Charles University in Prague

Third Faculty of Medicine

Interaction between adipocytes and immune cells in pathogenesis of obesity related pro-inflammatory state of adipose tissue

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PhD thesis

Interaction between adipocytes and immune cells in pathogenesis of obesity related pro-inflammatory state of adipose tissue

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Specialization: **Molecular and cell biology, genetics and virology**

Prague 2014

DECLARATION

I declare that this thesis has been written by me
and all sources of information
are reported in the list of literature.

Lucia Mališová

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CONTENT

LIST OF ABREVIATIONS

AC	adenylate cyclase
adipoR	adiponectin receptor
AMPK	AMP-activated protein kinase
AT	adipose tissue
ATF	activating transcription factor
BAs	bile acids
BMI	body mass index
CA	cholic acid
cAMP	cyclic adenosine monophosphate
CDCA	chenodeoxycholic acid
C/EBPs	family of CCAAT/enhancer-binding proteins
CYP7A1	cholesterol 7 alpha-hydroxylase
DCA	deoxycholic acid
DNAJC3	DnaJ homolog subfamily C member 3
eIF2α	eukaryotic initiation factor 2α
ER	endoplasmic reticulum
ERK1/2	extracellular signal-regulated protein kinase 1 and 2
ERS	endoplasmic reticulum stress
FA	fatty acids
FACS	fluorescence-activated cell sorting
FXR	farnesoid X receptor
GPCR	G protein-coupled receptor
HFM	high fat meal
HOMA-IR	homeostatic model assessment insulin resistance
HSL	hormone sensitive lipase
HSPA5	heat shock protein A5
IL	interleukin
IL1RA	interleukin 1 receptor antagonist
IKK	I-kappa-kinase
IR	insulin resistance
IRE1	inositol requiring enzyme 1
IRS	insulin receptor substrate
JNK	c-Jun NH 2-terminal kinase
KJ	kilojoules
LCD	low calorie diet
LPL	lipoprotein lipase
LPS	lipopolysaccharide
mRNA	messenger ribonucleic acid
MCP1	monocyte chemoattractant protein 1
NEFA	nonesterified fatty acids
NFκB	nuclear factor κ beta
PERK	protein kinase RNA-like endoplasmic reticulum kinase
PBMC	peripheral mononuclear cells
PI3K	phosphoinositide 3´kinase

PPARγ	peroxisome proliferator-activated receptor gamma
sAAT	subcutaneous abdominal adipose tissue
sGAT	subcutaneous gluteal adipose tissue
SOCS3	suppressor of cytokine signaling 3
SVF	stroma-vascular fraction
T2DM	type 2 diabetes mellitus
TG	triacylglycerides
TGR5	G protein bile acid coupled receptor 1
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TUDCA	tauroursodeoxycholic acid
UDCA	ursodeoxycholic acid
UCP1	uncoupling protein 1
UPR	unfolded protein response
VLDL	very-low-density-lipoprotein
VLCD	very low calorie diet
WM	weight maintenance
XBP1	X-box binding protein 1

1 INTRODUCTION

1.1 Obesity

Obesity is characterized by an excessive accumulation of adipose tissue (AT) and it is stratified into 3 categories according to body mass index (BMI) (Table 1) [1-2]. Although the mechanisms that lead to the body weight gain or loss are complex (behavioral, environmental, inherited and physiological) and not fully understood, the final determinant of alterations in body weight can be viewed most broadly as an energy imbalance driven by energy over-consumption, low energy expenditure or both [3]. At present, the prevalence of obesity has drastically escalated: the National Health and Nutrition Examination Survey (NHANES 2003-2004) reported that 66.2% of adults (20-74 years) in USA were classified as overweight or obese [4]. The prevalence of obesity in Europe has reached epidemic proportion as well, i.e. the highest prevalence of obesity (more than 25%) was found in Italy, Spain and Poland [5]. Increased prevalence of obesity was also recorded in the Czech Republic: the study by Kunešová et al. (2006) revealed that 35% of Czech adult population was overweight and 17% of Czechs were diagnosed as obese (www.obesitas.cz/download/dospeli_web.ppt).

Classification	BMI
underweight	<18.5 kg/m^2
overweight	25 to <30 kg/m^2
class I obesity	30 to <35 kg/m^2
class II obesity	35 to <40 kg/m^2
class III obesity	>40 kg/m^2

Table1: Body weight classification according to the BMI [1-2].

The excessive amount of AT is an important risk factor for a number of diseases including diabetes, cardiovascular and neurodegenerative diseases, hypertension, fatty liver disease, cancer, and airway disorders [3, 6-7]. All of these obesity-associated diseases contribute to the increased morbidity and mortality [3]. In fact, it has been estimated that 2.8 million people die every year worldwide due to diseases associated with overweight [8].

Therefore, there is a great need for obesity prevention or treatment. In present, three major strategies exist for treatment of obesity [9].

- lifestyle modification (dietary intervention, exercise)
- medical therapies (orlistat, phentermin)
- bariatric surgery

Lifestyle modifications, especially weight-reducing dietary interventions, are a first-choice strategy and key component of obesity treatment. As many studies showed, hypocaloric diets induce a fat mass reduction that is accompanied with an improvement of metabolic profile of the obese individuals [10-12]. Table 2 represents types of frequently used dietary interventions. Deciphering effects of hypocaloric diets on AT is a goal of many studies including those that are the basis of this PhD thesis.

	Daily energy intake in kilojoules (KJ)/day
Low calorie diet (LCD)	2100 – 2500 KJ per day lower than the estimated daily energy expenditure and > 3400 KJ
Very low calorie diet (VLCD)	< 3400 KJ
Hypocaloric balanced diet	≥ 5000 – 6300 KJ
Multiphase diet	combination of various types of diet

Table 2: Type of dietary interventions [13-14].

1.2 Adipose tissue

1.2.1 Adipose tissue characteristic

AT is a specialized, highly innervated and vascularized organ that functions as a main depot for energy storage and mobilization; it is also important for thermoregulation, lactation, immune responses and signaling [15-16]. The cells enabling the storage function of the adipose organ are adipocytes [17]. The remaining cell types of AT include preadipocytes, fibroblasts, pericytes, neuronal, endothelial and immune cells (macrophages, lymphocytes): these belong to the stroma-vascular fraction of AT (SVF, 30% of cells in AT) [18].

There are two main types of adipocytes, which are easily distinguished by morphology. Spherical white adipocytes are cells with variable diameter (20-200 μm) which comprise of nucleus and organelles localized on cell´s periphery and a single large cytoplasmic lipid

droplet (~90% of the cellular volume). On the other hand, brown adipocytes are polygonal cells with a roundish nucleus, several cytoplasmic lipid droplets [16] and numerous large mitochondria containing an uncoupling protein 1 (UCP1); a unique protein that uncouples oxidative phosphorylation from adenosine triphosphate (ATP) synthesis [19-20]. These facts point to the functional differences between the two types of adipocytes: white adipocytes store energy for times of energetic deficit, whereas brown adipocytes burn energy in order to generate heat in the process of thermogenesis. In humans, brown AT is found primarily in infants or young children [21-23] and during the life it is lost or replaced with white AT [21, 24-25]. Due to the fact, that this PhD thesis is focused on the white AT characteristics, all further information concern white AT biology.

1.2.2 Origin of adipose tissue

Adipocytes are thought to arise from multi-potent stem cells residing in the AT stroma. These mesenchymal stem cells become preadipocytes when they lose their ability to differentiate into other mesenchymal lineages and become 'committed' to the adipocytic lineage [26]. Differentiation into adipocytes, like in other cells, is accompanied by changes in the expression of numerous genes. Moreover, growth arrest or cell contacts are necessary for preadipocytes differentiation. Many genes and transcription factors play a role in adipogenesis. Especially the family of CCAAT/enhancer-binding proteins (C/EBPs: C/EBPα, C/EBPβ, and C/EBPδ) with peroxisome proliferator-activated receptor gamma (PPARγ) are crucial for adipogenesis [17, 20, 27-29]. Several loss-of-function studies with cells either lacking PPARγ or C/EBPα have shown an absolute requirement of PPARγ for the induction of differentiation, whereas cells lacking C/EBPα, but expressing PPARγ, can still differentiate and accumulate lipids [29]. Among the three isotypes of PPARγ family (3 splice variants PPARγ1-3), only PPARγ2 expression seems to be specific to AT. However, the data regarding the expression of PPARγ1 and PPARγ2 in human AT remain still conflicting [30].

During preadipocyte differentiation, a carefully coordinated chain of events occurs: in brief, one of the earliest events is the phosphorylation and DNA binding of C/EBPβ. C/EBPβ then induces C/EBPα and PPARγ2 expression [29, 31-32]. PPARγ, together with C/EBPα, then stimulates expression of genes responsible for fatty acids (FA) metabolism including adipocytes protein 2 (aP2), acyl CoA synthase (ACS), fatty acid transport protein-1 (FATP1) or lipoprotein lipase (LPL) [33]. Sterol regulatory element-binding protein

1, isoform 1c (ADD-1/SREBP1) is another important member of adipogenesis cascade responsible for cholesterol homeostasis and for FA metabolism [34].

Adipogenesis is a prerequisite for the hyperplastic growth of AT. AT expands also by increasing the size of pre-existing adipocytes (hypertrophy) [35]. Spalding et al. (2008) [36] revealed that adipocyte cell number is relatively fixed by early adulthood, and that the alterations in fat mass during adulthood are merely credited to alterations in adipocyte hypertrophy [36]. The mechanism of AT expansion may determine the development of metabolic syndrome [37]. While increased adipocyte size correlates with increased risk of type 2 diabetes mellitus (T2DM) [35], poorer metabolic parameters, AT dysfunction, inflammation and adipocyte death [38], similar relationship between hyperplastic AT and adverse metabolic outcomes was not documented. Different metabolic impact of AT hyperplasia and hypertrophy was confirmed by several clinical studies: obese subjects with few large adipocytes were more glucose intolerant and hyperinsulinemic than those having the same degree of obesity and many small fat cells [35, 39-41]. Differentiation of preadipocytes/ability of AT to expand by hyperplasia is impaired in obesity [42] and one of the goals of this thesis was to study the effect of weight loss on differentiation capacity of adipose precursors.

1.2.3 Adipose tissue depots

AT can be considered a multi-depot organ located primarily in three major anatomical areas – 1.subcutaneous (abdominal, gluteal and femoral fat depots), 2.intra-abdominal (mesenteric, omental, retroperitoneal, epiploic) fat depots associated with internal organ, and 3.numerous small AT depots (epicardial, intermuscular) with specialized function related to the neighboring tissue [16, 43] (Figure 1). In humans, size of various fat depots varies with sex, age and ethnic origin [44]: e.g. while increase of fat in the gluteofemoral region, is typical for women (gynoid AT distribution), the main subcutaneous AT in men accumulates in upper part of the body (abdominal distribution) [45]. The regional distribution of body fat is an important determinant of health complications: increased intra-abdominal adiposity is associated with insulin resistance (IR), progression of atherosclerosis, T2DM, hypertension, increased blood pressure and level of plasma triacylglyceride (TG) as well as premature death [46-49].

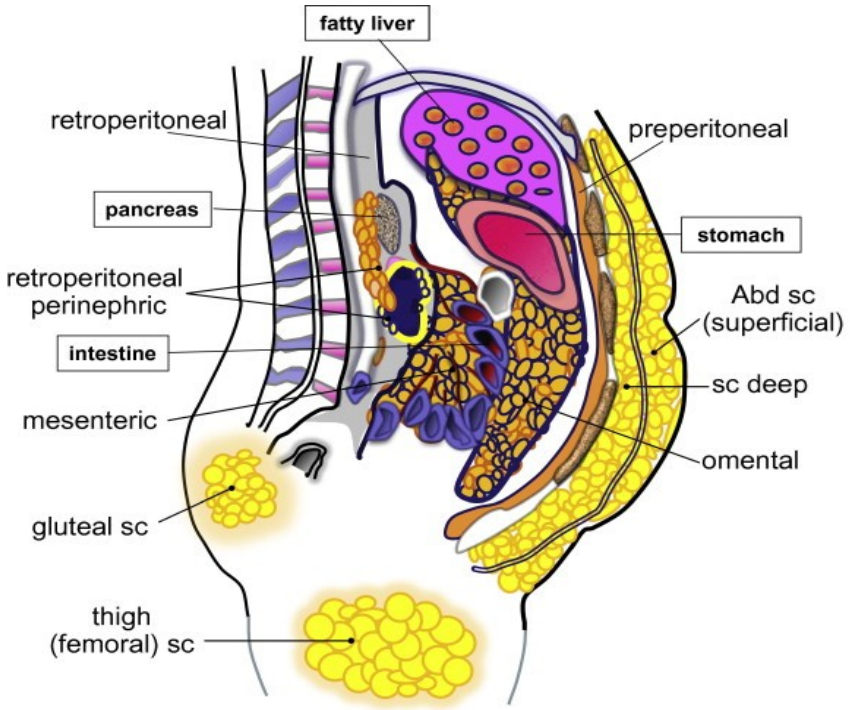

Figure 1: Human adipose depots. Sc, subcutaneous; Abd, abdominal [50].

One of the possible explanations for adverse effect of central obesity could be a high level of nonesterified fatty acids (NEFA) generated by active lipolysis in intra-abdominal fat that drains directly into the portal vein. The high level of NEFA interferes with glucose utilization – thus inducing IR [47]. On the other hand, increased amounts of AT in the gluteofemoral regions is associated with improved insulin sensitivity, lower risk of developing T2DM, dyslipidemia and atherosclerosis in comparison to central obesity [49, 51]. Protective nature of gluteofemoral fat includes also the direct effects on vascular health: increased gluteofemoral mass was associated with lower aortic calcification and arterial stiffness, as well as with decreased progression of aortic calcification [52]. Only few studies tried to decipher the basis of the protective character of subcutaneous gluteal AT (sGAT); different uptake and release of NEFA in the subcutaneous abdominal AT (sAAT) when compared to sGAT [53-54] or higher activity of LPL [55-56] suggest a higher capacity of sGAT for lipid accumulation and so could be candidates underlying cause. Nevertheless, reasons of the phenomenon have still not been fully elucidated and are solved in this PhD thesis.

Ectopic fat is defined as a deposition of lipids in locations not primarily dedicated to lipid storage (non-adipose tissue) like muscle, heart, liver and vasculature. Some researchers consider also intra-abdominal fat as an ectopic depot. There are several potential mechanisms that might explain the tendency to deposit lipids in ectopic depots: one

hypothesis suggests that, in states of positive energy balance, excess NEFA are initially stored subcutaneously, but once the capacity of subcutaneous AT is reached, storage shifts to ectopic sites [57]. Ectopic fat has a poor effect on health as confirmed by the association between ectopically deposited lipids and development of IR or cardiometabolic disorders [58].

1.2.4 Physiological function of adipose tissue – lipid metabolism

White AT serves functions like insulation, mechanical support, but its major physiological role lies in a fat storage where excess calories are deposited after a meal (lipogenesis) and in release of NEFA and glycerol as a fuel for other tissues during fasting or food deprivation (lipolysis) [59] (Figure 2).

In humans, majority of TG stored in adipocytes are derived from the dietary FA, circulating in blood either in the form of very-low-density-lipoprotein (VLDL) particles, chylomicrons or NEFA bound to albumin that are released from VLDL and chylomicrons by active LPL [50]. However, FA can be also synthesized by AT in the process of *de novo lipogenesis* where excess of carbohydrate are converted into lipids [60].

Lipolysis is defined as hydrolysis of TG into NEFA and glycerol. Adipose TG lipase (ATGL) hydrolyzes TG into diacylglyceride, which is further broken down to NEFA and glycerol by hormone sensitive lipase (HSL) and monoacylglyceride lipase (MAGL). Once released from adipocytes into the circulation, NEFA bound to albumin are delivered to muscle and liver for β oxidation [61]. Part of the NEFA released during lipolysis is re-esterified to TG in adipocytes. Glycerol is delivered to the liver, where it is used for glucose production, as adipocytes have only a low amount of glycerol kinase that would enable them recycling of glycerol for TG synthesis.

Fat mobilization is regulated by catecholamines (epinephrine and norepinephrine), natriuretic [61] peptides through adrenergic receptors and by insulin [62] through insulin receptor present in fat cells [61, 63]. Lipolytic activity of catecholamines is executed through binding to β–adrenergic receptors that leads to an activation of adenylate cyclase (AC). AC increases the level of cyclic adenosine monophosphate (cAMP) that subsequently activates protein kinase A (PKA) able to phosphorylate HSL [64]. Except for natriuretic peptides [63] also growth hormones [65] and cytokines like tumor necrosis factor alpha (TNFα), interleukin 6 (IL6) are able to stimulate lipolysis [66]. On the other hand, negative regulator of lipolysis, insulin, leads to the activation of protein kinase B (PKB) and

phosphodiesterase 3B which in turn decreases levels of cAMP and cyclic guanosine monophosphate (cGMP) and so reduces lipolysis [67].

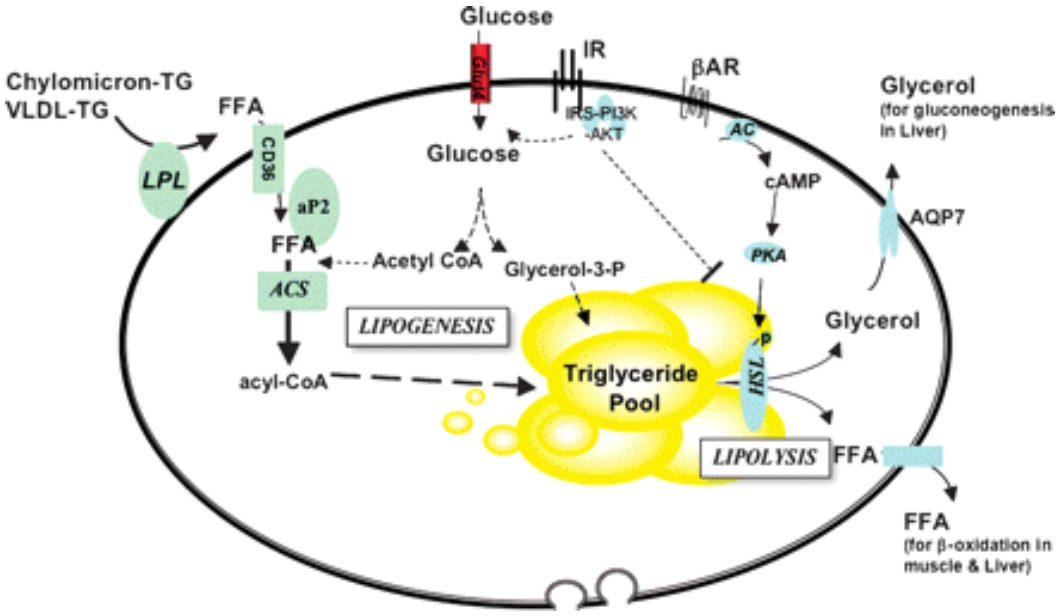

Figure 2: Lipid metabolism in adipocytes: lipogenesis and lipolysis:

AC, adenylate cyclase; ACS, acyl-CoA synthase; cAMP, cyclic adenosine monophosphate; AKT, protein kinase B; AQP7, aquaporin 7; aP2, activating protein 2; AR, adrenergic receptor; HSL, hormone sensitive lipase; IR, insulin receptor; IRS, insulin receptor substrate; LPL, lipoprotein lipase; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; TG, triacylglycerides; VLDL, very-low-density-lipoprotein [68].

1.2.5 Adipose tissue as an endocrine organ – adipokines and cytokines

The whole AT is an active metabolic tissue that secretes metabolically important hormones, cytokines and chemokines. Those of them produced specifically by adipocytes are called 'adipokines' [69]; cytokines and chemokines are produced by both adipocytes and cells of SVF. AT products modulate appetite, energy expenditure, endocrine and reproductive systems, bone metabolism, inflammation as well as immunity [70-71] and some of them also contribute to IR and cardiovascular complications associated with obesity [72-73]. The best known adipokines are leptin and adiponectin. Their secretion reflects the amount of AT: increased fat mass positively correlates with leptin production, while it negatively correlates with production of adiponectin [70.74]. Other adipokines secreted by adipocytes are visfatin [6, 75] and retinol-binding protein 4 (RBP4) [76].

1.2.5.1 Leptin

Leptin is a 16-kd protein encoded by the *ob* gene [75] that regulates body weight by signaling nutritional status to other organs especially the hypothalamus, which produces neuropeptides and neurotransmitters able to modulate food intake and energy expenditure [6, 77]. In general, higher levels of leptin stimulate energy expenditure while decrease food intake. Deficiencies in leptin production in human populations are very rare, but if they occur they are able to cause very severe cases of obesity and IR [78] alleviated only by leptin administration [79].

On the other hand, in diet-induced obesity, leptin levels are elevated. Injection of additional leptin in obese individuals however fails to counteract obesity [80]. These observations highlight that obese individuals are less responsive to the action of leptin due to so-called leptin resistance (Figure 3).

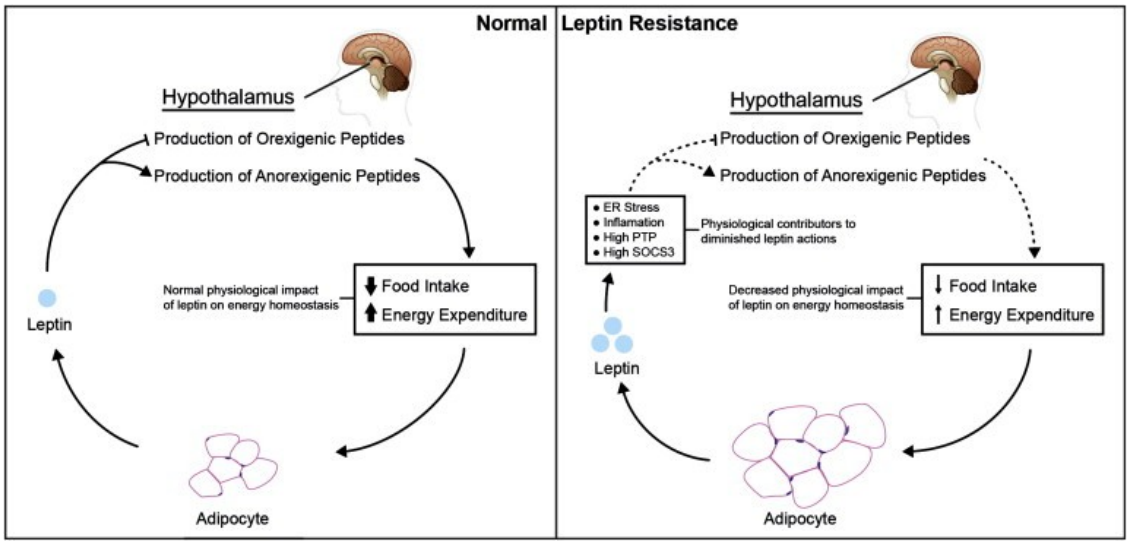

Figure 3: Leptin physiology:

In normal conditions, leptin leads to reduced appetite and increased energy expenditure through increased production of anorexigenic peptides. Leptin resistance is on the other hand induced by some pathological conditions where specific molecular events inhibit leptin signaling [81].

The extent to which leptin resistance is associated with the etiology of obesity or is a consequence of obesity is still unclear. Although, impaired transport of leptin across the blood brain barrier, endoplasmic reticulum stress (ERS), inflammation, as well as attenuation of leptin signaling by suppressor of cytokine signaling 3 (SOCS3) and protein tyrosine phosphatases (PTPs) are mechanisms involved in the development of mentioned resistance [81].

Beside these central effects, leptin is important for the crosstalk between AT and immune system: it is able to influence cytokine production from T lymphocytes and to switch their phenotype toward a T_H1 response [82]. Moreover, cytokine production from T lymphocytes is suppressed in leptin-deficient children and restored by leptin administration [83]. In addition to its effects on T lymphocytes, leptin also influences monocyte activation, phagocytosis and cytokine production. Most of the *in vivo* studies on the immune-modulating effects of leptin have been generated by using leptin-deficient *ob*/*ob* mice. In this setting leptin deficiency was associated with reduced inflammation in models of autoimmune disease but also with increased susceptibility to bacterial and viral infections. Nevertheless, the general consensus is that leptin such as exerts rather a pro-inflammatory role [74-75, 84,85].

1.2.5.2 Adiponectin

Adiponectin was discovered in 1996 in mice as a transcript abundant in AT [86]. It is a hormone secreted from adipocytes into bloodstream where it circulates in high concentration [87] (microgram per milliliter, while the range e.g. for leptin is nanograms per milliliter). Although adipocytes are the most important source of adiponectin, serum adiponectin levels do not increase with obesity as leptin levels do. In contrary, adiponectin levels are significantly reduced in obese and/or in patients with T2DM [75]. Adiponectin molecule is composed of a globular and collagenous domain that after synthesis in the endoplasmic reticulum (ER) form trimers (low molecular weight, LMW), hexamers (middle molecular weight, MMW) or high molecular weight (HMW) multimers (12-18 monomers) [75,88-89]. It seems that glycosylation of adiponectin is necessary for its full biologic activity [90].

Adiponectin plays important and pleiotropic roles in the whole body: 1. metabolic effect [91] includes improvement of insulin sensitivity [84] through AMP-activated protein kinase (AMPK) activation; 2. antiatherogenic effect through inhibition of macrophage adhesion to endothelial cells during atherosclerosis [92] and 3. anti-inflammatory effect [93] because it reduces the production of TNFα [94], IL6 and stimulates the production of anti-inflammatory cytokines interleukin 10 (IL10) and interleukin 1 receptor antagonist (IL1RA) [6]. Part of these effects might be explained by adiponectin capability to inhibit activity of nuclear factor κB (NFκB) at least [6, 75].

Adiponectin signaling is mediated by two adiponectin receptors, adipoR1 and adipoR2. Knockout of both receptors abrogates adiponectin binding and causes abnormal lipid accumulation, inflammation as well as IR. On the other hand, activation of adipoR1/2 in the liver or muscle tissues increases AMPK activity and thus mediates the insulin sensitizing effect of adiponectin, as mentioned earlier [6].

1.2.5.3 Cytokines

Cytokines produced by AT (by both adipocytes and SVF cells), can be divided into 3 groups: the first group is based on pro-inflammatory cytokines like TNFα, IL6, macrophage migration inhibitory factor (MIF) [95], IL1β [74] or transforming growth factor β1 (TGFβ1) [96]. The second group contains anti-inflammatory cytokines including IL10 and IL1RA that antagonizes binding of IL1α/β and interferon β (IFNβ). The third group are chemokines recruiting immune cells like MCP1, macrophage inflammatory protein 2 (MIP2), interleukin 8 (IL8) [74] and others.
The importance of cytokines lay in their direct effect on cellular metabolism as shown i.e. for TNFα that directly decreases insulin sensitivity by inactivating insulin receptor substrate (IRS) through serine/threonine phosphorylation and increases lipolysis in adipocytes [97] (see next Chapter).

1.2.6 Dysfunction of adipose tissue in obesity

Functional AT is essential for the metabolic health in humans and other mammals, as AT safely stores dietary FA lipotoxic for other tissues and contributes to the glucose homeostasis through several mechanisms [98-99] (partially discussed above). However, many physiological functions of AT are dysregulated in obesity [100]. Among them, IR and chronic inflammation of AT has been proposed to have an important role in the pathogenesis of obesity related diseases.

1.2.6.1 Insulin resistance

Insulin is a critical regulator of all aspects of adipocyte biology: it promotes adipocyte TG stores by a number of mechanisms, including stimulation of glucose transport and TG synthesis, inhibition lipolysis as well as increase the uptake of FA derived from circulating lipoproteins [101]. In normal, healthy tissue, the initial molecular signal for insulin action

involves activation of the insulin receptor tyrosine kinase, which results in phosphorylation of insulin receptor substrates 1-4 (IRSs1-4) on multiple tyrosine residues serving as docking sites for SH2 domain–containing proteins, including the p85 regulatory subunit of phosphoinositide 3′ kinase (PI3K) [102]. The activation of PI3K is necessary for full stimulation of glucose transport by insulin [101, 103] (Figure 4).

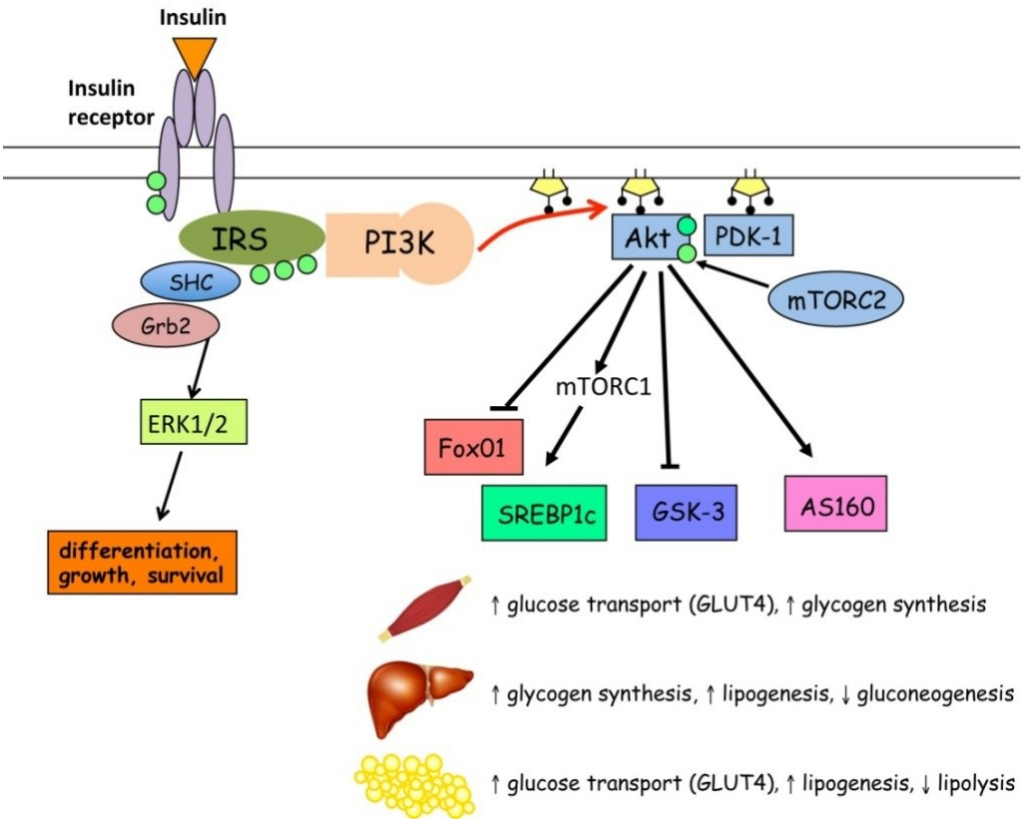

Figure 4: Insulin signaling pathway:

AKT, protein kinase B; GLUT4, glucose transporter type 4; IRS, insulin receptor substrate; SHC, Src Homology 2 domain; GRB2, growth factor receptor-bound protein 2; ERK, extracellular-signal-regulated kinases; PI3K, phosphoinositide 3-kinase; PDK1, phosphoinositide-dependent protein kinase 1; mTORC, mammalian target of rapamycin complex; FoxO1, forkhead box protein O1; SREBP1c, sterol regulatory element binding protein 1c; GSK-3, glycogen synthase kinase 3; AS160, 160 kDa Akt substrate [104].

Accumulation of AT during obesity is accompanied by IR development in AT, as well as in other tissues [105]. The main condition leading to the development of IR in AT is probably hyperglycemia or nutrient overload [106,107] associated with the inhibition of insulin signaling on many levels: i.e. the binding capacity of insulin receptor, activities of tyrosine/serine kinases and phosphorylation of IRS proteins that eventually lead to the decreased synthesis/translocation of glucose transporter type 4 (GLUT4) [92, 108]. The molecular mechanism of IR development includes the inhibitory serine phosphorylation of IRS, the key mediators of insulin receptor signaling [100, 109]. Among many kinases (c-Jun NH 2-terminal kinase: JNK, I-kappa-kinase betta: IKKβ, protein kinase C theta: PKCθ, SOCS3

and others) capable of serine phosphorylation of IRS, JNK is the most important one and therefore it is considered as a central mediator of IR. Indeed, genetic deletion of JNK1 gene in mice resulted in marked protection against IR, T2DM and fatty liver disease [110].

Other factors contributing to the development of IR in AT are diminished level of adiponectin [59] or enhanced levels of pro-inflammatory cytokines like MCP1, IL6 [111] and TNFα. TNFα was the first pro-inflammatory mediator produced by AT linked to development of IR in AT. TNFα and IL1β interfere with insulin signaling at the level of insulin receptor substrate 1 (IRS1), whereas the mechanisms of action of IL6 are still a matter of debate [112-113]. Resistance to insulin signaling also contributes to enhanced lipolysis of AT. Insulin resistant AT releases more NEFA that are then stored in the form of TG in ectopic areas [114] (see above, paragraph devoted to ectopic fat). Thus, IR of AT strongly influences insulin sensitivity of other tissues.

1.2.6.2 Inflammation

In obesity, circulating levels of inflammation markers, i.e. pro-inflammatory cytokines and acute phase proteins (haptoglobin and C-reactive protein: CRP) are substantially increased and this chronic state is defined as a low-grade inflammation. It has been shown that the source of these inflammatory molecules are hypertrophied adipocytes and immune cells (macrophages and lymphocytes) [115-116] infiltrated into obese AT [71-73, 75, 117-118]. Importantly, it is increasingly evident that the inflammatory state may be causal in the development of IR and the other disorders associated with obesity [118].

Although the cellular and molecular mechanisms involved in immune cells infiltration are poorly understood, it seems that MCP1 with its receptor are necessary for such accumulation (Figure 5) [119]. Kanda et al. (2006) proved, that MCP1 and MCP1 receptor knockout mice have fewer macrophages in AT and lower inflammatory gene expression than wild type mice [120].

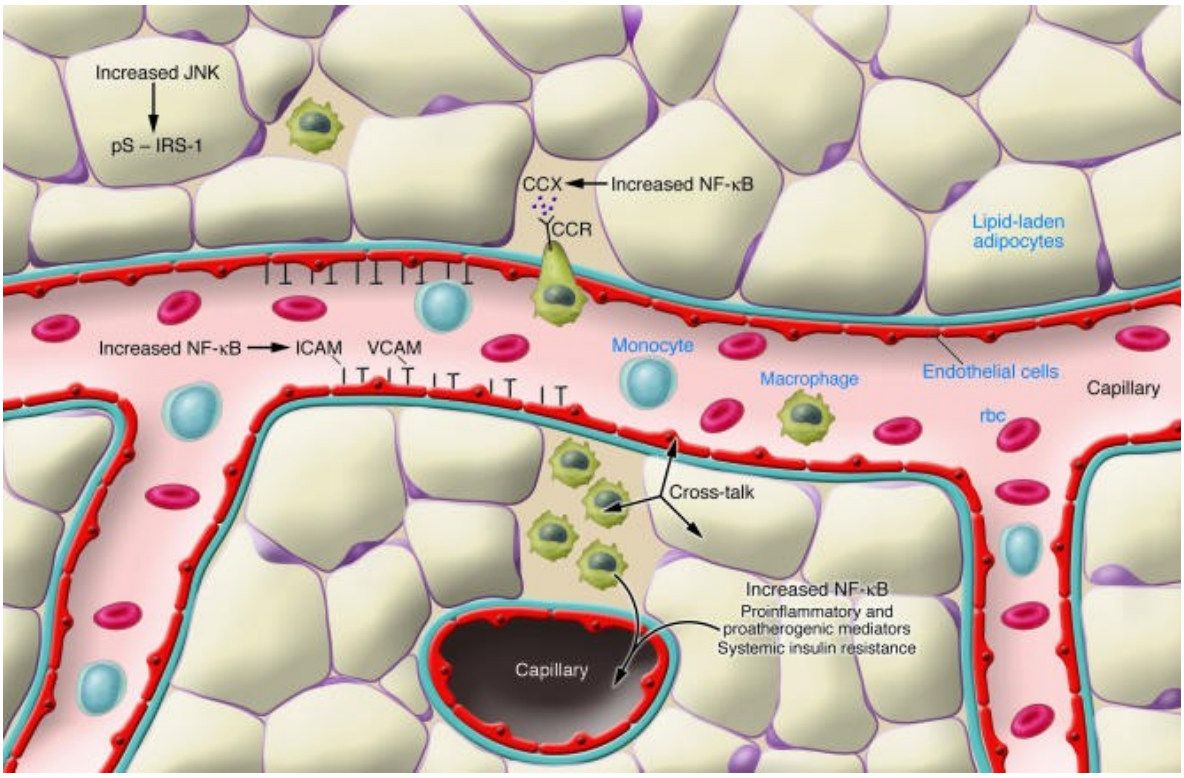

Figure 5: Inflammation in adipose tissue:

Excess of lipid accumulation is associated with activation of c-Jun NH 2-terminal kinase (JNK) and nuclear factor κ beta (NFkB) pathways, it increases level of pro-inflammatory cytokines as well as recruitment monocytes into the AT. The monocytes then differentiate into macrophages and produce more of the same inflammatory cytokines and chemokines [111].

Another possible trigger of immune cells infiltration is local hypoxia [121] or elevated levels of NEFA in circulation [122]. NEFA, released in large quantities from hypertrophied adipocytes during abnormal lipolysis, serve as a naturally occurring ligand for the family of toll-like receptors (TLRs) i.e. TLR4, thereby inducing the inflammatory changes in both adipocytes and macrophages through NFκB activation [123]. In humans, macrophage number in AT directly correlates with adiposity [72, 116, 124-125]. On the other hand, weight loss can induce the decrease of macrophage infiltration into AT, concentrations of inflammation-related products in the circulation, as well as inflammatory gene expression [117, 126-128].

1.3 Endoplasmic reticulum stress

One of mechanism which can contribute to the development of IR during obesity is a dysfunction of ER referred to as endoplasmic reticulum stress (ERS) [53].

The ER is an organelle responsible for the trafficking, synthesis and maturation of proteins as well as for transmitting of metabolic signals critical in cellular homeostasis and Ca^{2+} storage [10]. Conditions that challenge ER function (increased protein synthesis, the presence of mutant or misfolded proteins, inhibition of protein glycosylation, imbalance of ER calcium levels, glucose and energy deprivation, hypoxia, pathogens, toxins, NEFA [129]) lead to an adaptive response system known as the unfolded protein response (UPR) (Figure 6) [71, 130]. The UPR pathway is mediated by three proximal transmembrane proteins that in the absence of unfolded proteins or ERS are held inactive by the chaperone heat shock protein A5 (HSPA5 also known as GRP78 or BIP). The first branch of UPR is represented by an activation of protein kinase RNA-like endoplasmic reticulum kinase (PERK) that in turn phosphorylates eukaryotic initiation factor (eIF) 2α. Phosphorylated eIF2α consequently blocks general translation but favors translation of activating transcription factor (ATF) 4 and transcription of proapoptotic factors C/EBP homologous protein (CHOP) and activating transcription factor (ATF) 3. The second UPR branch is represented by an activated inositol requiring enzyme 1 (IRE1) which acts as a highly specialized ribonucleic acid (RNA) endonuclease that splices messenger ribonucleic acid (mRNA) of transcription factor X-box binding protein 1 (XBP1). Spliced form of XBP1 is then translated into a highly active transcription factor that activates transcription of down-stream chaperones [131]. The last branch of UPR consists of an activating transcription factor (ATF) 6 that is processed by two site specific proteases in Golgi apparatus. Released cytosolic fragment of ATF6 is a transcriptional coactivator that in turn increases transcription of down-stream chaperones, including HSPA5, hypoxia up-regulated protein 1 (HYOU1), calreticulin (CALR), DnaJ homolog subfamily C member 3 (DNAJC3) and glucose regulated protein 94kDa (GRP94) [130, 132].

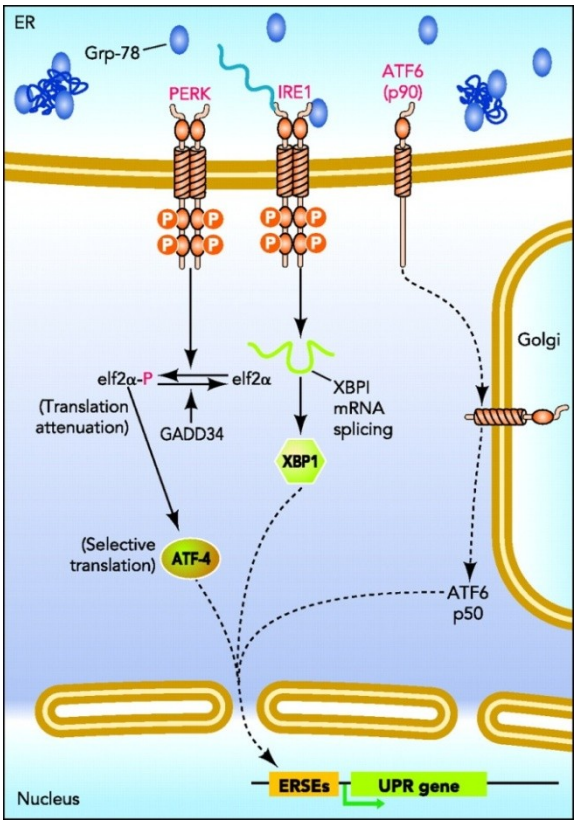

Figure 6: UPR pathway:

GRP78, glucose regulated protein 78; PERK, PKR-like ERS kinase; IRE1, inositol requiring enzyme 1; ATF6, activating transcription factor 6; XBP1, X-box binding protein 1; eIF2α, eukaryotic initiation factor 2α; ATF4, activating transcription factor 4; ERSE, ERS response elements; UPR, unfolded protein response [133].

Recent reports support the concept of a close dynamic relationship between obesity and ERS in human subjects: it has been demonstrated that ERS trigger the activation of several serine/threonine kinases, including JNK and IKK. For instance, IKK phosphorylates and inactivates nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha (IκBα), resulting in activation of NFκB as a key promoter of inflammation [134]. Phosphorylated and activated JNK kinase is able to induce the expression of pro-inflammatory cytokines but also IR via serine phosphorylation of IRS1 and insulin receptor substrate 2 (IRS2) [71, 135].

1.4 Chemical chaperones

As mentioned earlier, ERS plays an important role in the development of IR as well as inflammation [136]. An experimental approach exists to resolve ERS and to support the ER folding machinery by providing exogenous low molecular weight compounds –chemical

chaperones – that inhibit the formation of misfolded structures or aggregates [137] and thus improve insulin sensitivity in mouse model of obesity [138]. Recently, the short chain fatty acid derivative 4-phenylbutyrate (PBA) and the conjugated tertiary bile acid tauroursodeoxycholic acid (TUDCA) have been shown to have chaperone-like activity [138-140] and to alleviate signs of ERS in the liver and AT of obese mice accompanied with reduced IR and hyperglycemia [137, 141]. TUDCA is a bile acid (BA) derivative that has been used in Europe to treat cholelithiasis and cholestatic liver disease. Data from studies conducted in animal models and cell systems demonstrate beneficial metabolic effects of BAs and the effect of TUDCA on insulin action has been confirmed also in human subjects [142-143]. Regarding the chaperone activity of BAs, the most data concern animal studies, while clinical data are insufficient. Therefore one of the aims of this PhD thesis was to determine the effect of BAs on ERS in human adipocytes and blood cells.

1.4.1 Bile acids

1.4.1.1 Bile acids synthesis

BAs are the end products of cholesterol catabolism, which constitutes a major route for the elimination of surplus cholesterol and absorption of lipids with the fat-soluble vitamins (A, D, E, K) from the intestine [144].

Two distinct pathways of BA synthesis (restricted to hepatocytes) exist: the classic/neutral and the alternative/acidic. The neutral BA biosynthetic pathway is initiated by cholesterol 7 alpha-hydroxylase (CYP7A1**)** in the smooth ER and leads to the formation of cholic acid (CA) and chenodeoxycholic acid (CDCA). The neutral pathway of BA biosynthesis appears to be the major pathway of BA synthesis in humans under normal conditions. During pathophysiological conditions, the alternative pathway of BA synthesis is the most active [145]. It is initiated by mitochondrial sterol 27-hydroxylase (CYP27A1) and the rate-limiting step in this pathway appears to be a transport of cholesterol to the inner mitochondrial membrane through steroidogenic acute regulatory protein (StarD1 protein) [145].

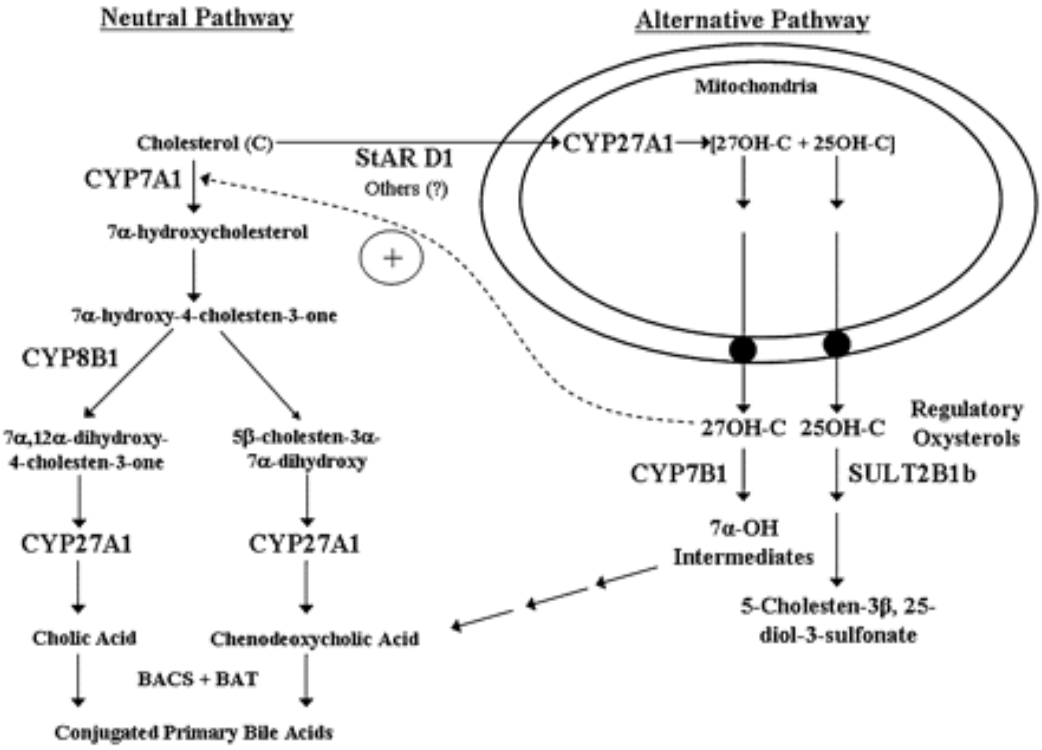

Figure 7: Synthesis of bile acids [145].

After synthesis (Figure 7), BAs are transported to and stored in the gallbladder and their secretion (into the small intestine) depends on the dietary intake. Approximately 95% of BAs are reabsorbed and transported back to the liver from the ileum of the small intestine, in so-called enterohepatic circulation. Due to toxic effects of high concentration of BAs, levels of BAs need to be tightly regulated. For example, higher levels of BAs in the liver results in a development of cholestasis [146], malignant tumors of gastrointestinal tract or colorectal adenocarcinomas [147-148].

There are four major types of BAs in humans; primary BAs: CA and CDCA and secondary BAs: deoxycholic acid (DCA) and lithocholic acid (LCA). Secondary or conjugated BAs are products of bacterial metabolism with various effect on cells [146, 149]: DCA is able to stimulate the expression of inflammatory factors as well as abnormal proliferation of epithelial cells of colon through activation of transcription factors NFκB and activating protein 1 (AP1) [150]. Another conjugated BA, ursodeoxycholic acid (UDCA), is routinely

used for treatment of primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), cystic fibrosis [147] or chronic hepatitis C [149, 151]. Anti-inflammatory effect of UDCA confirmed animals models of carcinogenesis [147] and clinical studies [148]. Another member of conjugated BAs, TUDCA, is able to suppress apoptosis in rat hepatocytes [152] or increases insulin sensitivity in human adipocytes [142].

1.4.1.2 Bile acids as signaling molecules

In addition to the critical roles of BAs in lipid and vitamin absorption, they are ligands for many receptors as well. BAs play very important role in intracellular signaling [146]. Receptors through which BAs activate are depicted in the Figure 8.

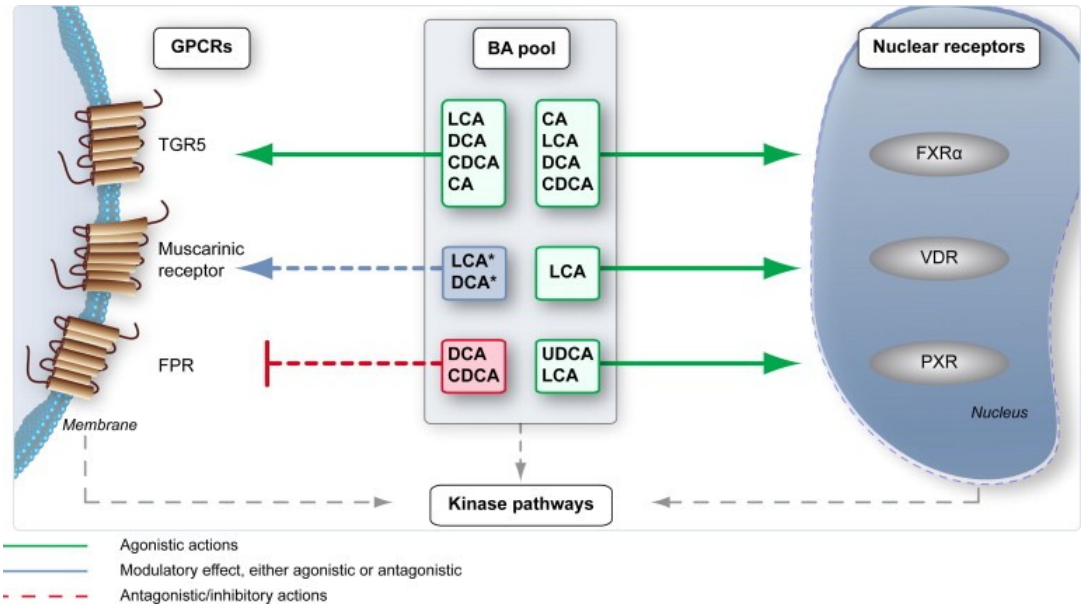

Figure 8: Bile acids receptors:

GPCRs, G protein-coupled receptor; FPR, formyl peptide receptor; TGR5, G protein bile acid coupled receptor; BA, bile acids; LCA, litocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid; FXRα, farnesoid X receptor; VDR, vitamin D receptor; PXR, pregnane X receptor [144].

1.4.1.2.1 Nuclear receptor signaling pathways

The best known nuclear receptor of BAs, farnesoid X receptor (FXR), consists from two primary domains, the DNA binding and the ligand binding domain [150]. Whilst rodents have two FXR family members, FXRα and FXRβ, in humans only FXRα is expressed. FXRβ is present in the human genome only as a nonexpressed pseudogene [144]. FXR was first cloned in 1995 and was found to be highly expressed in the liver, intestine, kidney and adrenal

glands [146]. Signaling through this receptor has pleiotropic effect based on the regulation of liver regeneration, hepatic inflammation or carcinogenesis [153], moreover, it is able to modulate the expression of genes whose products are critically important for BAs and cholesterol homeostasis [154-155]. For instance, in target tissues, FXR ligands negatively regulate BAs synthesis because they decrease the expression of Cyp7A1, the rate-limiting enzyme of the BAs synthetic pathway [153].

Furthermore, FXR may control AT biology [156], its activation *in vitro* stimulates adipocyte differentiation by promoting PPARγ activity and inhibiting the Wnt/β-catenin pathway. Conversely, $FXR^{-/-}$ mice exhibit a decrease in AT mass. [153]. Characterization of $FXR^{-/-}$ mice further revealed an important function of FXR in the control of glucose homeostasis; $FXR^{-/-}$ mice display peripheral IR with an impaired insulin signaling response in AT and skeletal muscle [157-159].

Studies on human blood cells revealed that FXR nuclear transcription factors have an immuno-modulatory effect. All peripheral mononuclear cells (PBMC) that include population of monocytes, natural killers (NK) and T and B cells express FXR and animal experiments demonstrate that mice lacking FXR are characterized by pro-inflammatory phenotype [160]. Moreover, BAs like DCA or CDCA successfully inhibit the lipopolysaccharide (LPS) induced production of pro-inflammatory cytokines (IL1, IL6, TNFα) in macrophages [161].

1.4.1.2.2 Kinase-signaling pathway

In the last few years, BAs have also been discovered to activate JNK pathway that has been demonstrated to be involved in the regulation of CYP7A1[145, 149]. Except for JNK pathway, BAs could also activate the group of receptor tyrosine kinases [162] and different isoforms of protein kinase C (PKC) in colonic cells by poorly defined mechanisms [145].

1.4.1.2.3 G protein-coupled receptor signaling pathway

The total G protein-coupled receptor (GPCR) family comprises over 800 receptors including glucagon, formyl peptide (FPR), insulin, muscarinic receptors and others [162]. The most studied GPCR in relation to BAs is G protein bile acid coupled receptor 1 (TGR5), also known as M-BAR, GPBAR, or GPR131. TGR5 was identified as BA receptor by two different groups in 2002 [163] and 2003 [161]. Expression of TGR5 was detected in gallbladder

epithelium, in the intestine, human monocytes and in brown AT [144]. The activation of TGR5 in brown AT increases energy expenditure [164] thus preventing the obesity [149] and T2DM development [144]. In details, TGR5 signaling leads to the conversion of thyroxine (T4) to triiodothyronine (T3) and thus to stimulation of UCP1 and uncoupling protein 2 (UCP2) production, β oxidation, oxidative phosphorylation and energy uncoupling [165]. TGR5 ligands are therefore potential drug candidates for treatment of obesity and its adverse effects [146].

One of the initial studies on TGR5 concerns its role in immune cells and has linked TGR5 to the immuno-modulatory properties of BAs. The inhibition of inflammation through TGR5 activation is caused by elevated levels of cAMP and can be considered beneficial for atherosclerosis, steatosis, and obesity [144, 146, 160, 166]. The precise mechanism leading to cAMP synthesis starts by TGR5 activation which is followed by release of the $G\alpha_s$ subunit and activation of AC. Increased intracellular concentration of the cAMP leads to the phosphorylation of cAMP-response element-binding protein (CREB) that mediates the activation of cAMP response elements (CREs) of target genes [165] (Figure 9). In accordance with this, BAs were found to increase cAMP and thus reduced the phagocytic activity of immune cells and inhibited LPS-induced production of several pro-inflammatory cytokines (TNFα, IL1α, IL1β, IL6, and IL8) [84, 144, 160].

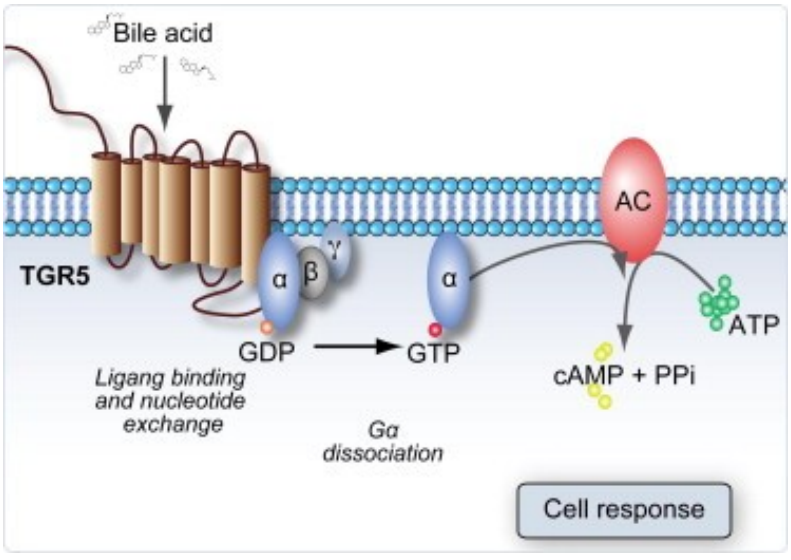

Figure 9: BAs, as ligands for TGR5 are able to induce cAMP:

GDP, guanosin diphosphate; GTP, guanosin triphosphate; PPi, pyrophosphate; ATP, adenosine triphosphate; AC, adenylate cyclase [144].

2 AIMS OF THE THESIS

Obesity is associated with a state of low-grade inflammation on systemic and AT level and it is believed that play a role in the development of obesity-related metabolic disorders. The source of inflammatory mediators (molecules inducing the state of low-grade inflammation) within obese AT is not known, but hypertrophic adipocytes, resident or infiltrating macrophages as well as other cells of SVF fraction may contribute to increased level of these molecules. It has been shown previously that also distribution of AT determinates the metabolic complications related to obesity. While the excess of AT in the upper part of the body is associated with low-grade inflammatory state and other pathologies related with obesity, the accumulation of fat in the lower part of the body appears to be protective and associated with lower morbidity and mortality. The underlying cause of beneficial effect of gluteal AT in obese body is not elucidated. Only few studies tried to shed light on the issue of protective mechanism of gluteofemoral fat in the body: decreased basal lipolysis or higher capacity for fat storage (higher activity of LPL) in gluteofemoral adipocytes are possible explanations. Another potential but not studied mechanism could be the lower production of pro-inflammatory mediators by gluteal AT when compared to abdominal AT. Moreover, nothing is known about the effect of calorie restriction, an important treatment of obesity, on this inflammatory status of gluteal AT.

AT expands through either hypertrophy or hyperplasia. It is assumed that from these two mechanisms of AT growth, hyperplasia is more salubrious: people with higher number of small adipocytes are metabolically healthier compared to obese with hypertrophic adipocytes. However, the differentiation of new (small) adipocytes is limited in majority of obese humans and it remains unknown whether this adipogenic capacity of adipose precursors can be modified by calorie restriction-induced weight loss.

In animal models it has been shown that AT functions can be also influenced by naturally occurring BAs, the end-products of cholesterol synthesis in hepatocytes. Except for their role in treatment of liver´s diseases and lipid metabolism, BAs are able to modulate AT metabolic state like energy expenditure, adipogenesis and IR, modulate inflammatory state and act as chaperones in cells during the ERS. Nevertheless, available data on their effect on adipogenic capacity or inflammatory status of human adipocytes are very limited.

Not only the excess of AT but also food intake are associated with transiently elevated concentrations of inflammatory mediators in the bloodstream, so-called postprandial inflammation. Despite the fact that this inflammation is in healthy individuals resolved within several hours, it is exaggerated and prolonged in obese and T2DM subjects. The orally administered nutrients have a direct impact on the activation of blood cells that are the source of immune cells infiltrating AT. Thus, prolonged postprandial inflammation may further worsen inflammation of AT in obese and T2DM subjects. The exact molecular triggers that lead to the postprandial inflammation are however not fully elucidated.

Specific aims therefore were:

Part I (studies evaluating the effects of long term dietary interventions)

- To compare the expression profile of pro-inflammatory markers (cytokines and macrophage markers) between subcutaneous abdominal and gluteal AT in basal state and during the dietary intervention.
- To compare the secretory profiles and the adipogenic capacities of precursors of adipose cells derived from AT before and after weight loss.

Part II (studies focused on ERS and chemical chaperons)

- To determine whether chemical chaperones UDCA and its taurine conjugate, TUDCA, are able to suppress the stress of endoplasmic reticulum in human adipose cells To analyze effects of these BAs on human preadipocytes and differentiated adipocytes derived from paired samples of subcutaneous abdominal and gluteal AT.
- To determine whether inflammation induced by consumption of high fat meal is linked with stress of endoplasmic reticulum and whether UDCA is able to modify/prevent the formation of the pro-inflammatory state and stress of endoplasmic reticulum *in vivo*.

3 RESULTS

List of publications

Part I

1. Expression of inflammation-related genes in gluteal and abdominal subcutaneous adipose tissue during weight-reducing dietary intervention in obese women **(*page 32)***

Lucia Mališová, Lenka Rossmeislová, Zuzana Kováčová, Jana Kračmerová, Michaela Tencerová, Dominique Langin, Michaela Šiklová-Vítková and Vladimír Štich

Physiological Research, 2014, March, 63(1): 73-82

2. Weight loss improves the adipogenic capacity of human preadipocytes and modulates their secretory profile **(*page 43*)**

Lenka Rossmeislová, Lucia Mališová, Jana Kračmerová,Michaela Tencerová, Zuzana Kováčová, Michal Koc, Michaela Šiklová-Vítková, Nathalie Viquerie, Dominique Langin, and Vladimír Štich

Diabetes, 2013, June, 62(6): 1990-1995

Part II

3. Ursodeoxycholic Acid but not Tauroursodeoxycholic Acid inhibits proliferation and differentiation of human subcutaneous adipocytes **(*page 50)***

Lucia Mališová, Zuzana Kováčová, Michal Koc, Jana Kračmerová, Vladimír Štich, Lenka Rossmeislová

Plos One, 2013, December, 8(12):e82086

4. Postprandial inflammation is not associated with endoplasmic reticulum stress in PBMC from healthy lean men **(*page 62*)**

Jana Kračmerová, Eva Czudková, Michal Koc, Lucia Mališová, Michaela Šiklová, Vladimír Štich and Lenka Rossmeislová

British Journal of Nutrition, 2014, April, in press

COMENTS TO THE RESULTS

According to the aims, four clinical studies were performed and the results were published as original papers presented below (the last one has been accepted for publication in April 2014). In the first part, two longitudinal studies were designed to examine the effects of diet-induced weight loss on 1. inflammation status of gluteal AT in comparison with abdominal AT and 2. differentiation and secretory capacity of preadipocytes and differentiated adipocytes (part 1, study: 1-2). In the second part, two clinical studies were performed to reveal 3. the effect of BAs on AT cells (part 2, study 3) *in vitro* and 4. the relationship between stress of endoplasmic reticulum and postprandial inflammation in blood cells *in vivo* in humans (part 2, study 4).

Part I

Body fat distribution is an important determinant of the risk of cardiovascular and metabolic diseases. The adverse effect of upper body fat accumulation has been described in numerous studies. Despite the fact that the protective role of the lower body fat accumulation has been confirmed in many studies [167-169], the underlying cause of metabolic differences between these two depots have not been fully elucidated. Based on the link between pro-inflammatory state of AT and obesity-related metabolic disturbance [118, 170-172], we examined, **in the first study,** the hypothesis that the different clinical impact of gluteal and abdominal AT could be based on lower pro-inflammatory profile of gluteal AT. Till now only a limited number of studies were dedicated to the comparison of inflammatory profile of these two depots and no data were provided on inflammatory status of gluteal AT during dietary intervention.

Therefore, 14 pre-menopausal women underwent dietary intervention consisting of 3 periods: very low calorie diet (VLCD), low calorie diet (LCD), followed by weight maintenance (WM) phase. The whole dietary intervention lasted 6 months and led to significant weight loss and improvement of clinical parameters including insulin sensitivity, assessed by homeostatic model assessment insulin resistence (HOMA-IR) index. Paired samples of gluteal and abdominal subcutaneous AT were acquired by needle biopsy in 3 phases of the dietary intervention and mRNA expression of cytokines and macrophage markers, considered as markers of inflammatory status of AT, was analyzed

by real-time polymerase chain reaction (RT PCR). Regarding the gene expression in basal state, there were no major differences in mRNA levels of analyzed inflammatory markers between abdominal and gluteal AT. This result is in line with previous report [173]. On the other hand, contrary to the expectations, the difference was found only in the pattern of 3 cytokines in gluteal AT compared to abdominal AT during the dietary intervention. Moreover, lack of correlation in gene expression between both depots underlines the facts that no direct quantitative association of the changes in gene expression exists between compared depots. Therefore, our results suggest that the differences between the clinical impact of the subcutaneous gluteal AT (sGAT) and subcutaneous abdominal AT (sAAT) are not associated with pro-inflammatory features of both depot.

To sum up, this study was focused on the comparison of inflammatory status of two depots before, during and after the weight loss. The data showed that weight loss induced by 6 months lasted dietary intervention led to the similar changes in gene expression of pro-inflammatory markers in both sAAT and sGAT. Moreover, there were no major differences in gene expression in basal state between both examined depots. These results are presented in *Paper 1* (page 32).

Among two mechanisms which lead to AT expansion, hyperplasia is considered as metabolically beneficial: small adipocytes are more insulin-sensitive and show a normal adipokine and cytokine production when compared to hypertrophic adipocytes [174]. The subjects with hyperplastic obesity show less metabolic abnormalities when compared to hypertrophic obesity. The process prerequisite for AT hyperplasia is the adipogenic conversion of preadipocytes but AT cells derived from obese patients exhibit impaired adipogenic capacity [42, 159]. The factors influencing sensitivity of human preadipocytes to adipogenic stimuli remain mostly unknown. Moreover, data concerning the adipogenic capacity of adipocytes after moderate weight loss are missing.

Therefore, **the second study** was focused on investigation of the effect of diet induced weight loss on preadipocyte characteristics.

To analyze the differentiation capacity and secretion profile of adipocytes, 23 obese, pre-menopausal women were included into 5-6 months lasting 2 phases dietary intervention: 3 months' LCD and subsequent 3 months´ WM. Adipocyte characteristics were examined under *in vitro* conditions: cell cultures were derived from

adipocyte precursors acquired by needle biopsy in abdominal region before and after dietary intervention.

In line with the results of other studies, our data showed that weight loss leads to the improvement of metabolic parameters of all patients [117, 175]. The oil red O staining of neutral lipids and gene expression analysis showed that the adipogenic potential of preadipocytes increased after weight loss. The enhanced adipogenic capacity was confirmed also on the mRNA levels of PPARγ and lipogenic genes. Notably, preadipocytes derived after weight loss exhibited a marked down-regulation of runt-related transcription factor 2 (RUNX2), a transcription factor favoring osteogenic differentiation [176], suggesting that weight loss favors the adipogenic differentiation through inhibition of alternative lineage programs (e.g., osteogenesis).

The second aim of this study was to estimate the effect of weight loss on the secretory capacity of *in vitro* differentiated adipocytes. Adipocytes derived from cells after weight loss exhibited lower pro-inflammatory profile, as they expressed and secreted less MCP1 or IL8, two chemokines associated with AT inflammation in obesity. Moreover, weight loss was associated with the enhanced secretion of insulin sensitizing adipokine, adiponectin, by *in vitro* differentiated adipocytes.

Taken together, we found that weight loss increased adipogenic capacity of preadipocytes and shifts their secretion profile to less pro-inflammatory state. These results are presented in *Paper 2* (page 43).

Part II

Stress of endoplasmic reticulum has been suggested to be one of the molecular triggers of adipocyte dysfunction [177] and chronic low-grade inflammation accompanying obesity [130]. It can be alleviated by chemical chaperons such as BAs [138]. BAs currently used to treat cholestasis, might potentially lessen the adverse metabolic effects of obesity. They could regulate gene expression through pathways different from their chaperon action, namely through activation of their own receptors. Regarding the effect of BAs on adipocytes metabolism, the data from animal studies are available but studies on human adipocytes are missing. Therefore, in the **third paper** we investigated effects of ursodeoxycholic acid (UDCA) and tauroursodeoxycholic acid (TUDCA) on preadipocytes and adipocytes derived from human AT.

For the study, 10 pre-menopausal obese women were recruited. In order to study differences between sAAT and sGAT, preadipocytes used to study the effect of BAs were

isolated from AT acquired by needle biopsy of both depots. All experiments were then performed *in vitro*.

Proliferation of preadipocytes estimated by colorimetric assay MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)) revealed that UDCA, but not TUDCA, has strong anti-proliferative effects, as it almost completely blunted the division of cells from both depots. To investigate how UDCA inhibits the growth of cells, fluorescence-activated cell sorting (FACS) analysis of cell cycle was performed. UDCA induced arrest of cell cycle in G2/M phase and so inhibited the proliferation capacity of examined cells. Also the effect of UDCA on adipocyte differentiation was strikingly inhibitory, as confirmed both, oil red O staining of neutral lipids and the expression of key adipogenic markers. Because the inhibitory effect of UDCA on adipogenesis was unexpected, the mechanism of this phenomenon was further researched. One of the hypotheses was that UDCA acts through FXR receptor. Despite the fact, that we documented for the first time that human adipocytes express FXRα, anti-adipogenic effect of this BAs was not dependent on FXR activation. However, we brought the evidence that UDCA treatment leads to sustained phosphorylation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) which has been implicated in phosphorylation and consequently inhibition of master adipogenic regulator PPARγ [178].

The chaperone like properties indicated BAs as a potential therapy of obesity-related comorbidities. However, we could not confirm this beneficial effect of BAs in human adipocytes as neither of them was able to alleviate acute ERS induced by tunicamycin in these cells; none from 3 pathways of ERS was decreased as documented on the expression levels of XBP1, HSPA5 and ATF4.

Finally, we tested whether BAs are able to reduce the pro-inflammatory status of adipocytes and thus eventually to decrease attraction of monocytes/macrophages into AT. Quite contrary, UDCA strongly up-regulated mRNA levels of chemokines and cytokines independently of activation of NFκB pathway.

To summarize, the effect of individual BAs was different: not only proliferation of preadipocytes but also differentiation of adipocytes was inhibited specifically by UDCA but not by TUDCA. The effect of both BAs on adipocytes and its precursors were similar in cells from both examined depots. These results are presented in *Paper 3* (page 50).

The fourth study was focused on the investigation of molecular triggers that can lead to postprandial inflammation characterized by elevated level of cytokines and activated leukocytes [179-180]. Several studies [177, 181-182] have shown that treatment of cells with saturated FA and high level of glucose is accompanied by ERS development [177, 181-182] and that ERS-associated inflammation may be alleviated by chemical chaperons [139].

Therefore, 10 healthy men were recruited to a randomized blind crossover trial consisting of two one-day studies. The participants were given lactose (placebo-control group) or ursodeoxycholic acid (URSOSAN) and consequently high fat meal (HFM) containing high content of lipids and sugars (test meal from McDonalds, 6151 kJ, 32.8% carbohydrates, 47.4% lipids, 11.3% proteins). Blood and PBMC were analyzed before meal and after 3 hours postprandially. Quantification of mRNA expression of selected genes involved in the lipid metabolism/signaling and coding pro-inflammatory cytokines as well as ERS markers was performed in two populations of PBMC, monocytes and lymphocytes. The two populations were separated according to the expression of CD14 marker.

First, we documented the effect of „fast-food„ type of diet on postprandial changes of basic metabolites: while glycemia remained unchanged, levels of TG, glycerol or NEFA concentration increased postprandially. FACS analysis of white blood cells revealed that HFM induced a postprandial inflammation; absolute number of all measured cells, granulocytes, monocytes and leukocytes increased regardless of treatment to placebo or URSOSAN. Moreover, postprandial inflammation was accompanied by elevated level of CD11c, a marker of monocyte activation [183]. However, several hours of exposition to dietary lipids was not sufficient to induce substantial expression changes of genes involved in lipid metabolism/signaling (TLR2, TLR4, PPARγ, PPARα) in CD14+ cells.

To determine whether postprandial inflammation could be triggered by enhanced ERS, we analysed mRNA level of ERS markers representing of all 3 arms of UPR. Following HFM challenge, mRNA expression of tested cytokines (IL1β, IL8, MCP1 and TNFα) and miR181 was elevated while majority of ERS markers (HSPA5, ATF4, EDEM1, XBP1 and DNAJC3) was not altered in PBMC in both placebo and UDCA conditions.

Thus, HFM-induced inflammation detectable on the level of gene expression in PBMC was not associated with elevated ERS and could not be prevented by UDCA. These results are presented in *Paper 4* (page 62).

Paper 1

Expression of inflammation-related genes in gluteal and abdominal subcutaneous adipose tissue during weight-reducing dietary intervention in obese women

Lucia Mališová, Lenka Rossmeislová, Zuzana Kováčová, Jana Kračmerová, Michaela Tencerová, Dominique Langin, Michaela Šiklová-Vítková and Vladimír Štich

Physiological Research, 2014, March, 63(1): 73-82

Physiol. Res. 63: 73-82, 2014

Expression of Inflammation-Related Genes in Gluteal and Abdominal Subcutaneous Adipose Tissue During Weight-Reducing Dietary Intervention in Obese Women

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Summary

Accumulation of adipose tissue in lower body lowers risk of cardiovascular and metabolic disorders. The molecular basis of this protective effect of gluteofemoral depot is not clear. The aim of this study was to compare the profile of expression of inflammation-related genes in subcutaneous gluteal (sGAT) and abdominal (sAAT) adipose tissue at baseline and in response to multiphase weight-reducing dietary intervention (DI). 14 premenopausal healthy obese women underwent a 6 months' DI consisting of 1 month very-low-calorie-diet (VLCD), subsequent 2 months' low-calorie-diet and 3 months' weight maintenance diet (WM). Paired samples of sGAT and sAAT were obtained before and at the end of VLCD and WM periods. mRNA expression of 17 genes (macrophage markers, cytokines) was measured using RT-qPCR on chip-platform. At baseline, there were no differences in gene expression of macrophage markers and cytokines between sGAT and sAAT. The dynamic changes induced by DI were similar in both depots for all genes except for three cytokines (IL6, IL10, CCL2) that differed in their response during weight maintenance phase. The results show that, in obese women, there are no major differences between sGAT and sAAT in expression of inflammation-related genes at baseline conditions and in response to the weight-reducing DI.

Key words

Body fat distribution • Hypocaloric diet • Macrophages • Cytokines

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Introduction

Obesity is associated with higher risk of metabolic and cardiovascular diseases. In addition to body mass index (BMI), body fat distribution plays a major role in the development of the above mentioned diseases. While upper body fat accumulation is associated with increased obesity-related health risk, the lower body fat accumulation was shown to be linked with the reduction of metabolic (Snijder *et al.* 2004), cardiovascular risk (Canoy *et al.* 2007, Faloia *et al.* 2009, Seidell *et al.* 2001) and with lower morbidity and mortality (Folsom *et al.* 1993, Pischon *et al.* 2008). The increased amount of lower body fat – expressed as hip circumference – was associated with lower triacylglycerol and higher HDL cholesterol levels (Ruige and Van Gaal 2009).

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Possible mechanisms that may contribute to the subcutaneous gluteal adipose tissue (sGAT) protective role have not been fully elucidated. Different uptake and release of fatty acids in the subcutaneous abdominal (sAAT) when compared to sGAT have been suggested as a candidate underlying cause (Berman *et al.* 1998, Berman *et al.* 2004). In several studies, the activity of lipoprotein lipase (LPL) was found to be higher (Arner *et al.* 1991, Ferrara *et al.* 2002) in sGAT in obese women. This might suggest a higher capacity of sGAT for lipid accumulation.

In addition to alteration of adipose tissue (AT) fatty acid handling, the obesity-related metabolic disturbances are linked with pro-inflammatory state of AT characterized by enhanced recruitment of macrophages in AT and modified AT secretion of cytokines (Klimcakova *et al.* 2011, O'Hara *et al.* 2009, Suganami and Ogawa 2010, Trayhurn and Wood 2004). Thus, it may be hypothesized that differences in the inflammation-related characteristics underlie the differences in metabolic role of sGAT when compared with sAAT. Few studies paid attention to this topic. Recently, Evans *et al.* (2011) showed that sGAT had, contrary to the expectation, greater mRNA expression of a set of pro-inflammatory genes than sAAT.

Thus the first aim of our study was to compare, in obese women, expression of wider range of cytokines and macrophage markers in sGAT vs. sAAT (selected according to our previous studies of Capel *et al.* 2009, Klimcakova *et al.* 2011, Siklova-Vitkova *et al.* 2012) and their association with indices of metabolic syndrome. Our second aim was to explore the regulation of expression of the above mentioned genes in a dynamic condition that was realized by a dietary intervention (DI) using hypocaloric diet. It was shown previously that hypocaloric diet-induced changes in adipocyte lipolysis (Mauriege *et al.* 1999) or in adipocyte size (Bjorntorp *et al.* 1975) were less pronounced in sGAT when compared with sAAT. We hypothesized that this impaired responsiveness, or "inflexibility", of sGAT might also appear in respect to the diet-induced modulation of gene expression of immunity-related genes. Thus, we measured mRNA expression of the respective genes in the paired samples of sGAT and sAAT obtained in obese women before and during multiple phases of a 6 months' hypocaloric DI.

Materials and Methods

Subjects

14 premenopausal obese women (BMI 34.2±2.6 kg/m^2, range 27-49 years) without medication and diseases except for obesity participated in this study. Their body weight had been stable for 3 months prior to the examination. The informed consent was obtained from each patient before the study. The study was performed according to the Declaration of Helsinki protocols and was approved by Ethical Committee of the Third Faculty of Medicine, Charles University in Prague.

Dietary intervention and clinical investigation

The entire DI lasted 6 months. During the first dietary period, obese subjects received a very low calorie diet (VLCD) of 800 kcal/day (liquid formula diet, Redita, Promil, Czech Republic) for 1 month. The subsequent period consisted of a 2 months' low-calorie diet (LCD) followed by 3 months' weight maintenance (WM) diet. LCD was designed to provide 600 kcal/day 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. Patients consulted a dietician once a week during the first 3 months of the program and once a month during subsequent phase. They provided a written 3 days' dietary record at each dietary consultation.

Complete clinical investigation including anthropometric measurements, blood sampling and AT biopsies was performed in the morning in the fasting state before the beginning of the diet and at the end of VLCD and WM periods.

The whole body composition was evaluated by multi-frequency bioimpedance (Bodystat, Quad scan 4000, Isle of Man, UK). The blood was collected and centrifuged at 1300 RPM, 4 °C, separated plasma was stored at −80 °C until analysis. The paired samples of subcutaneous AT were obtained from the subcutaneous abdominal (10 cm lateral to the umbilicus) and gluteal (right upper quadrant) region using needle biopsy under local anesthesia (1 % Xylocaine). AT samples were obtained from superficial sAAT, as we verified on several occasions using ultrasonography. AT was washed in physiological saline, aliquoted, snap-frozen in liquid nitrogen and stored at −80 °C until processing.

Laboratory measurements

Plasma glucose was determined using the glucose-oxidase technique (Beckman Instruments, Fullerton, CA). Plasma insulin was measured using an Immunotech Insulin Irma kit (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

Table 1. Clinical characteristics of subjects and plasma levels of cytokines before dietary intervention (basal) and at the end of VLCD and weight maintenance (WM) phase of the dietary intervention.

	Basal	VLCD	WM
Age (year)	27-49		
BMI (kg/m²)	34.2±0.2	31.5±0.2***	30.4±0.2†††
Weight (kg)	93.5 ± 0.6	86.0±0.6***	83.1±0.7†††
Waist circumference (cm)	102.3±0.4	95.4±0.6***	93.6±0.6†††
Hip circumference (cm)	119.1±0.5	114.1±0.5***	112.1±0.5†††
Waist to hip ratio (cm)	0.861±0.0	0.838±0.0*	0.836±0.0††
Fat mass (%)	41.9±0.3	39.4±0.4***	37.2±0.4†††
FFM (%)	58.1±0.3	60.1±0.4*	63.0±0.5†††
Glucose (mmol/l)	4.9±0.4	4.6±0.0*	4.7±0.0
Insulin (mIU/l)	11.7±0.8	8.0±0.3*	9.0±0.4†
FFA (μmol/l)	842±60.1	1190±28.2*	676±13.5†
Triglycerides (mmol/l)	1.6±0.0	1.2±0.0	1.5±0.0
HDL cholesterol (mmol/l)	2.0±0.0	1.2±0.0	1.6±0.0
Total cholesterol (mmol/l)	5.2±0.0	4.3±0.0***	5.2±0.0
HOMA-IR	2.6±0.1	1.7±0.1*	1.9±0.1††
hs-CRP (mg/l)	5.8±0.4	4.2±0.3	4.0±0.3†
IL10 (pg/ml)	1.4±0.4	1.5±0.6	1.5±0.6
IL6 (pg/ml)	3.7±0.1	3.1±0.1	2.6±0.1
TNF (pg/ml)	2.0±0.1	2.3±0.1**	2.0±0.1
CCL2 (pg/ml)	104.3±4.7	103.1±3.2	86.4±2.7†

Values are means ± SEM, n=14. Significance was set as follows: VLCD vs. basal: * p<0.05, ** p<0.01, *** p<0.001, WM phase vs. basal: † p<0.05, †† p<0.01, ††† p<0.001. BMI, body mass index; CCL2, chemokine (C-C motif) ligand 2; FFM, fat-free mass; HOMA-IR, homeostasis model assessment of the insulin resistance index; hs-CRP, high-sensitivity C-reactive protein; IL6, interleukin 6; IL10, interleukin 10; FFA, free fatty acids; TNF, tumor necrosis factor

mmol/l) / 22.5). Plasma levels of other relevant substances were determined using standard clinical biochemistry methods. Plasma levels of cytokines were determined using multiplex human cytokines Milliplex panels (Millipore-Merck, Bedford, MA, USA).

Gene expression analysis

Total RNA was isolated from 100-300 mg aliquots of AT using RNeasy Lipid Tissue RNA Mini kit (Qiagen, Hilden, Germany). RNA concentration was measured by Nanodrop1000 (Thermo Fisher Scientific, Wilmington, Delaware, USA). Genomic DNA was removed by DNAse I treatment (Invitrogen, Carlsbad, CA, USA). cDNA was obtained by reverse transcription (High Capacity cDNA Reverse Transcription Kit, Applied Biosystem, Carlsbad, CA, USA) using 200 ng of total RNA. 1 ng of cDNA was then preamplified to improve detection of target genes during subsequent Real Time qPCR (16 cycles, TaqMan Pre Amp Master Mix Kit, Applied Biosystem). For the preamplification, 20 x TaqMan gene expression assays of all target genes were pooled together and diluted with 1x TE buffer to the final concentration 0.2x (each probe). The RT-qPCR was performed on Biomark Real Time qPCR system and 96x96 chip (Fluidigm, USA) in triplicates. This part of analysis was carried out as a paid service by Biotechnology Institute, AS CR.

Expression of 17 genes grouped according to their origin or function was measured: cytokines (IL6, TNF, CCL2, CXCL1, IL10, TGFβ1, IL10RA) and cytokine receptor (CCR2), macrophage markers (SPP1, CD68, MSR1, PLA2G7, ACP5, FCGBP, CD14, TLR4, TLR2). Macrophage markers were selected according to the work of Capel *et al.* (2009) and Klimcakova *et al.* (2011) and cytokines produced predominantly by cells of stromavascular fraction were chosen according to study of Siklova-Vitkova *et al.* (2012) so that comparisons of outcomes between this and our previous studies were enabled. Expression data were normalized to expression of reference gene, PPIA, and delta Ct was

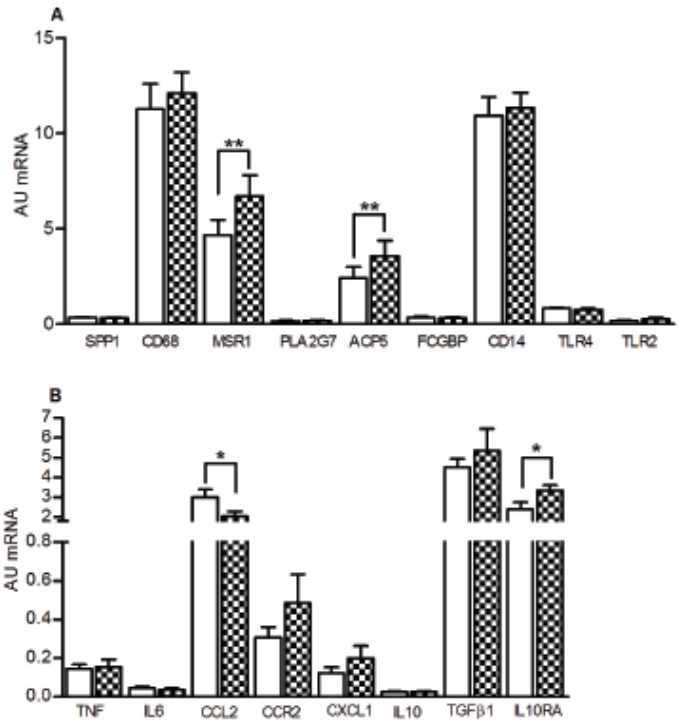

Fig. 1. Adipose tissue gene expression in basal state, before the diet. **A:** macrophage markers, **B:** cytokines. Subcutaneous abdominal adipose tissue (open columns), subcutaneous gluteal adipose tissue (cross-hatched columns). Each column represents mean of gene expression levels calculated as delta Ct (normalized to PPIA). * $p<0.05$, ** $p<0.01$

log-transformed for statistical analysis.

Statistical analysis

The data from RT-qPCR were analyzed with Genex software (MultiD Analysis AB, Sweden) and SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). For all the analyses the data were log-transformed. One way ANOVA was used for comparison of gene expression separately in each depot, the effect of sAAT vs. sGAT on the diet-induced changes was estimated by two way ANOVA with repetitive measures and Tukey´s posthoc analysis. The level of significance was set at $p<0.05$. Clinical and anthropometrical data were analyzed with GraphPad Prism 5.0. (La Jolla, CA, USA). Correlations were performed using Pearson's parametric test.

Results

Effect of dietary intervention on anthropometrical and plasma variables

The clinical data of the entire group of subjects at baseline and during the DI are presented in Table 1. When compared to baseline, the subjects' body weight decreased by 8.0 % after the VLCD and decreased further during subsequent period so that the weight loss at the end of WM represented 11.1 % of the original weight. BMI, fat mass, waist and hip circumference showed a similar pattern. The relative decrease of waist circumference was greater than that of hip circumference at the end of VLCD as well as at the end of the entire DI (VLCD: waist: −6.8±0.3 %, hip: −4.2±0.3 %, $P<0.05$, the end of DI: waist: −8.6±0.2 %, hip: −5.9±0.3 %, $P<0.01$). Plasma levels of insulin were lower at the end of VLCD when compared to baseline condition and remained lower at the end of WM. Glycemia and total cholesterol decreased after VLCD and returned to the baseline levels at WM. Free fatty acid levels were increased after VLCD and decreased below the baseline values at the end of the WM. Insulin resistance assessed by HOMA-IR decreased during VLCD and remained reduced at the end of WM phase. The changes of plasma levels of cytokines IL10, IL6, TNF, CCL2 were in line with our previous study (Siklova-Vitkova *et al.* 2012).

Comparison of gene expression in gluteal and abdominal subcutaneous AT in obese women at pre-diet condition (Fig. 1)

To compare gene expression profile in sAAT and sGAT, we measured mRNA expression of 17 genes divided into 2 functional groups: macrophage markers (9 genes), cytokines (8 genes), and reference gene PPIA. The expression of macrophage markers was similar in

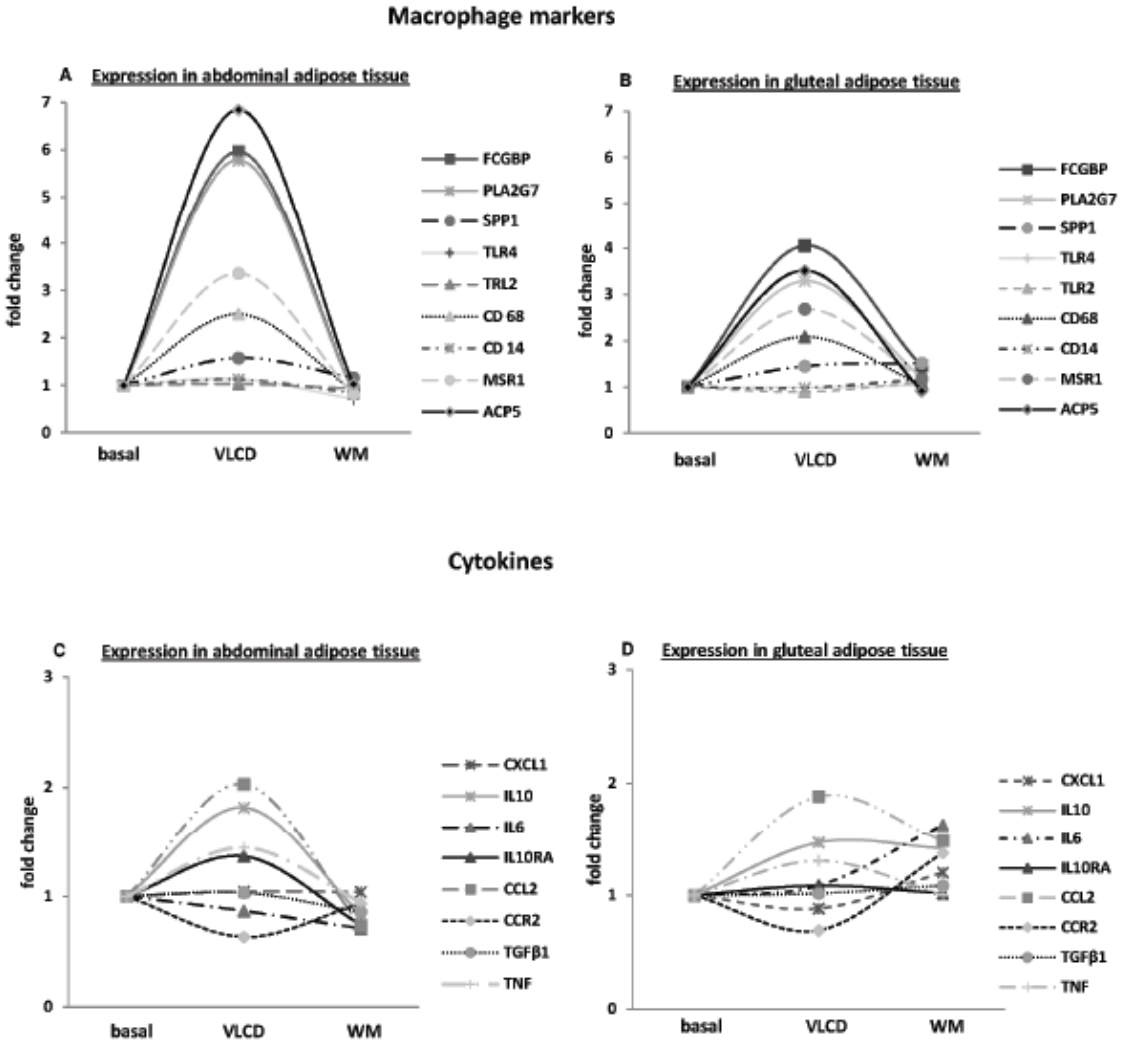

Fig. 2. Profile of gene expression in subcutaneous abdominal (sAAT) and gluteal (sGAT) adipose tissue during the two phases (VLCD and WM) of dietary intervention. **A** and **B:** Expression of macrophage markers in sAAT (**A**) and sGAT (**B**). **C** and **D:** Expression of cytokines in sAAT (**C**) and sGAT (**D**). The data are presented as fold change in respect to the pre-diet (basal) levels. Data are presented as means ± SEM.

both depots except for two genes, ACP5 and MSR1 that had higher expression in sGAT compared to sAAT (Fig. 1A). There were no depot-related differences in the expression of all measured cytokines (TNF, IL6, CCR2, CXCL1, IL10, TGFβ1) with exception of IL10RA (higher in sGAT) and CCL2 (lower in sGAT) (Fig. 1B).

Effect of dietary intervention on gene expression in gluteal and abdominal subcutaneous AT (Fig. 2, Table 2)

The evolution of the mRNA expression for individual genes during DI is shown in Figure 2. Schematic representation of the direction and significance of the diet-induced changes of gene expression during VLCD and during the entire DI in each depot is presented in Table 2.

Macrophage markers (Fig. 2A-2B)

The expression of 6 macrophage markers (CD68, ACP5, FCGBP, MSR1, PLA2G7, SPP1) increased during VLCD in both depots while expression of 3 remaining markers (CD14, TLR4, TLR2) was not changed in both depots. At the end of WM, the mRNA levels of all macrophage markers were not different from the baseline values in both depots (Fig. 2A-2B).

Cytokines (Fig. 2C-2D)

During VLCD, the mRNA levels of 5 cytokines (TNF, IL6, IL10RA, TGFβ1, CXCL1) did not change in either depot, CCL2 increased and CCR2 decreased in both depots and IL10 showed a significant increase

Table 2. Changes of gene expression in subcutaneous abdominal (sAAT) and subcutaneous gluteal (sGAT) adipose tissue during VLCD and weight maintenance (WM) phases of dietary intervention in relation to the pre-diet (basal) level.

Name of gene	sAAT		sGAT	
	VLCD	WM	VLCD	WM
Macrophage markers				
CD68	**↑	↔	*↑	↔
ACP5	***↑	↔	**↑	↔
CD14	↔	↔	↔	↔
FCGBP	***↑	↔	**↑	↔
MSR1	***↑	↔	**↑	↔
PLA2G7	**↑	↔	*↑	↔
SPP1	*↑	↔	*↑	↔
TLR4	↔	↔	↔	↔
TLR2	↔	↔	↔	↔
Cytokines				
TNF	↔	*↓	↔	↔
IL10	*↑	*↓	↔	*↑ (##)
IL6	↔	*↓	↔	↔ (##)
IL10RA	↔	**↓	↔	↔
CCL2	*↑	*↓	*↑	*↑ (##)
CCR2	**↓	↔	**↓	↔
TGFβ	↔	↔	↔	↔
CXCL1	↔	↔	↔	↔

↑ denotes a higher level of gene expression at the end of respective dietary phase (VLCD or WM) in relation to the pre-diet (basal) level; ↓ denotes a lower level of gene expression in relation to the pre-diet (basal) level; ↔ denotes no change in gene expression in relation to the pre-diet (basal) level; *,**,*** denotes the level of significance ($p<0.05$, $p<0.01$, $p<0.001$, respectively) of the difference between the value at the end of respective dietary phase vs. pre-diet (basal) level; ## denotes the level of significance ($p<0.01$) of the difference between sGAT and sAAT in respect to the change of gene expression during respective dietary phase (as assessed by two-way ANOVA)

selectively in sAAT.

At the end of DI, IL10 and CCL2 mRNA levels were higher than baseline values in sGAT while, in sAAT, the mRNA levels of these two cytokines as well as those of IL6, IL10RA and TNF were lower when compared with the baseline.

The diet-induced changes of mRNA levels were similar in the two depots for all of the cytokine genes with 3 exceptions: during the entire DI, expression of IL10, IL6, and CCL2 decreased in sAAT while it increased or was unaltered in sGAT.

Summary of the comparison of the diet-induced responses of the gene expression in sGAT and sAAT

For majority of measured genes no differences in the diet-induced changes between sGAT and sAAT were found. Different responses were found only for the 3 cytokines (IL10, IL6, CCL2): their decrease during the entire DI was pronounced in sAAT but not in sGAT.

Correlations

No correlations between the diet-induced changes of mRNA expression of examined genes in sGAT vs. sAAT were found. No correlations were found between the diet-induced changes of plasma levels of IL10, IL6, TNF, CCL2 and the changes in the expression of these genes in either sGAT or sAAT. In addition, no correlations between the diet-induced changes of mRNA expression of examined genes and those of BMI or HOMA-IR were found.

Discussion

It was hypothesized that the protective role of

AT accumulation in the lower body, in respect to cardiovascular risk and metabolic disturbances, might be based on the lower pro-inflammatory profile of sGAT. However, recent work of Evans *et al.* (2011) reported that the expression of several pro-inflammatory markers was higher in sGAT compared to sAAT in a mixed group of lean and obese black and white South African women. Our work extended the range of explored genes and showed that, in a wide group of cytokines and macrophage markers, there were, with exception of 4 genes, no differences between sAAT and sGAT at baseline. Taken together, our and Evans' results do not support the hypothesis of the lower pro-inflammatory profile of sGAT. This is in line with the finding reported by Tchoukalova *et al.* (2010) that subcutaneous abdominal and femoral fat depot did not differ in number of macrophages in lean men and women.

The main interest of this study lies in the comparison of gene regulation in sAAT vs. sGAT in dynamic condition represented by two phases of a 6 months' dietary intervention. The pattern of the expression of macrophage markers observed in this study, including the increase during initial VLCD phase is in agreement with our previous work carried out in sAAT in another cohort of subjects (Capel *et al.* 2009). The pattern is bi-phasic, characterized by an increased expression during VLCD and a decrease towards baseline values during subsequent weight maintenance phase of the diet. Increased expression of macrophage markers during VLCD might be associated with enhanced fatty acids release from adipocytes as a possible trigger of macrophage activation and infiltration mediated by TLR4 signaling as shown before (Kosteli *et al.* 2010, Suganami *et al.* 2005). Importantly, this bi-phasic response of macrophage markers expression was similar in sGAT when compared with sAAT (Fig. 2, Table 2) and the magnitudes of the diet-induced changes were not different in the two fat depots (Table 2). This finding suggests the same regulation of macrophage infiltration in sGAT and sAAT during weight-reducing dietary intervention.

The bi-phasic pattern of the diet-induced regulation was found also in the expression of cytokines derived predominantly from the cells of stromavascular fraction. This pattern was in accordance with previous results obtained in sAAT in a different cohort of subjects (Siklova-Vitkova *et al.* 2012). The magnitudes of the diet-induced changes were not different between sGAT and sAAT – except for three cytokines – IL6, IL10 and CCL2. The observed variability in respect to the individual cytokine genes is, again, in agreement with the above mentioned study of Siklova-Vitkova *et al.* (2012): in that study the same three cytokine genes were the only ones that showed a significant decrease in sAAT at the end of dietary intervention. This partial differential depot-related response – limited to the expression of the three cytokines – might be linked to differential response of endocannabinoid system as observed in the study of Bennetzen *et al.* (2011). In fact, during the weight-reducing diet the authors found different change of expression of cannabinoid receptor type 1 in sGAT when compared with sAAT. And endocannabinoids were shown to inhibit production of several proinflammatory cytokines in primary human Muller cells (Krishnan and Chatterjee 2012). This mechanism could be taken into account, although no such regulation has been reported in adipose tissue. Moreover, in resting condition, Rantalainen *et al.* (2011) found differential expression of 12 % of measured microRNA (e.g. miR146-5b, miR-21, miR155) in sGAT when compared with sAAT which could imply a differential expression of targets of these microRNA such as IL10 (Quinn and O'neill 2011) in the two fat depots.

The lack of correlations in gene expression changes between sAAT and sGAT suggests that, in spite of the similar pattern of the diet-induced response of the gene expression in the two fat depots, there is no direct quantitative association of the magnitude of the change between these two depots. In light of our and others results demonstrating the absence of major differences between sGAT and sAAT it has been suggested that the deleterious effect of upper body obesity could be mediated by the excess of visceral adipose tissue (VAT) and not excess of sAAT. Nevertheless, several studies showed that both, VAT and sAAT, are associated with the increased risk of metabolic profile and pro-inflammatory status (although the association was stronger with VAT) (Fox *et al.* 2007, Oka *et al.* 2010, Pou *et al.* 2007).

Furthermore, it should be noted that the present study compared sGAT and sAAT on transcriptional level. Due to the lack of sufficient amount of adipose tissue we did not explore the protein levels of cytokines in adipose tissues or their levels of secretion. It is not excluded that the underlying causes of different physiological impact of sGAT vs. sAAT may be at the level of translation or post-translational regulations of cytokine production or release.

It is to be noted that the results of this study are limited to women. Female adipose tissue shows different metabolic and endocrine characteristics (Kern *et al.* 2003, Montague *et al.* 1997) when compared with men. Moreover, the initial fat distribution in our set of women (mean WHR=0.861±0.0) might play a role in the diet-induced response of the two fat depots although the reports on the effect of initial fat distribution on the body fat reduction are not unequivocal (Svendsen *et al.* 1995, Jones and Edwards 1999).

In conclusion, we did not find major differences in mRNA levels of macrophage markers and cytokines between sAAT and sGAT at baseline condition or in the pattern of their regulation in response to two phases of hypocaloric weight-reducing dietary intervention (with exception of 3 cytokines that were differentially regulated during weight maintenance phase). Therefore, our results do not bring evidence of an altered pro-inflammatory status or an altered "responsiveness" of immune cells in sGAT when compared with sAAT. Further research elucidating the molecular base of the protective role of sGAT fat depot is therefore warranted.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Paper 2

Weight loss improves the adipogenic capacity of human preadipocytes and modulates their secretory profile

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BRIEF REPORT

Weight Loss Improves the Adipogenic Capacity of Human Preadipocytes and Modulates Their Secretory Profile

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Calorie restriction–induced weight loss is accompanied by profound changes in adipose tissue characteristics. To determine the effect of weight loss on differentiation of preadipocytes and secretory capacity of in vitro differentiated adipocytes, we established cultures of these cells from paired subcutaneous adipose tissue biopsies obtained before and at the end of weight-reducing dietary intervention (DI) in 23 obese women. Based on lipid accumulation and the expression of differentiation markers, in vitro adipogenesis increased after weight loss and it was accompanied by enhanced expression of genes involved in de novo lipogenesis. This effect of weight loss was not driven by changes of peroxisome proliferator–activated receptor γ sensitivity to rosiglitazone. Weight loss also enhanced the expression of adiponectin and leptin while reducing that of monocyte chemoattractant protein 1 and interleukin-8 by cultured adipocytes. Thus, the weight-reducing (DI) increased adipogenic capacity of preadipocytes and shifted their secretion toward lower inflammatory profile. Reprogramming of preadipocytes could represent an adaptation to weight loss leading to partial restoration of preobese adipose tissue traits and thus contribute to the improvement of metabolic status. However, enhanced adipogenesis could also contribute to the unwanted weight regain after initial weight loss. ***Diabetes* 62:1990–1995, 2013**

Worsening of metabolic health in obesity is associated with the hypertrophy of adipocytes (1). Indeed, the recruitment of new and small adipocytes improves insulin sensitivity (2). These cells have a high potential to store lipids and therefore alleviate peripheral lipotoxicity associated with whole-body insulin resistance. However, adipose stromavascular cells derived from obese donors exhibit impaired adipogenic capacity (3), and the factors influencing sensitivity of human preadipocytes to adipogenic stimuli in vivo remain unknown.

Weight reduction induced by hypocaloric diet is the key approach for treatment of obesity-related metabolic disturbances. A moderate loss of body weight induces an adaptation of human adipose tissue associated with improved whole-body metabolic status (4,5). We hypothesized that cell cultures of preadipocytes established from subcutaneous adipose tissue collected before and after a weight loss–inducing dietary intervention (DI) correspond to two distinct metabolic and nutritional stages of the donor. The current knowledge on intrinsic adipogenic and endocrine potential of these cells is based on and limited to cross-sectional studies. Here, we show that DI-induced weight loss increased the differentiation capacity of preadipocytes and shifted their secretion toward less inflammatory profile. This reprogramming of preadipocytes by weight loss could represent a cellular mechanism leading to the restoration of preobese traits of adipose tissue and correction of inflammatory status.

RESEARCH DESIGN AND METHODS

Subjects. Obese premenopausal women (n = 23) were recruited at the Third Faculty of Medicine of Charles University and University Hospital Kralovske Vinohrady, Prague, Czech Republic. Exclusion criteria were set as previously described (6). The study was performed according to the Declaration of Helsinki and was approved by the ethics committee of the Third Faculty of Medicine of Charles University. Volunteers signed informed consent before participation in the study.

DI and clinical investigation. The DI lasted 5–6 months. Participants reduced their calorie intake by 600 kcal/day in relation to the individually estimated energy requirement (initial resting metabolic rate multiplied by 1.3, the coefficient of correction for physical activity). Weight loss was achieved within the first 3 months, and then women were advised to keep the diet leading to the weight maintenance. Subjects consulted a dietitian once a week during the first 3 months and once a month during the weight-maintenance phase.

Clinical investigation was performed after an overnight fast before and at the end of DI. Anthropometric measurements, blood sampling, and needle biopsy of adipose tissue were performed as previously described (6). Briefly, after administration of local anesthesia (1% xylocaine), a 1- to 2-mm incision was made 10 cm laterally from umbilicus and a 12G needle coupled with syringe was used to aspirate fragments of superficial subcutaneous adipose tissue. On average, 1.5 g tissue was obtained (0.6–2.5 g).

Isolation and culture of preadipocytes. Adipose tissue was digested in 1.5 volume of collagenase I (300 units/mL; Biochrom, Berlin, Germany) for 60 min in 37°C shaking water bath and processed as previously described (7). Digested tissue was diluted with PBS/gentamicin and spun at 1,300 rpm for 5 min. Cells were then shaken forcefully to complete the dissociation from mature adipocytes and centrifuged. Pellet containing cells from the stromavascular fraction was incubated in erythrocyte lysis buffer for 10 min at room temperature. Cells were centrifuged, and without any filtration step, they were resuspended in PM4 medium (8) with 132 nmol/L insulin. PM4 was replaced every other day. Cells were subcultivated at 70% confluence; experiments were performed at passage 3. Differentiation of 2-day postconfluent cells was induced by Dulbecco's modified Eagle's/F12 medium supplemented with 66 nmol/L insulin, 1 μmol/L dexamethasone, 1 nmol/L T3, 0.1 μg/mL transferrin, 0.25 mmol/L isobutylmethylxanthine, and 1 μmol/L rosiglitazone. After 6 days, rosiglitazone and isobutylmethylxanthine were omitted and dexamethasone was replaced with 0.1 μmol/L cortisol. The differentiation continued until day 12. Medium conditioned for 24 h was then collected, and cells were harvested for RNA and protein analysis. Protein concentration was determined by BCA assay (Pierce Biotechnology, Rockford, IL). For experiments focused on the

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effect of peroxisome proliferator–activated receptor (PPAR)γ activation, cells were induced to differentiate in the media containing either 1 μmol/L rosiglitazone or DMSO. As nondifferentiating controls, preadipocytes switched to serum-free medium supplemented with transferrin and insulin were used.

Gene expression analysis. Total RNA was isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany). mRNA levels were measured using reverse transcription quantitative PCR (Applied Biosystems, Carlsbad, CA) (9). GUSB (glucuronidase beta) was used as an endogenous control. Results are expressed as $\Delta\Delta C_t$ (threshold cycle) values.

Cytokine analysis. Cytokines were measured by ELISA (interleukin [IL]-6 and monocyte chemoattractant protein [MCP]1, Ready-Go-sets, eBioscience, San Diego, CA; IL6 Quantikine HS, R&D Systems, Abingdon, U.K.; and adiponectin DuoSet, R&D Systems, Minneapolis, MN). Detection of adiponectin isoforms was performed by native polyacrylamide gel electrophoresis and Western blot (10). Chemiluminescent signal was detected on Kodak Image Station 4000R and analyzed by associated software.

Oil Red O and BODIPY staining. Cells were fixed, stained with Oil Red O (ORO), and analyzed as previously described (11) or stained with 1 μmol/L BODIPY 493/503 (Life Technologies) and DAPI. Standard curve from ORO stock was used to normalize data. The OD of eluates from 100% differentiated cells reached values of 40% of stock ORO. Images for BODIPY analysis were acquired on fluorescent microscope DMI6000 coupled with CCD camera (Leica Microsystems, Wetzler, Germany).

Statistical analysis. Data were analyzed using GraphPad Prism 5.0 software with Wilcoxon matched-pair signed rank or Mann-Whitney *U* test, as appropriate. The level of significance was set at $P < 0.05$.

RESULTS

Clinical characteristics of obese subjects. The clinical data of subjects before and after DI are listed in Table 1. Compared with baseline values, the subjects' body weight decreased by 9.7% and insulin resistance assessed by homeostasis model assessment of insulin resistance was reduced.

Preadipocytes derived from adipose tissue after weight loss exhibit increased adipogenesis. The stromavascular cells from adipose tissue samples were isolated, expanded, and differentiated into adipocytes. Cell cultures derived from the samples obtained after weight loss exhibited increased differentiation as evaluated by the ORO staining and mRNA expression of differentiation markers *aP2* and *PPARγ* (Fig. 1*A* and *B*). Moreover, the expression of stearoyl-CoA desaturase 1 (*SCD1*), diacylglycerol *O*-acyltransferase 2 (*DGAT2*), fatty acid synthase (*FASN*), ATP citrate lyase (*ACLY*), *ChREBPα*, and *GLUT4* was also upregulated, confirming higher capacity of cells for lipogenesis (Fig. 1*B*). Although mRNA levels of markers of mitochondrial biogenesis nuclear respiratory factor 1 and PPARγ coactivator 1α were not altered, *UCP1* expression was notably upregulated (Fig. 1*B*). Interestingly, cell differentiation into adipocytes was associated with an increase in total protein content that was more pronounced after weight loss (Fig. 1*C*). The ratio between protein content in adipocytes versus preadipocytes correlated with the degree of differentiation measured by ORO (Fig. 1*D*). Notably, there was a positive link between changes in protein content and mRNA levels of differentiation markers, lipogenic genes, and *UCP1* (Fig. 1*E* and Supplementary Table 1). The enhancement of adipogenesis was not caused by the alteration of proliferative capacity of preadipocytes, since there was no difference in the yield of the cells at passage 3 and length of cultivation period preceding the experiments (Supplementary Table 2).

To decipher the putative role of PPARγ in the reprogramming of preadipocytes induced by weight loss, preadipocytes were stimulated with differentiation medium supplemented either with DMSO (control) or 1 μmol/L rosiglitazone (PPARγ ligand). As expected, rosiglitazone enhanced markedly the expression of *FASN*, *SCD1*, and *aP2* compared with control cells (Fig. 2*A*); however, the upregulation of lipogenic markers *FASN* and *SCD1* in cells derived after the DI was more pronounced in the absence of rosiglitazone (Fig. 2*A*). Furthermore, the ratio of FASN and SCD1 expression under rosiglitazone versus control treatment was not different between the cells obtained before and after the DI (Fig. 2*B*). In addition, the percentage of cells accumulating neutral lipids after 6 days of differentiation in the presence or absence of PPARγ ligand was in both cases higher after weight loss (Fig. 2*C*). Again, the ratio between the numbers of cells differentiated in the presence of rosiglitazone versus DMSO was unchanged after the DI (not shown). These data suggest that weight loss did not alter the sensitivity of cells to rosiglitazone and, rather, affected pathways upstream of PPARγ. Indeed, the expression of *PPARγ* itself as well as the expression of *KLF9* (Kruppel-like factor 9), the transcription factors that regulate PPARγ transcription, was not different in preadipocytes derived before or after weight loss (Fig. 2*D*). However, cells derived after weight loss exhibited a marked downregulation of expression of runt-related transcription factor 2 (*RUNX2*), the transcription factor favoring osteogenic differentiation (12), both prior to and during differentiation (Fig. 2*D*).

Expression and secretion of cytokines by in vitro differentiated preadipocytes are altered after weight loss. For determination of whether weight loss affects the intrinsic secretory potential of adipocytes, the secretion and mRNA expression of several cytokines were measured in in vitro differentiated preadipocytes derived from adipose tissue before and after weight loss. Both expression and secretion of adiponectin and its high–molecular weight form were higher in adipocytes after DI compared with baseline (Fig. 3). However, when the secretion of total adiponectin was adjusted to the degree of differentiation assessed by ORO, the effect of DI was lost, suggesting a close relationship between adiponectin secretion and the differentiation state of adipocytes (not shown). Leptin mRNA levels were also elevated in adipocytes after weight loss (Fig. 3*A*), and this change was not related to the degree of differentiation. In contrast, *MCP1* and *IL-8* mRNA levels

TABLE 1
Clinical characteristics of the subjects before and after dietary intervention

	Baseline	DI	*P*
Age (years)	40.7 ± 1.79		
Weight (kg)	91.49 ± 2.12	82.5 ± 1.93	**<0.001**
BMI (kg/m^2)	32.97 ± 0.91	29.71 ± 0.82	**<0.001**
Fat mass (%)	39.71 ± 1.1	36.47 ± 1.11	**<0.001**
Waist (cm)	102.6 ± 2.24	93.04 ± 2.13	**<0.001**
WHR	0.86 ± 0.02	0.84 ± 0.02	**0.011**
Glucose (mmol/L)	5.42 ± 0.11	5.06 ± 0.13	**0.012**
Insulin (mIU/L)	9.62 ± 0.97	7.27 ± 0.92	**0.002**
HOMA-IR	2.36 ± 0.27	1.71 ± 0.27	**0.002**
Total cholesterol (mmol/L)	5.38 ± 0.27	4.63 ± 0.18	**0.006**
HDL-C (mmol/L)	1.66 ± 0.09	1.44 ± 0.07	**0.007**
Triglycerides (mmol/L)	1.19 ± 0.09	0.78 ± 0.05	**<0.001**
IL-6 (pg/mL)	0.86 ± 0.1	0.82 ± 0.09	0.381
MCP1 (pg/mL)	25.61 ± 2.72	23.73 ± 2.82	0.075
Adiponectin (μg/mL)	1.89 ± 0.12	1.92 ± 0.14	0.721

Data are means ± SEM. n = 23. HDL-C, HDL cholesterol; HOMA-IR, homeostasis model assessment of the insulin resistance index; WHR, waist-to-hip ratio. *P* values in bold reached the level of significance.

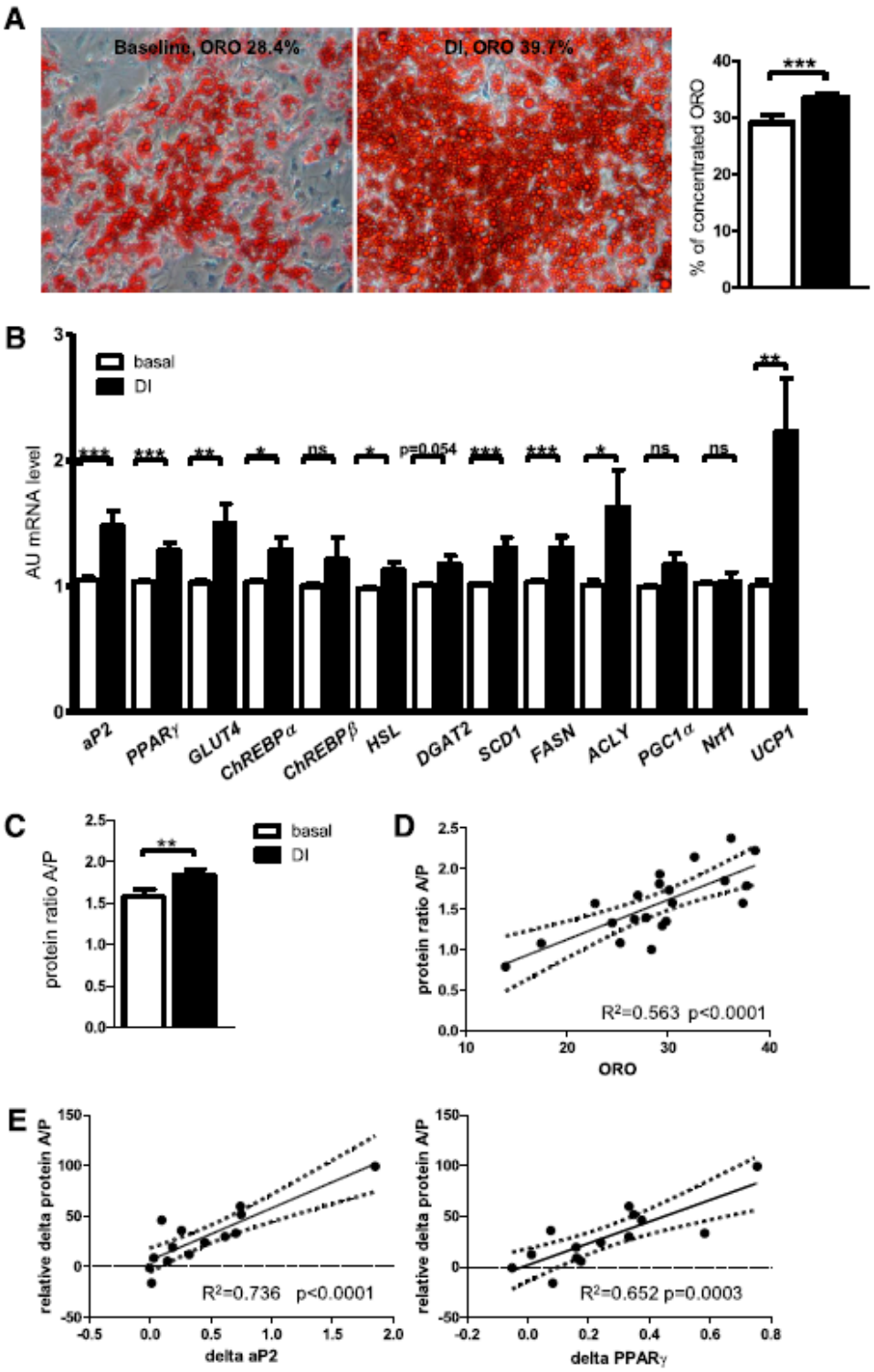

FIG. 1. Weight loss improves in vitro adipogenesis. Cells were differentiated for 12 days, and then accumulation of lipids, protein content, or gene expression was analyzed. *A*: Effect of weight loss on lipid accumulation. Representative images of adipocytes from one donor before and after DI stained with ORO and quantification of neutral lipid accumulation expressed as percent of stock ORO ($n = 22$). *B*: Effect of weight loss on gene expression. mRNA expression (arbitrary units [AU]) in adipocytes normalized to *GUSB* expression ($n = 15$). *C*: Effect of weight loss on protein content. Ratio between total protein content in adipocytes vs. preadipocytes ($n = 22$). Data are means ± SE; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. *D*: Linear regression between ORO accumulation and protein content in adipocytes (A) vs. preadipocytes (P) at baseline. *E*: Linear regression between relative Δprotein (adipocytes vs. protein) and ΔmRNA expression of *aP2* and *PPARγ*.

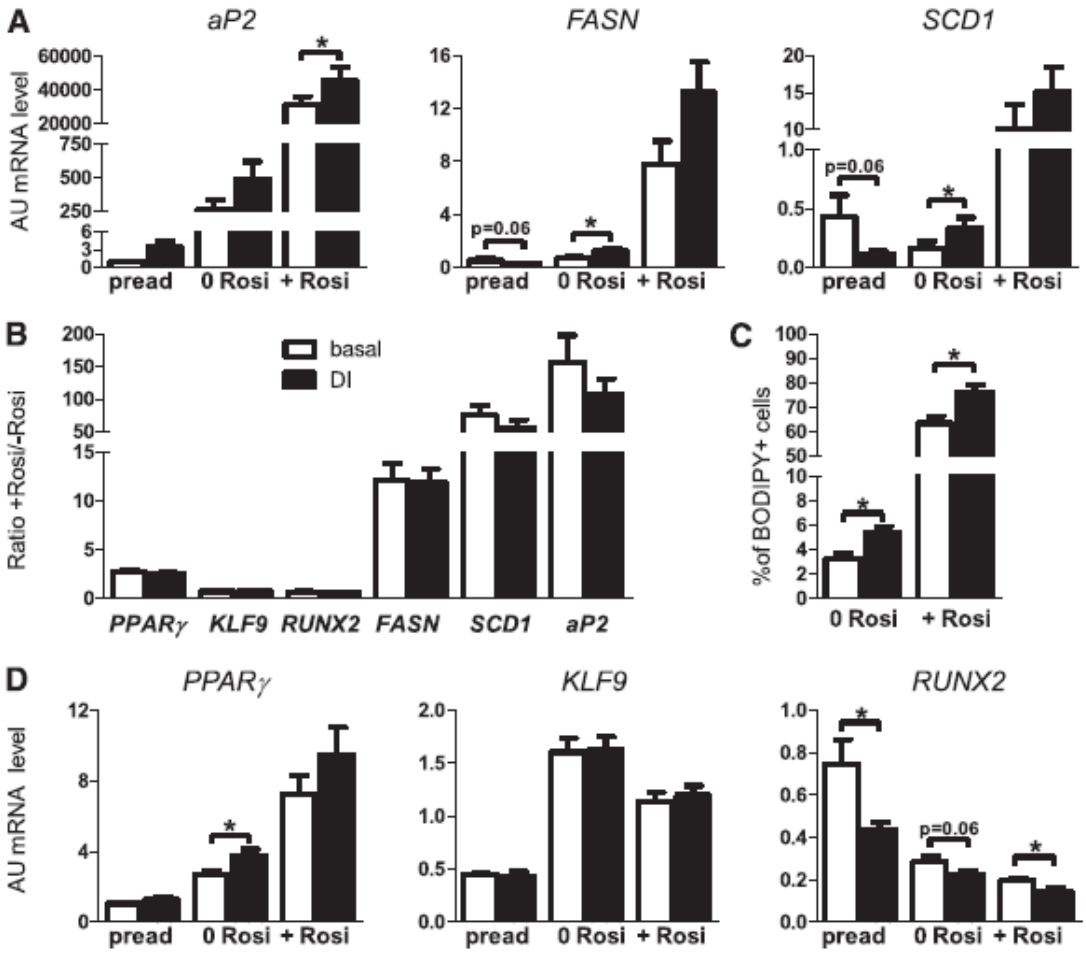

FIG. 2. Weight loss does not enhance the sensitivity to PPARγ ligand rosiglitazone (Rosi) but is connected with the suppression of *RUNX2* expression. *A* and *B*: Cells were differentiated for 3 days in the presence or absence of 1 mmol/L rosiglitazone. Cells kept in serum-free medium supplemented with transferrin and insulin that did not undergo adipogenesis were used as controls. *A*: Relative mRNA levels of selected genes were detected by quantitative RT-PCR ($n = 6$). □, Baseline; ■, DI. Data are means ± SE; *$P < 0.05$. *B*: The ratio between expression of selected genes in cells differentiated in the presence and absence of rosiglitazone was calculated in cells derived before and after the DI. *C*: Cells were differentiated for 6 days in the presence or absence of 1 mmol/L rosiglitazone ($n = 4$). After staining with BODIPY, the 45 microscopy images encompassing an average of 2,700 cells were analyzed and numbers of BODIPY$^+$ cells were counted. Chart represents the percentage of BODIPY$^+$ cells within analyzed populations (each on average from 2,700 cells). Data are means ± SE; *$P < 0.05$. AU, arbitrary units; pread, preadipocytes.

in adipocytes obtained after DI were reduced compared with baseline (Fig. 3*A*). Secretion of MCP1 was lower (Fig. 3*B*) compared with baseline even after adjustments to the degree of differentiation (not shown), but no significant changes in secretion or expression of IL-6 were observed (Fig. 3*A* and *B*).

DISCUSSION

It has been hypothesized that worsening of metabolic health in obesity is related to dysfunction of hypertrophic adipocytes or diminished ability of adipose tissue to react to energetic surplus by the enhanced adipogenesis from available precursors. The latter is evidenced by *1*) insulin-resistant subjects exhibiting lower expression of adipogenic genes (13) and *2*) the insulin-sensitizing drugs thiazolidinediones alleviating insulin resistance by the recruitment of new adipocytes with a high potential to store lipids (14,15).

In this study using cells derived from paired subcutaneous adipose tissue biopsies from obese women undergoing long-term DI, we showed that adipogenic potential of preadipocytes was increased by moderate weight loss. Obesity was shown to be associated with lower differentiation capacity of preadipocytes (3,16). Our data obtained in the prospective study therefore not only are in agreement with the cross-sectional observations but also provide evidence that the lowering of adipose tissue mass is associated with higher preadipocyte differentiation capacity and sensitivity to adipogenic stimuli. This implies that the effect of weight loss on adipose tissue function should be ascribed not only to changes in size and metabolism of mature adipocytes and in proinflammatory potential/numbers of infiltrated immune cells (6,17) but also to reprogramming of preadipocytes. Lower *RUNX2* expression in cells derived after weight loss suggests that weight loss inhibits alternative lineage programs

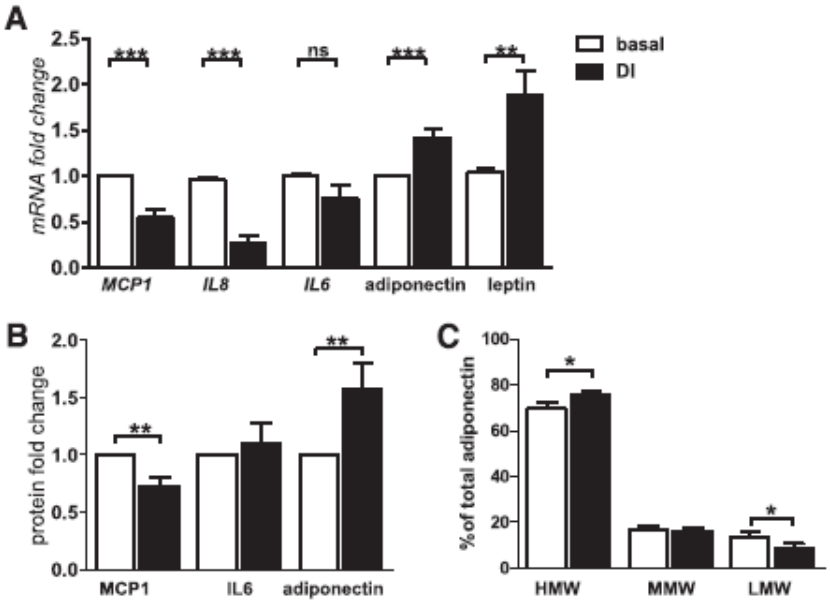

FIG. 3. Weight loss alters the expression and secretion of cytokines in adipocytes differentiated in vitro. Cells were differentiated for 12 days, and conditioned media and cells were collected after 24 h of incubation in freshly added media for analysis of cytokine expression and secretion. *A*: Effect of weight loss on cytokine gene expression. Quantitative RT-PCR analysis of mRNA for selected cytokines, normalized to *GUSB* expression ($n = 15$). *B*: Effect of weight loss on cytokine secretion. Fold change over the basal values for MCP1, IL-6, and adiponectin in conditioned media measured by ELISA, normalized to protein content ($n = 22$). *C*: Effect of weight loss on adiponectin isoform secretion. Quantification of adiponectin isoforms by native polyacrylamide gel electrophoresis and Western blot analysis ($n = 21$). Data are means ± SE; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. HMW, high molecular weight; LMW, low molecular weight, MMW, medium molecular weight.

(e.g., osteogenesis), which in turn favors the adipogenic differentiation.

Nevertheless, the impact of in vivo changes of preadipocytes, which are important for the maintenance or development of AT (17), remains unknown. It is tempting to speculate that upon fat mass reduction, a higher sensitivity of precursor cells to adipogenic stimuli could enhance fatty acid storage and therefore indirectly lower lipotoxicity at the whole-body level while improving insulin sensitivity. On the other hand, increased adipogenesis after previous weight loss could compromise long-term weight loss maintenance. Indeed, studies on obese and then calorie-restricted rats showed that short overfeeding after calorie restriction was accompanied with appearance of small adipocytes (18).

Development of mature adipocytes is dependent on active lipogenesis. In serum-free culture conditions, all accumulated lipids are synthesized de novo (19). De novo lipogenesis in adipose tissue, possibly orchestrated by ChREBPβ (20), was downregulated in subjects with hypertrophied adipocytes who are more insulin resistant compared with subjects with smaller adipocytes (21). Since we observed that weight loss was accompanied with a higher expression of lipogenic genes *FASN*, *DGAT2*, *SCD1*, *ACLY*, and *ChREBPα* (a regulator of ChREBPβ expression) in in vitro differentiated cells, it can be suggested that de novo lipogenesis capacity linked to higher insulin sensitivity represents intrinsic characteristics of adipocytes reprogrammable by weight loss.

In obesity, adipocytes produce more proinflammatory cytokines and chemoattractants while secretion of insulin-sensitizing adiponectin is diminished (22). In our study, we show that weight loss altered the capacity of in vitro differentiated adipocytes to express *IL-8*, *MCP1*, leptin, and adiponectin. Lower secretion of MCP1 from adipocytes reprogrammed by weight loss could contribute to a lower infiltration of macrophages into AT. Selective increase of high–molecular weight adiponectin secretion might underlie beneficial effects of weight loss on insulin sensitivity.

Studies performed on cell culture models may be influenced by culture conditions. Although we cannot completely exclude possible effects of subcultivation on adipogenic and secretory potential of cells, it has been shown previously that in vitro conditions preserve the original phenotype of a donor in preadipocytes and adipocytes (13,23). Moreover, subcultivation of stromavascular cells eliminates contaminating cells like macrophages and results in a more homogenous population than primary cells (3,24). It is also unlikely that the observed differences were based on dissimilar starting numbers of cells, as there was no difference in the length of cultivation or yield of cells before and at the end of DI.

In conclusion, our study shows that weight loss improves the adipogenic capacity of preadipocytes and alters their secretory potential. This effect may be associated with the improvement of the metabolic status of obese as well as with an increased tendency for weight regain. We believe that the analysis of a distinct cellular population, such as preadipocytes subjected to uniform in vitro conditions, can offer a focused and unique image of an intrinsic adaptation of AT to weight loss.

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L.R. designed the study, performed experiments and data analysis, and wrote the manuscript. L.M., J.K., M.T., Z.K., and M.K. performed experiments and contributed to discussion. M.S.-V., N.V., and D.L. contributed to discussion and to the writing of the manuscript. V.S. designed the study, organized the clinical part of the study, and contributed to discussion and the writing of the manuscript. L.R. 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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Paper 3

Ursodeoxycholic Acid but not Tauroursodeoxycholic Acid inhibits proliferation and differentiation of human subcutaneous adipocytes

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Ursodeoxycholic Acid but Not Tauroursodeoxycholic Acid Inhibits Proliferation and Differentiation of Human Subcutaneous Adipocytes

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Abstract

Stress of endoplasmic reticulum (ERS) is one of the molecular triggers of adipocyte dysfunction and chronic low inflammation accompanying obesity. ERS can be alleviated by chemical chaperones from the family of bile acids (BAs). Thus, two BAs currently used to treat cholestasis, ursodeoxycholic and tauroursodeoxycholic acid (UDCA and TUDCA), could potentially lessen adverse metabolic effects of obesity. Nevertheless, BAs effects on human adipose cells are mostly unknown. They could regulate gene expression through pathways different from their chaperone function, namely through activation of farnesoid X receptor (FXR) and TGR5, G-coupled receptor. Therefore, this study aimed to analyze effects of UDCA and TUDCA on human preadipocytes and differentiated adipocytes derived from paired samples of two distinct subcutaneous adipose tissue depots, abdominal and gluteal. While TUDCA did not alter proliferation of cells from either depot, UDCA exerted strong anti-proliferative effect. In differentiated adipocytes, acute exposition to neither TUDCA nor UDCA was able to reduce effect of ERS stressor tunicamycin. However, exposure of cells to UDCA during whole differentiation process decreased expression of ERS markers. At the same time however, UDCA profoundly inhibited adipogenic conversion of cells. UDCA abolished expression of PPARγ and lipogenic enzymes already in the early phases of adipogenesis. This anti-adipogenic effect of UDCA was not dependent on FXR or TGR5 activation, but could be related to ability of UDCA to sustain the activation of ERK1/2 previously linked with PPARγ inactivation. Finally, neither BAs did lower expression of chemokines inducible by TLR4 pathway, when UDCA enhanced their expression in gluteal adipocytes. Therefore while TUDCA has neutral effect on human preadipocytes and adipocytes, the therapeutic use of UDCA different from treating cholestatic diseases should be considered with caution because UDCA alters functions of human adipose cells.

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Introduction

Obesity develops when the storage of surplus energy requires excessive expansion of the adipose tissue (AT). Expansion of AT occurs through hyperplasia or hypertrophy that is in adult obesity prevailing. Hypertrophy of adipocytes is connected with their dysfunction manifested by lower insulin sensitivity, higher basal lipolysis and altered production of cytokines contributing to a development of chronic low-grade inflammation [1,2]. Even though the exact molecular insult leading to such adipocyte dysfunction is not clear, it appears that the nutrient overload creating excessive demands on the endoplasmic reticulum (ER) could be an important if not central contributor [3,4]. ER is an organelle with the direct control over the cytokine production and lipid storage and its overload initiates processes that should enhance ER capacity but also potentiate typical pro-inflammatory pathways [5]. Indeed, ER stress (ERS) is higher in obese insulin resistant subjects that at the same time show evidence of low grade inflammation [6,7]. On the other hand, the resolution of ERS by chemical chaperones has been shown to alleviate inflammation [5,8]. One class of the chemical chaperones is represented by bile acids (BAs), natural products of cholesterol catabolism [9]. BAs were shown to prevent ERS in AT of obese mice [10]. Apart from their chaperone capacity, BAs may influence metabolic state of AT also by regulating other pathways as evidenced by

Table 1. Clinical characteristics of subjects.

Age (years)	43±0.7
BMI (kg/m^2)	32.8±0.3
Weight (kg)	91.3 ± 1.1
Waist circumference (cm)	100.9±1.0
Hip circumference (cm)	120.1±0.9
Fat mass (%)	41.0±0.6
FFM (%)	59.1±0.6
Glucose (mmol/l)	5.2 ±0.1
Insulin (mIU/l)	8.9±0.5
NEFA (mmol/l)	0.5±0.0
Triglycerides (mmol/l)	1.2±0.1
HDL cholesterol (mmol/l)	1.4±0.0
Total cholesterol (mmol/l)	4.7±0.1
HOMA-IR	2.1±0.1

Values are means ± SEM, n = 10.

BMI, body mass index; FFM, fat-free mass; HOMA-IR, homeostasis model assessment of the insulin resistance index; NEFA, nonesterified fatty acids, HDL, high-density lipoprotein

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animal studies, i.e. BAs were shown to regulate adipocyte functions through the activation of nuclear farnesoid X receptor (FXR) and specific G protein-coupled membrane surface receptor TGR5 [11,12]. In 3T3-L1 cells, FXR cooperates with PPARγ and in addition to that it stimulates adipogenesis also through inhibition of Wnt pathway [11,13].In brown adipocytes, TGR5 pathway regulates energy expenditure through the induction of mitochondrial uncoupling protein (UCP1) expression [12]. However, these findings have not yet been confirmed in humans and effects of BAs on properties of human preadipocytes, resp. adipocytes remain mostly unknown. Indeed, this study aimed to evaluate and compare the effects of two common species of BAs, ursodeoxycholic (UDCA) and tauroursodeoxycholic acid (TUDCA), on proliferation and adipogenic conversion of human preadipocytes as well as on their inflammatory status. Since adipocytes characteristics differ in respect to the fat depot, the effects of BAs were evaluated in cells derived from abdominal (sAAT) and gluteal (sGAT) subcutaneous AT.

Materials and Methods

Subjects

10 premenopausal obese women (body mass index [BMI] 32.8 ± 3.2 kg/m^2) without medication and diseases except for obesity participated in this study. The written informed consent was obtained from each patient before the study. The study was performed according to the Declaration of Helsinki protocols and was approved by Ethical Committee of the Third Faculty of Medicine, Charles University in Prague.

Clinical investigation and laboratory measurements

Complete clinical investigation including anthropometric measurements, blood sampling and AT biopsies was performed in the morning in the fasting state. The whole body composition was evaluated by multi-frequency bioimpedance (Bodystat, Quad scan 4000, Isle of Man, UK). The blood was collected and centrifuged at 1300 RPM, 4°C, separated plasma was stored at -80°C until analysis. The paired samples of subcutaneous AT were obtained from the subcutaneous abdominal (10 cm lateral to the umbilicus) and gluteal (right upper quadrant) region using needle biopsy under local anesthesia (1% Xylocaine). Plasma glucose was determined using the glucose-oxidase technique (Beckman Instruments, Fullerton, CA). Plasma insulin was measured using an Immunotech Insulin Irma kit (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). Plasma levels of other relevant substances were determined using standard clinical biochemistry methods. Anthropometrical and biochemical characteristics of subjects are shown in Table 1.

Isolation, cultivation and differentiation of preadipocytes

Samples of AT were washed with PBS supplemented with gentamycin and then digested with collagenase I (300 U/ml, Biochrom, Berlin, Germany) for 40-60 minutes in shaking water bath at 37°C. The digested AT was centrifuged twice (1300 RPM, 5 min), adipocytes were discarded and pellet containing stroma-vascular fraction (SVF) was incubated in erythrocyte lysis buffer for 10 min at room temperature. Cells were collected by centrifugation and, without any filtration step, they were resuspended in PM4 medium (base medium-DMEM/F12, L-glutamine, Panthotenate, biotin, gentamycin, Pen/strep, supplemented with 2.5% MSC qualified FBS, Invitrogen, 1 ng/ml FGFβ, 10 ng/ml EGF, 132 nM insulin). The proliferation medium (PM4) was change every 2 days until the cells reached 70% confluence, then they were subcultivated. Two additional

subcultivations were performed and then cells (passage 4) were plated for proliferation assay at density specified bellow or 10 000 cells/cm^2 for experiments on differentiated adipocytes.

To induce adipogenic differentiation, two days postconfluent cells were washed with PBS with Ca^{2+}/Mg^{2+} and fed with DIFM + medium (base medium supplemented with 2.5% MSC qualified FBS, Invitrogen, 66 nM insulin, 1 µM dexamethasone, 1 nM T3, 0.1 µg/ml transferin, 0.25 mM IBMX, 1 µM Rosiglitazone). The medium was changed after 3 days. At day 6th, Rosiglitazone and IBMX were omitted and dexamethasone replaced with 0.1 µM cortisol. The differentiation continued until day 12 with one change of media.

For experiments with BAs, proliferation and differentiation medium were supplemented with 200 µM UDCA (Sigma Aldrich, St Louis, MO, USA), 500 µM TUDCA (Calbiochem, San Diego, CA, USA) or PBS as control. The effective concentrations of BAs were based on previously published observations [14,15]. To create ERS, cells were treated with 1 mg/ml tunicamycin (LKT Laboratories, St. Paul, MN, USA). To activate FXR, cells were treated with 10 µM GW4064 (Sigma Aldrich). To activate NFκB pathway, cells were treated with 10 ng/ml TNFα (Immunotools, Friesoythe, Germany). To prevent phosphorylation of Erk1/2, cells were pretreated with 50 µM PD98059 (Enzo, Farmingdale, NY, USA).

Proliferation assay

MTS assay-2000 cells/cm^2 were plated onto 96 well plate, in triplicates for each condition and cultivated in PM4 medium supplemented with either UDCA, TUDCA or PBS. Medium was changed on day 2 and 4. BAs were present in medium during the whole proliferation assay. Numbers of adherent cells were estimated using MTS assay (CellTiter96 aqueous MTS reagent powder, Promega, Madison, WI, USA; Phenazine methosulfate, Sigma) by assessing the absorbance of formazan measured at 490 nm.

Cell cycle - Cells were cultured and treated as described for proliferation assays. At day 5, they were trypsinized and fixed in 70% ethanol at 4°C overnight. Then cells were washed with PBS two times, stained with 50 µg/ml Propidium Iodide and treated with 0.1 mg/ml RNAse I diluted in PBS for 30 minutes at 37°C. Cell cycle analysis was performed on FACSCalibur and analyzed with FlowJo 8.2 (BD Biosciences, Franklin Lakes, NJ, USA).

Gene expression analysis

For RNA analysis, cells were lysed in RLT buffer and total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentration was measured by Nanodrop 1000 (Thermo Fisher Scientific, Wilmington, USA). Genomic DNA was removed by DNAse I treatment (Invitrogen, Carlsbad CA, USA). cDNA was obtained by reverse transcription (High Capacity cDNA Reverse Transcription Kit, Applied Biosystem, Carlsbad, CA, USA) of 600 ng of total RNA. cDNA equivalent of 5 or 25 ng of RNA was used for Real Time PCR reactions using Gene Expression Master Mix or Universal Master Mix II and Gene expression assay of PPARγ, SCD1, FASN, b2ADR, HSPA5, ATF4, FXR, TGR5, TLR4, GROα, MCP1, IL8, UCP1 (Applied Biosystem). aP2 was detected by specific primers by Sybr Green technology (Power Sybr Green Master Mix). All samples were run in duplicates on 7500 Fast ABI PRISM instrument (Applied Biosystem). Gene expression of target genes was normalized to expression of GUSB (glucuronidase, beta) or to same input of cDNA (in case of time course of differentiation when all tested control genes exhibited substantial shifts in Ct value) and fold change of expression was calculated using ΔΔ Ct method.

Oil Red O (ORO) staining

12 days differentiated cells were fixed by direct addition of buffered formaline into media (1v:1v), after 10 minutes medium was discarded and replaced by fresh undiluted formaline for another 20 minutes. Cells were washed several times in PBS and once with 60% isopropanol then stained with 60% ORO for 20 minutes. After extensive washing with water, ORO was eluted with 100% isopropanol and absorbance of eluates was measured at 500 nm. Standard curve from working stock of ORO was performed to normalize data and decrease inter-experimental variation.

Western blotting

Cells were washed two times with PBS and lysed on ice for 30 minutes in RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Complete, PhoStop, Roche Diagnostics, Mannheim, Germany). Lysates were then centrifuged for 15 minutes at 15,000x g, 4°C. Protein concentrations were determined using the bicinchoninic assay, Pierce (Rockford, IL, USA). Samples were loaded to a 10% acrylamide minigel and electrotransferred onto the nitrocellulose membrane. Membranes were blocked with 5% BSA. Antibodies against actin, IκBα, NFκB, Erk1/2 and their phosphorylated forms were from Cell Signaling (Danvers, MA, USA). Antigen-antibody complexes were detected using secondary antibodies coupled with horseradish peroxidase and the ECL detection system (Pierce).

Statistical analysis

The data from RT-qPCR were analyzed with GraphPad Prism 5.0. (La Jolla, CA, USA). Wilcoxon paired t-test was used for comparison of gene expression of paired samples between sAAT vs. sGAT, the effect of various treatments separately in each depot was estimated by Main-Whitney test. The levels of significance was set at $p<0.05$.

Results

Effect of BAs on proliferation of preadipocytes

To evaluate effect of BAs on preadipocytes and adipocyte properties we have established the cultures of preadipocytes from paired samples of sAAT and sGAT of 10 obese women. Under standard growth conditions, proliferation of preadipocytes from both depots was similar (Figure 1A). The concentration of formazan, read out of MTS proliferation assay, increased after 6 days of proliferation 14.53±3.11 times for sAAT and 13.7±2.76 times for sGAT preadipocytes. TUDCA did not affect proliferation of preadipocytes from either depot.

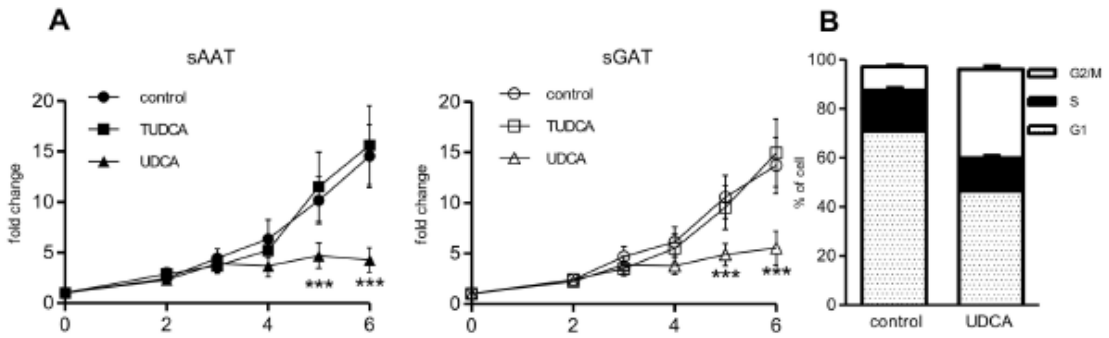

Figure 1. Effect of BAs on proliferation of preadipocytes. (A) Preadipocytes were seeded at density 2000 cells/cm^2 and cultivated for 6 days under control conditions or in the presence of 200 µM UDCA or 500 µM TUDCA. MTS assay was performed at indicated days. Fold changes of measured absorbance over the control was calculated for each donor (n=10). Data are means ±SE, *** p<0.001. (B) Cell cycle analysis. sAAT preadipocytes were seeded at density 2000 cells/cm^2 and cultivated for 5 days under control conditions or in the presence of 200 µM UDCA. Then cells were harvested, stained with propidium iodide and analyzed by flow cytometry (n=5). The percentage of cells in G1, S and G2/M cell cycle phases is shown.

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UDCA had however strong anti-proliferative effects, as it almost completely blunted the division of cells from both depots. To investigate how UDCA inhibits growth, we analyzed the cell cycle of cells from sAAT after 5 days of growth under control condition or in medium supplemented with UDCA. Based on FACS analysis, percentage of apoptotic cells in control and UDCA-treated cells was negligible. However, the distribution of cells in cell cycle was substantially affected by UDCA, i.e. percentage of cells in G2/M phase increased from 9.55±0.75% to 36.16±1.3% , while percentage of cells in G1 phase decreased (control v. UDCA 70,91 ±0.44 v. 46.5±2.3) (Figure 1 B).

Effect of BAs on key ERS proteins

To confirm previously described potential of BAs to prevent development of ERS in adipocytes, in vitro differentiated human adipocytes pretreated with BAs for 2 hours were exposed to ERS inducer tunicamycin (1µg/ml) for additional 24 hours (BAs were still present in medium) and then, the mRNA expression of five ERS markers, HSPA5, ATF4, DNAJC3, XBP1 (spliced versus total) and EDEM1, representing targets of all three arms of unfolded protein response (UPR), was evaluated. Unexpectedly, pretreatment of adipocytes with BAs did not prevent upregulation of ERS markers mRNA induced by tunicamycin (Figure 2A). This prompted us to determine whether BAs exert effects on expression of ERS markers when present chronically during whole time course of adipogenesis. The process of adipogenic differentiation in the absence of BAs was accompanied with the modest increase of HSPA5 expression and upregulation of ATF4, DNAJC3 mRNA at the end of differentiation (Figure 2B). Differentiation in the presence of BAs decreased mRNA expression of ERS marker ATF4, but only UDCA lowered expression of HSPA5 and DNAJC3 (Figure 2C). At the same time, we noticed that UDCA affected the process of differentiation and this effect was studied in the subsequent series of experiments.

Effect of BAs on adipogenic conversion of preadipocytes

The ORO staining after 12 days of adipocytes cultivation with BAs showed that accumulation of neutral lipids in cells stimulated to adipogenesis was not altered by TUDCA but was substantially reduced by UDCA treatment when compared with control conditions (Figure 3 A, B). This anti-adipogenic potential of UDCA was more pronounced in preadipocytes derived from sGAT (Figure 3C). Inhibitory effect of UDCA on adipogenesis and lipogenesis was confirmed on mRNA levels of several genes typical for mature adipocytes- expression of two markers of differentiation process, PPARγ and aP2, two markers of lipogenesis, FASN and SCD1, and β2 adrenergic receptor was decreased after 12 days of UDCA treatment (Figure 3D). Notably, aP2 mRNA level was significantly decreased also in the presence of TUDCA in cells from both depots.

Effect of BAs on expression of BA receptors and activity of Erk1/2

Based on the fact that UDCA can modulate activity of FXR and TGR5 - two known BAs receptors - we hypothesized that anti-adipogenic effect of UDCA might be mediated through these two factors. However, expression of neither FXRα nor TGR5 in human white adipocytes was reported previously. Therefore, we analyzed evolution of expression of FXRα and TGR5 during adipogenesis. FXRα mRNA was undetectable in preadipocytes but was induced during adipogenic conversion of cells (Figure 4A). TGR5 was expressed already in preadipocytes and its expression was strongly upregulated during the induction phase of differentiation, i.e. up to day 6, and then its mRNA levels decreased gradually (Figure 4A).

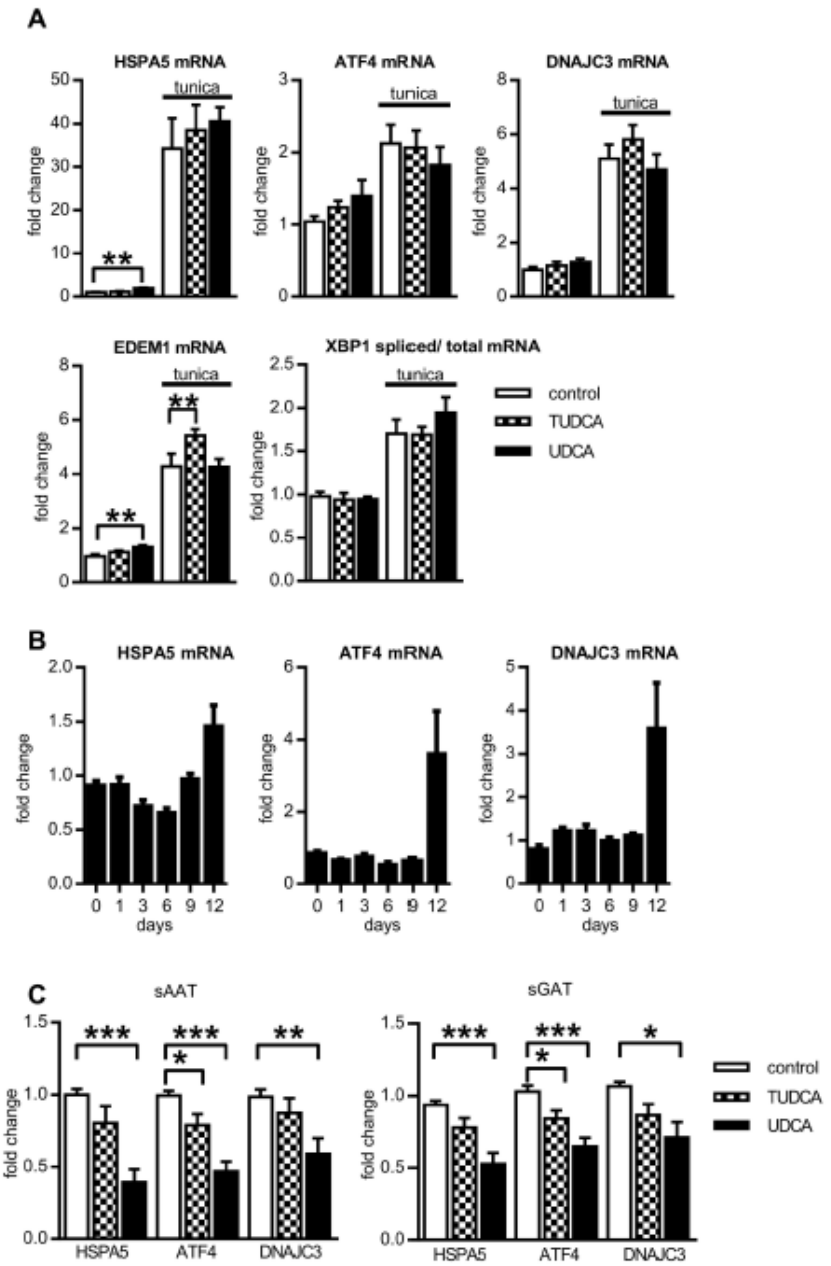

Figure 2. Effect of BAs on ERS markers. (A). Cells were differentiated for 12 days, and after 2 hr pretreatment with BAs they were exposed to 1µg/ml tunicamycin for 24 hrs. Then gene expression of ERS markers was analyzed and expressed as fold change in mRNA expression normalized to GUSB expression (n=3). (B) Cells were differentiated for indicated days and then gene expression of ERS markers was analyzed. Fold change in mRNA expression in adipocytes was normalized to input of cDNA (n=3) (C) Cells were differentiated for 12 days in the absence or presence of 200 µM UDCA or 500 µM TUDCA and then gene expression of ERS markers was analyzed. Fold change in mRNA expression in adipocytes was normalized to GUSB expression (n=10). Data are means ±SE, *p<0.05, *** p<0.001.

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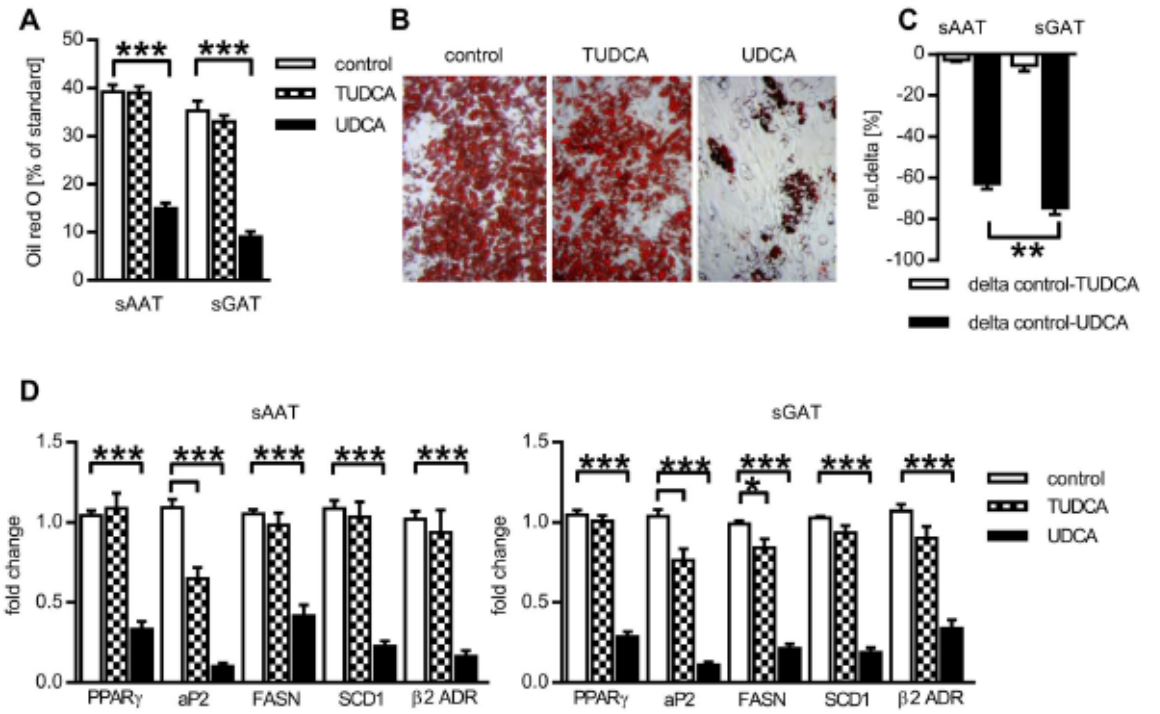

Figure 3. Effect of BAs on adipogenic differentiation of preadipocytes. Cells were differentiated for 12 days in the absence or presence of 200 μM UDCA or 500 μM TUDCA and then accumulation of lipids or gene expression was analyzed. (A) Effect of BAs on lipid accumulation. Quantification of neutral lipid accumulation expressed as a % of stock ORO (n=10). (B) Representative images of adipocytes from one donor stained with ORO. (C) Comparison of sensitivity of adipocytes from sAAT and sGAT depots to BAs treatment. Relative delta between control and BAs treatment was assessed for each donor (n=10). (D) Effect of BAs on gene expression in adipocytes. Fold change in mRNA expression in adipocytes was normalized to GUSB expression (n=10). Data are means ±SE, *p<0.05, ** p<0.01, *** p<0.001.

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When the adipocytes were differentiated in the presence of BAs, the expression of FXRαand TGR5 mRNA was suppressed in UDCA- and to lesser extent also in TUDCA-treated adipocytes from both depots (Figure 4B). To evaluate the involvement of FXR in UDCA-induced inhibition of adipogenesis, cells were induced to differentiate in the presence or absence of UDCA or specific FXR ligand GW4064 for 3 days when we expected major impact on the cascade of adipogenic transcription factors. UDCA treatment and the activation of FXR receptor with specific agonist GW4064 resulted in the similar suppression of expression of differentiation markers PPARγ, aP2 and FASN but had opposite effect on FXR expression itself (Figure 4C).

Both, TUDCA and UDCA, can also activate TGR5 receptor. In brown AT, TGR5 activation leads to upregulation of UCP1 expression [12]. However, even though UCP1 was expressed in sAAT and sGAT adipocytes, its expression was not altered by TUDCA and strongly suppressed by UDCA (data not shown). Moreover, expression of both, TGR5 and UCP1, was significantly repressed as early as after 3 days after induction of adipogenesis (Figure 4C).

Another pathway putatively activated by UDCA and also capable to regulate early steps of adipogenesis involves Erk1/2 activation [16]. Sustained activation of Erk1/2 may lower PPARγ transcriptional potency [17]. Therefore, to evaluate immediate effects of UDCA on Erk1/2 phosphorylation levels, we exposed cells to adipogenic medium in the presence or absence of UDCA or PD98059 (Erk1/2 inhibitor) for 1 hour and 24 hours. The adipogenic medium itself and in combination with UDCA induced sharp increase in Erk1/2 phosphorylation levels that was prevented by pretreatment with PD98059. Phosphorylation degree of Erk1/2 returned to basal levels after 24 hrs in control cells while UDCA treated cells maintained high Erk1/2 phosphorylation (Figure 4D).

Effect of BAs on expression of cytokines

Finally, we tested whether BAs can positively influence the inflammatory status of adipocytes since BAs were reported to have immunomodulatory properties [18]. Adipogenesis itself

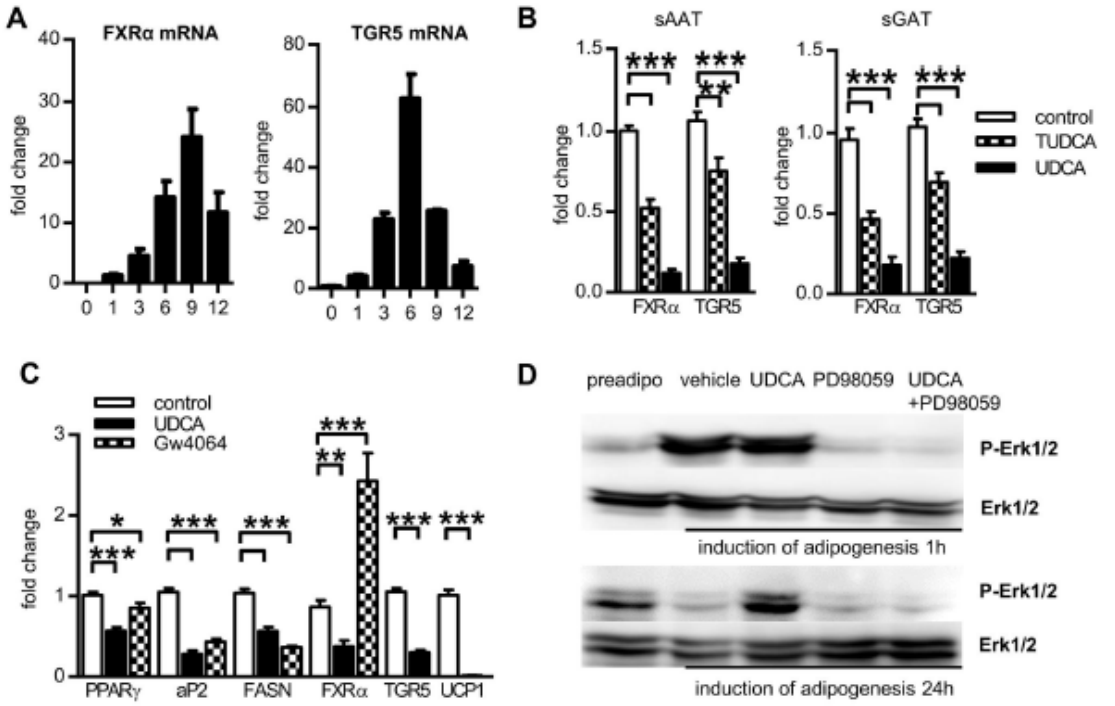

Figure 4. Expression of BA receptors and Erk1/2 in human adipocytes and their modulation by BAs. (A) Cells were differentiated for indicated days and then gene expression of FXRα and TGR5 was analyzed. Fold change in mRNA expression in adipocytes was normalized to input of cDNA (n=3) (B) Cells were differentiated for 12 days in the absence or presence of 200 μM UDCA or 500 μM TUDCA and then gene expression of FXRα and TGR5 was analyzed. Fold change in mRNA expression in adipocytes normalized to GUSB expression (n=10). Data are means ±SE, *** p<0.001. (C) Effect of UDCA and FXR agonist GW4046 on early phases of adipogenesis. Cells were induced to differentiate in the presence of 200 μM UDCA or 10 μM GW4046 for 3 days and then gene expression was analyzed. Fold change in mRNA expression in adipocytes was normalized to GUSB expression (n=3). Data are means ±SE, *p<0.05, ** p<0.01, *** p<0.001. (D) Western blotting analysis of Erk1/2 activation. Preadipocytes switched to adipogenic medium were treated with 200 μM UDCA and/or 50 μM PD98059 (PD98059 was added 30 minutes before the start of differentiation) for indicated times.

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was connected with mild induction of TLR4 expression (Figure 5A). TLR4 pathway is coupled with activation of NFκB that is directly responsible for enhancement of cytokine expression stimulation. We hypothesized that adipocytes differentiated in the presence of BAs would exert lower basal expression of inflammatory cytokines regulated by TLR4 pathway, i.e. IL8 [19], GROα and MCP1 [20]. However, chronic supplementation of adipogenic medium with TUDCA did not decrease expression of GROα, IL8, MCP1 or TLR4. Moreover, differentiation of adipocytes in the presence of UDCA led to enhanced mRNA expression of GROα and MCP1 in adipocytes from both depots and of IL8 and TLR4 selectively in sGAT adipocytes (Figure 5B). Surprisingly, despite this pro-inflammatory potential of UDCA, exposition of preadipocytes to UDCA did not initiate phosphorylation of NFκB and degradation of IκBα (Figure 5C).

Discussion

Chronic surplus of energy leading to development of obesity can, on cellular level, disrupt the function of ER and thus induce ERS. ERS is suggested as one of the first steps leading to deterioration of AT functions [5]. Thus, it was hypothesized that improvement of ER function in stressed adipocytes could restore their metabolic and endocrine profile to pre-obese conditions. Two naturally occurring BAs, UDCA and TUDCA, are considered as potent alleviators of ERS as they prevent aggregation of proteins and inhibit activation of UPR pathway [21-23]. Importantly, both UDCA and TUDCA are powerful detergents with low toxicity, and as such they have been already approved for clinical use for treatment of several cholestatic diseases [24]. However, BAs may modulate the metabolic and endocrine function of AT not only through their

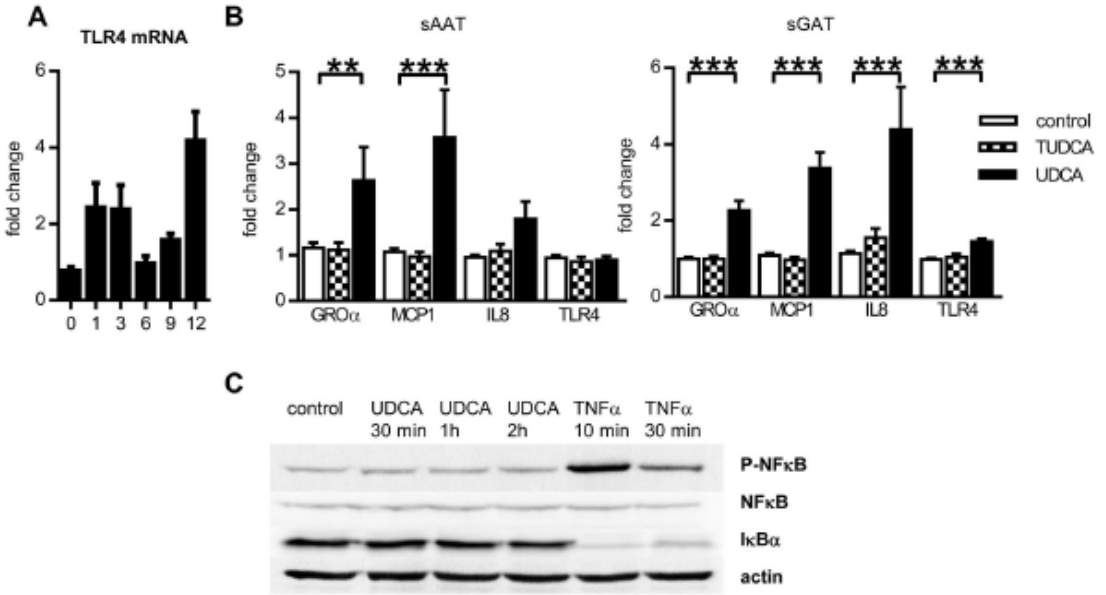

Figure 5. Effect of BAs on target genes of TLR4 pathway. (A) Cells were differentiated for indicated days and then gene expression of TLR4 was analyzed. Fold change in mRNA expression in adipocytes normalized to input of cDNA (n=3). (B) Cells were differentiated for 12 days in the absence or presence of 200 μM UDCA or 500 μM TUDCA. Fold changes in mRNA expression in adipocytes normalized to GUSB expression (n=10). Data are means ±SE, *p<0.05, ** p<0.01, *** p<0.001. (C) Western blotting analysis of NFκB activation. Preadipocytes were treated with 200 μM UDCA or 10 ng/ml TNFα (positive control) for indicated times.
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action on ER but also through direct activation of BA-specific receptors. Thus, this study evaluated effects of UDCA and TUDCA on several characteristics of human adipose cells derived from two subcutaneous depots, which differ in their metabolic function [25,26] and type of expansion [27].

Proliferation of preadipocytes is prerequisite for maintenance and also hyperplasic expansion of AT. In adulthood, hyperplasia is stimulated preferentially in sGAT, whereas adipocytes from sAAT depot become in response to excessive caloric intake rather hypertrophic [27]. Therefore we tested effects of BAs on proliferation of preadipocytes isolated from these two depots. Our data showed that although TUDCA had no apparent effect on proliferation of preadipocytes, UDCA exhibited unexpectedly strong anti-proliferative potential. UDCA was previously shown to inhibit proliferation of several types of carcinoma cells [28] as well as the growth of normal intestinal cells in vivo when administered to mice for 3 weeks [16]. Anti-proliferative effect of UDCA was associated with cell cycle arrest, apoptosis or cell senescence [29-31]. Our FACS analysis excluded apoptosis as possible mechanism of UDCA anti-proliferative effect in preadipocytes (Figure 1 C). Moreover, we did not observe an increased appearance of senescent cells with large nuclei and flattened morphology following the UDCA treatment. On the other hand the substantial enrichment of G2/M peak supports previous observations showing that UDCA can block cell cycle progression at the G2/M phase [32].

The sensitivity to anti-proliferative effects of UDCA as well as the proliferation under standard culture conditions was not different between preadipocytes from the two depots. Thus the increased proliferation of sGAT preadipocytes observed in vivo during overfeeding [27] is probably dependent more on the local tissue milieu than on the intrinsic properties of cells derived from this depot.

The chaperone like property of BAs observed in several cellular and animal models [10,22,33] indicated BAs as a potential therapy of obesity-associated comorbidities. However, we could not confirm this beneficial effect of BAs in human adipocytes as neither of BAs was able to alleviate acute ERS induced by tunicamycin in these cells. Indeed, in support of our observation in human adipocytes, in vivo treatment of obese subjects with TUDCA did not alter the level of ER chaperone expression in AT [34]. It is assumed that adipocytes cannot readily uptake certain BAs because expression of transporters responsible for BAs tissue uptake is very low in most extrahepatic tissues [35,36]. In fact, BAs are effectively metabolized by the liver, intestine and other cells that are naturally exposed to BAs [9] but little is known about metabolism of BAs in adipose cells. In addition, in the study of Berger et al. [21] proving BAs protective effect in epithelial

cells, supraphysiological amount of TUDCA were used (1-10 mM) compared to 200 and 500 μM UDCA and TUDCA resp. used in this study. Thus, it is possible that higher concentration or prolonged exposition of already differentiated adipocytes to BAs might be needed to achieve adequate effect on ERS levels. In fact, the prolonged exposition of cells to UDCA did lower expression of HSPA5, ATF4 and DNAJC3 (Figure 2C). This UDCA treatment, however, blocked adipogenic conversion of adipose precursors.

The distinct effects of TUDCA and UDCA on both ER proteins and adipogenesis could be based also on the fact that UDCA and TUDCA differ in their hydrophility and affinity to receptors. TUDCA cannot pass the cellular membrane of cells lacking specific transporters [35]. Major effects of TUDCA in cells of non-hepatic origin could be therefore ascribed to its binding to TGR5 receptor [35]. UDCA, on the other hand, can readily diffuse through membranes and apart from TGR5 [37] can weakly activate also nuclear FXR [38]. Both, TGR5 and FXR, seem to play important role in AT physiology. FXR was implicated in the regulation of the AT insulin sensitivity and of adipocytes differentiation and function [11,13,39]. Although expression of FXR in human adipose cells was not detected [40], we confirmed that FXRα was expressed in human adipocytes. Similarly to major adipogenic transcription factor, PPARγ, FXRα expression was elevated with the adipogenic conversion, which does suggest an involvement of FXRα in human adipogenesis. Even early stages of adipogenesis in the presence of UDCA were accompanied with reduced expression of PPARγ, aP2 and FASN similarly to the effects of specific ligand FXR GW4064 (Figure 4C). However, the fact that GW4064 and UDCA had completely opposite effect on the expression of FXRα mRNA itself does not support the assumption that UDCA inhibitory effect on adipogenesis might be dependent on activation of FXR.

Surprisingly, while TUDCA presence during differentiation of cells did not modify expression of PPARγ and other genes involved in lipid handling, it reduced expression of aP2 (Figure 3D). This selective effect on aP2 without impact on adipogenesis and lipid accumulation is potentially beneficial, as inhibition of aP2 activity in mice decreased macrophage infiltration and inflammation in AT [41].

Another known bile acid receptor, TGR5, is preferentially expressed in brown AT, skeletal muscle and immune cells [42] and very recently it has been found also in whole white AT [43]. In addition to these previous findings, we have detected TGR5 mRNA in both sAAT and sGAT adipocytes/preadipocytes (Figure 3A). TGR5 mRNA levels were strongly elevated when adipogenic medium containing IBMX and rosiglitazone was used. It was therefore possible that not the adipogenesis itself but one of these compounds induces TGR5 expression. However, short exposition of cells to differentiation medium lacking either dexamethasone or IBMX with rosiglitazone did not stimulate TGR5 expression (not shown). Therefore full activation of adipogenic process is necessary for upregulation of TGR5 expression. Activation of TGR5 in brown AT and skeletal muscle leads to increased UCP1 activity, oxidative phosphorylation and energy expenditure [12,44]. However, presence of BAs during the differentiation of preadipocytes did not enhance UCP1 expression, quite contrary, UDCA strongly inhibited both TGR5 and UCP1 expression (Figure 4C). Therefore, the lower accumulation of lipids in adipocytes treated with UDCA cannot be ascribed to enhanced energy expenditure through mitochondrial uncoupling.

In Figures 3D and 4C, we shown that addition of UDCA to adipogenic medium suppressed PPARγ expression. In order to provide possible mechanism of UDCA on adipogenesis, we investigated activity of Erk1/2 that is both upstream of PPARγ induction [45] and putatively activated by UDCA [16]. Erk1/2 activation has both pro- and anti-adipogenic outcomes, depending on the exact timing [45]. Brief activation or Erk1/2 is in 3T3-L1 prerequisite for mitotic clonal expansion and when it is prevented in this specific time window, 3T3-L1 fail to differentiate into adipocytes [46]. In contrast, prolonged activity of Erk1/2 is linked with lowered PPARγ transcriptional activity [47] and supports osteogenic differentiation pathway in expense of adipogenic program [48]. We brought the evidence that Erk1/2 phosphorylation is sustained in the presence of UDCA for at least 24 hours, when it was minimal in control cells. Thus, it can be suggested that UDCA may lower sensitivity of cells to adipogenic stimuli by Erk1/2 – dependent inhibition of PPARγ.

Finally, we tested whether UDCA and TUDCA may decrease expression of cytokines by adipocytes and thus inhibit attraction of monocytes and macrophages into AT. Exposition to BAs did not lead to reduction of expression of cytokines in adipocytes. On the contrary, UDCA strongly upregulated mRNA levels of IL8, GROα, MCP1 and TLR4 in sGAT adipocytes. The mechanism of UDCA-induced expression of chemokines in adipocytes remains, however, to be investigated, because the classic inflammatory pathway including NFκB activation was unaltered by UDCA treatment in human preadipocytes, similarly as was shown earlier in cancer cells [49]. Selective activation of IL8 and TLR4 by UDCA in sGAT adipocytes was rather unexpected. It could suggest that sGAT adipocytes may under certain stimulation attract more macrophages compared to sAAT adipocytes. Recently published comparison of sAAT and sGAT depots revealing higher expression of pro-inflammatory cytokines and macrophage markers in sGAT [50] supports this hypothesis.

Together, our data showed that UDCA inhibits both proliferation and differentiation of human preadipocytes derived from two distinct subcutaneous AT depots. Therefore the potential therapeutic use of UDCA different from treatment of cholestatic diseases should be considered with caution. Nevertheless, its taurine conjugate TUDCA does not have the same negative impact on the function of human preadipocytes and it lowers partially the demands on ER function. Thus, we suggest TUDCA as a preferred chemical chaperone for modulation of ER function in vivo.

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Author Contributions

Conceived and designed the experiments: LR VS. Performed the experiments: LM ZK JK MK. Analyzed the data: LM MK LR. Wrote the manuscript: LM LR VS.

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Paper 4

Postprandial inflammation is not associated with endoplasmic reticulum stress in PBMC 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 whether (i) inflammation induced by a single high energy high fat meal (HFM) is linked with stress of endoplasmic reticulum (ERS) in peripheral blood mononuclear cells (PBMC) and (ii) could be prevented by chemical chaperone ursodeoxycholic acid. 10 healthy lean men were recruited to a randomized blind crossover trial. Participants were given two doses of placebo (lactose) or ursodeoxycholic acid prior to HFM (6151 kJ, 47.4% lipids) consumption. Blood was collected at baseline and 4 hours after HFM. Cell populations and their activation were analysed using flow cytometry; plasma levels of inflammatory cytokines were assessed by ELISA and Luminex technology. Gene expression of inflammation and ERS markers was analysed in CD14+ and CD14- PBMC using quantitative RT-PCR. HFM induced an increase in mRNA expression of proinflammatory cytokines (IL1β, 2.1 fold; IL8, 2.4 fold; TNFα, 1.4 fold; MCP1 2.1fold) and a decrease of miR181 (0.8 fold) in CD14+ monocytes. HFM did not upregulate expression of ERS markers (XBP1, HSPA5, EDEM1, DNAJC3, ATF4) except for ATF3 (2.3 fold) in either CD14+ or CD14- PBMC. Administration of ursodeoxycholic acid before HFM consumption did not alter the HFM-induced change of expression of ERS or inflammation markers. In conclusion, HFM-induced inflammation detectable on the level of gene expression in PBMC was not associated with concomitant increase of ERS and could not be prevented by ursodeoxycholic acid.

Introduction

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. The palatability is based on a high content of lipids and simple sugars. However, the over-consumption of lipids and simple sugars is associated with the exaggeration of postprandial blood glucose and lipid levels [184]. The protracted elevations of blood metabolites are signs of postprandial dysmetabolism associated with so called postprandial inflammation [184-186]. Postprandial inflammation is manifested by increased plasma levels of inflammatory cytokines and leukocyte activation [187-188] although the precise contribution of blood monocytes and lymphocytes to these proinflammatory changes remains unknown. While in healthy people postprandial inflammation is transient, it is prolonged in obese people and subjects with Type 2 Diabetes [185-186, 189]. Thus, augmented postprandial inflammation has been suggested to promote insulin resistance and atherosclerosis. The exact molecular trigger of postprandial inflammation is not fully elucidated. Nevertheless, it has been shown previously that exposure of cells to saturated lipids and a high concentration of glucose may cause stress of endoplasmic reticulum (ERS) as documented by the increased mRNA levels of several ERS markers or by increased activity of an ERS-responsive LacZ reporter system. [190-192]. ERS leads to activation of pathways that primarily decrease the burden of endoplasmic reticulum or eliminate the affected cell. At the same time, however, the classic inflammatory regulatory molecules, NFκB and JNK, are stimulated [193]. Thus, postprandial inflammation could be triggered by ERS. Notably, ERS-associated inflammation may be alleviated by chemical chaperones such as bile acids [194]. One such a chemical chaperone, ursodeoxycholic acid (UDCA), currently used therapeutically for the treatment of cholestasis, has been shown to prevent chemically induced ERS in vitro [195-196]. In view of 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 linked with ERS. Furthermore, we investigated whether the inflammatory response may be modified or prevented by a non-toxic chemical chaperone UDCA.

Experimental methods

Subjects and study design

10 healthy lean male subjects were recruited to a randomized blind crossover trial consisting of two one-day studies, separated by at least 1 week (when subjects followed their habitual diet and level of exercise). Exclusion criteria were weight changes of more than 3 kg within the 3 months before the start of the study, participation in other trials, hyperbilirubinemia, smoking, and alcohol or drug abuse. The characteristics of the subjects are provided in Table 1. Participants were given 10 mg/kg placebo (lactose) or ursodeoxycholic acid (Ursosan, PRO.MED.CS, Prague, Czech Republic) in gelatine capsules with the last evening meal (20:00 h) prior to experimental day. Upon admission (08:00 h), a catheter was placed in the antecubital vein. After baseline blood sampling, subjects were given 15 mg/kg placebo or Ursosan. Within 15 minutes they consumed a high energy, high-fat meal (HFM) consisting of a breakfast sandwich with pork meat and egg omelette, French fries, ketchup, Nutella spread, croissant, Ice tea (McDonalds, Prague, Czech Republic, 6151 kJ, 32.8% carbohydrates, 47.4% lipids, 11.3% proteins). After the meal was consumed, blood was drawn each hour up to the 4^{th} hour. During the intervention, subjects had free access to drinking water. This 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 prior the study.

Determination of plasma levels of biochemical parameters

Plasma glucose levels were determined using the glucose-oxidase technique (Beckman Instruments, Fullerton, CA, USA). Plasma insulin was measured using an Immunotech Insulin Irma kit (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). Plasma levels of glycerol, free fatty acids and triglycerides were measured by colorimetric enzymatic assays using kits from Randox (Crumlim, United Kingdom).

Flow cytometry analysis

To determine the absolute numbers of cells in blood, Trucount tubes containing defined numbers of beads detectable by flow cytometry were used according to manufacturer's

protocol (BD Biosciences, Bedford, MA, USA). The subpopulation of blood cells representing lymphocytes, granulocytes and monocytes were analysed according to their size and granularity. To detect specific surface antigens, the whole blood samples were stained with fluorescence-labelled monoclonal antibodies (FITC-conjugated antibody CD4, CD14, CD16, CD36; PE-conjugated antibody CD3, CD11c, CD14, TLR2, TLR4; APC-conjugated antibody CD8, 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 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. Background was set up to 5% of positive cells of isotype control.

Isolation of PBMC and CD14+ cells

PBMC were isolated by gradient centrifugation. Briefly, 9 ml of uncoagulated blood was diluted in PBS to 16 ml and applied onto Leucosep tubes (Greiner Bio-One, Solingen, Germany) filled with 3 ml of Histopaque1077 separation medium (Sigma Aldrich, St Louis, MO, USA). After centrifugation for 15 min at 800 g, plasma was discarded and PBMC located above the frit were transferred to a tube containing ECBM medium (PromoCell, Heidelberg, Germany). Cells were washed 3 times, diluted in isolation buffer (PBS supplemented with 0.1% BSA and 2 mM EDTA, pH 7.4) and counted. Up to 10 million cells were mixed with 25 μl CD14 Dynabeads (Invitrogen, Carlsbad, CA, USA) and incubated on a rotator for 20 minutes at 4^{o}C, then CD14+ cells were separated by magnet and lysed in RLT (Qiagen, Hilden, Germany). CD14- PBMC were collected by centrifugation and lysed in RLT. Both fraction of PBMC were then used for RNA isolation. The separation efficiency was confirmed by both FACS and qRT-PCR analysis (not shown).

Gene expression analysis

Total RNA was isolated using a miRNeasy Mini Kit (Qiagen). Genomic DNA was removed by DNAse I treatment (Invitrogen). cDNA was obtained by reverse transcription (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Carlsbad, CA, USA) of 300 or 600 ng of total RNA. cDNA equivalent of 5 ng of RNA was used for Real Time PCR reactions using Gene Expression Master Mix and Gene expression assay of HSPA5 (Hs99999174_m1), ATF4 (Hs00909569_g1), ATF3 (Hs00231069_m1), EDEM1

(Hs00976004_m1), DNAJC3 (Hs00534483_m1), RANTES (Hs00174575_m1), IL1β (Hs01555410_m1), IL8 (Hs00174103_m1), MCP1 (Hs00234140_m1), PPARα (Hs00947539_m1), PPARγ (Hs01115513_m1), TLR2 (Hs00152932_m1), TLR4 (Hs01060206_m1) (Applied Biosystems). TNFα, XBP1 total and XBP1 spliced were detected by specific primers (TNFα forward 5'-TCTCGAACCCCGAGTGACA-3', reverse5'-GGCCCGGCGGTTCA-3'; XBP1 total, forward 5'-CGCTGAGGAGGAAACTGAA-3', reverse 5'-CACTTGCTGTTCCAGCTCACTCAT-3'; XBP1 spliced, forward 5'-GAGTCCGCAGCAGGTGCA-3', reverse 5'-ACTGGGTCCAAGTTGTCCAG-3') by Sybr Green technology (Power Sybr Green Master Mix). miRNA were transcribed by miScript II RT kit (Qiagen) without prior DNAse I treatment. cDNA equivalent of 1 ng of RNA was used for Real Time PCR reactions using miScript SYBR Green PCR Kit and miScript Primer Assay of miR146a and miR181a (Hs_miR-146a*_1,Hs_miR-181a*_1, Qiagen). All samples were run in duplicates on a 7500 Fast ABI PRISM instrument (Applied Biosystems). Gene expression of target genes was normalized to expression of RPS13 (mRNA, Hs01011487_g1) or RNU6-2 (miRNA, Hs_RNU6-2_1, Qiagen) and expressed as fold change calculated using ΔΔCt method.

Cytokine analysis

Leptin and adiponectin were measured in plasma samples by ELISA (DuoSets, R&D Systems, Minneapolis, USA), with a limit of detection of 62.5 pg/ml. TNFα, IL6, IL1β, IL8 were measured by MILLIPLEX MAP Human High sensitivity Cytokine Panel (Merck, Whitehouse Station, NJ, USA), with a limit of detection of 0.13 pg/ml.

Statistical analyses

Statistical analysis was performed using GraphPad Prism 6 and SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA). The data of plasma metabolites, gene expression (ΔCt) and flow cytometry-derived variables were log transformed and the normality of the data was assessed by Shapiro-Wilk normality test. The effects of HFM in placebo and UDCA conditions were tested using one way and two way ANOVA with Bonferroni post-hoc analysis. Correlations among relative mRNA levels were analysed using Spearman`s correlation. Data are presented as mean ± SEM. Differences at the level of $P < 0.05$ were considered statistically significant.

Results

Postprandial changes of plasma metabolites

Evolution of plasmatic postprandial levels of glycerol, non-esterified fatty acids (NEFA), triacylglycerols (TAG), glucose and insulin in response to the experimental meal are shown in Fig. 1. NEFA levels dropped after consumption of HFM and then gradually increased during the time course of the experiment but not above fasting levels (Fig. 1(a)). Glycerol and TAG concentration reached peak values 3 hours after HFM ingestion (Fig. 1(b, c)). The glucose levels did not change significantly during the whole intervention (Fig. 1(d)), whereas insulin levels increased 1 hour after HFM ingestion and remained elevated above fasting levels (Fig. 1(e)). Basal plasma levels of NEFA and glycerol were lower in UDCA condition, though this difference did not reach a significant level. Thus, no differences in basal or postprandial plasma levels of tested metabolites between placebo and UDCA conditions were detected.

Postprandial changes of blood cell populations

Fasting leukocyte numbers per μl of blood were not different between placebo and UDCA treatments (placebo: 9821±704 cells/μl, UDCA: 9380±763 cells/μl). HFM significantly increased absolute numbers of monocytes, lymphocytes, granulocytes and total numbers of leukocytes (Fig. 2(a)). This increase was similar in the presence of UDCA. In addition, relative distribution of two main leukocyte populations in blood, namely lymphocyte and granulocytes, changed postprandially, i.e. relative lymphocyte proportion decreased while relative granulocyte proportion reciprocally decreased in response to the test meal in placebo treatment (not shown). The relative proportion of monocytes within the whole leukocyte population remained unaltered in response to HFM. Given that both the relative distribution of the leukocyte population and the absolute counts of cells were affected by HFM consumption, the numbers of events representing gated cells were normalized by Trucount data (percentage of positive cells was multiplied with the absolute number of events in either the monocyte, lymphocyte or granulocyte gate).

HFM increased the counts of CD14+/CD11c+ and CD14+/TLR2+ monocytes in both placebo and UDCA conditions. Counts of CD14+/TLR4+ monocytes were increased after HFM in placebo condition only. On the other hand, only in UDCA condition, HFM elevated counts of CD4+ and CD8+ lymphocytes (Fig. 2(b)).

Evaluation of expression levels of individual surface markers (expressed as geometric mean fluorescence intensity, MFI) revealed that HFM enhanced expression of activation marker CD11c in monocytes. This increase was significant in both placebo and UDCA condition (Fig. 2(c)).

Postprandial plasma changes in adipokines and inflammatory cytokines

Plasma levels of leptin, adiponectin, IL8 and TNFα did not change during the HFM intervention in either placebo or UDCA condition (data not shown). Plasma IL6 levels increased gradually over the 4 hours in both placebo and UDCA conditions (Fig.3). IL1β levels were, in most samples, under the detection limit of the method used.

Postprandial changes of gene expression of cytokines in PBMC

In basal conditions, CD14+ cells expressed substantially higher levels of IL1β, IL8, MCP1 and TNFα and lower levels of RANTES mRNA compared to the CD14- population (Fig. 4(a)). Therefore, HFM effect on expression of IL1β, IL8, MCP1 and TNFα was analysed in CD14+ and the HFM effect on expression of RANTES in CD14- cells.

In CD14+ cells, expression of all measured cytokines was increased in response to HFM challenge (Fig. 4(b)). This increase was similar in both conditions except for TNFα that was not altered in response to HFM in UDCA treatment. Next, expression of two miRNAs implicated in the negative regulation of expression of TLR2/4 pathway members was analysed. Expression of miRNA181a but not that of miRNA146a was decreased by HFM in both placebo and UDCA conditions. Expression of RANTES, a cytokine produced by CD8+ lymphocytes, was decreased in CD14- cells after HFM in UDCA condition only (Fig. 4(b)). This result was also confirmed when expression of RANTES was normalized to the pan T lymphocyte marker CD3g (not shown). However, the changes of all cytokine mRNA expression in response to HFM were not different between placebo and UDCA conditions when analysed by two-way ANOVA.

Expression of other genes potentially activated by dietary fatty acids-i.e. TLR4, TLR2, PPARα and PPARγ was not altered significantly in response to HFM challenge (Fig. 4(c)).

Postprandial changes of gene expression of ERS markers in CD14+ and CD14- PBMC

First, we compared the level of expression of ERS markers between the two populations of PMBC. Compared to the CD14- cell population, CD14+ cells expressed more ATF4,

HSPA5 and DNAJC3 mRNA, while EDEM1 and XBP1 were expressed in both cellular populations to the same degree (Fig. 5(a)). Expression of ATF3 was restricted to CD14+ cells. In response to HFM, PBMC did not alter their expression of HSPA5, ATF4, EDEM1, XBP1 (spliced vs. total) and DNAJC3 in either placebo or UDCA condition (Fig. 5(b)). Nevertheless, HFM challenge led to significant elevation of ATF3 mRNA in CD14+ cells in both placebo and UDCA condition (Fig. 5(b)). Relative change of ATF3 expression caused by HFM correlated with the relative change of IL8 expression (R=0.745, *P=0.017*) but not with the change of other cytokines. In addition, basal levels of mRNA DNAJC3, EDEM1, ATF4, XBP1s and HSPA5 correlated with mRNA levels of RANTES (Fig. 5(c), all correlations reached R>0.7, *P<0.03*).

Discussion

The aims of the present study were to (i) examine a potential association between the inflammatory and ERS response to a HFM meal in two subpopulations of PBMC representing cells of innate and adaptive immunity and (ii) to 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 subjects to model the situation that precedes and could contribute to the development of obesity and 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 of major metabolites. The evolution of NEFA plasma concentration followed a known pattern in response to a single mixed meal, i.e. immediate sharp decrease of NEFA due to insulin antilipolytic action, and a following increase of plasmatic NEFA levels dependent on the spill over fatty acids from chylomicron-TAG [197]. On the other hand, the glycaemia remained unchanged in response to HFM as described before [198-201], even though some published studies [202-204] showed peak glucose levels after 30-60 minutes following a mixed meal challenge. The observed blunted hyperglycaemic response could be caused by significant absolute and relative amounts of fat and proteins in the test meal that have been shown to reduce postprandial glucose metabolism probably due to delayed gastric emptying [200, 205]. Thus, the complexity of meal, despite its high absolute (not relative) carbohydrate content, may lead to paradoxical suppression of postprandial glucose plasma concentration.

In accordance with previous studies [187, 206], postprandial leukocytosis was observed in this study. In line with results by Hansen et al [187], our experimental meal increased absolute numbers of granulocytes in blood. These fast changes 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) [207]. We have also observed an increase in absolute lymphocyte and monocyte counts in blood. It is to be noted that the increase of lymphocyte counts may be associated with circadian rhythm [208-209] . Nevertheless, the meal used in this study had higher total energy, carbohydrate and protein content than meals used in the previously cited studies by van Oostrom et al [208-209], and thus these metabolic variables may have a more important role in the observed lymphocyte and monocyte activation than the circadian rhythm.

Postprandial inflammation was previously characterized by the increased circulating levels of several inflammatory cytokines [188]. We confirmed the postprandial elevation of IL6.

Postprandial increases of IL6 plasma levels were reported by others [210-211]. As IL6 mRNA levels were barely detectable in CD14+ or CD14- cells (not shown), the elevation of IL6 in circulation was driven by other IL6 producing cells or tissues.

Concerning HFM-induced changes in blood cells, we confirmed the finding by Gower et al showing increased CD11c expression on the surface of monocytes after HFM in healthy volunteers [212]. CD11c is considered an activation marker of monocytes because it enhances their adhesion to endothelial cells and potential to migrate into target tissues. Importantly, high fat diet feeding results in CD11c+ monocyte infiltration into adipose tissue in mice [213-214] and these monocytes/macrophages exhibit a proinflammatory M1 phenotype. CD11c expression is also increased in blood monocytes of obese subjects and positively correlates with HOMA-IR [215]. Therefore, a single HFM may activate monocytes in a similar direction as long term overfeeding or obesity. This observation is important in 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 potentially harmful condition long before they become obese.

We then focused on gene expression in CD14+ (monocytes) and CD14- (lymphocytes) PBMC, i.e. the cells that are intimately exposed to metabolite fluctuations but upon the activation also contribute to the development of inflammation in adipose tissue in response to overfeeding. Until now, changes of gene expression induced by a meal were analysed only in whole PBMC population [198-199, 201]. 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 prior to gene expression analysis. Remarkably, expression of all tested proinflammatory cytokines was enhanced after HFM in CD14+ monocytes. Moreover, we also detected decreased expression of miR181a, a negative regulator of the TLR4/NFκB pathway [216]. This decrease of miRNA181a expression following HFM challenge could reinforce the synthesis of proinflammatory cytokines. Observed downregulation of miR181a may be specific for inflammation induced postprandially, given that expression of another miRNA involved in negative regulation of several proinflammatory cytokines, miR146a [217], remained unaltered. As noted already for CD11c expression, postprandial expression changes of miRNA181a and proinflammatory cytokines were similar to the alterations in their expression associated with obesity [216, 218].

Interestingly, we did not detect any alterations of expression of genes potentially activated by dietary fatty acids (PPARγ, 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 leukocytes [206] suggesting that leukocytes uptake NEFA. However, our data suggest that several hours of exposure to dietary lipids is not sufficient to induce substantial expression changes of regulators of lipid metabolism in CD14+ cells. Levels of TLR2 and TLR4 mRNA were not changed in CD14+ monocytes by HFM challenge even though we detected higher counts of CD14/TLR2 and CD14/TLR4 positive monocytes in blood. Nevertheless, the level of fluorescence (MFI) of TLR2 and TLR4 on the monocyte surface was not changed (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 3 arms of unfolded protein response (UPR). IRE1 activation leads to XBP1 splicing and XBP1s then stimulates expression of DNAJC3 and EDEM1 and partially HSPA5 [219]. HSPA5 is primarily a target of ATF6 UPR arm [220]. Activation of PERK is linked with ATF4 upregulation that then induces expression of ATF3 [221]. Following 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 postprandial increase of cytokine expression by CD14+ monocytes. Absence of XBP1 splicing was rather surprising as it can be stimulated by insulin [222] and insulin levels were raised in response to HFM challenge. It was also reported that higher activation of XBP1 is detectable in monocytes from obese subjects and subjects with metabolic syndrome [192]. The finding that HFM challenge does not initiate ERS in PBMC also explains minor effects of UDCA on expression of inflammatory cytokines. These minor effects could not be based on low blood bioavalailability of UDCA as pharmacokinetics data show that UDCA reaches peak concentration at 60 minutes after oral administration and its half-life is more than 3 days. The ability of UDCA to modulate cytokine expression observed in the case of TNFα in CD14+ and RANTES in CD14- PBMC is therefore probably unrelated to its chaperone-like properties. Of note, UDCA was shown to have immunosuppressive potential different from its effect on ERS due to its ability to activate glucocorticoid receptors and inhibit TLR signalling pathway [195]. UDCA may also influence blood cells through binding to the G-protein-coupled receptor TGR5 [223]. 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 that was postprandially elevated was ATF3. ATF3 mostly acts as a transcriptional repressor and may thus be part of a counterbalance system in healthy individuals, protecting them from over activation of pathways induced by stress [224-226]. 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 [185-186, 189]. Indeed, careful evaluation of differences in the expression 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 of ATF3 expression induced by HFM correlated specifically with a change of IL8 expression. IL8 was recently described as a cytokine whose expression is altered specifically by HFM [198]. ATF3 is, however, activated by various stresses not only by ERS, [227] and the absence of ATF4 upregulation in analysed CD14+ cells of ATF3 in classic UPR suggests that ATF3 upregulation does not involve UPR activation. Moreover, the lack of rise in blood glucose after HFM digestion suggests that hyperglycaemia-induced oxidative stress is not the trigger of ATF3 expression.

Although we did not find a relationship between HFM-induced changes of inflammatory cytokines and most ERS markers, striking coregulation of mRNA expression of RANTES and all ERS markers opens the question as to whether the higher ERS levels in CD14- PBMC (probably CD8+ T cells that are main producers of RANTES [228]) could be a marker of their activation as was previously suggested for the conditions of acute pathogen infection [229].

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

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Conflict of Interest

Authors declare no conflicts of interest.

Authorship

L.R. 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. L.R. designed the study, performed data analysis and wrote the manuscript, J.K, E.C, M.K. and L.M. performed experiments, data analysis and contributed to discussion, E.C. organized clinical part of the study, M.S. and V.S. contributed to discussion and writing of the manuscript. All authors read and approved the final manuscript.

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Figure legends

Fig. 1. Evolution of plasma levels of non-esterified fatty acids (NEFA) (a), glycerol (b), triacylglycerols (TAG) (c), glucose (d) and insulin (e) following a high fat meal (HFM) challenge. Open circles-placebo, black squares- ursodeoxycholic acid (UDCA). Data represent means ±SE, * *P<0.05*, ** *P<0.01,* *** *P<0.001* values different from basal levels in placebo condition, † *P<0.05*, †† *P<0.01,* ††† *P<0.001* values different from basal levels in UDCA condition.

Fig. 2 The effect of the test meal on numbers and activation of leukocytes. (a) Fasting (basal) absolute numbers of leukocytes were compared with leukocyte numbers 4 hours after high fat meal (HFM) challenge in placebo and ursodeoxycholic acid (UDCA) conditions. M-monocytes, L-lymphocytes, G-granulocytes, leuko-total leukocytes (b) Number of cells in subpopulations of monocytes and lymphocytes out of 10 000 events in both, placebo (Plac) and UDCA conditions. (c) Mean fluorescence intensity for CD11c in monocytes. White bars - baseline, black bars -HFM. Data represent means ±SE, **P<0.05*, ** *P<0.01*, *** *P<0.001*.

Fig. 3 Evolution of plasma levels of IL6 following a high fat meal (HFM) challenge. Open circles-placebo, black squares- ursodeoxycholic acid (UDCA). Data are means ±SE, * *P<0.05*, values different from basal levels in placebo condition, † *P<0.05* values different from basal levels in UDCA condition.

Fig. 4 The 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 cytokines between CD14+ and CD14- PBMC. (b) qRT-PCR analysis of cytokines and miRNAs implicated in regulation of inflammatory pathways in PBMC collected before and 4 hours after a high fat meal (HFM) challenge. (c) qRT-PCR analysis of genes potentially activated by non-esterified fatty acids (NEFA) in CD14+ PBMC collected before and 4 hours after HFM challenge. White bars - baseline, black bars -HFM. Data represent means ±SE, **P<0.05*, ** *P<0.01,* *** *P<0.001*..

Fig. 5 The 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- PBMC. (b) qRT-PCR analysis of ERS markers in PBMC collected before and 4 hours after a high fat meal (HFM) challenge. Data represent means ±SE, ** *P<0.01*. (c) Linear regression between mRNA levels of RANTES and HSPA5 in CD14- PBMC in fasting conditions.

Table 1. Characteristics of the subjects (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

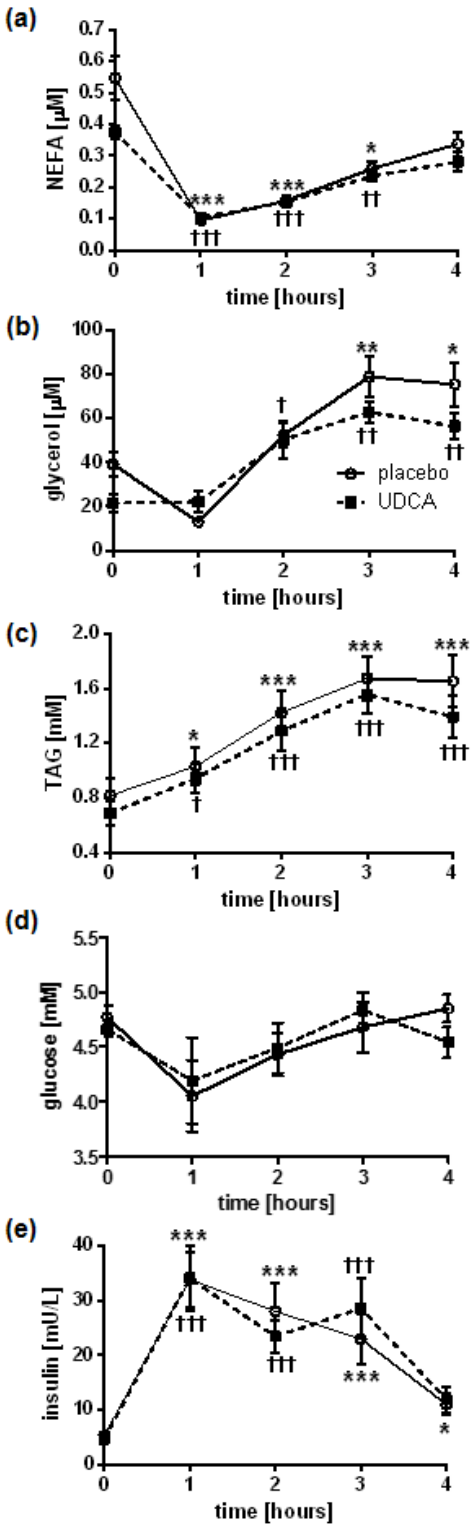
(a)
NEFA [µM]
time [hours]
(b)
glycerol [µM]
placebo
UDCA
time [hours]
(c)
TAG [mM]
time [hours]
(d)
glucose [mM]
time [hours]
(e)
insulin [mU/L]
time [hours]

Figure 1

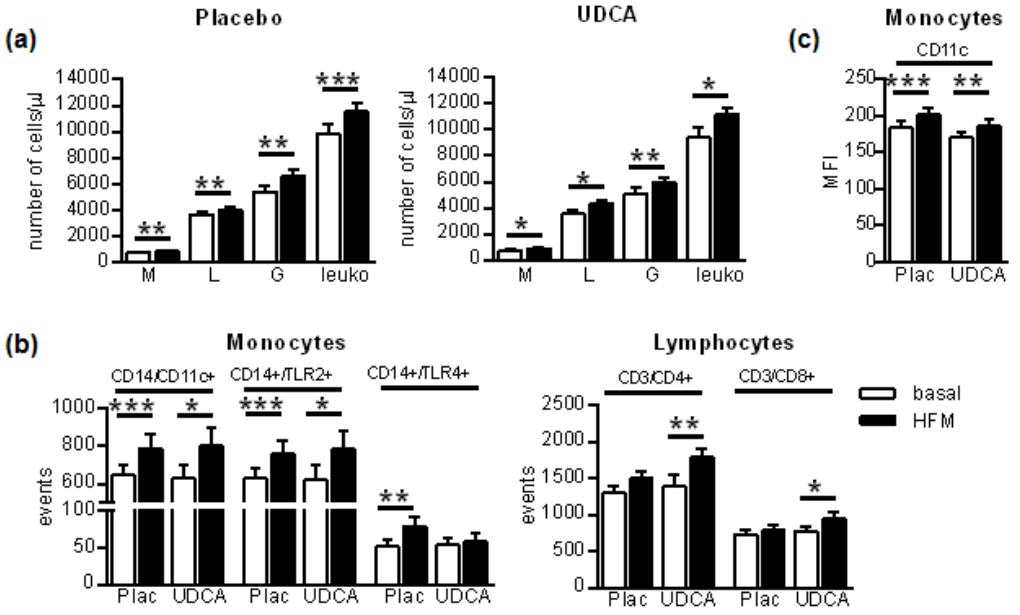
(a)
Placebo
UDCA
number of cells/µl
14000
12000
10000
8000
6000
4000
2000
0
M
L
G
leuko
(c)
Monocytes
CD11c
MFI
250
200
150
100
50
0
Plac
UDCA
(b)
Monocytes
CD14/CD11c+
CD14+/TLR2+
CD14+/TLR4+
events
1000
800
600
100
50
0
Lymphocytes
CD3/CD4+
CD3/CD8+
2500
2000
1500
1000
500
basal
HFM

Figure 2

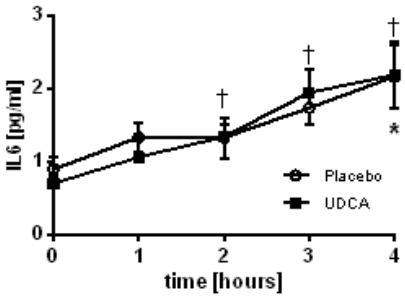

Figure 3

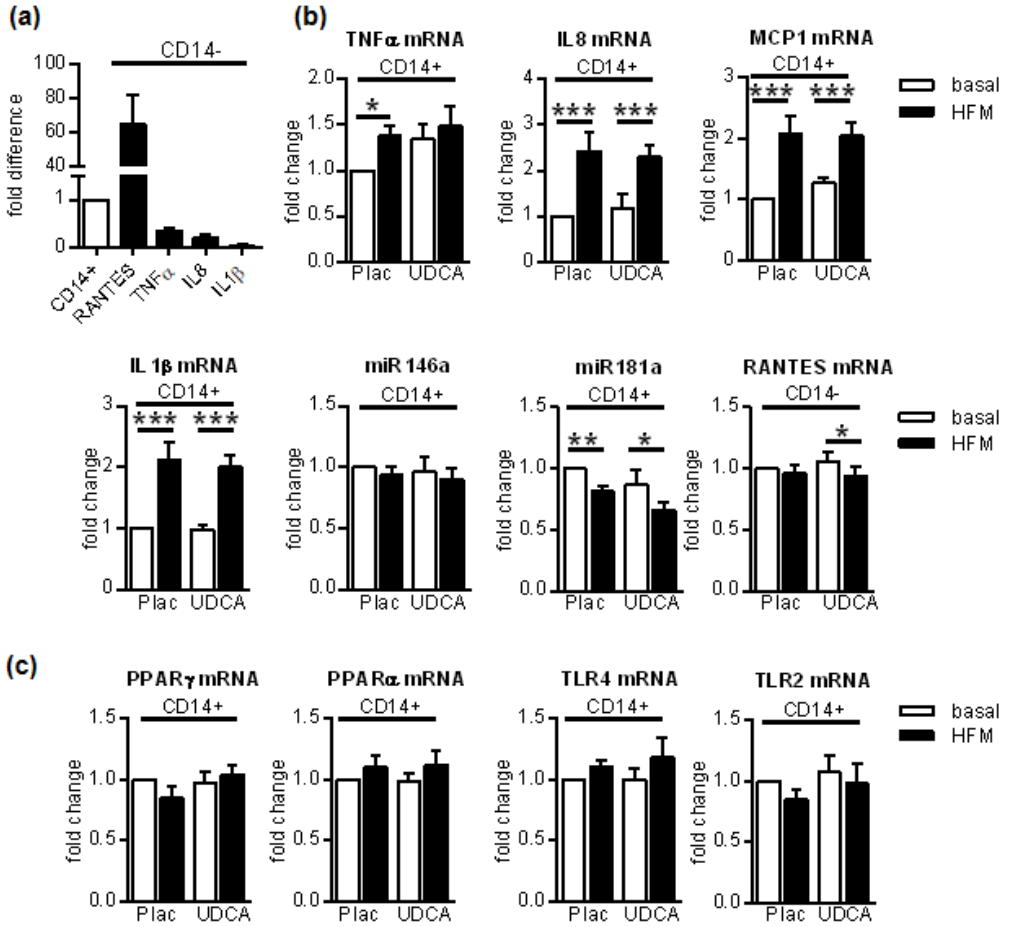
(a)
CD14-
fold difference
100
80
60
40
1
0
CD14+
RANTES
TNFα
IL8
IL1β
(b)
TNFα mRNA
IL8 mRNA
MCP1 mRNA
IL 1β mRNA
miR 146a
miR181a
RANTES mRNA
CD14+
CD14-
fold change
Plac
UDCA
basal
HFM
(c)
PPARγ mRNA
PPARα mRNA
TLR4 mRNA
TLR2 mRNA

Figure 4

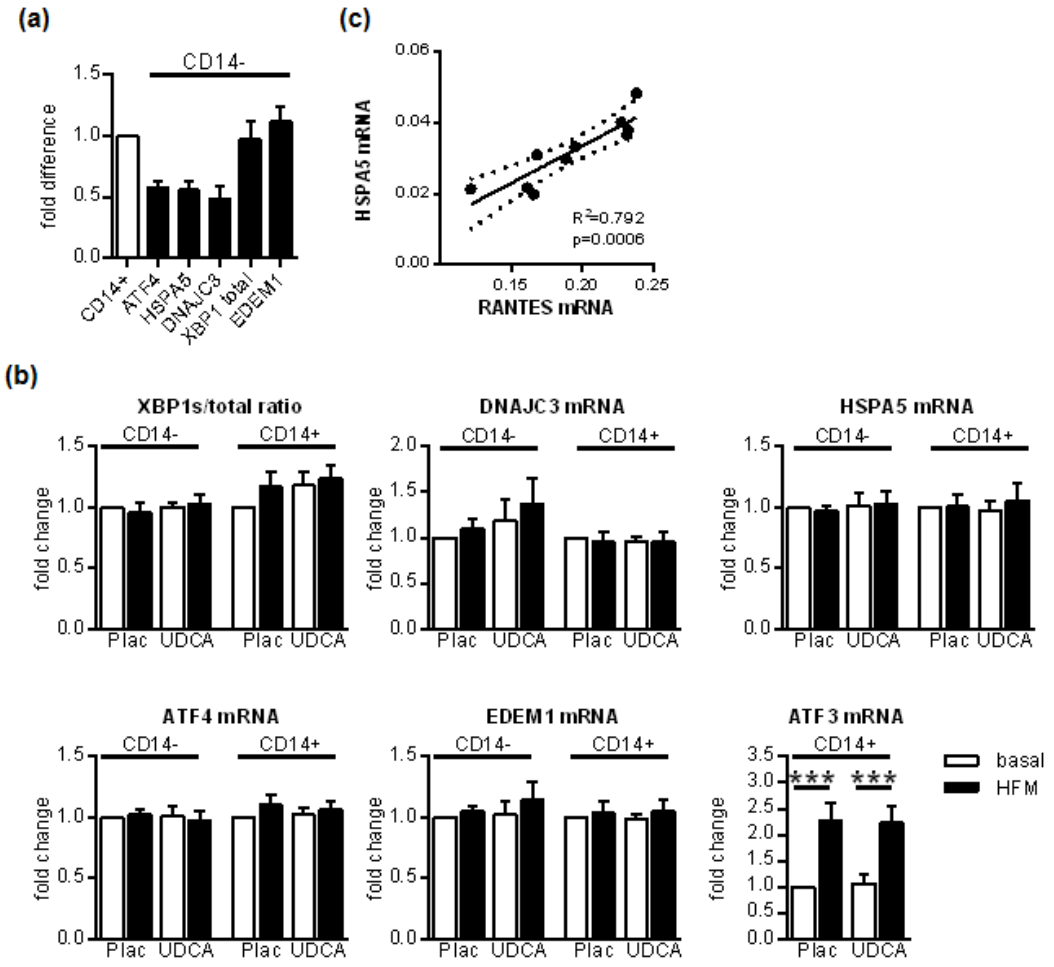
(a)
CD14-
fold difference
CD14+
ATF4
HSPA5
DNAJC3
XBP1 total
EDEM1
(c)
HSPA5 mRNA
RANTES mRNA
R²=0.792
p=0.0006
(b)
XBP1s/total ratio
DNAJC3 mRNA
HSPA5 mRNA
ATF4 mRNA
EDEM1 mRNA
ATF3 mRNA
fold change
Plac
UDCA
basal
HFM
***

Figure 5

4 DISCUSSION

Increased incidence of obesity and obesity-related disorders pointed at the importance of understanding how excessive adiposity perturbs metabolic functions. The discovery of increased level of the pro-inflammatory cytokine TNFα in AT of obese mice was the first evidence that the increased level of pro-inflammatory mediators was associated with obesity [230]. At present, it is well accepted that changes in inflammatory signaling by adipocytes or infiltration of AT by immune cells are key steps in the development of IR as well as other metabolic disorders associated with obesity [111]. Further, recent studies have suggested that ERS activated under obese condition can mediate both metabolic and immune responses to obesity [136, 177 193, 231] especially in the liver and AT [177]. Intriguingly, inflammation itself can induce the ERS [232] and, thus, inflammation and ERS may potentiate each other. Moreover, increasing number of evidences suggests that BAs, the products of cholesterol metabolism, are chemical chaperones capable to influence the ERS, as a suggested key link between obesity, IR and T2DM.

The key treatment of obesity is a modification of lifestyle including restriction of calorie intake. It has been shown that weight loss induced by calorie restriction leads to the improvement of systemic metabolic characteristics including reduction of IR, in hand with the decrease of pro-inflammatory state in obese individuals: i.e. diminution of expression of vascular inflammation markers [233] or pro-inflammatory cytokines produced by immune cells and adipocytes [234] or an increase of circulating anti-inflammatory cytokines and adiponectin [235-236].

Therefore, four studies included in this thesis were focused to investigate the AT or blood cells inflammatory state and other characteristics in relation to A) weight loss and B) ERS.

Part 1

Two studies were focused on the effect of dietary intervention on AT properties. The first one revealed, contrary to the original hypothesis, that the differences in the clinical impact of abdominal and gluteal AT were not based on their inflammatory status either in basal state or in response to dietary intervention. Due to the association between low-grade inflammation state and obesity-related metabolic disturbances (primarily insulin resistance) [118, 170-172], we hypothesised that differences in inflammatory states might explain striking metabolic differences between ,,good gluteal,, and ,,bad abdominal,, AT. A few studies paid attention to this topic and revealed that the diet-induced changes in lipolysis or adipocyte size of AT in response to hypocaloric diet were less pronounced in gluteal AT when compared with subcutaneous abdominal adipose tissue (sAAT) [237-238]. Therefore, we hypothesized that this impaired responsiveness of subcutaneous gluteal adipose tissue (sGAT) might also appear in respect to the diet-induced modulation of gene expression of immunity-related genes.

Both, gluteal and abdominal, AT were analyzed in basal state, (i.e. before dietary intervention) and during and at the end of calorie restriction. Contrary to recent work of Evans et al. [239] that reported higher expression of inflammatory genes in sGAT compared to sAAT, we reported no major differences in gene expression of pro-inflammatory markers between both depots in basal state. Discrepancies between our and Evans´s study could be based on differences in subjects recruited in the two studies: our study included only obese white women in contrast with mixed group of lean and obese black and white South African women examined in the study of Evans et al. [239].

The main interest of this study lay in the comparison of gene regulation of inflammatory markers in sAAT *versus* sGAT in dynamic condition represented by two phases of a 6 months´ dietary intervention. Similarly to our previous study [240] we observed enhancement of gene expression of macrophage markers in both depots during VLCD phase of diet. This phenomenon could be explained by enhanced FA release from adipocytes, as a possible trigger of macrophage activation and infiltration mediated by TLR4 signaling as shown before [122, 241].

Despite the fact that no major differences were found in mRNA level of inflammatory markers in response to the diet, 3 cytokines exerted different expression pattern. The differential response of these cytokines might be linked to different response of sGAT

(when compared to sAAT) to one of the up-stream regulators of pro-inflammatory cytokine production [242], endocannabinoid system, as described in the study of Bennetzen et al. [243].

Limitation of our study lies in its focusing specifically on transcriptional level of inflammatory markers. It would be interesting to study the secretion level of pro-inflammatory cytokines before, during and after the whole dietary intervention in both depots, but limited amount of AT obtained by needle biopsy prevents this type of analysis. Moreover, the results of this study are limited to women. It is to be noted that female AT shows different metabolic and endocrine characteristics [244-245] when compared with men.

In conclusion, we did not find major differences in mRNA levels of macrophage markers and cytokines between sAAT and sGAT at baseline condition or in the pattern of their regulation in response to two phases of hypocaloric weight-reducing dietary intervention. Thus, our results do not bring evidence of an altered pro-inflammatory status or an altered "responsiveness" of immune cells in sGAT when compared with sAAT, what suggests the similar regulation of macrophage infiltration in sGAT and sAAT during weight reducing dietary intervention.

As shown previously, development of obesity in the prepubertal age is associated with appearance of new small adipocytes, whiles obesity in adults is linked mainly with the hypertrophy of existing adipocytes [246-247]. Isakson et al. [248] and others brought evidence that number of preadipocytes capable of undergoing differentiation is lowered in obesity [142-143] and negatively correlates with BMI of the donor [42, 248-249]. The lack of information about the association between weight loss and adipogenic capacity of adipocytes give a basis for the formation of the second study. We hypothesized that cell cultures established from AT before and after the diet-induced weight loss would reflect two distinct metabolic and nutritional stages of the donor and could provide the information about the adipogenic and endocrine potential of obese and post obese AT.

We revealed that moderate weight loss improved adipogenic potential of preadipocytes, as earlier shown in rat models [250-251]. This finding extends current understanding of the impact of weight loss on the function of AT that has been until now attributed mainly to decreased size of mature adipocytes and pro-inflammatory potential/numbers of infiltrated immune cells [240, 252].

Maturation of adipocytes is dependent on lipogenesis. Indeed, enzymes for lipogenesis in general were found to be down-regulated in obese patients [253].

Our study showed that adipocytes derived after the weight loss exhibited higher expression of lipogenic enzymes: fatty acid synthese (FASN), diacylglycerol O-acyltransferase 2 (DGAT2), stearoyl-CoA desaturase (SCD1) and ATP citrate lyase (ACLY). This observation based on *in vitro* differentiated adipocytes is in line with the results of the study on isolated mature adipocytes that showed increased incorporation of labeled glucose into lipids after the weight loss induced by gastric banding or lifestyle modification [254].

Limitation of this study is that we cannot completely exclude the possible effects of subcultivation on adipogenic potential of cells. However, the *in vitro* conditions are unlikely to entirely suppress the original phenotype of the donor as it has been shown several times in preadipocytes and adipocytes [255]. Moreover, subcultivation of SVF cells gives more homogenous population than primary cells because conditions of the cultivation eliminate contaminating cells as e.g. macrophages [248, 256-257].

The profile of secreted adipokines by AT becomes altered in obesity: pro-inflammatory molecules are increased whereas adiponectin, a molecule with several beneficial actions, is reduced [246]. In our study, we documented that the weight loss *in vivo* altered capacity of the differentiated adipocytes to secrete pro-inflammatory cytokines as well as adiponectin *in vitro*. We observed A) decreased secretion of cytokines in AT *in vitro* at the end of 6 months´ dietary intervention when compared with the pre-diet condition and B) enhanced adiponectin production.

To sum up, since metabolically active, newly differentiated adipocytes contribute to an improved physiological status [258] of the body and adipogenesis is an important mechanism in the prevention of hypertrophy development [259], our data suggest that beneficial effect of weight loss lies in the improvement of enhanced adipogenic capacity as well as in the amelioration of inflammatory profile of adipose tissue cells.

Part 2

As mentioned earlier, obese state can disrupt the function of ER in AT, induce its stress and so deteriorate AT functions [110]. Endogenous BAs including UDCA and its taurine conjugate TUDCA that are successfully used to treat liver diseases [151] are considered as chemical chaperones capable to modulate functions of ER [138]. Chaperone activity of BAs was observed in several cellular and animal models [138-140]. Contrary to these results we

could not prove a capacity of UDCA or TUDCA to prevent ERS (assessed by expression of HSPA4, ATF4, DNAJC3, EDEM1 and XBP markers), induced by commonly used stressor, tunicamycin [260]. Indeed, in support of our observation in human adipocytes, *in vivo* treatment of obese subjects with TUDCA did not alter the expression of ER chaperone in AT [142]. This might be caused by low expression of BAs transporters in most extrahepatic tissues including AT [261-262]. Nevertheless, BAs can influence tissue metabolism through some of their receptors.

The effect of BAs on human adipocyte metabolism is still not fully elucidated. Therefore, in addition, we investigated the effect of BAs on proliferation and differentiation of adipocytes derived from human AT. We found an inhibition of preadipocyte proliferation and differentiation under UDCA treatment. In agreement with our data, recent studies of Krishna-Subramanian et al. (2012) and Peiró-Jordán et al. (2012) confirmed anti-proliferative potential of UDCA in several type of carcinoma [263] and normal intestinal cells [147]. From 3 possible mechanisms that could underlie the UDCA´s anti-proliferative effect-senescence [264], apoptosis [265] or cell cycle arrest [266]- cell cycle arrest in G2/M phase was confirmed in our experiments [267]. Treatment of preadipocytes with taurine conjugate of UDCA did not reveal any changes in their proliferation.

In respect to differentiation, BAs (TUDCA, UDCA) influence signaling pathways through binding to the TGR5 [261] receptors or activation of nuclear FXR receptor [154]. Both, TGR5 and FXR, seem to play important role in AT physiology, especially FXR was implicated in the regulation of adipocytes differentiation and function [153, 268-269]. Since UDCA is able to weakly bind the FXR receptor, we hypothesised that its activation could explain UDCA´s inhibitory effect on adipocytes differentiation. In our study, we showed, for the first time, that FXRα is expressed in human adipocytes. Similarly to major adipogenic transcription factor, PPARγ, FXRα expression was elevated with the adipogenic conversion, which does suggest an involvement of FXRα in human adipogenesis. The observation that agonist of FXRα receptor, GW4064, and UDCA had completely opposite effect on the expression of FXRα mRNA itself does not, however, support the assumption that UDCA inhibitory effect on adipogenesis might be dependent on activation of FXR. Therefore, another mechanism of anti-adipogenic activity of UDCA must play a role. Erk1/2 was shown previously to be activated by UDCA [147] and at the same time Erk1/2 can inhibit PPARγ activity [270]. We brought the evidence that Erk1/2 phosphorylation was sustained in the

presence of UDCA for at least 24 hours. Thus, it can be suggested that UDCA may lower sensitivity of cells to adipogenic stimuli by Erk1/2 – dependent inhibition of PPARγ.

Regarding the effect of taurine conjugate of UDCA, TUDCA, on cells differentiation, the data are inconsistent. We and Kim at al. [271] described neutral effect of TUDCA, while very recently Cha et al. [272] described its inhibitory effect on adipocytes differentiation. Since adipogenesis is very sensitive process, differences in conditions of adipocytes cultivation including the exact composition of differentiation medium could underlay the discrepancies that have been seen in both studies.

The third aim of this study was to determine the effect of both BAs on cytokine expression. We observed that exposition to BAs did not lead to reduction of expression of cytokines in adipocytes, in contrary, UDCA up-regulated mRNA levels of selected pro-inflammatory cytokines selectively in gluteal adipocytes. The mechanism why UDCA up-regulated the expression of cytokines in adipocytes remains unknown, but as confirmed previously [148], activation of other pathway than NFκB might play a role. Selective activation of IL8 and TLR4 by UDCA in gluteal adipocytes was rather unexpected but it could reflect higher attraction of macrophages compared to abdominal adipocytes.

To summarize, our data revealed that while TUDCA has neutral effect on human preadipocytes and adipocytes, the therapeutic use of UDCA different from treating cholestatic diseases should be considered with caution because UDCA alters functions of human adipose cells.

The increased circulating lipid and glucose levels induced by high fat-high calorie diet, as seen in obesity and T2DM, appear to elevate ERS [273] in the liver [136], in AT [274] as well as in atherosclerotic lesions [275-276].

Postprandial inflammation induced by HFM was confirmed in this study by increased levels of circulating IL6 that was previously reported also by others [210-211]. Concerning HFM-induced changes in blood cells, we revealed elevated amount of monocytes activation marker CD11c after HFM in healthy volunteers, which is in line with data of Gower et al. that showed increased mRNA level of CD11c after HFM [212]. Notably, it was proved that high fat diet results in CD11c+ monocytes infiltration into AT in mice [213-214]. Moreover, CD11c expression is also increased in blood monocytes of obese subjects and positively correlates with HOMA-IR [215] what could link the IR development with immune cells infiltration.

Until now, changes of gene expression in postprandial inflammation state were analyzed only in whole PBMC population [198-199, 201] which did not provide information on the behaviour of individual populations of blood cells. Therefore, this study was focused on gene expression analysis in CD14+ (monocytes) and CD14- (lymphocytes) PBMC populations.

HFM led to the increased mRNA (TNFα, IL8, MCP1 and IL1β) level of all tested pro-inflammatory cytokines in CD14+ monocytes as well as to the decreased expression of miR181a, negative regulator of TLR4/NFκB pathway [216]. These postprandial changes of expression of miRNA181a and pro-inflammatory cytokines were similar to the alterations in their expression associated with obesity [216, 218].

To determine whether the postprandial inflammation could be triggered by enhanced ERS and alleviated by potential chemical chaperone UDCA, we analyzed ERS markers representing all 3 arms of UPR pathway (see Chapter 1). Contrary to the expectation, mRNA expression of majority of ERS markers (except for mRNA of ATF3 [227] that is activated by various stresses not only by ERS) was not altered in PBMC. This indicates that classic activation of UPR does not seem to be the driver of postprandial increase of cytokine expression by CD14+ monocytes. The finding that HFM challenge does not initiate ERS in PBMC explains also the minor effects of UDCA on the HFM-induced expression of inflammatory cytokines.

Briefly, we brought the evidence that HFM induced an inflammation in two populations of PMBC that was not accompanied by activation of majority of investigated ERS markers. Acute effect of UDCA prior to HFM consumption did not affect expression of ERS markers but modified partially the HFM-induced activation of blood cells. The putative molecular trigger of postprandial inflammation thus remains to be established.

5 CONCLUSIONS

This thesis was focused on the analysis of the effect of weight loss and of modulation of ERS on the pro-inflammatory status and other characteristics of AT. In addition, the link between ERS and postprandial inflammation was studied in blood cells. Two prospective dietary studies and two studies with pharmacological intervention were included into the thesis to fulfil aims defined in Chapter 2.

- Different clinical impact of subcutaneous gluteal and abdominal adipose tissue is not associated with differences in the inflammatory state of the respective adipose tissue: profile of mRNA expression of inflammation-related genes was not different in gluteal compared to abdominal adipose tissue in basal state or in response to dietary intervention.

- Weight loss induced by calorie restriction shifted the cytokine secretion profile of differentiated adipocytes *in vitro* towards a less pro-inflammatory one and improved preadipocyte adipogenic capacities.

- Neither ursodeoxycholic, nor tauroursodeoxycholic acid, the potential chaperones of stress of endoplasmic reticulum, were able to suppress the stress of endoplasmic reticulum in human adipose cells. Ursodeoxycholic acid negatively affected both proliferation and differentiation of adipose cells.

- Postprandial inflammation induced by consumption of high fat meal was not linked with the stress of endoplasmic reticulum in blood cells. Accordingly, chemical chaperone, ursodeoxycholic acid, was not able to prevent the postprandial inflammation *in vivo*.

6 SUMMARY

Obesity is considered to be a worldwide epidemic disease characterized by an accumulation of adipose tissue. Increased adiposity can perturb normal metabolic functions and lead to the development of diseases like insulin resistance, atherosclerosis, cardiovascular or metabolic disorders. A large amount of clinical studies have been shown that changes in inflammatory signaling in adipose tissue cells, increased infiltration of immune cells into adipose tissue, low-grade inflammatory state as well as stress of endoplasmic reticulum belong to the key molecular steps leading to the development of insulin resistance and other metabolic disturbances associated with this disease.

Adverse metabolic effects of adipose tissue accumulation can be diminished by calorie restriction resulting in weight loss. In addition, stress of endoplasmic reticulum could be alleviated by chemical chaperones including bile acids that were shown in animal studies or cell culture models, to modulate not only the stress of endoplasmic reticulum but also inflammatory state of cells. These two approaches for the treatment of obesity or the obesity-associated disturbances were basis for this PhD thesis.

We performed four clinical studies. In the first part of this work, we studied inflammation status of gluteal adipose tissue in comparison with abdominal adipose tissue and differentiation and secretory capacity of adipocytes after weight loss in obese patients. We revealed that inflammatory profile of gluteal adipose tissue, estimated by mRNA level of macrophages and cytokines as markers of inflammatory status of the body, did not explain the different clinical impact of subcutaneous abdominal and gluteal adipose tissue. We proved that weight loss is associated with an improvement of adipogenic capacity of preadipocytes in obese women as well as with change of the inflammatory status of adipocytes from more to less pro-inflammatory profile.

The second part of this PhD study consists from two clinical studies focused on the determination of the role of stress of endoplasmic reticulum and bile acids in adipose tissue and blood cells exposed to different experimental conditions.

We investigated effects of ursodeoxycholic acid (UDCA) and tauroursodeoxycholic acid (TUDCA) on preadipocytes and adipocytes derived from human adipose tissue of obese patients. Our results demonstrate that from two studied bile acids, only UDCA, is able to

influence physiology of adipose tissue cells and neither from two tested bile acids were able to suppress the stress of endoplasmic reticulum.

Except for the excess of adipose tissue, also food intake is accompanied by transiently elevated concentrations of inflammatory markers in the bloodstream, so-called postprandial inflammation. We hypothesised that stress of endoplasmic reticulum could be one of the possible triggers of postprandial inflammation. However, our results showed that postprandial inflammation was not accompanied by the stress of endoplasmic reticulum and was not preventable by UDCA.

To sum up, results of this thesis contributed to the understanding of the impact of weight loss and chemical chaperones on the molecular characteristics of adipose tissue. At the same time, the outcomes of the thesis stressed the need of further investigation of the individual adipose tissue depots, role of bile acids in human adipose tissue physiology and triggers of postprandial inflammation.

7 ANNEXES

Soluble CD163 is associated with CD163 mRNA expression in adipose tissue and with insulin sensitivity in steady state condition but not in response to calorie restriction

Kračmerová J, Rossmeislová L, Kováčová Z, Klimčáková E, Polák J, Tencerová M, Mališová L, Štich V, Langin D, Šiklová M.

J Clin Endocrinol Metab., 2014, Jan, in press

Adaptation of human adipose tissue to hypocaloric diet

Rossmeislová L, Mališová L, Kračmerová J, Štich V.

Int J Obes. (Lond), 2013, May, 37(5):640-50, Review

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