) implicated in the negative regulation of the expression of TLR2/4 pathway members were analysed (Fig. 4(f) and (g)). The expression level of *miR181a*, but not *miR146a*, was decreased by the consumption of the test meal in both placebo and UDCA treatments. The mRNA expression level of *RANTES*, a cytokine produced by $CD8^+$ lymphocytes, was decreased in $CD14^-$ cells after ingestion of the HFM in the UDCA treatment only (Fig. 4(h)). This result was also confirmed when the expression of *RANTES* was normalised to the pan T-lymphocyte marker *CD3g* (data not shown). However, the changes in the mRNA expression levels of all the measured cytokines in response to the HFM challenge were not different between the placebo and UDCA treatments as revealed by the two-way ANOVA.

The expression levels of other genes potentially activated by dietary fatty acids (i.e. *TLR4*, *TLR2*, *PPARα* and *PPARγ*) were

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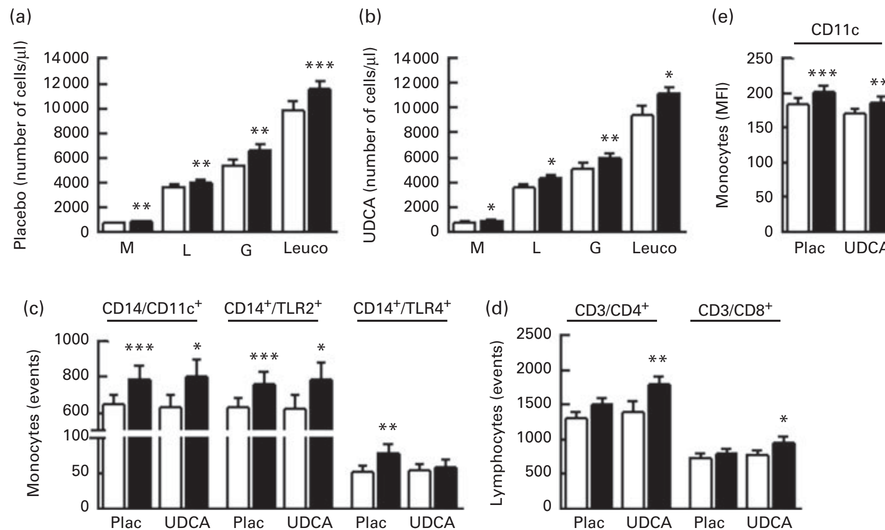

Fig. 2. Effect of the test meal on the numbers and activation of leucocytes. The absolute numbers of leucocytes at the fasting (baseline, □) state were compared with the numbers of leucocytes 4 h after a high-fat meal (■) challenge in the (a) placebo (Plac) and (b) ursodeoxycholic acid (UDCA) treatments. The number of cells in the subpopulations of (c) monocytes and (d) lymphocytes out of 10 000 events in both Plac and UDCA treatments. (e) Mean fluorescence intensity (MFI) for CD11c in monocytes. Values are means, with their standard errors represented by vertical bars. Mean value was significantly different from that of baseline levels: * $P<0.05$, ** $P<0.01$, *** $P<0.001$. M, monocytes; L, lymphocytes; G, granulocytes; leuco, total leucocytes; TLR, Toll-like receptor.

not altered significantly in response to the HFM challenge (Fig. 4(i)–(l)).

Postprandial changes in the gene expression of endoplasmic reticulum markers in CD14$^+$ and CD14$^-$ peripheral blood mononuclear cells

First, we compared the expression levels of ERS markers between the two subpopulations of PBMC. Compared with the CD14$^-$ cell population, CD14$^+$ cells expressed higher mRNA levels of *ATF4*, *HSPA5* and *DNAJC3*, while both cell populations expressed the levels of *EDEM1* and *XBP1* to the same degree (Fig. 5(a)). The expression of *ATF3* was restricted to CD14$^+$ cells. In response to the HFM challenge, PBMC did not alter the expression levels of *HSPA5*, *ATF4*, *EDEM1*, *XBP1* (spliced *v.* total) and *DNAJC3* in either the placebo or UDCA treatment (Fig. 5(b)–(f)). Nevertheless, the HFM challenge led to a significant increase in the mRNA levels of *ATF3* in CD14$^+$ cells in both placebo and UDCA treatments (Fig. 5(g)). The relative change in *ATF3* expression induced by the test meal correlated with that in *IL-8* expression (R 0·745, P=0·017), but did not correlate with the change in the expression of the other cytokines. In addition, baseline mRNA levels of *DNAJC3*, *EDEM1*, *ATF4*, *XBP1s* and *HSPA5* correlated with those of *RANTES* (all correlations reached $R>0.7$, $P<0.03$; Fig. 5(h)).

Discussion

The aims of the present study were to (1) examine a potential association between inflammatory and ERS responses to a HFM in two subpopulations of PBMC representing cells of innate and adaptive immunity and (2) assess the potential of UDCA, a chemical chaperone, to modify or prevent these responses. Postprandial responses to the test meal were studied in healthy lean male subjects to model the situation that precedes and could contribute to the development of obesity and the metabolic syndrome.

First, we documented the effects of the test meal, which was selected as a typical example of a Western 'fast food' type of diet, on postprandial plasma changes in major metabolites. The evolution of NEFA plasma concentration followed a known pattern in response to a single mixed meal, i.e. an immediate sharp decrease in NEFA levels due to the antilipolytic action of insulin, followed by increased NEFA levels dependent on the spillover fatty acids from chylomicron TAG[14]. In contrast, glucose levels remained unaltered in response to the HFM challenge, as described previously[15–18], even though some published studies[19–21] have shown peak glucose levels after a 30 to 60 min period following a mixed meal challenge. The observed blunted hyperglycaemic response could be caused by significant absolute and relative

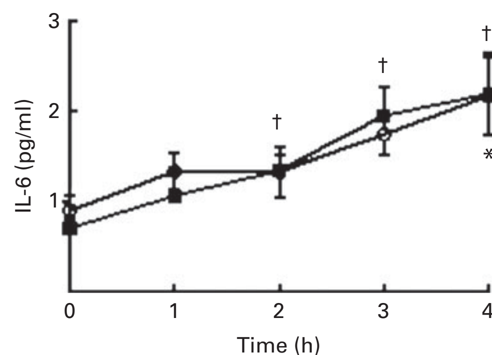

Fig. 3. Evolution of plasma levels of IL-6 following a high-fat meal challenge. Values are means, with their standard errors represented by vertical bars. * Mean value was significantly different from that of baseline levels in the placebo (-○-) treatment ($P<0.05$). † Mean value was significantly different from that of baseline levels in the ursodeoxycholic acid (-■-) treatment ($P<0.05$).

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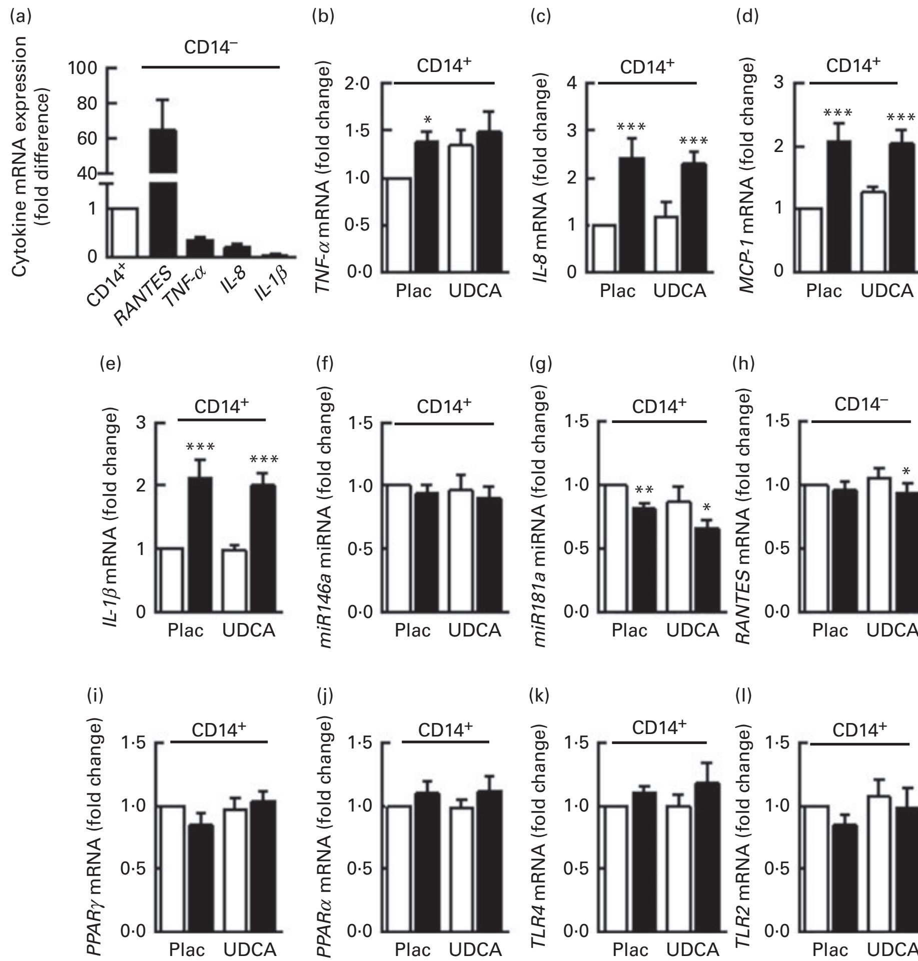

Fig. 4. Effect of the test meal on gene expression in CD14$^+$ and CD14$^-$ peripheral blood mononuclear cells (PBMC). (a) Comparison of mRNA expression levels of selected inflammatory cytokines between the CD14$^+$ and CD14$^-$ cells. Quantitative RT-PCR (qRT-PCR) analysis of cytokines (b–e, h) and miRNA (f, g) implicated in the regulation of inflammatory pathways in PBMC collected before and 4 h after a high-fat meal (HFM, ■) challenge. (i–l) qRT-PCR analysis of genes potentially activated by NEFA in CD14$^+$ cells collected before and 4 h after the HFM challenge. Values are means, with their standard errors represented by vertical bars. Mean value was significantly different from that of baseline (□) levels: * $P<0·05$, ** $P<0·01$, *** $P<0·001$. *RANTES*, regulated on activation, normal T-cell expressed and secreted; Plac, placebo; UDCA, ursodeoxycholic acid; *MCP1*, monocyte chemoattractant protein 1; miRNA, microRNA; TLR, Toll-like receptor.

amounts of fat and proteins in the test meal that have been shown to reduce postprandial glucose metabolism probably due to delayed gastric emptying[17,22]. Thus, the complexity of the meal, despite its high absolute (not relative) carbohydrate content, may lead to the paradoxical suppression of postprandial glucose plasma concentration.

In accordance with previous studies[4,23], postprandial leucocytosis was observed in the present study. In line with the results by Hansen *et al.*[4], the test meal used in the present study increased the absolute numbers of granulocytes in the blood. These fast changes observed in granulocyte numbers are probably caused by the release of cells from the marginal pool (cells residing in the slow-flowing lining fluid of the vasculature)[24]. We have also observed an increase in the absolute counts of lymphocytes and monocytes in the blood. It should be noted that the increase in lymphocyte counts may be associated with the circadian rhythm[25,26]. Nevertheless, the meal used in the present study had higher total energy, carbohydrate and protein contents than meals used in the previously cited studies by van Oostrom *et al.*[25,26]. Thus, these metabolic variables may have a more important role in the observed activation of lymphocytes and monocytes than in the circadian rhythm.

Postprandial inflammation was previously characterised by the increased circulating levels of several inflammatory cytokines[5]. We confirmed the postprandial elevation of IL-6 levels. Postprandial increases in plasma IL-6 levels were reported by others[27,28]. As mRNA levels of *IL-6* were barely detectable in CD14$^+$ or CD14$^-$ cells (data not shown), the elevation of IL-6 levels in the circulation was driven by other IL-6-producing cells or tissues.

Concerning HFM-induced changes in blood cells, we confirmed the finding by Gower *et al.*[29] showing increased CD11c expression on the surface of monocytes after ingestion of the HFM by healthy volunteers. CD11c is considered as an activation marker of monocytes because it enhances their adhesion to endothelial cells and the potential to migrate into target tissues. Importantly, high-fat diet feeding results

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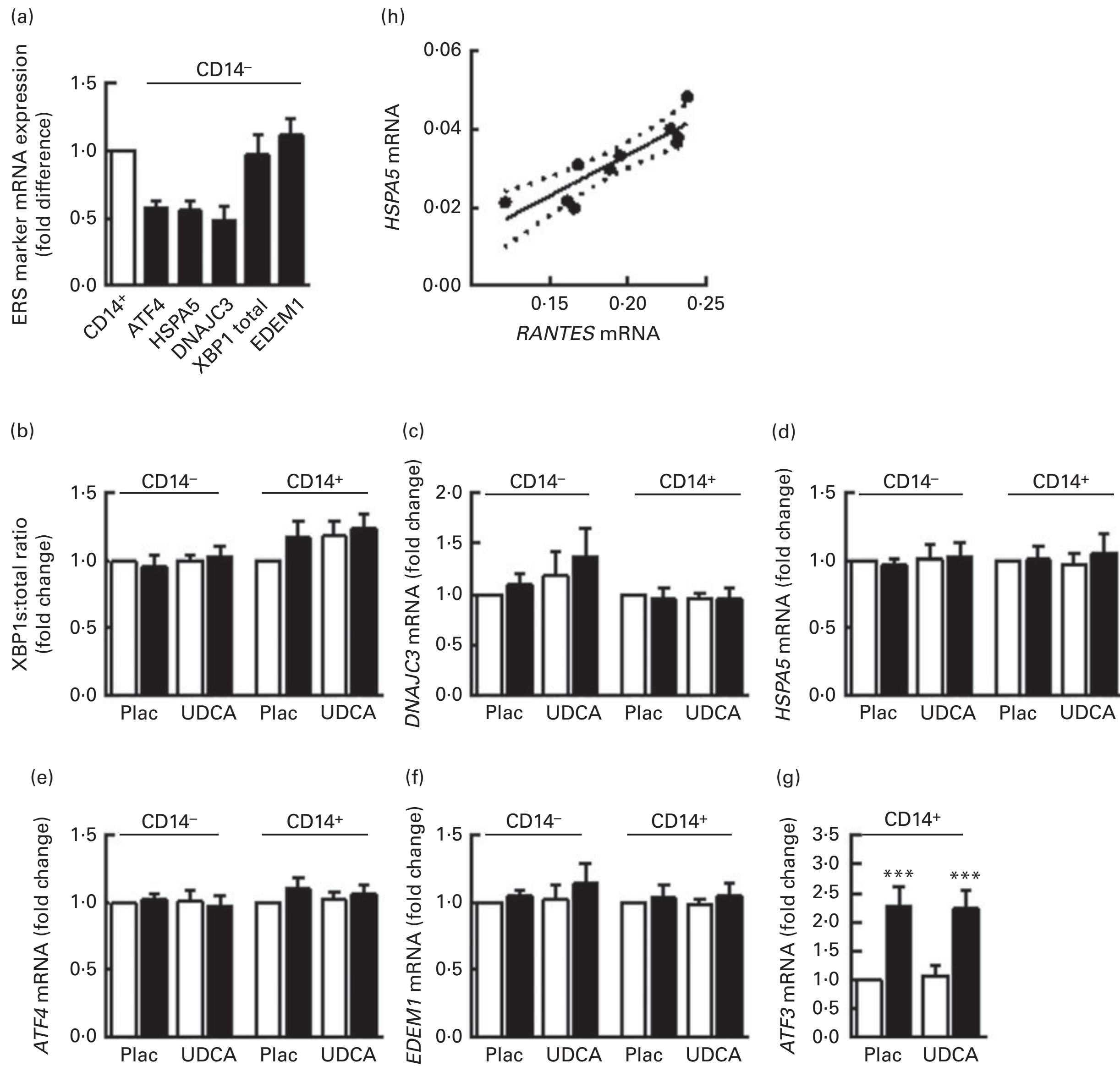

Fig. 5. Effect of the test meal on gene expression in $CD14^+$ and $CD14^-$ peripheral blood mononuclear cells (PBMC). (a) Comparison of mRNA expression levels of selected endoplasmic reticulum stress (ERS) markers between $CD14^+$ and $CD14^-$ cells. Quantitative RT-PCR analysis of ERS markers (b–g) in PBMC collected before and 4 h after a high-fat meal (■) challenge. Values are means, with their standard errors represented by vertical bars. *** Mean value was significantly different from that of baseline levels (□) ($P<0·001$). (h) Linear regression between mRNA levels of regulated on activation, normal T-cell expressed and secreted (*RANTES*) and *HSPA5* in $CD14^-$ cells at the fasting state (R^2 0·792, $P=0·0006$). ATF, activating transcription factor; HSPA5, heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa); DNAJC3, DnaJ (Hsp40) homolog, subfamily C, member 3; XBP1, X-box binding protein 1; EDEM1, ER degradation enhancer, mannosidase alpha-like 1; XBP1s, X-box binding protein 1 spliced; Plac, placebo; UDCA, ursodeoxycholic acid.

in the infiltration of $CD11c^+$ monocytes into adipose tissue in mice[30,31], and these monocytes/macrophages exhibit a pro-inflammatory M1 phenotype. CD11c expression has also been found to increase in blood monocytes of obese subjects and to positively correlate with homeostasis model assessment of the insulin resistance index[32]. Therefore, a single HFM may activate monocytes in a similar direction to long-term overfeeding or obesity. This observation is important with respect to the fact that a majority of European and North American people are in a postprandial state most of the day, and therefore they might be exposed to a potentially harmful condition long before they become obese.

We then focused on gene expression in $CD14^+$ (monocytes) and $CD14^-$ (lymphocytes) PBMC, i.e. cells that are intimately exposed to metabolite fluctuations, but upon activation also contribute to the development of inflammation in adipose tissue in response to overfeeding. Until now, changes in gene expression induced by a meal were analysed only in the whole-PBMC population[15,16,18]. Analysis of such a mixture of cell types could mask the possible differences between the postprandial responses of mononuclear cells of innate and adaptive immunity. Therefore, we opted to separate these two categories of PBMC before gene expression analysis. Remarkably, the expression levels of all the tested pro-inflammatory

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cytokines were enhanced after the HFM challenge in CD14$^+$ monocytes. Moreover, we also detected decreased expression levels of *miR181a*, a negative regulator of the TLR4/NF-κB pathway[33]. This decrease in *miR181a* expression following the HFM challenge could reinforce the synthesis of pro-inflammatory cytokines. The observed down-regulation of *miR181a* expression may be specific for inflammation induced postprandially, given that the expression level of another miRNA, *miR146a*[34], involved in the negative regulation of several pro-inflammatory cytokines remained unaltered. As noted already for CD11c expression, postprandial changes in the expression of *miR181a* and pro-inflammatory cytokines were similar to the changes in their expression associated with obesity[33,35].

Interestingly, we did not detect any changes in the expression of genes potentially activated by dietary fatty acids (*PPARγ* and *PPARα*) in CD14$^+$ cells, although these cells were postprandially exposed to high levels of lipids. Indeed, it was reported previously that a fatty meal induced an increase in the content of TAG in leucocytes[23], suggesting the uptake of NEFA by leucocytes. However, the present data suggest that several hours of exposure to dietary lipids are not sufficient to induce substantial expression changes in the regulators of lipid metabolism in CD14$^+$ cells. The mRNA levels of *TLR2* and *TLR4* were not altered in CD14$^+$ monocytes by the HFM challenge, even though we detected higher counts of CD14/TLR2- and CD14/TLR4-positive monocytes in the blood. Nevertheless, the level of fluorescence (mean fluorescence intensity) of TLR2 and TLR4 on the monocyte surface was not altered (data not shown), which confirms the results of mRNA analysis.

To determine whether postprandial inflammation could be triggered by enhanced ERS, we analysed ERS markers representing all three arms of unfolded protein response (UPR). The activation of inositol-requiring enzyme 1 (IRE) leads to XBP1 splicing, which in turn stimulates the expression of *DNAJC3* and *EDEM1* and partially *HSPA5*[36]. HSPA5 is primarily a target of the ATF6 UPR arm[37]. The activation of PRKR-like endoplasmic reticulum kinase (PERK) is associated with the up-regulation of *ATF4*, which in turn induces the expression of *ATF3*[38]. Following the HFM challenge, mRNA expression of a majority of ERS markers was not altered in PBMC. Thus, the classic activation of UPR does not seem to be the driver of the postprandial increase in the expression levels of inflammatory cytokines in CD14$^+$ monocytes. The absence of XBP1 splicing was rather surprising as it can be stimulated by insulin[39], and insulin levels were raised in response to the HFM challenge. It was also reported that higher activation of XBP1 is detectable in monocytes from obese subjects and subjects with the metabolic syndrome[9]. The finding that the HFM challenge does not initiate ERS in PBMC also explains the minor effects of UDCA on the expression levels of inflammatory cytokines. These minor effects could not be based on the low bioavailability of UDCA in the blood as pharmacokinetic data show that UDCA reaches a peak concentration at 60 min after oral administration and its half-life is more than 3 d. The ability of UDCA to modulate the expression levels of inflammatory cytokines observed in the case of *TNF-α* in CD14$^+$ cells and *RANTES* in CD14$^-$ cells is therefore probably unrelated to its chaperone-like property. Importantly, UDCA has been shown to have an immunosuppressive potential different from its effect on ERS due to its ability to activate glucocorticoid receptors and to inhibit the TLR signalling pathway[12]. UDCA may also influence blood cells through binding to the G-protein-coupled bile acid receptor TGR5[40]. However, these effects were tested mostly *in vitro* or in patients with primary biliary cirrhosis, and therefore they cannot be easily extrapolated to an *in vivo* condition in healthy men.

The only ERS marker whose expression was postprandially elevated was *ATF3*. It mostly acts as a transcriptional repressor and may thus be part of a counterbalance system in healthy individuals, protecting them from overactivation of pathways induced by stress[41–43]. Therefore, it could be envisioned that this counterbalance system is impaired in obese and/or diabetic subjects who suffer from intensified and prolonged postprandial inflammation[2,3,6]. Indeed, careful evaluation of differences in the expression levels of any putative regulator of postprandial inflammation between lean and obese subjects will be crucial for identification of mechanisms leading to pathological deregulation of this process in metabolically impaired individuals.

Interestingly, the change in *ATF3* expression induced by the HFM challenge correlated specifically with a change in *IL-8* expression. IL-8 has recently been described as a cytokine whose expression is altered specifically by the HFM challenge[15]. ATF3 is, however, activated not only by ERS but also by other various stresses[44], and the absence of the up-regulation of ATF4 in the analysed CD14$^+$ cells of ATF3 in the classic UPR pathway suggests that the up-regulation of *ATF3* is not associated with the activation of UPR. Moreover, the lack of an increase in blood glucose concentration after the HFM challenge suggests that hyperglycaemia-induced oxidative stress is not the trigger of *ATF3* expression.

Although we did not find a relationship between HFM-induced changes in the expression levels of inflammatory cytokines and most ERS markers, the striking co-regulation of mRNA expression levels of *RANTES* and all ERS markers opens the question as to whether the higher ERS levels in CD14$^-$ cells (probably CD8$^+$ T cells that are the main producers of RANTES[45]) could be a marker of their activation as was previously suggested for the conditions of acute pathogen infection[46].

In conclusion, we demonstrate the evidence that inflammation induced by the HFM challenge in CD14$^+$ monocytes was not accompanied by an activation of a majority of the investigated ERS markers (*HSPA5*, *XBP1*, *DNAJC3*, *EDEM1* and *ATF4*). Administration of UDCA before the consumption of the HFM did not alter the expression levels of these ERS markers. The putative molecular trigger of postprandial inflammation remains to be established.

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The authors' contributions were as follows: L. R. was the guarantor of the study and, as such, had full access to all the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis, and also designed the study, performed the data analysis and wrote the manuscript; J. K., E. C., M. K. and L. M. performed the experiments and data analysis and contributed to the discussion; E. C. organised the clinical part of the study; M. S. and V. S. contributed to the discussion and the writing of the manuscript. All authors read and approved the final manuscript.

The authors declare that there are no conflicts of interest.

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Metformin Does Not Inhibit Exercise-Induced Lipolysis in Adipose Tissue in Young Healthy Lean Men.

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Metformin Does Not Inhibit Exercise-Induced Lipolysis in Adipose Tissue in Young Healthy Lean Men

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Objective: Metformin was shown to exert an antilipolytic action in adipose tissue (AT) that might mediate beneficial effects on lipid metabolism in diabetic patients. However, during exercise, the inhibition of induced lipolysis in AT would limit the energy substrate supply for working muscle. Thus, the aim of this study was to investigate whether metformin exerts inhibitory effect on exercise-induced lipolysis in subcutaneous adipose tissue (SCAT) (Moro et al., 2007) in humans.

Approach: Ten healthy lean men underwent two exercise sessions consisting of 60 min of cycling on bicycle ergometer combined with (a) orally administered metformin and (b) metformin locally administered into SCAT. Microdialysis was used to assess lipolysis *in situ* in SCAT. Glycerol, metformin and lactate were measured in dialysate and plasma by enzyme colorimetric kits and capillary electrophoresis.

Results: Metformin levels increased continuously in plasma during 3 h after oral administration, and peaked after 3.5 h (peak concentration 4 μg/ml). Metformin was detected in dialysate outflowing from SCAT and showed a similar time-course as that in plasma with the peak concentration of 1.3 μg/ml. The lipolytic rate in SCAT (assessed as glycerol release) increased in response to exercise (4.3 ± 0.5-fold vs. basal; $p = 0.002$) and was not suppressed either by local or oral metformin administration. The lactate levels increased in plasma and in dialysate from SCAT after 30–60 min of exercise (3.6-fold vs. basal; $p = 0.015$; 2.75-fold vs. basal; $p = 0.002$, respectively). No effect of metformin on lactate levels in SCAT dialysate or in plasma during exercise was observed.

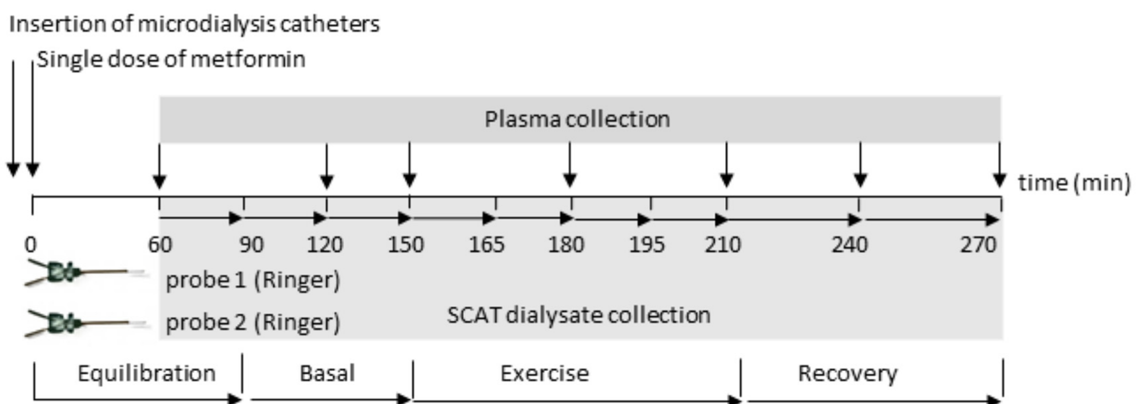
Conclusion: Metformin did not reduce the exercise-induced lipolysis in SCAT. This suggests that metformin administration does not interfere with the lipid mobilization and energy substrate provision during physical activity.

Keywords: metformin, microdialysis, lipolysis, human adipose tissue, exercise, lactate

INTRODUCTION

Metformin and physical activity are recommended as the first therapeutic agents to improve glycemic control in prediabetes and type 2 diabetes patients (Ortega et al., 2014). Metformin improves various features of systemic metabolism, such as insulin sensitivity, glycated hemoglobin levels, or plasma cholesterol in patients with type 2 diabetes (Robinson et al., 1998). It has been shown that metformin also reduces plasma concentration of free fatty acids (FFA) and it was hypothesized that this reduction was caused partially by inhibition of lipolysis in adipose tissue (AT) (Flechtner-Mors et al., 1999). In 3T3-L1 cells as well as in human primary adipocytes metformin inhibited catecholamine- and ANP-stimulated lipolysis through activation of AMP-activated protein kinase (AMPK) (Zhang et al., 2009; Bourron et al., 2010). Thus, it could be hypothesized that metformin might inhibit the exercise-induced lipolysis in AT as this is mediated – among other pathways – by catecholamines and ANP. As the inhibitory effect of metformin for both of these pathways was demonstrated either *in vitro* or *in situ* condition with pharmacological doses of metformin, we aimed in this study to verify whether metformin

Protocol 1) oral administration of metformin



Protocol 2) administration of metformin locally to AT

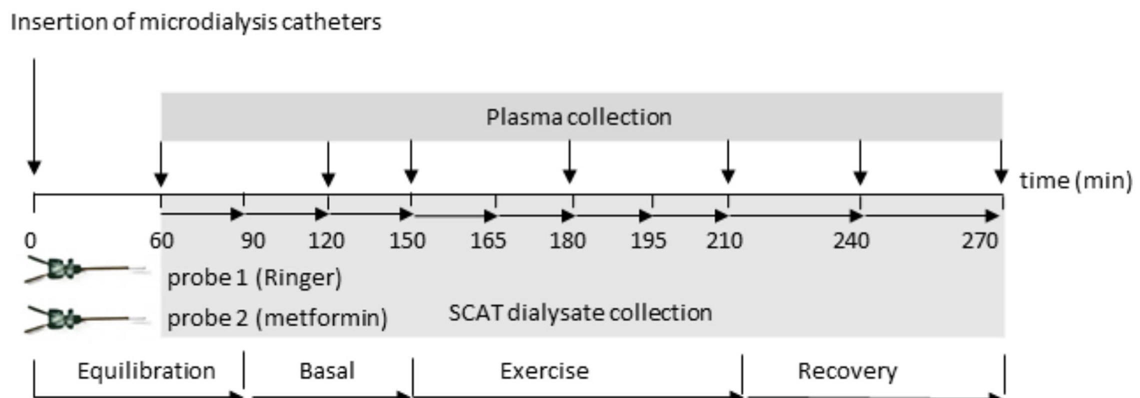


FIGURE 1 | Scheme of the experimental protocols: two probes were connected to a microperfusion pump and perfused at a flow rate 2.5 µl/min. In the Protocol 1: oral metformin administration, both probes were perfused by sterile Ringer's solution; after 90 min of equilibration, two basal dialysate fractions were collected in 30 min interval, then the subjects performed 60-min exercise bout at 50–55% V̇O2peak followed by 60 min of recovery period. Dialysate was collected in 15 min intervals during exercise and in 30 min intervals at the recovery period. Blood samples were taken in 30 or 60 min intervals. Glycerol and lactate was measured in dialysate collected from the first probe, metformin pharmacokinetics was analyzed in the dialysate from the second probe. In the Protocol 2: local metformin administration, the first probe was perfused by sterile Ringer's solution, the second probe was perfused by Ringer's solution with 1 mM metformin, (flow rate 2.5 µL/min). After 90 min of equilibration two basal dialysate fractions were collected in 30 min interval, then the subjects performed 60-min exercise bout at 50–55% V̇O2peak followed by 60 min of recovery period. Dialysate was collected in 15 min intervals during exercise and in 30 min intervals at the recovery period. Blood samples were taken in 30 or 60 min intervals. Glycerol and lactate were measured in dialysate collected from the both probes.

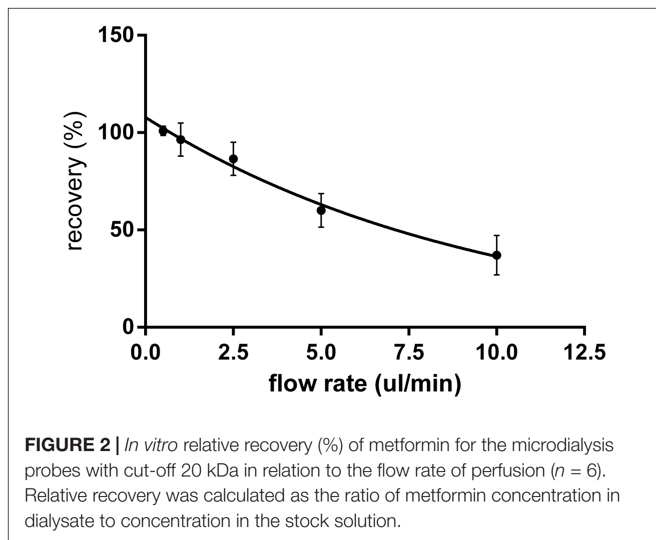


FIGURE 2 | *In vitro* relative recovery (%) of metformin for the microdialysis probes with cut-off 20 kDa in relation to the flow rate of perfusion (*n* = 6). Relative recovery was calculated as the ratio of metformin concentration in dialysate to concentration in the stock solution.

will inhibit lipolysis also in a physiological condition (i.e., exercise).

However, the inhibition of AT lipolysis during physical activity would affect fatty acids mobilization necessary to supply energy for working muscle and heart. In this respect, inhibition of lipolysis by metformin might be considered as undesirable effect of this drug, and thus metformin administration might influence the tolerance of an exercise of long duration. Although metformin was shown to enhance the insulin-sensitizing effect of exercise in insulin resistant patients (Ortega et al., 2014), or to induce higher oxygen consumption and lower lactate response during exercise (Johnson et al., 2008), the effects of metformin on exercise-induced lipolysis in AT has not been investigated, yet. Thus, in this study, we examined the effect of metformin on lipolysis in subcutaneous adipose tissue (SCAT) during an acute bout of exercise using microdialysis technique *in situ* in healthy lean men. It should be mentioned, that this study was carried out in young healthy lean men, as in obese or in diabetic patients the exercise–induced or catecholamine-stimulated lipolysis is impaired (Stich et al., 2000b; Verboven et al., 2016) and the possible antilipolytic effect of metformin could be masked. Thus, we aimed to demonstrate the antilipolytic effect of metformin during exercise in standard physiological condition. Moreover, we have assessed pharmacokinetics of metformin in the dialysate outflowing from SCAT after a single oral administration of metformin. The study could bring important evidence based recommendations to combination of physical activity and metformin therapy.

MATERIALS AND METHODS

Subjects

Ten lean men (age 27.2 ± 0.4 years; BMI 23.6 ± 0.5 kg/m^2) were recruited for the study. Exclusion criteria were: no weight change within 3 months before the study, smoking, hypertension, impaired fasting glucose, diabetes, hyperlipidemia, drug, or alcohol abuse. This study was carried out in accordance with the recommendations of The Ethical committee of the Third Faculty of Medicine (Charles University, Prague, Czechia). The protocol was approved by the Ethical committee of the Third Faculty of Medicine (Charles University, Czechia). All participants provided written informed consent prior the start of the study.

Experimental Protocol

The subjects underwent complete clinical investigation in the fasted state, including anthropometric and body composition measurement (Bodystat QuadScan 4000; Bodystat Ltd., British Isles). After that the catheter was placed in their antecubital vein, and two microdialysis probes (20 mm × 0.5 mm; 20 kDa cutoff; Carnegie Medicine, Stockholm, Sweden) were inserted percutaneously after epidermal anesthesia (1 mL of 1% Mesocain, Zentiva, Czechia) into the SCAT at a distance of 10 cm from the umbilicus. The probes were connected to microperfusion pump (Harvard Apparatus, France) and perfused at a flow rate 2.5 μl/min. Subjects performed two protocols consisting of a 60 min exercise bout with at least 1 week interval between them, in random order. The exercise intensity corresponding to 55–60% of coronary heart rate reserve calculated according to Karvonen formula (Karvonen et al., 1957) was chosen as it represents optimal intensity for significant increase of lipolysis in healthy lean men (Moro et al., 2007). In protocol 1, metformin (Teva Pharmaceuticals, Czechia) was administered orally (2250 mg/single dose) 2.5 h before the start of the exercise. Both microdialysis probes were perfused with Ringer solution. In the dialysate from one probe glycerol and lactate concentrations were measured. In the dialysate from the second probe metformin concentration was measured. In protocol 2, metformin was administered locally into SCAT by perfusion into one microdialysis probe (1 mM, 2.5 μl/min). The second probe was perfused by Ringer solution (control). Glycerol and lactate concentrations were measured in the dialysate collected from both probes. According to reported pharmacokinetics of metformin in plasma, the exercise bout was subjected at expected peak levels after one-dose metformin administration (FDA; 2.64 ± 0.82 h). The detailed protocol description is depicted in **Figure 1**.

In *in vitro* experiment, the recovery of metformin through microdialysis probe was assessed using zero-flow method as described before (Siklova-Vitkova et al., 2009).

Plasma Analyses

Plasma levels of glucose, insulin, and lipid parameters were determined using standard methods in certified laboratories. FFA in plasma, and glycerol in plasma and dialysate were measured using enzymatic colorimetric kits (Randox, Crumlin, United Kingdom). Lactate and metformin in dialysate and plasma were analyzed by capillary electrophoresis, as described before (Tuma, 2014).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, United States). The differences in the concentration of analytes (glycerol, lactate,

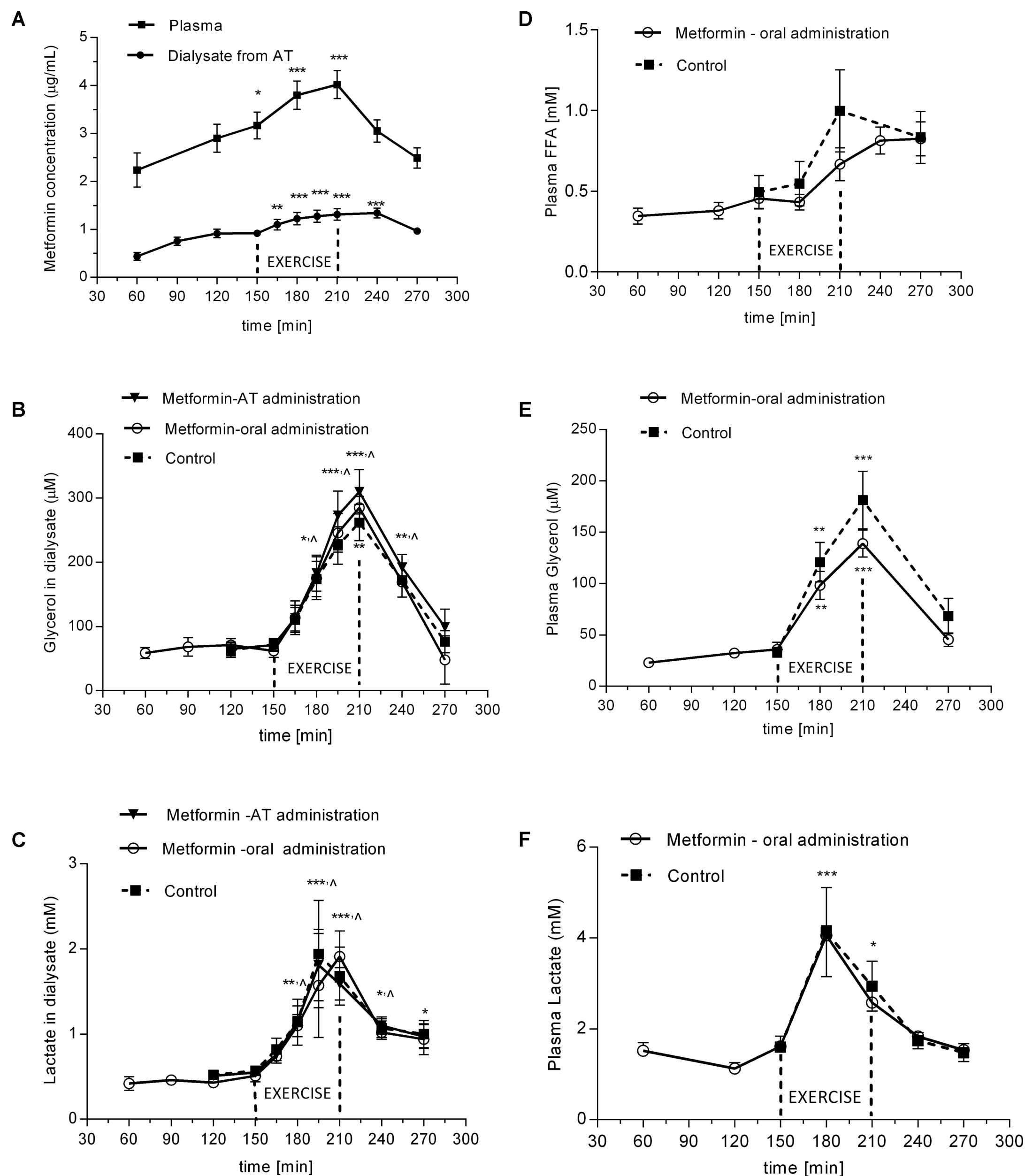


FIGURE 3 | Time-course of metformin, glycerol, FFA, and lactate during experimental exercise protocols with or without metformin treatment: **(A)** metformin levels in plasma and in dialysate outflowing from SCAT after oral administration of metformin; **(B)** glycerol levels in dialysate outflowing from SCAT; **(C)** lactate levels in dialysate outflowing from SCAT; **(D)** FFA levels in plasma; **(E)** glycerol levels in plasma; **(F)** lactate levels in plasma. Values are means ± SEM represented by vertical bars. Significant difference from of pre-exercise levels (baseline): *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (One-way ANOVA with Bonferroni *post hoc* analysis). ^significant values apply to all three curves. SCAT, subcutaneous adipose tissue; FFA, free fatty acids.

metformin, FFA) in time during experimental protocols were analyzed by One-way ANOVA with Bonferroni *post hoc* analysis. The difference in concentration of analytes (glycerol, lactate, metformin, FFA) in plasma or dialysate between the Protocol 1 and 2 or between the two probes in Protocol 1 was analyzed using Two-way ANOVA with Bonferroni *post hoc* analysis. Data are presented as mean ± SEM. Differences at the level of $p < 0.05$ were considered statistically significant.

RESULTS

In Vitro Relative Recovery of Metformin

Relative recovery (Bourron et al., 2010), defined as the ratio of "*metformin concentration in dialysate outflowing from the probe*" and "*metformin concentration in stock solution,*" was determined for different flow rates. The relationship between RR and the perfusion flow rates is presented in **Figure 2**. The flow rate of

TABLE 1 | Anthropometric and biochemical parameters of the subjects.

	healthy lean men (n = 10)
Age (years)	27.2 ± 1.4
Weight (kg)	79.1 ± 7.7
BMI (kg/m^2)	23.6 ± 1.6
Fat mass (%)	11.5 ± 3.6
Fat-free mass (%)	88.5 ± 3.6
Cholesterol (mmol/L)	3.7 ± 0.6
HDL-C (mmol/L)	1.3 ± 0.2
Triglycerides (mmol/L)	0.7 ± 0.1
Glucose (mmol/L)	5.0 ± 0.3
Insulin (mU/L)	3.7 ± 1.8
HOMA-IR	0.8 ± 0.4

Data are presented as mean ± SD; BMI, body mass index; HOMA-IR, homeostasis model assessment of the insulin resistance index; HDL-C, HDL cholesterol.

2.5 μl/min was chosen as the best compromise between the magnitudes of RR and flow rate for all further experiments.

Pharmacokinetics of Metformin in the Dialysate From Adipose Tissue After Single Oral Dose

After administration of a single dose of 2250 mg the metformin levels were steadily increasing in plasma as well as in SCAT dialysate for 3.5 h and declined thereafter (**Figure 3A**). Concentration of metformin in dialysate outflowing from SCAT ranged between 0.44 and 1.34 μg/mL, which represent approximately 30% of the concentration in plasma (**Figure 3A**).

Effect of Locally and Orally Administered Metformin on Lipolysis in SCAT During Exercise

Ten healthy lean men underwent two experimental protocols with acute bout of exercise and metformin administration: (1) orally (2250 mg/acute dose) 2.5 h before the start of exercise; (2) locally directly into AT by microdialysis probe (1 mM, 2.5 μl/min). Anthropometric and biochemical parameters of the subjects, who underwent the experimental protocol are shown in **Table 1**.

The glycerol concentration in the dialysate from SCAT increased during exercise (4.3 ± 0.5-fold vs. basal; p = 0.002). There was no difference in dialysate glycerol response after oral administration of metformin in Protocol 1 when compared with the glycerol response in the control (i.e., probe perfused with Ringer) in Protocol 2. In Protocol 2, there was no difference in the glycerol response in the probe with locally perfused metformin when compared with the response in control probe perfused with Ringer (**Figure 3B**). Thus, neither type of metformin administration had inhibitory effect on lipolysis induced in SCAT by a single bout of exercise. Similarly, the oral administration of metformin had no effect on circulating glycerol (p = 0.46) and fatty acid levels in plasma (p = 0.18) (**Figures 3D,E**) at the end of exercise in 210 min.

The lactate levels increased in plasma and in dialysate from SCAT after 30–60 min of exercise (3.6-fold vs. basal; p = 0.015; 2.75-fold vs. basal; p = 0.002; respectively) (**Figures 3C,F**). Metformin administration did not affect lactate concentration in dialysate (**Figure 3C**) or in plasma (**Figure 3F**).

DISCUSSION

In this study we have demonstrated that metformin is distributed in SCAT after single oral dose administration, and that metformin administration exerts no inhibitory effect on exercise-induced lipolysis in healthy lean men.

Pharmaco-kinetics measurements have shown that maximal metformin plasma concentrations are typically reached after 2.64 ± 0.82 h (FDA) after orally administered dose. Similarly, in the current study we observed maximal concentrations between 180 and 210 min after the oral administration in SCAT and in plasma. Mean dialysate concentrations were approximately 30% of those in plasma. According to *in vitro* metformin recovery (80%) the concentration in SCAT interstitium might be estimated as 0.55 – 1.68 μg/mL. Nevertheless, the recovery of the probe *in vivo* may differ from that *in vitro* as we have shown before (Siklova-Vitkova et al., 2009), thus these values have to be considered with caution. The limitation of the present study is that it was not possible to measure precise *in vivo* recovery (i.e., concentration) of metformin in SCAT in our subjects, as the "stable-in time" concentration of metformin in SCAT is a necessary condition for the zero-flow method.

It was shown previously, in human and rodent isolated adipocytes, that metformin treatment inhibited catecholamine and ANP-stimulated lipolysis through its action on AMPK (Zhang et al., 2009; Bourron et al., 2010). Metformin activation of AMPK led to the inhibition of phosphorylation of HSL preventing HSL translocation to lipid droplet (Bourron et al., 2010). However, the inhibition of stimulated lipolysis during exercise might be regarded as unfavorable process as fatty acids released by adipose tissue serve as energy substrate for other organs, especially for working muscle.

Importantly, no inhibition of exercise-stimulated lipolysis in SCAT by *in situ* or oral metformin administration was found in the present study. It should be emphasized that previously reported studies dealt with *in vitro* systems (isolated adipocytes) (Zhang et al., 2009; Bourron et al., 2010) and employed supraphysiological (2 mM) concentration of metformin, i.e., approximately 200 times higher than that found in adipose tissue in this study. *In vivo* inhibition of lipolysis in SCAT was shown only after local stimulation of the adrenergic pathway in one study (Flechtner-Mors et al., 1999). Our protocol employs physical activity, which represents physiological and more complex trigger of lipolysis. During local administration, we used high concentration of metformin in perfusate, similar as in published study (Flechtner-Mors et al., 1999). It was much higher than during orally given dose (2550 mg – submaximal recommended dose). Nevertheless, on both occasions no antilipolytic effect was detected in SCAT, which shows that the

lack of effect is independent on the local concentration of the metformin in AT.

We used the given exercise intensity as it represents an optimal model to increase lipolysis in SCAT in healthy lean men as shown by Moro et al. (2007). The similar intensity was used in our previous studies focused on lipolysis regulation in SCAT during exercise (Stich et al., 2000a,b).

It should be considered, when interpreting this study, that it was performed in young healthy lean men, while the study Bourron et al. (2010) was carried out in normal to moderately overweight women and the study of Flechtner-Mors et al. (1999) in severely obese women. The impact of metformin on exercise-induced lipolysis in diabetic patients would be warranted in future studies. Furthermore, taken into account that exercise-induced lipolysis is gender-specific (Arner et al., 1990; Moro et al., 2007), it may not be excluded that metformin effect during exercise would be gender-specific, too.

A wide range of regulatory and signaling pathways may play a role in the lipolysis regulation, such as cytokines/myokines IL-6, IL-15 (Ajuwon and Spurlock, 2004), insulin, or FGF21 (Hotta et al., 2009). Indeed, metformin may interfere with these pathways, as it is able to inhibit IL-6 and insulin signaling (Kisfalvi et al., 2009; Li et al., 2014), or FGF21 expression (Kim et al., 2013). Also the role of AMPK in exercise stimulated lipolysis remains controversial (Gaidhu and Ceddia, 2011). Thus, the detailed effects of metformin on AMPK and/or other signaling pathways during physiological conditions, such as physical activity, needs to be investigated in future studies.

It was reported that metformin administration may increase lactate production in diabetic subjects, which may lead to the development of lactate acidosis (Wills et al., 2010). The SCAT is one of the sources of lactate production (Jansson et al., 1990), therefore we analyzed its levels in dialysate and in plasma during exercise. The increase of lactate levels in plasma was observed after 30 min and in SCAT after 60 min of exercise, which suggests that the exercise work-loads applied in this study were close to the individual anaerobic thresholds. However, there was no effect of orally or locally administered metformin on evolution of lactate levels in plasma or in SCAT. This is in line with previous reports, which observed that metformin did not alter circulating lactate concentration during acute bout of exercise (Gudat et al., 1997). But it is difficult to generalize from previous studies as variable results were reported including increased (Hansen et al., 2015) or reduced lactate response to exercise after metformin administration (Johnson et al., 2008).

CONCLUSION

We have demonstrated that metformin is distributed in adipose tissue and it shows a similar time-course to plasma following single oral administration. Metformin did not inhibit physiologically increased lipolysis induced by a single bout of exercise in healthy lean men. This suggests that metformin does not play detrimental role in mobilization of lipid energy substrates during exercise.

AUTHOR CONTRIBUTIONS

EK performed the experiments, researched the data, and wrote the manuscript. PT performed the experiments, researched the data, and edited the manuscript. IdG performed the experiments. VŠ designed the study, and reviewed and edited the manuscript. MŠ designed the study, researched the data, and wrote the manuscript. EK is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Expression of lipolytic genes in adipose tissue is differentially regulated during multiple phases of dietary intervention in obese women.

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Expression of Lipolytic Genes in Adipose Tissue Is Differentially Regulated During Multiple Phases of Dietary Intervention in Obese Women

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Summary

The aim of this study was to investigate the time-course of the expression of key lipolysis-regulating genes in the subcutaneous adipose tissue (SCAT) during different phases of a 6-month dietary intervention. Fifteen obese women (BMI 34.7±1.0 $kg \cdot m^{-2}$) underwent a 6-month dietary intervention consisting of 1 month very low calorie diet (VLCD), followed by 2 months low calorie diet (LCD) and 3 months weight maintenance diet (WM). At each phase of the dietary intervention, a needle microbiopsy of the abdominal SCAT was obtained to evaluate mRNA expression of key lipolysis-regulating genes and a hyperinsulinemic euglycemic clamp (HEC) was performed. Dietary intervention induced a body weight reduction of 9.8 % and an improvement of insulin sensitivity as assessed by a HEC. Compared to pre-diet levels, mRNA levels of the adrenergic β_2-receptor in SCAT were higher at the end of VLCD and not different at the end of LCD and WM. In contrast, the expression of the adrenergic α_2-receptor was lower at the end of VLCD and LCD compared to the pre-diet levels and did not differ at WM. Adipose triglyceride lipase and hormone-sensitive lipase levels were lower than the pre-diet levels at the end of LCD only, while phosphodiesterase-3B and the insulin receptor levels did not change throughout the dietary intervention. The results suggest that the regulation pattern of the genes that are involved in the control of lipolysis is different at the respective phases of the dietary intervention and depends on the duration of the diet and the status of energy balance.

Key words

Insulin • Adipocytes • Hypocaloric diet

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Introduction

Traditional weight-reducing hypocaloric diets as well as bariatric surgery interventions lead to an improvement in metabolic status of obese subjects already during the early stages of the intervention. It has been suggested that the early improvement in metabolic status is associated with calorie restriction *per se* – eventually combined with a mild initial weight loss – while the later beneficial metabolic effects are linked to sustained weight loss only (Hensrud 2001, Gumbs *et al.* 2005). Adipose tissue lipolysis has been suggested as one

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of the candidate mechanisms underlying the obesity-related metabolic disturbances, namely insulin resistance (Reynisdottir *et al.* 1994a,b). Moreover, impaired catecholamine-mediated lipolysis was demonstrated in first-degree relatives of obese subjects (Hellström *et al.* 1996a) which suggests an association between blunted catecholamine-induced lipolysis and excessive accumulation of body fat.

Therefore, in the present study we aimed to follow the evolution of whole-body insulin sensitivity and the evolution of expression of genes involved in lipolysis regulation in adipose tissue (AT) during a dietary intervention (DI) which consisted of multiple phases. The first phase of the diet consisted of one month severe calorie restriction using a very low calorie diet (VLCD) of 3000 kJ/d. The subsequent phase consisted of 2 months low calorie diet (LCD) with a calorie intake of 5000-6000 kJ, followed by a 3 month weight maintenance diet (WM). This model provides an opportunity to study the "lipolysis – metabolic status – energy status" relationship.

Previous studies that reported mRNA data of genes involved in lipolysis regulation in AT during dietary interventions yielded various results, possibly related to differences in duration and severity of the diet as well as to the characteristics of the study populations. For example, the mRNA and protein levels of hormone-sensitive lipase (HSL) in subcutaneous AT (SCAT) of obese subjects were reduced after 8-12 weeks of diet (Reynisdottir *et al.* 1995, Jocken *et al.* 2007), while no differences or an increase in the HSL protein level were reported for dietary interventions with a shorter duration (4 weeks of VLCD) (Hellström *et al.* 1996b, Stich *et al.* 1997). Capel *et al.* (2009) showed that the regulation of adipocyte and macrophage genes during a multi-phase diet was dependent on the phase of the diet, namely the metabolism-related genes predominantly expressed in adipocytes were downregulated during energy restriction and upregulated during weight stabilization, while the genes related to immune functions that were predominantly expressed in macrophages were not changed or upregulated during energy restriction and downregulated during weight stabilization. Similar results were demonstrated regarding the regulation of lipolysis *in situ*: in obese women it was shown that the responsiveness of SCAT to adrenergic regulation of lipolysis varied during the respective phases of the diet (Koppo *et al.* 2012).

In the present study, we explored the lipolysis regulation at the transcriptional level and measured mRNA expression of key lipolytic genes in needle biopsy-derived samples of SCAT in a group of obese women that followed the above mentioned multiple-phase dietary intervention. In each individual, the SCAT samples were obtained at baseline and at the end of each dietary phase. At each phase of the diet, the whole-body insulin sensititivity was measured using a hyperinsulinemic euglycemic clamp. We demonstrated that the lipolysis is regulated at the transcriptional level during a multiple-phase dietary intervention and that the regulation during the early phase of the diet differs from that during the later periods. No straightforward relationship between the evolution of gene expression and that of insulin sensitivity was found.

Methods

Subjects

Fifteen obese premenopausal women were recruited for the study (Table 1). They all had a stable weight during the 3 months that preceded the study. Exclusion criteria were hypertension, diabetes, hyperlipidemia treated by drugs, drug-treated obesity, drug or alcohol abuse, pregnancy or participation in other studies. All subjects were fully informed about the aim and the protocol of the study and signed an informed consent approved by the Ethics committee of the Third Faculty of Medicine of the Charles University (Prague, Czech Republic).

Dietary intervention

During the first month of the dietary intervention program, subjects received a 3000 kJ/d VLCD (liquid formula diet; Redita, Promil, Czech Republic). During the next 2 months, a LCD was designed to provide 2500 kJ/d less than the individually estimated energy requirement based on an initial resting metabolic rate multiplied by 1.3 (the coefficient of correction for physical activity level; Toubro *et al.* 1996.). The final 3 months consisted of a WM diet during which subjects kept a stable weight. Subjects consulted a dietician once a week during the first 3 months and once a month during the WM phase. They provided a written 3-days dietary record at each consultation. Subjects were instructed not to change the level of their leisure time physical activity during the intervention. This was monitored by self-reported questionnaires.

Table 1. Subjects' characteristics.

	Pre-diet	VLCD	LCD	WM
Weight (kg)	94.9 ± 3.4	87.9 ± 3.2 [a]	85.2 ± 3.2 [a,b]	85.6 ± 3.2 [a,b]
BMI (kg/m²)	34.7 ± 1.0	32.1 ± 1.0 [a]	31.1 ± 1.0 [a,b]	31.3 ± 1.0 [a,b]
Fat mass (kg)	38.2 ± 2.7	32.7 ± 2.4 [a]	29.7 ± 2.0 [a,b]	30.6 ± 2.4 [a]
Fat free mass (kg)	56.8 ± 1.4	55.2 ± 1.5 [a]	55.6 ± 1.7	55.1 ± 1.5 [a]
Waist circumference (cm)	102.9 ± 3.1	97.2 ± 3.3 [a]	95.4 ± 3.3 [a,b]	95.1 ± 3.2 [a,b]
Cholesterol (mmol/l)	4.8 ± 0.1	3.9 ± 0.2 [a]	4.3 ± 0.2 [a,b]	4.6 ± 0.2 [b]
HDL cholesterol (mmol/l)	1.10 ± 0.09	0.94 ± 0.07 [a]	1.12 ± 0.08 [b]	1.22 ± 0.06 [a,b,c]
Triglycerides (mmol/l)	1.57 ± 0.21	1.12 ± 0.10 [a]	1.11 ± 0.10 [a]	1.07 ± 0.06 [a]
Glycerol (μmol/l)	222 ± 21	157 ± 10 [a]	140 ± 17 [a]	142 ± 12 [a]
NEFAs (μmol/l)	653 ± 45	684 ± 46	553 ± 50 [a,b]	515 ± 53 [a,b]
Fasting glucose (mmol/l)	5.6 ± 0.1	5.4 ± 0.2	5.5 ± 0.1	5.3 ± 0.2
Fasting insulin (mU/l)	12.4 ± 2.0	6.7 ± 0.6 [a]	5.6 ± 0.6 [a]	7.3 ± 0.7 [a,c]
GDR (mg/kg.min)	3.1 ± 0.4	3.9 ± 0.5 [a]	4.3 ± 0.5 [a]	4.3 ± 0.4 [a]

BMI: body mass index, HDL: high-density lipoprotein, NEFAs: non-esterified fatty acids and GDR: glucose disposal rate normalized to kg of body weight. [a] = significantly different from pre-diet values ($P<0.05$), [b] = significantly different from VLCD ($P<0.05$), [c] = significantly different from LCD ($P<0.05$).

Experimental protocol

On four occasions (i.e. before the start of the dietary intervention (baseline) and at the end of the VLCD, LCD and WM phase, respectively) subjects entered the laboratory at 8.00 a.m. after an overnight fast. A complete clinical investigation was performed, anthropometric parameters were measured and body composition was determined with multifrequency bioimpedance (Bodystat QuadScan 4000; Bodystat Ltd., Isle of Man, British Isles). Subsequently, subjects were placed in a semi-recumbent position. Venous blood samples were taken from an antecubital vein in order to determine the blood parameters related to lipid and carbohydrate metabolism. A needle microbiopsy of the abdominal SCAT (14-20 cm lateral to the umbilicus) was performed under local anesthesia (1 % Xylocaine; AstraZeneca PLC, London, UK) as previously described (Klimcakova *et al.* 2006) to evaluate mRNA of key genes that are involved in SCAT lipolysis regulation. The samples were frozen immediately in liquid nitrogen and stored at –80 °C until analysis. Finally, a 3-h hyperinsulinemic euglycemic clamp (HEC) was performed to determine the glucose disposal rate.

Hyperinsulinemic euglycemic clamp

HEC was performed according to the DeFronzo method (DeFronzo *et al.* 1979). Priming plus continuous infusion of crystalline human insulin (Actrapid Human; Novo, Bagsvaerd, Denmark), 40 mU/m² body area · min, was given for 180 min. Euglycemia (the fasting blood glucose concentration) was maintained by a variable 20 % glucose infusion. The infusion rate was determined by measuring arterialized plasma glucose every 5 min (Beckman Glucose Analyzer; Beckman Coulter Inc., Fullerton, CA, USA).

Quantification of mRNA analysis

Total RNA was extracted from the adipose tissue biopsy samples with an RNeasy Mini kit (Qiagen, Valencia, CA) and reverse transcribed using random hexamers (Promega Corp., Madison, WI) and SuperScript II Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA). Real-time quantitative PCR was performed with TaqMan probe-based gene expression assays (Applied Biosystems, Foster City, CA). An 18S ribosomal RNA was used as control to normalize gene expression (Ribosomal RNA Control TaqMan Assay kit; Applied Biosystems). Each sample was performed in duplicate and 10 ng cDNA was used as a template for real-time PCR. The relative expression was calculated using the ΔΔCt method (Livak and Schmittgen 2001).

Drugs and biochemical determinations

Plasma glucose was determined with a glucose hexokinase technique (Konelab 60i, Labsystems CLD, Konelab, Finland). Plasma insulin concentrations were

Table 2. Expression of lipolytic genes before and during the respective phases of the multi-dietary intervention.

	Pre-diet	VLCD	LCD	WM
ADRA2A	0.079 ± 0.008	0.058 ± 0.008 [a]	0.050 ± 0.005 [a]	0.065 ± 0.008
ADRB2	0.17 ± 0.01	0.21 ± 0.02 [a]	0.19 ± 0.01	0.18 ± 0.01
PDE3B	0.94 ± 0.06	0.82 ± 0.07	0.87 ± 0.06	0.87 ± 0.05
INSR	0.57 ± 0.04	0.63 ± 0.04	0.62 ± 0.04	0.63 ± 0.03
HSL	4.71 ± 0.29	4.53 ± 0.29	4.11 ± 0.31 [a]	4.31 ± 0.20
ATGL	4.15 ± 0.23	3.61 ± 0.24 *	3.67 ± 0.20 [a]	3.99 ± 0.16

ADRA2A: adrenergic α_{2A}-receptor, ADRB2: adrenergic β_2-receptor, PDE3B: phosphodiesterase-3B, INSR: insulin receptor, HSL: hormone-sensitive lipase and ATGL: adipose triglyceride lipase. Values shown in the table represent mRNA levels ($x \cdot 10^4$). [a] = significantly different from pre-diet values (P<0.05), * p=0.076.

measured using a chemiluminescent immunometric assay (Immulite 2000 Insulin, DPC Czech sro, Brno, Czech Republic). Other laboratory analyses (lipids) were performed using standard biochemical laboratory methods.

Statistical analysis

All values are presented as means ± SEM. Statistical evaluation of the data was performed using ANOVA for repeated measures with an LSD *post-hoc* test (SPSS statistical software, version 19, SPSS Inc, Chicago). Correlations between mRNA gene expression and the diet-induced change in body fat mass were examined with the Pearson correlation coefficient. Significance was determined at P<0.05.

Results

Anthropometric and plasma parameters of subjects during the different phases of the diet

Pre-diet values and values measured at different phases during the weight reduction program (i.e. at the end of VLCD, LCD and WM) are reported in Table 1. Subjects' body weight progressively decreased during VLCD and LCD and stabilized during WM. The loss in body weight was mainly due to a decrease in fat mass. Plasma triglycerides were significantly lower at the end of VLCD, LCD and WM compared to the pre-diet values. Similarly, baseline insulin levels were significantly lower at the end of VLCD, LCD and WM (Table 1). The glucose disposal rate (GDR) normalized to body weight significantly increased at the end of VLCD and the increase was maintained at the end of LCD and WM (Table 1).

Effect of the dietary intervention on gene expression

SCAT mRNA levels of key genes involved in adipose tissue lipolysis regulation, i.e. the adrenergic β_2-receptor (ADRB2), adrenergic α_{2A}-receptor (ADRA2A), adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), phosphodiesterase-3B (PDE3B) and insulin receptor (INSR), were quantified (Table 2). ADRB2 expression significantly increased at the end of VLCD when compared with the pre-diet baseline level and then, at the end of LCD and WM, returned to the pre-diet baseline levels. The expression of ADRA2A was significantly lower at the end of VLCD and LCD compared to the pre-diet baseline and increased back towards the pre-diet levels during WM. Expression of PDE3B and INSR did not significantly change throughout the intervention. The diet-induced adaptation of HSL and ATGL was similar: mRNA levels of the two lipases were significantly lower at the end of LCD compared with pre-diet levels and returned to the pre-diet levels at the end of WM. At the end of VLCD, ATGL levels were lower than the pre-diet levels but the difference was not significant (P=0.076).

Associations between the diet-induced changes of clinical variables and mRNA levels

No significant correlations between the pre-diet baseline mRNA expression of the genes explored and the diet-induced change in body fat mass during VLCD were observed: r = 0.076 for ADRA2A, r = –0.159 for ADRB2, r = –0.001 for PDE3B, r = –0.147 for INSR, r = –0.266 for HSL and r = –0.174 for ATGL. Similarly, no significant correlations between the pre-diet baseline mRNA expression of the genes and the diet-induced change in body fat mass during the entire 6-month intervention were observed: r = –0.018 for ADRA2A,

r = 0.064 for ADRB2, r = 0.052 for PDE3B, r = –0.060 for INSR, r = –0.056 for HSL and r = –0.004 for ATGL. When attention was paid to the predictive power of the diet-induced changes of mRNA levels during the early phase of the intervention (VLCD), no significant correlations were found between VLCD-induced changes of mRNA levels of the respective genes and the diet-induced reduction of body fat mass either during VLCD (r = 0.278 for ADRA2A, r = 0.192 for ADRB2, r = 0.240 for PDE3B, r = 0.073 for INSR, r = 0.143 for HSL and r = 0.147 for ATGL) or during the entire 6-month intervention (r = –0.085 for ADRA2A, r = –0.101 for ADRB2, r = –0.128 for PDE3B, r = –0.304 for INSR, r = –0.254 for HSL and r = –0.200 for ATGL).

Discussion

Results of the present study demonstrate that multi-phase dietary intervention modifies the expression of key genes involved in SCAT lipolysis control and that the status of energy balance during the dietary intervention is an important determinant of the lipolysis genes regulation. These findings are in agreement with previous research in humans (Vitkova *et al.* 2007, Capel *et al.* 2009, Kováčiková *et al.* 2010) and in experimental models (Kosteli *et al.* 2010) that showed that the diet-induced changes of adipose tissue expression of genes related to metabolism or immune function are strongly dependent on the phase of the diet.

One of the regulators of lipolysis that received substantial attention in dietary intervention studies are catecholamines as they are believed to be one of the major players that regulate lipid mobilization in adipose tissue. Most of the studies that examined the lipolytic response to β-adrenergic stimulation after 3-12 weeks VLCD reported an increased lipolytic response *in situ* (Barbe *et al.* 1997, Flechtner-Mors *et al.* 1999, Sengenes *et al.* 2002) and *in vitro* (Stich *et al.* 1997, Wahrenberg *et al.* 1999). After 15-weeks LCD, the maximal lipolytic response to isoprenaline (a β-adrenoreceptor agonist) was unchanged when compared to the pre-diet level, while the β-adrenoreceptor lipolytic sensitivity was increased in isolated adipocytes (Mauriège *et al.* 1999). The α_2-adrenoceptor mediated anti-lipolytic action in isolated adipocytes was found to be unchanged after 4-12 weeks VLCD (Kather *et al.* 1985, Stich *et al.* 1997, Wahrenberg *et al.* 1999) and to be reduced after 12-15 weeks LCD *in vitro* (Mauriège *et al.* 1999) and *in situ* (Stich *et al.* 2002). Results of the above mentioned single-phase dietary intervention studies were in agreement with a recent study in which the SCAT lipolysis was examined during a long-term multi-phase diet (Koppo *et al.* 2012). The authors reported that the adrenaline-induced SCAT lipolysis *in vivo* was enhanced during the calorie-restricted phases of the diet and returned back to the pre-diet levels during the WM phase. The diet-induced changes in SCAT lipolysis were rather related to a decreased α_2-adrenergic regulation than to an increased β-adrenergic regulation (Koppo *et al.* 2012). Changes in postreceptor signaling pathways as well as changes in density of the adrenoceptors should be considered as possible mechanisms that underlie the changes in lipolytic responsiveness during the dietary intervention. It was found that in SCAT of obese women the β-adrenoreceptor density increased after VLCD compared with the pre-diet levels, while the α_2-adrenoreceptor density remained unchanged (Mauriège *et al.* 1999, Wahrenberg *et al.* 1999). On the transcriptional level, it was reported that, after 12 weeks of LCD, SCAT ADRA2A mRNA levels decreased and ADRB2 mRNA levels did not change compared with the pre-diet condition in obese women (Stich *et al.* 2002). The present study investigated the changes in adrenoceptor gene expression during different phases of the dietary intervention in the same individual. The lipolytic ADRB2 gene was up-regulated after VLCD, and not after LCD and WM, while the anti-lipolytic ADRA2A gene was down-regulated after VLCD and LCD. After WM, the adrenoceptor mRNA levels returned back to the pre-diet values. Although there are many intermediate steps between the adrenergic regulation of SCAT lipolysis *in vivo* and the transcriptional level of the adrenoceptor gene expression, these findings parallel the results of our previous study mentioned above (Koppo *et al.* 2012) in which adrenergic regulation of lipolysis was investigated *in situ* using microdialysis. Indeed, in that study, the α_2-mediated antilipolytic action was reduced *in situ* at VLCD and LCD, similarly to the evolution of ADRA2A mRNA levels. In contrast, the β-adrenoceptor-mediated stimulation of lipolysis *in situ* increased at VLCD and went back to pre-diet levels, similarly to ADRB2 mRNA levels.

Another important lipolysis-regulating pathway is that of insulin. In the present study mRNA levels of PDE3B and INSR were explored. The anti-lipolytic effect of insulin is mediated through activation of PDE3B; more specifically, insulin activates phosphatidylinositol 3-kinase serine kinase, the kinase that activates PDE3B,

through binding to INSR (Rondinone *et al.* 2000). The activation of PDE3B promotes cAMP degradation, which in turn inactivates protein kinase A (PKA) that reduces phosphorylation of lipases and perilipins. In the present study, no changes in mRNA levels of PDE3B and INSR were observed throughout the dietary intervention. It cannot be excluded that a change in the activation of PDE3B might have occurred. Nevertheless, *in situ* data also suggest a lack of change in insulin sensitivity of adipose tissue during this dietary intervention: it was reported that, during a hyperinsulinemic euglycemic clamp performed at different time points of a multi-phase diet, the decrease in SCAT dialysate glycerol was similar at the respective phases of the diet. This demonstrated that the anti-lipolytic effect of insulin did not change throughout the dietary intervention during which subjects' whole-body insulin sensitivity improved (Koppo *et al.* 2012).

In the present study we also focused on the two lipases that are involved in the lipolytic pathway. ATGL and HSL are cytosol lipases that hydrolyze intracellular triacylglycerols (TAGs) into glycerol and NEFA. HSL has been considered to be the enzyme that catalyzes the rate-limiting step of adipose tissue lipolysis. However, more recently it was reported that the overexpression of ATGL may increase basal lipolysis while its inhibition suppresses stimulated lipolysis (Bezaire *et al.* 2009). Regarding the factors that influence HSL gene expression, it was reported that glucose deprivation (Raclot *et al.* 1998) as well as a sustained activation of the PKA pathway (Plée-Gautier *et al.* 1996) down-regulated the HSL gene. Taking into account that during the calorie-restricted phases of the diet, glucose intake is limited and the stimulated SCAT lipolysis (and thus the activation of the PKA pathway) is enhanced, it can be hypothesized that the HSL gene would be down-regulated during the calorie-restricted phases. Indeed, mRNA levels of HSL were significantly lower at the end of LCD (12th week of diet) compared with pre-diet levels, but no changes were observed at the end of VLCD (4th week of diet). In accordance with these findings, the HSL mRNA and protein levels in SCAT of obese subjects were reduced after 8-12 weeks of different types of diet (Reynisdottir *et al.* 1995, Jocken *et al.* 2007), while no differences or an increase in HSL protein level were reported for dietary interventions with a shorter duration (4 weeks of VLCD) (Hellström *et al.* 1996b, Stich *et al.* 1997). The duration of the calorie restriction seems to play an important role in the regulation of the lipase expression. Similarly to HSL, ATGL mRNA and protein level were shown to be decreased in SCAT of obese subjects that had followed a 10 week low-fat or medium-fat diet (Jocken *et al.* 2007). Also in the present study, mRNA levels of ATGL were significantly lower at the end of LCD compared with pre-diet levels. Interestingly, the pattern of evolution of ATGL mRNA levels during this dietary intervention paralleled the evolution of basal lipolysis *in situ* observed in our previous microdialysis study (Koppo *et al.* 2012). In contrast, no such parallel was found for the pattern of evolution of mRNA HSL: it was different when compared with either basal lipolysis or adrenaline-stimulated lipolysis *in situ*.

As mentioned above, results of this study and of previous ones show that the diet-induced changes of adipose tissue gene expression are dependent on the phase of the diet. Capel *et al.* (2009) showed that the regulation of adipocyte and macrophage genes during a multi-phase diet was dependent on the phase of the diet. The metabolism-related genes (predominantly expressed in adipocytes) were downregulated during energy restriction and upregulated during weight stabilization, while the genes related to immune functions (predominantly expressed in macrophages) were not changed or upregulated during energy restriction and downregulated during weight stabilization (Capel *et al.* 2009). Furthermore, recent studies showed the dependence of lipolysis on the phase of the hypocaloric diet in mice (Kosteli *et al.* 2010) and in humans (Koppo *et al.* 2012). In the former study (Kosteli *et al.* 2010) it was suggested that lipolysis drives the adipose tissue macrophage accumulation since the peak in macrophage number coincided with the peak in adipose tissue lipolysis. Furthermore, after an extended period of weight loss, adipose tissue lipolysis decreased, as did the macrophage content. It would be tempting to hypothesize that the same regulatory effect could occur in humans. It was reported that markers of macrophage infiltration in human SCAT show a similar time-pattern, i.e. a slight or no increase during one month of VLCD and a decrease under the pre-diet levels at the end of a 6 month diet (Capel *et al.* 2009, Kováčiková *et al.* 2010). This would suggest that the biological role of SCAT lipolysis goes beyond serving as an energy substrate and that it may play a role in the regulation of macrophage infiltration and the inflammation of the adipose tissue.

Similarly to the studies of Mutch *et al.* (2007, 2011) it might be hypothesized that the baseline levels of the lipolytic genes or their diet-induced changes are

predictors of the diet-induced loss in fat mass. We tested specifically whether subjects with higher "responsiveness", i.e. larger modifications of mRNA levels of the respective genes during the initial phase of the diet (i.e. VLCD), would be those with higher diet-induced fat loss either during VLCD or during the entire 6-month intervention. This association was not confirmed in the present study. Similarly, no associations between the pre-diet mRNA expression of lipolysis-regulating genes and the diet-induced loss in fat mass during either VLCD or entire intervention were observed.

In conclusion, this study demonstrates that the dietary intervention associated with an improvement in insulin sensitivity modifies the mRNA expression of key genes that are involved in SCAT lipolysis regulation. The pattern of this diet-induced modification varies during the course of the diet and is related to the status of energy balance. No straightforward relationship between the evolution of expression of the respective genes and that of insulin sensitivity was found.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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PŘÍLOHA 6

Comparison of Early (2 Days) and Later (28 Days) Response of Adipose Tissue to Very Low-Calorie Diet in Obese Women.

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Comparison of Early (2 Days) and Later (28 Days) Response of Adipose Tissue to Very Low-Calorie Diet in Obese Women

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Context: Beneficial metabolic effects of calorie restriction found in the early stage of hypocalorie diets may be caused by the modulation of metabolic and endocrine function of adipose tissue.

Objective: The objective of the study was to compare metabolic and inflammation-related characteristics of sc adipose tissue (SAAT) in the early (2 d) and later (28 d) phase of a very low calorie diet (VLCD).

Design, Setting, Intervention, and Patients: Seventeen moderately obese premenopausal women followed an 800 kcal/d VLCD for 28 days. Anthropometric measurements, blood sampling, and a biopsy of SAAT were performed before the diet and after 2 and 28 days of the VLCD.

Main Outcome Measure(s): mRNA expression of 50 genes related to lipid metabolism, inflammation, and fibrosis were analyzed in SAAT. Secretion of adipokines was determined in SAAT explants and adipokines, fibroblast growth factor 21 (FGF21) and C-reactive protein were measured in plasma.

Results: In the early phase of the VLCD, the expression of lipolytic genes was increased, whereas the expression of lipogenic genes was significantly suppressed. The inflammatory markers in SAAT remained unchanged. At the later phase, expression of genes involved in lipogenesis and β-oxidation was markedly suppressed, whereas the expression of inflammatory markers was increased. The changes of lipogenic genes after 28 days of the VLCD correlated with FGF21 changes.

Conclusion: The early and later phases of a VLCD differ with respect to metabolic and inflammatory responses in SAAT. The expression changes in SAAT in the early phase of the VLCD could not explain the effect of short calorie restriction on the improvement of insulin sensitivity. An interplay of SAAT with liver function during VLCD mediated by FGF21 might be suggested. ***(J Clin Endocrinol Metab* 101: 5021–5029, 2016)**

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Abbreviations: AT, adipose tissue; ATGL, adipose triglyceride lipase; CRP, C-reactive protein; DGAT2, diacylglycerol acyltransferase 2; FFA, free fatty acid; FGF21, fibroblast growth factor 21; GLUT4, glucose transporter 4; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; HSL, hormone-sensitive lipase; IS, insulin sensitivity; LOX, lysyl oxidase; MCP-1, monocyte chemoattractant protein-1; PPAR, peroxisome proliferator-activated receptor; qPCR, quantitative PCR; SAAT, sc AT; SREBP1c, sterol regulatory element-binding protein-1c; VLCD, very low-calorie diet.

Very low-calorie diets (VLCDs) are often prescribed in obesity treatment to achieve rapid weight loss. Generally, this type of dietary intervention consists of 500–800 kcal/d during 1–2 months and leads to an improvement in metabolic profile (such as plasma total cholesterol, triglycerides, high-density lipoprotein [HDL]cholesterol, insulin, etc) and insulin sensitivity (IS) (1). A study that compared the effects of VLCD and bariatric surgery has shown that VLCD drives almost the same improvements of IS, β-cell function, and lipid parameters as bariatric surgery when the same reduction of body weight and fat mass is achieved (2). However, some of the positive effects of severe calorie restriction are observed already before the loss of fat mass is accomplished. Whole-body/hepatic insulin resistance measured by the homeostasis model assessment for insulin resistance (HOMA-IR) or quantitative insulin sensitivity check index improved as soon as after 2 days of a VLCD (3, 4). Similarly the beneficial effects of bariatric surgery on carbohydrate metabolism were observed within several days after bariatric operation in type 2 diabetic patients, before significant weight loss has occurred (5). Mechanism of the beneficial metabolic effects of the calorie restriction per se are not well understood. It might be hypothesized that modifications of immune and metabolic characteristics of adipose tissue (AT) might occur and play a role in this process despite the fact that there is no change in AT mass. Whereas the response of inflammation-related cytokines during a 1-month VLCD was investigated in a number of studies (reviewed in references 1 and 6), the effects of a very short calorie restriction was studied rarely (3). Similarly, it was shown that the expression of metabolism-related genes in AT was reduced after 1 month of a VLCD (7), but the response to a shorter calorie restriction (eg, several days) was not thoroughly studied.

Therefore, in this longitudinal study, we compared the effects of 2 days and 28 days of a VLCD on metabolic and inflammation-related indices in sc adipose tissue (SAAT) and their possible relationship with systemic inflammatory and metabolic status in moderately obese women. We investigated the expression of the respective genes in SAAT as well as the secretion of cytokines in SAAT explants.

According to recent studies, the diet-induced metabolic changes might be partially controlled by fibroblast growth factor 21 (FGF21). FGF21 is released by the liver and stimulates fatty acid oxidation and ketogenesis (8). Recently it was shown, in mice and in cell cultures, that FGF21 may affect adipose tissue metabolic pathways (lipogenesis, lipolysis) (9, 10). Thus, FGF21 levels and their association with changes in SAAT were also investigated.

Subjects and Methods

Subjects, dietary protocol, and clinical examination

Seventeen metabolically healthy obese women (aged 35 ± 7 y, mean body mass index 32.6 ± 3.6 kg/m^{-2}) were recruited for the study. All subjects were drug free and healthy, as determined by medical history and laboratory findings. All patients had a stable weight for at least 3 months prior to inclusion. All subjects underwent a VLCD intervention program, during which they received 800 kcal/d (liquid formula diet; Redita; representing an intake of 52 g of protein, 118 g of carbohydrates, and 12.9 g of fat per day). Patients consulted a dietitian once a week. The evaluation of physical activity was performed before the start of the study by the International Physical Activity Questionnaire, and the subjects were recommended not to change their habitual activity during the study. The design of the study is shown in Figure 1. Clinical investigation was carried out at day 0 (baseline), day 2 (2 d of the VLCD), and day 28 of the VLCD. During these investigations the subjects were examined at 8:00 AM after overnight fasting. Body weight and waist and hip circumferences were measured, and body composition was assessed by bioimpedance (QuadScan 4000; Bodystat). The needle biopsy of SAAT and the samples of peripheral blood were taken. The study was approved by Ethical Committee of the Third Faculty of Medicine, Charles University in Prague, and all subjects gave their informed consent before the start of the study.

Secretion of cytokines from SAAT explants

AT samples obtained by biopsy were processed as previously described (11). Briefly, AT was washed in saline and separated to several aliquots. Two aliquots (200–500 mg) were snap frozen in liquid nitrogen for subsequent gene expression analysis. Another aliquot of approximately 400 mg was cut into small pieces and the explants were incubated in 4 mL of Krebs/Ringer phosphate buffer (pH 7.4) supplemented with 20 g/L of BSA and 1 g/L of glucose at 37°C in a shaking water bath with air as the gas phase. After 4 hours of incubation, the conditioned medium was collected, the cellular debris was removed by centrifugation, and the cell-free supernatant was stored at −80°C until analysis.

Analysis of plasma and SAAT conditioned media

Plasma samples were prepared from uncoagulated peripheral blood by centrifugation. Plasma glucose was determined with a glucose oxidase technique (Beckman Instruments). Plasma insulin was measured using an Immunotech insulin immunoradiometric assay kit (Immunotech).

β-Hydroxybutyrate, glycerol, and free fatty acids (FFAs) were analyzed by enzymatic colorimetric assays (Randox Laboratories Ltd). A multiplex immunoassay at the MagPIX or Luminex 200 was used to analyze the following: 1) plasma cytokines IL-6, IL-8, IL-10, and TNF-α (high sensitivity human cytokine Milliplex panel; Merck-Millipore); 2) cytokines in conditioned media, IL-6, IL-8, IL-10, monocyte chemoattractant protein-1 (MCP-1), TNF-α, and leptin (human adipocyte kit; Merck-Millipore). The circulating levels of MCP-1, leptin, FGF21, and C-reactive protein (CRP) were quantified by ELISA kits (eBioscience and R&D Systems).

Gene expression analysis

Total RNA was isolated from 200- to 500-mg aliquots of AT using an RNeasy lipid tissue RNA minikit (QIAGEN). The RNA

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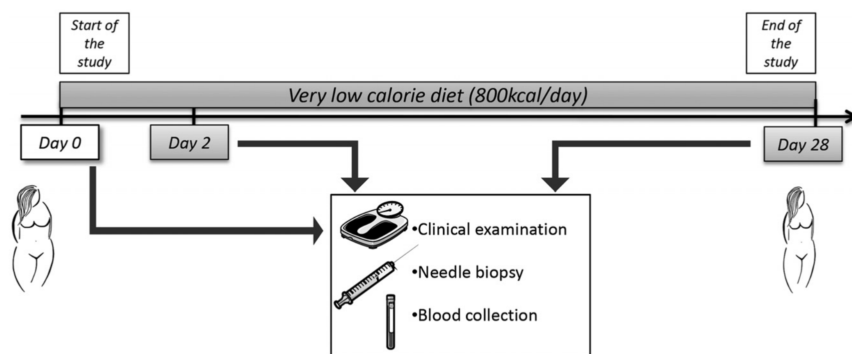

Figure 1. The design of the study. Seventeen obese premenopausal women were included in the study. Clinical examination, needle biopsy of SAAT, and blood collection were performed at indicated days (d 0, before the start of VLCD; d 2, after 2 d of VLCD; d 28, at the end of 1 mo of VLCD). Samples from needle biopsies and plasma were used for further analysis of inflammatory and metabolic characteristics during the intervention.

concentration was measured using Nanodrop1000 (Thermo Fisher Scientific). To remove genomic DNA, deoxyribonuclease I (Invitrogen) treatment was applied. Six hundred nanograms of total RNA were reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems). For microfluidics, 4 ng of cDNA was preamplified within 16 cycles to improve detection of target genes during subsequent real-time quantitative PCR (qPCR; TaqMan Pre Amp master mix kit; Applied Biosystems). For the preamplification, 20× TaqMan gene expression assays of all target genes (the list of genes in Supplemental Table 1) were pooled together and diluted with water to the final concentration 0.2× for each probe. The real-time qPCR was performed in duplicates on Biomark real-time qPCR system using 96 × 96 array (Fluidigm). In addition, the mRNA expression of *CD36*, peroxisome proliferator-activated receptor (*PPAR*)-γ, adipose triglyceride lipase (*ATGL*), hormone-sensitive lipase (*HSL*), diacylglycerol acyltransferase (*DGAT*)-2, *IL-6*, *IL-8*, *IL-10*, *MCP-1*, *TNF-α*, and *leptin* was quantified by qPCR without preamplification on an ABI PRISM 7500 (Applied Biosystems). Data were normalized to reference gene *PPIA*, which proved to be superior over two other measured reference genes, *PUM1* and *GUSB* (not shown). The method of $2^{(-\delta Ct)}$ was calculated for statistical analysis, and the final values for the figures were expressed as fold change related to mean basal value.

Statistical analysis

Data are presented as means ± SEM. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc). The comparison of the anthropometric, biochemical, and other variables before the diet, at day 2, and at day 28 of the VLCD was done using a one-way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing. Correlations of fold changes of all parameters during 2 days and 28 days of the VLCD (value at d 2/baseline) or (value at d 28/baseline) were assessed by Pearson's correlation. The difference of $P < .05$ was considered as statistically significant.

Results

Effect of dietary intervention on clinical and laboratory characteristics of obese women

Anthropometric and biochemical parameters of subjects before and during two stages of the diet are presented in Table 1. At day 2 of the VLCD, the subjects´ body weight was reduced by 1.4%, whereas fat mass was not changed. After 28 days of the VLCD, a body weight loss of 9.2% was achieved, associated with a decrease of 16.5% of fat mass (kilograms).

Plasma glucose levels and triglycerides were not changed significantly during any phase of the intervention. FFA and β-hydroxybutyrate levels were elevated after both 2 days and 28 days of the VLCD. Total cholesterol and HDL cholesterol levels decreased after 28 days of the VLCD. Insulin and insulin resistance estimated by HOMA-IR decreased after 2 days of the diet by 13.7% and 16.4%, whereas at day 28 these variables decreased by 40% and 44%, respectively (Table 1).

Effect of dietary intervention on mRNA gene expression in sc abdominal adipose

Genes regulated after 2 days of VLCD

Among all the genes analyzed, those that were down-regulated at day 2 were as follows: three lipogenic genes (*SCD1*, *FASN*, and *ELOVL6*), the lipogenic transcription factor sterol regulatory element-binding protein-1c (*SREBP1c*), and fibrotic enzyme-lysyl oxidase (*LOX*).

Up-regulated genes at day 2 were as follows: lipases (*ATGL*, *HSL*), ATGL coactivator *CGI58*, transcription factor *PPARγ*, and fatty acid translocase *CD36*. mRNA expression of glucose transporter *GLUT1* had a tendency to increase after 2 days of the VLCD ($P = .09$).

All other genes were not changed at day 2 of the VLCD; explicitly we would mention the genes involved in β-oxidation (*CPT1β*, *ACOX*, *ACADM*, *PPARα*, *PCG1*) (Figure 2C), the genes involved in fibrosis (*TLR4*, collagens, *TGFβ1*, *MMP9*) (Figure 2E) and in inflammation (macrophage markers and cytokines) (Figure 2F), and several genes related to lipogenesis and lipolysis.

Genes regulated after 28 days of VLCD

Genes down-regulated after 28 days of VLCD were as follows: all lipogenic enzymes (*SCD1*, *FAS*, *DGAT2*, *ACLY*, *ACACA*, *ELOVL6*) and two lipogenic transcription factors (*SREBP1c*, carbohydrate-responsive element binding protein) (Figure 2A); lipolytic genes and regulators, *MGL*, *G0S2* (an inhibitor of ATGL), *PLIN1* (an inhibitor of HSL), and *DGAT1* (an enzyme involved in the reesterification of fatty acids and in lipogenesis) (Figure 2B); genes associated with β-oxidation of fatty acids, *CPT1*, *ACOX1*, and *ACAD* (Figure 2C); insulin-stimu-

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Table 1. Clinical Characteristics of 17 Obese Women Before the Diet and After 2 Days and After 28 Days of VLCD

	Before Diet	2 Days of VLCD	28 Days of VLCD
Weight, kg	93.5 ± 2.3	92.1 ± 2.3[a]	84.9 ± 2.3[a]
BMI, kg/m^{-2}	32.7 ± 0.9	32.2 ± 0.9[a]	29.7 ± 0.8[a]
Fat mass, kg	38.9 ± 2.0	38.6 ± 1.9	32.3 ± 1.6[a]
Fat-free mass, %	59.6 ± 1.2	59.3 ± 1.2	63.5 ± 1.1[b]
Waist circumference, cm	99.9 ± 1.7	98.6 ± 1.6[b]	92.6 ± 1.6[a]
Glucose, mmol/L^{-1}	5.0 ± 0.1	5.1 ± 0.1	4.9 ± 0.2
Insulin, mU/L^{-1}	10.2 ± 1.0	8.0 ± 0.6[c]	5.3 ± 0.6[a]
FFAs, μmol/L^{-1}	820 ± 56	1156 ± 119[c]	1115 ± 70[b]
Glycerol, μmol/L^{-1}	124 ± 16	147 ± 14	113 ± 10
Triglycerides, mmol/L^{-1}	1.12 ± 0.12	1.04 ± 0.07	0.93 ± 0.10
HDL, mmol/L^{-1}	1.25 ± 0.05	1.21 ± 0.06	1.06 ± 0.04[a]
Total cholesterol, mmol/L^{-1}	4.82 ± 0.20	4.86 ± 0.18	3.87 ± 0.13[a]
β-Hydroxybutyrate, mmol/L^{-1}	114 ± 19	379 ± 63[b]	603 ± 124[b]
HOMA-IR	2.3 ± 0.2	1.8 ± 0.1[c]	1.3 ± 0.2[a]
QUICKI	0.342 ± 0.005	0.354 ± 0.004[c]	0.386 ± 0.009[a]

Abbreviations: BMI, body mass index; QUICKI, quantitative insulin sensitivity check index. Data are presented as mean ± SEM.

[a] $P < .001$ when compared with baseline (before the diet) values (one way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing)

[b] $P < .01$ when compared with baseline (before the diet) values (one way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing).

[c] $P < .05$ when compared with baseline (before the diet) values (one way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing).

lated glucose transporter 4 (*GLUT4*) (Figure 2D); leptin (Figure 2D); and fibrotic enzyme *LOX* (Figure 2E).

Genes up-regulated after 28 days of VLCD were some macrophage markers, namely *CD163*, *MSR1*, *IRF5*, and *CCR2*. The increase of mRNA expression of other markers (*ACP5*, *FCGBP*, *ITGAX*) and cytokines (*IL-8*, *MCP-1*, *TNFα*, *IL-6*, and *IL-10*) was observed, but it did not reach statistical significance (Figure 2F).

Expression of all other genes was not significantly modified at the end of 28 days of the dietary intervention, specifically the following: genes involved in lipolysis (*HSL*, *ATGL*, *CGI58*, *CD36*) (Figure 2B), transcription factors *PPAR*. *PPARγ*, and *PPARγ* coactivactor 1α, insulin receptor substrate 1, and genes involved in fibrosis (Figure 2E).

Correlations of the diet-induced changes in gene expression in SAAT and in metabolic parameters during VLCD intervention

Two-day changes

Changes of circulating FFAs and glycerol after 2 days of the VLCD correlated with changes in mRNA expression of *CGI58* (Supplemental Figure 1). The changes of glycerol after 2 days of the VLCD correlated with expression changes of *HSL* and *ATGL* (Supplemental Figure 1, data not shown). The changes of the HOMA-IR after 2 days of the VLCD tended to correlate with changes of *HSL* and *ATGL* expression (data not shown).

Twenty-eight-day changes

The changes in mRNA expression of *leptin* and *LOX* correlated positively with the changes of mRNA expression of lipolytic and lipogenic enzymes, β-oxidation, and insulin receptor substrate 1 during 28 days of the VLCD (Supplemental Table 2). The changes of the HOMA-IR correlated with changes of plasma levels and the secretion of leptin (Supplemental Figure 1 and Supplemental Table 2). Changes of cholesterol, insulin, and triglycerides correlated with the changes of expression of several lipolytic and lipogenic genes (ie, *HSL*, *SCD1*, *FASN*, *DGAT2*) (Supplemental Figure 1).

Changes of plasma FGF21 correlated positively with corresponding changes of β-hydroxybutyrate ($r = 0.537$, $P = .048$) and negatively with corresponding fold changes of *ATGL*, *DGAT2*, *PPAR*, and *GLUT4* expression (Supplemental Figure 1, data not shown).

Secretion of cytokines/adipokines in sc abdominal adipose tissue during VLCD

In vitro secretion of cytokines IL-6 and MCP-1 from SAAT explants did not change after 2 days of VLCD but increased after 28 days of VLCD. Secretion of IL-8 and TNFα was not significantly changed after 2 days and tended to be increased after 28 days of the VLCD ($P = .053$ and $P = .066$, respectively). Secretion of IL-10 was not significantly changed in either VLCD phase (Figure 3). Secretion of leptin was not changed after 2 days of the

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Figure 2. mRNA expression of genes in SAAT of obese women before the diet (white), 2 days after the VLCD (gray), and 28 days after the VLCD (black). Lipogenesis (A), lipolysis (B), β-oxidation (C), insulin/glucose receptors and leptin (D), fibrosis (E), and inflammation (F) are shown. Data are presented as fold change ± SEM, related to mean basal (before diet) gene expression, normalized to *PPIA* expression (n = 16). *, $P < .05$, **, $P < .01$, ***, $P < .001$, compared with prediet levels or values at 2 days of the VLCD (one way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing).

VLCD but decreased significantly after 28 days of the VLCD (Figure 3).

Plasma levels of cytokines, CRP, FGF21, and leptin during VLCD

Plasma concentration of cytokines IL-6, and MCP1 increased after 2 days of the VLCD and returned to baseline after 28 days of the VLCD (Figure 4). Similarly, CRP concentration had a tendency to increase after 2 days of the VLCD ($P = .07$) and decreased under the baseline values after 28 days of the VLCD (Figure 4). IL-8, IL-10, TNFα, and cortisol levels were not significantly changed after either 2 or 28 days of the VLCD. The average plasma leptin levels did not change significantly after 2 days' VLCD (decrease by 21%, $P = .21$); however, the response showed a high interindividual variability. After 28 days of the VLCD, the decrease of leptin was markedly pronounced (by 49%, $P < .001$). FGF21 was not changed

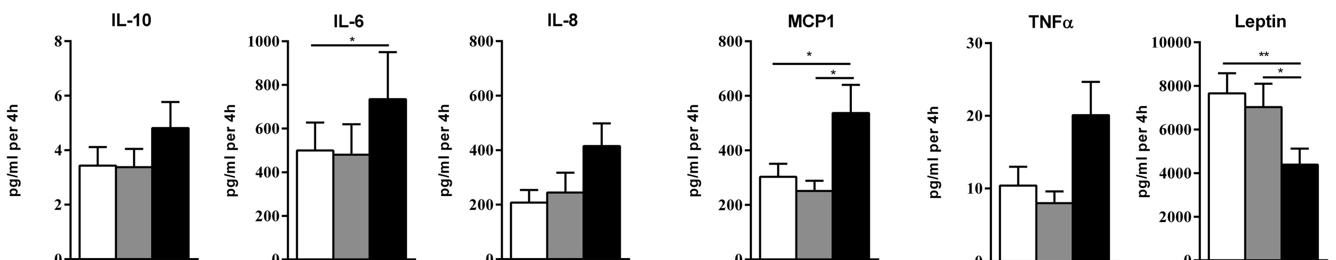

Figure 3. Secretion of cytokines/adipokines from SAAT of obese women before the diet (white), 2 days after the VLCD (gray), and 28 days after the VLCD (black). Data are presented as a concentration of secreted protein (picograms per milliliter per 4 h) ± SEM (n = 16). *, $P < .05$, **, $P < .01$, ***, $P < .001$, compared with prediet levels or values at 2 days of the VLCD (one way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing).

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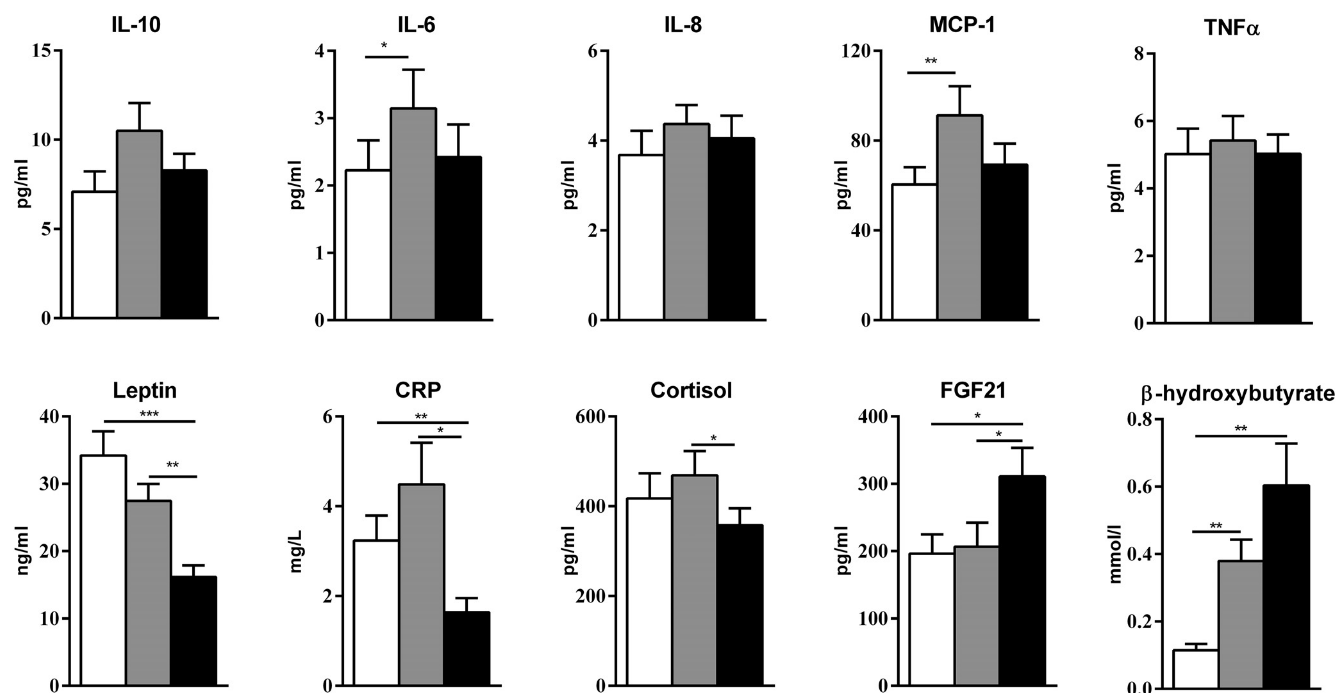

Figure 4. Plasma levels of cytokines and hormones in obese women before the diet (white), 2 days after the VLCD (gray), and 28 days after the VLCD (black). Data are presented as mean ± SEM (n = 17). *, $P < .05$, **, $P < .01$, ***, $P < .001$, compared with prediet values or values at 2 days of the VLCD (one way ANOVA with repeated measures, followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple testing).

after 2 days of the VLCD and was elevated after 28 days of the VLCD (Figure 4).

Discussion

The aim of this study was to elucidate metabolic and immune effects of the calorie restriction per se, when compared with the restriction accompanied with the body fat mass loss. Therefore, in moderately obese women, whole-body and AT characteristics were compared between the early (2 d) and later phase (28 d) of VLCD. The main finding is that the responses of metabolic and inflammation-related characteristics in SAAT and in plasma differed markedly between the two phases of the diet.

The 2 days of calorie restriction modified genes involved in lipolysis and lipogenesis in SAAT, whereas the inflammatory status of AT was not changed. The increased levels of FFAs and β-hydroxybutyrate in plasma reflect enhanced AT lipolysis and hepatic ketogenesis, similarly as seen in fasting (12). Increased expression of major AT lipases in SAAT, *HSL*, *ATGL*, with its cofactor *CGI58*, could contribute to a higher release of FFAs from AT to plasma, as reviewed by Nielsen and Moller (13). Indeed, in this study the increase in plasma FFAs and glycerol after 2 days of the VLCD correlated with the changes in expression of *CGI58*, and the changes in plasma glycerol correlated with SAAT lipases, *ATGL* and *HSL*. In addition, increased mRNA expression of *CD36*, a fatty acid transporter in SAAT, was found at the early phase of the VLCD, and this expression correlated with increased FFA plasma levels. This is in line with published studies showing that the expression of *CD36* in AT increased in response to the acute increase of FFA plasma levels induced by lipid infusion (14) and also in response to chronic elevation of FFA plasma levels in obese subjects with metabolic syndrome (15). On the other hand, in the study of Hames et al (16), CD36 was shown to facilitate the FFA uptake by AT when the levels of FFAs in plasma are low, ie, after consumption of a carbohydrate-containing meal. Thus, the higher expression of *CD36* observed after 2 days of calorie restriction could provide AT better FFA absorption capacities for the anticipated refeeding phase. Also, it was suggested that FFAs released from adipocyte by lipolysis are immediately taken back by CD36 to secure cycling of FFA, and this mechanism may prevent excessive release of FFA under condition of stimulated lipolysis (17). The up-regulation of *CD36* in our study is likely driven by *PPAR* activation. Indeed, *PPARγ* mRNA expression was elevated during the early VLCD phase, and this change correlated with the changes of mRNA expression of *CD36* and with changes of lipolytic genes (*HSL*, *ATGL*) in this study. Up-regulated *PPARγ* expression and activity might be associated by the enhanced availability of lipolysis-de-

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rived fatty acids (PPARγ ligands), as suggested by Haemmerle et al (18, 19).

In contrast to the effect of 2 days of calorie restriction on lipolytic genes, the expression of lipogenic enzymes in SAAT was reduced. This reduction was likely linked to a down-regulation of *SREBP1c*, one of the lipogenic transcription factors, which is regulated by insulin (20). The improvement in IS during the early phase of the VLCD was not associated with the metabolic gene expression in SAAT, except a borderline correlation with several lipolytic genes (ie, *HSL*, *ATGL*). Thus, changes occurring in AT in response to short calorie restriction do not appear to play major role in the metabolic improvement induced by 2 days of a VLCD. Therefore, the potential role of other insulin -sensitive organs in the diet-induced metabolic changes must be taken into account. Lara-Castro et al (21) demonstrated that the decrease of the intramyocellular lipids after 6 days of a VLCD was closely related to insulin resistance. In contrast to that, Jazet et al (22) found no changes in the markers of insulin signaling and glucose transport in skeletal muscle after 2 days of a VLCD, but they found a diet-induced decrease of endogenous glucose production. The latter points to the role of the liver in the very short VLCD-induced metabolic changes.

Because no significant change in gene expression and secretion of proinflammatory (*IL-6*, *IL-8*, *TNF*α, *MCP-1*) and antiinflammatory (*IL-10*, *IL-1Ra*, *TGF*β1) cytokines, and macrophage markers (*CD68*, *CD163*, *IRF5*, *MSR1*, *ACP5*, *CCR2*, *FCGBP*, *ITGAX*) in SAAT were observed after 2 days of the VLCD, it may be concluded that the early improvement of IS (metabolic changes) was not related to changes of immune status of adipose tissue. Indeed, several studies using opposite dietary intervention, ie, short overfeeding in healthy men, showed similar dichotomy between AT inflammation and IS under conditions of mild weight gain when IS was impaired despite no induction of inflammatory cytokines and macrophage activation in SAAT (23, 24).

In contrast to the changes induced by 2 days of a VLCD, after 28 days of severe calorie restriction mRNA expression of lipolytic genes (*ATGL*, *HSL*, *MGL*, *PLIN1*, *CGI58*) returned to the prediet levels. The no change in *ATGL* or *HSL* mRNA expression after 1 month of the VLCD, when compared with the prediet condition, is in agreement with some of the previous studies of our and other teams (1, 25). However, the decrease in expression of lipolytic genes on day 28 compared with day 2 does not essentially mean that lipolysis is attenuated. FFA levels in plasma were elevated at 28 day similarly to day 2, indicating maintenance of higher lipolytic rate. The decrease to baseline in expression of lipases at the late phase of the VLCD was accompanied by a decrease of *G0S2*, which has been shown as a dominant inhibitor of ATGL in adipocytes (26), and by a reduction in *DGAT1*, which is responsible for FFA reesterification. Reduced inhibition of *ATGL* together with reduced reesterification can thus still ensure the FFA release needful to cover the energy demand of the organism. Thus, our data support the hypothesis that G0S2 acts predominantly as a long-term regulator of ATGL, whereas CGI58 is more important for the regulation of acute lipolytic response (13). Furthermore, at day 28 a marked decrease of lipogenic genes, genes of β-oxidation, and insulin-stimulated glucose transport (*GLUT4*) in SAAT was observed. These processes probably prevent excessive breakdown as well as storage of FFAs in adipocytes during longer-term shortage and secure its adequate release from AT to serve as substrates for other organs.

The increase in a SAAT inflammatory state after 28 days of the VLCD is in agreement with published results of our and other groups (3, 11, 27, 28). Previously we have shown that 28 days of a VLCD was not associated with an increase in macrophage content in SAAT (29). Thus, it might be assumed that the observed increase of macrophage marker expression in response to strong calorie restriction reflects stimulation of macrophage activation rather than their accumulation. The increase of proinflammatory (*IL-6*, *IL-8*, *MCP-1*, *TNF*) but also antiinflammatory (*IL-10*, *IL-1Ra*) cytokines together with M1 (*IGTAX*, *CCR*) and M2 (*CD163*) macrophage markers is in accordance with the published findings that macrophages in SAAT are of a mixed phenotype (30). The role of the increase in the inflammatory state in SAAT after 28 days of severe calorie restriction is still unclear.

Interestingly, the only gene identified to be significantly down-regulated in both phases of the VLCD was *LOX*, one of the genes involved in fibrosis (and extracellular matrix remodeling. LOX catalyzes the cross-linking of collagens in AT and, thus, it is one of a key factors contributing to the fibrosis of AT observed in obesity (31). Inhibition of LOX also resulted in the improvement of several metabolic parameters, ameliorated glucose and insulin levels, decreased HOMA index, and reduced plasma triglyceride level in obese rats (31). Indeed, the decrease of *LOX* expression found in our study was associated with changes in SAAT metabolism genes (Supplemental Table 2). Importantly, the reduction of lipogenesis and fibrosis observed during weight loss in our study represent the opposite processes to those found in the overfeeding studies (23, 24).

Leptin appeared as the only adipokine for which a quantitative relationship with the diet-induced changes of HOMA-IR was found. In addition, leptin changes were correlated also with a number of metabolic-related genes

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in AT and with plasma FGF21 levels. Leptin was shown to act on peripheral tissues by the regulation of fatty acid oxidation and energy expenditure through activation of AMP-activated protein kinase, induction of FFA oxidation genes, and increased transport of FFAs to mitochondria (32). It might be suggested that this adipokine is a sensor of metabolic changes in SAAT and the signal that mediates the metabolic interplay with other organs during calorie restriction. Further studies in this issue in obese humans should be warranted.

After 28 days of the VLCD, the diet-induced improvement of metabolic indices (TG, insulin, cholesterol) was correlated with changes in lipogenic genes (eg, *SCD1*, *FASN*, *DGAT2*). It was shown in mice that a decrease of *SCD1* expression (in liver or systemic level) was correlated with an improvement of the metabolic profile and insulin sensitivity (33, 34). Thus, we may hypothesize that the decrease of lipogenic genes in AT is paralleled with the decrease of lipogenesis in liver, which is probably one of the important contributors to the improvement of metabolic profile and IS during the VLCD.

FGF21 was shown to act as a metabolic regulator during fasting through stimulating ketogenesis and fatty acid oxidation in liver in mice (8). In addition, in mice and 3T3-L1 adipocytes, the role of FGF21 in the down-regulation of lipogenesis and lipolysis in SAAT was shown (8–10). In line with this, we observed negative correlation between the increase of plasma FGF21 during the 28 days of the VLCD and the changes in mRNA expression of genes involved in SAAT lipogenesis (*FASN*, *DGAT2*) and glucose uptake (*GLUT4*). Because FGF21 plasma levels are increased only in later phase of the diet (35), it might be suggested that FGF21 plays a role in the regulation of a switch from a short-term to a longer-term calorie restriction. The down-regulation of lipogenesis in association with insulin-stimulated glucose transport into AT supports the role of FGF21 in the saving of substrates for other organs during famine.

In conclusion, our findings show that the early (2 d) and later (28 d) phases of the VLCD differ with respect to metabolic and inflammatory response in SAAT. Although in both phases the effects of severe calorie restriction represent the reaction to shortage of calories/nutrients (ie, induced lipolysis, reduced storage of lipids), the expression of regulatory cofactors involved in these processes is different in the early and later phase of the VLCD. The diet-induced modifications in metabolic and inflammation-related functions of AT did not appear to play a pivotal role in the improvement of IS at the early phase of the VLCD. The processes observed after 28 days of the VLCD probably contribute to adaptation of SAAT to prolonged calorie restriction through the saving of substrates. Moreover, the correlation of the changes in metabolic genes in SAAT with metabolic indices and FGF21 suggest the possible cross talk of SAAT with liver function during the VLCD.

Acknowledgments

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