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Mechanisms of insulin resistance in humans with focus on adipose tissue

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SUMMARY

Background and Aims: Endocrine activity of adipose tissue is implicated in the development of insulin resistance (IR). The thesis aimed to extend the knowledge of mechanisms contributing to IR.

Study I – To investigate the effect of acute hyperinsulinaemia and acute angiotensin II type 1 receptor blockade (ARB) on plasma concentrations and subcutaneous adipose tissue (SAT) expressions of selected adipokines in patients with type 2 diabetes and healthy controls

Study II - To investigate the effect of 3-week telmisartan treatment on insulin resistance and plasma concentrations and SAT expressions of selected adipokines in subjects with metabolic syndrome and impaired fasting glucose (IFG)

Study III - To investigate the effect of prolonged hypertriglyceridaemia on plasma concentrations and SAT expressions of selected adipokines in patients with type 2 diabetes and healthy control subjects

Study IV - To assess the plasma concentrations and SAT expressions of selected adipokines in subjects with different categories of glucose intolerance

Methodology: Hyperinsulinaemic-euglycaemic clamp, Intralipid infusion and saline infusion were used to simulate specific metabolic conditions in vivo in 4 groups: 8 young healthy men, 11 overweight/obese patients with type 2 diabetes, 12 age-matched healthy controls and 12 overweight/obese patients with IFG.

Results:

Study I – In diabetic patients, plasma concentrations and/or SAT expressions of selected adipokines differ from those in healthy subjects. Insulin differentially regulates circulating resistin and leptin in diabetes and healthy subjects, while plasma adiponectin and TNFα are not acutely regulated by insulin. Stimulatory effect of insulin on SAT expressions was demonstrated only for TNFα and adiponectin. Suppressive effect of losartan on plasma resistin and leptin but no changes of the adipokines' expression were shown in diabetic patients following acute treatment. Importantly, losartan-induced increase in plasma adiponectin and its expressions suggests a potential mechanism for metabolic effects of losartan. Changes in plasma adipokines cannot be explained by changes in their SAT expressions. Circulating A-FABP and its expressions are closely related to obesity, IR and hyperglycaemia. Hyperinsulinaemia suppresses plasma A-FABP but does not influence its expression. Acute ARB stimulates basal A-FABP plasma concentrations without any effect on its expression.

Study II – In IFG, a short-term telmisartan treatment increases plasma adiponectin, leptin and resistin and decreases plasma TNFα levels. These effects appear to be important during hyperinsulinaemia. The changes in plasma concentrations of adipokines cannot be explained by their expressions in SAT.

Study III – Prolonged hyperlipidaemia stimulates an increase in plasma TNFα and resistin, while it results in decline in plasma leptin and A-FABP and it does not affect expressions of adipokines in SAT.

Study IV – The selected adipokines including A-FABP display differential regulations on the level of circulating concentrations and SAT expressions during the progression of glucose intolerance. The between-group differences in plasma concentrations are not mirrored in SAT expressions.

Conclusions: Providing a comprehensive evaluation of adipose tissue endocrine activity in vivo under different experimental conditions, the presented studies broaden the recent knowledge on the role of adipose tissue in pathophysiology of insulin resistance in humans. Type 2 diabetic patients, healthy subjects and patients with IFG differed in terms of baseline plasma concentrations and SAT expressions of selected adipokines. We have also demonstrated differential group-specific regulations of adipokines' concentrations and expressions in response to hyperinsulinaemia and hypertriglyceridaemia as well as to treatment with losartan or telmisartan. The presented results support the role of adipokines in the pathogenesis of IR.

INTRODUCTION

Insulin resistance – background and classification

Insulin resistance (IR) was originally defined as a reduced response of target tissues to metabolic actions of insulin, manifested by decreased insulin-stimulated glucose transport and metabolism in skeletal muscle and adipocytes and by impaired suppression of hepatic glucose output [1].

IR, impairment of glucose homeostasis, essential hypertension and dyslipidaemia are closely linked to obesity, forming a cluster of abnormalities well known as metabolic syndrome [2] that represents a major risk factor for accelerated atherosclerosis and cardiovascular disease [3] and has become one of the major public-health challenges worldwide. Recently, the mechanisms linking insulin resistance as a key feature with other metabolic and atherosclerotic defects are being intensively explored.

Understanding of insulin signalling cascade (summarized in Figure 1) has been essential for exploring of potential mechanisms of IR. IR usually relies on altered post-receptor actions but generally, defects at any level of insulin signalling may be involved in development of IR.

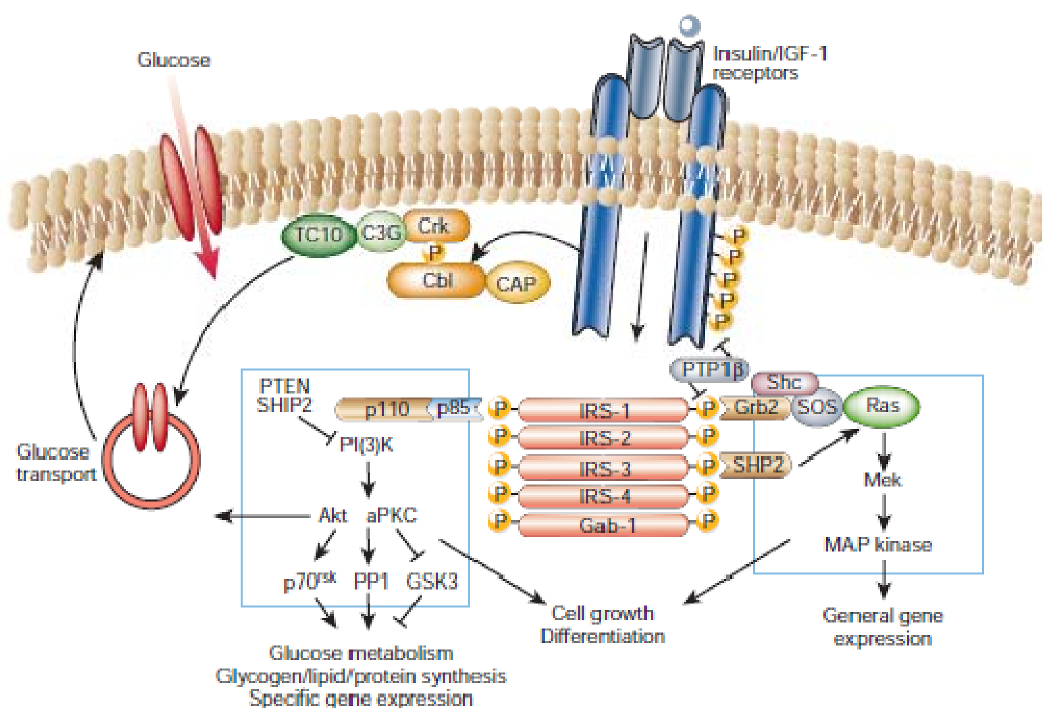

Figure 1 *Signal transduction in insulin action (adopted from [4]). The insulin receptor is a tyrosine kinase that undergoes autophosphorylation after binding of insulin and catalyses phosphorylation of cellular signal protein IRS 1-4 (insulin receptor substrate), Shc and Cbl. Upon tyrosine phosphorylation, these proteins interact with signalling molecules through their SH2 domains resulting in a diverse series of signalling pathways, including activation of PI3-kinase (phosphatidylinositol 3-kinase) and downstream PtdIns(3,4,5)P3-dependent protein kinases, Ras and the MAP kinase cascade and Cbl/CAP and the activation of TC10. These pathways act in a concerted fashion to coordinate the regulation of vesicle trafficking, protein synthesis, enzyme activation and inactivation and gene expression. In IR state, the decreased activation of PI3-kinase pathway leads to an inhibition of metabolic actions (mainly decline in insulin-dependent glucose transport). Resistance to insulin action increases the demand on insulin secretion by beta cells. Consequently, the resulting hyperinsulinaemia and increased insulin binding to its receptor significantly activates the growth-factor-like pathway mediated by MAP-kinase with negative consequences in lipid metabolism, mitotic activity and regulation of gene expression.*

IR is considered to be a multifactorial abnormality that has both genetic background (primary causes) and secondary causes that can be schematically divided in metabolic, humoral and neural disorders.

Metabolic causes include impaired lipid metabolism with increased concentrations of circulating non-esterified fatty acids (NEFAs) or hyperglycaemia (glucotoxicity). Elevated NEFAs result from increased release from the adipose tissue mass (which is resistant to antilipolytic effect of insulin), impaired tissue utilisation or combination of both processes. NEFAs impair the ability of insulin to suppress hepatic glucose output and to stimulate glucose uptake into skeletal muscle, as well as to inhibit insulin secretion from pancreatic beta cells [4]. NEFAs are also implicated in the central regulation of glucose production [5]. NEFA overflow may further lead to free radical formation during oxidative phosphorylation and production of toxic lipid metabolites that reflect oxidative damage (lipotoxicity) and interfere with the insulin signalling cascade in target tissues as well as with beta cell function [6]. Increased NEFA fluxes in IR and obesity lead to ectopic accumulation of triglycerides in other tissues (muscle, liver, Langerhans' islets of pancreas), which promotes the development of IR. The increased intramyocellular and intrahepatic lipid contents are consistently found already in early stages of IR development, such as in lean, normal glucose tolerant off-springs of type 2 diabetic parents [7, 8], as well as in manifest type 2 diabetes and obesity. Further studies have documented reduced lipid oxidation and reduced expression of key mitochondrial genes involved in regulation of oxidative metabolism in skeletal muscle [7] and provided thus support for the hypothesis of the role of mitochondrial dysfunction in the pathogenesis of IR.

Despite of elevated NEFAs in fasting state, a greater reliance on glucose oxidation and reduced efficiency of fat oxidation has been shown in type 2 diabetes and obesity, which is accompanied by blunted stimulation of glucose oxidation and impaired suppression of NEFAs in response to insulin (e.g. postprandially or during experimental hyperinsulinaemic clamp) [9, 10]. This impaired ability to efficiently switch between oxidizing fat and glucose has been described as metabolic inflexibility and represent one key mechanisms of skeletal muscle IR.

Hyperglycaemia *per se* can also down-regulate glucose transport system and other intracellular events involved in both insulin action and secretion from beta cell (glucose toxicity).

Among humoral causes of IR, hyperinsulinaemia represents a dominant and well-investigated factor, being not only a compensatory response to insulin resistance, but also a self-perpetuating cause of the defect in insulin action via down-regulation of insulin receptor number and intracellular signalling [7]. Other humoral factors involved in IR include an increase in insulin-contraregulatory hormones, insulin antibodies or a variety of cytokines and factors produced by adipocytes, endothelial cells and immunocompentent cells. The latter ones are recently subject of intensive investigation and the current knowledge on them is summarized in chapter Endocrine activity of adipose tissue.

Neural factors involved in IR pathogenesis are represented by insulin-stimulated sympathetic activation influencing intermediary metabolism, as well as by central regulation of food intake and energy expenditure.

Where does IR start?

Another approach to classify IR is to define the contribution of particular organs and tissues involved in the IR pathogenesis, namely skeletal muscle, adipose tissue, liver, beta cells and central nervous system. In this context, a great scientific debate is dealing with the questions: Where does insulin resistance start? Where and which is the primary defect and what changes are secondary? Although increasing experimental evidence can be found for the priority of all tissues mentioned, there is probably no simple answer. The development of IR clearly relies on interactions and cross-talk

between the involved tissues and represents a very complex multifactorial process. Another noteworthy consideration is that not all insulin-dependent processes and tissues are equally resistant to insulin.

Skeletal muscle is responsible for 80-90% of insulin-stimulated glucose uptake under euglycaemic hyperinsulinaemic conditions and postprandially and thus it has been traditionally investigated and considered as the primary site of IR. There is indeed a great body of evidence that muscle IR is the initial metabolic defect in the development type 2 diabetes [7]. As a model of IR pathogenesis, lean, normal glucose tolerant (NGT) offspring of type 2 diabetic parents were investigated. Similarly to patients with manifest type 2 diabetes, a decreased glucose uptake has been demonstrated in offspring, for which reduced non-oxidative glucose metabolism, e.g. a defect in insulin-stimulated glycogen synthesis accounted. Additionally, an increase in intramyocellular lipid content and mitochondrial dysfunction in myocytes has been documented in this cohort [11], accompanied with elevated NEFAs and metabolic inflexibility during hyperinsulinaemia. These findings however indicate the presence of marked adipocyte resistance to insulin action [7] and do not disclose the parallel involvement of other tissues.

Even if adipose tissue plays a minor role in insulin-stimulated glucose uptake postprandially, it has been shown to be a key player in the development of IR, particularly due to production of variety of factors (such as NEFAs, adipokines, cytokines etc.), known to modulate insulin sensitivity not only within adipose tissue but especially in other organs including skeletal muscle. The detailed description can be found in chapter Endocrine activity of adipose tissue.

Liver plays a central role in regulation of nutrient metabolism and it is also the primary site of insulin degradation. With the increasing experimental and clinical knowledge on non-alcoholic fatty liver disease (NAFLD), which is characterized by triglyceride accumulation within hepatocytes accompanied by features of both central (impaired insulin-mediated inhibition of hepatic glucose production) and peripheral IR (reduced insulin-mediated glucose uptake in muscle and decreased inhibition of lipolysis by insulin), there is more evidence available supporting the primacy of liver in the IR pathogenesis [8]. Ectopic fat in liver may be more important than visceral fat, since the intrahepatic fat content is more strongly related to peripheral IR than visceral, subcutaneous or intramyocellular lipid contents in obesity and type 2 diabetes [12-14]. Fatty liver might interfere with insulin degradation and resulting hyperinsulinaemia contributes to impairment of peripheral insulin action [15]. NAFLD is also associated with chronic low-grade inflammation with increased NF-κB (nuclear factor kappa B) activation and macrophage infiltration [16], changes that are postulated to be secondary to triglycerides accumulation in hepatocytes. However, longitudinal data showing the development of NAFLD (as it is the case for skeletal muscle) are lacking.

Brain and particularly hypothalamus play an important role in the regulation of energy balance and glucose homeostasis [17]. Insulin receptors and components of the insulin signalling pathways are widely distributed in the brain. Insulin crosses the blood-brain barrier through a receptor-mediated and saturable transport mechanism. Insulin in association with other nutrient and adiposity signals, such as NEFAs, amino acids or leptin directly regulate neuropeptide expression in hypothalamic nuclei and are involved in the feedback loop that is necessary for regulation of food intake. Additionally, insulin and leptin also regulate neuronal electrical activity via stimulation of ATP-sensitive potassium channel. Thus, insulin in central nervous system modulates glucose homeostasis

not only by increasing hypothalamic anorexigenic stimuli but also by activation of hypothalamic neurons leading to decreased hepatic glucose production [18] and stimulation of lipogenesis in adipose tissue [19], effects that are mediated by the autonomous nervous system. Diet, rather than obesity per se, seems to play a greater role in inducing a state of central IR [20]. In turn, the ensuing obesity may further reduce the neuronal sensitivity to peripheral signals, such as insulin and leptin, which further exacerbates obesity and insulin resistance.

Adipose tissue

Adipose tissue is no longer considered as an inert tissue devoted to storage of energy-rich triglycerides. Besides its major role in the regulation of nutrient and energy homeostasis, it is involved in the modulation of neuroendocrine and immune responses, reproductive function, bone mass growth and thermogenesis. It is recognized as the largest endocrine organ in the body secreting a variety of bioactive peptides, so called adipokines, growth factors and cytokines, which act at both the local level within adipose tissue (autocrine/paracrine) and systemic (endocrine) level [21].

Adipose tissue composition, inflammation, perfusion and innervation

White adipose tissue is a heterogeneous organ composed of adipocytes and stromal-vascular fraction, in which pre-adipocytes, macrophages, nerves, fibroblasts, endothelial and vascular cells are present. Pre-adipocytes originate from pluripotent mesodermal stem-cell with life-long potential to generate new adipocytes [22]. Several adipokines are secreted exclusively by adipocytes, while in production of other factors cells of the stromal-vascular fraction are substantially involved.

In obesity and IR, macrophages are more abundant in adipose tissue [23]. Interestingly, resident macrophages share certain characteristics with adipocytes, such as lipid accumulation or secretory activity and thus play an important role in initiation and maintaining of adipocyte dysfunction and the status of low-grade inflammation. It has been documented that inflammation and macrophage infiltration intensifies with increasing obesity and can be reversed by weight loss [24]. Moreover, the increased macrophage content in adipose tissue seen in obesity is composed predominantly of classically activated pro-inflammatory M1 macrophages, whereas the proportion of anti-inflammatory M2 macrophages is substantially smaller than in lean state [25]. Several mechanisms of the macrophage recruitment into adipose tissue were postulated: increased NEFA concentrations activate cellular pro-inflammatory pathways (NF-κB) in adipocytes and residing macrophages, which lead to release of chemotactic and pro-inflammatory cytokines (e.g. monocyte chemoattractant protein 1 or osteopontin) from both cell types. A local paracrine loop between adipocytes and macrophages establishes a vicious cycle [23]. Adipocyte apoptosis represents another stimulus for macrophage accumulation [26]. Recently, a role of T lymphocytes in the initiation of macrophage infiltration in adipose tissue has been recognized in animal models. Large numbers of CD8+ T cells are present in adipose tissue in obesity and are able to promote recruitment and activation of macrophages [27]. On the other hand, CD4+ helper and regulatory T cells that are able to down-regulate the inflammatory state and control glucose homeostasis, are substantially reduced in models of obesity [28, 29].

In the context of chronic energy imbalance and nutrient excess, adipose tissue capacity and plasticity to buffer the lipid load is essential for regulation of nutrient supply to other organs. In both obesity

and lipodystrophy, the ability of adipocytes to dispose of increased lipid load is impaired [30] which is a sign of IR in adipose tissue. Increased fat mass has been suggested to partly compensate for the defect in insulin action [31]. The expansion of adipose tissue can be attributed to both adipocyte hypertrophy and hyperplasia [32]. Adipocyte can store 0.8µg lipid per cell as a maximum. Large hypertrophic adipocytes (140-180 µm in diameter) characterized by reduced glucose and triglycerides clearance and increased lipolytic activity, can be found not only in obese and type 2 diabetes patients, but also in lean NGT offspring of type 2 diabetic parents [6]. On the other hand, adipocyte differentiation is a sign of insulin-sensitive adipose tissue and is exactly regulated by means of sequential activation of transcription factor cascade including PPARγ (peroxisome proliferator-activated receptor γ) and SREBP1c (sterol response element binding protein 1c) [33]. Interventions reducing adipocyte size either by recruitment of new small adipocytes (e.g. treatment with thiazolidinediones and potentially also with angiotensin receptor blockers) or by depleting triglyceride stores in existing adipocytes (e.g. exercise, diet) are able to reverse the features of IR [6].

Adipose tissue blood flow increases with prolonged fasting and during exercise in order to ensure supply of released NEFAs in the circulation, as well as after feeding when there is a need to increase substrate delivery for triglyceride clearance [30]. The close relation of angiogenesis and adipogenesis during adipose tissue expansion known from experimental models cannot be ensured as the adipocyte hypertrophy endures [6, 23]. Reduced adipose tissue blood flow documented in obesity leads to local hypoxia since the diameter of enlarged fat cell is greater than the diffusion limit of oxygen, thus limiting the exchange between blood and adipocyte cytoplasm. Hypoxia further promotes adipocyte dysfunction by inhibition of differentiation, inhibition of adiponectin gene expression, formation of free radicals and promotion of inflammation and thus may lead to cell death [34]. Apoptotic adipocytes promote macrophage infiltration in adipose tissue further stimulating the vicious cycle.

Autonomic nervous system is involved in the regulation of adipose tissue function and mass through both sympathetic and parasympathetic activation [30]. Sympathetic innervation is known to stimulate lipolysis and lipid mobilisation and to negatively regulate proliferation of pre-adipocytes [30]. It also influences adipose tissue metabolism indirectly through regulation of blood-flow: an increase in postprandial perfusion is dependent on sympathetic activation induced by insulin [30]. Changes in adrenoreceptor numbers and sympathetic drive have been documented in obesity, which suggests the modulation of autonomic signals to adipose tissue in response to energy stores and adipocyte size. Autonomic innervation appears to have a sensory afferent component that conveys adiposity information from the periphery to the brain. Parasympathetic innervation was historically considered to be less important but recent studies have shown that vagal innervation controls anabolic processes with decreased lipolysis in adipose tissue [35].

Adipose tissue topography and depot specificity

White adipose tissue is distributed through the body in different depots, each of them having specific gene expression, different responsiveness to nutrients, hormones and temperature that reflect their specific functions. It is found in subcutaneous, visceral, epicardial, extramyocellular, perivascular, retroorbital, facial and lymphnodal regions, as well as in bone marrow and mammary gland.

Visceral, epicardial, intermuscular and perivascular fat depots are physiologically more metabolically active and less insulin sensitive, showing higher lipolytic activity and thus ensuring direct energy

supply to the vital organs (i.e. liver, heart, skeletal muscle, vessels). These depots share similar adipokine, cytokine and NEFA release patterns or higher density of adrenoreceptors [6]. Omental fat appears to be important for regulation of disposal of ingested nutrients in the liver and periphery, with feedback to the brain via autonomic neurons. It has been suggested that relative IR of intra-abdominal, intrathoracic and intermuscular fat optimizes their ability to release energy to proximal organs, while expansion upon surrounding structures is limited [6]. There is a well documented evidence of association between increased visceral fat mass and IR, development of type 2 diabetes and cardiovascular risk [36, 37].

On the other hand, subcutaneous adipose tissue (SAT) primarily serves as a storage depot responding to anabolic action of insulin and showing more efficient proliferation in vitro [38]. Despite being less metabolically active, subcutaneous fat can substantially contribute to NEFA and adipokine release and imbalance, since subcutaneous fat mass is at least 10-fold larger than the visceral one. It has been postulated that impaired storage capability and defective expandability of subcutaneous fat, independent of body weight or adiposity, might be the primary cause of IR rather than solely enlargement of visceral fat depot [6]. The defect in SAT storage capacity is accompanied by increased NEFA fluxes, compensatory enlargement of non-subcutaneous fat depots and ectopic deposition of triglycerides in non-adipose tissues, starting thus a vicious cycle of IR. This hypothesis is supported e.g. by evidence in lipodystrophy (severe IR despite the lack of visceral fat), in treatments enhancing the ability to take up and store fat (treatment with thiazolidinediones in humans or subcutaneous fat re-implantation in animals) that reverse insulin resistance without influencing or even increasing fat mass. In accordance with this, liposuction fails to improve IR. Hyperplastic obesity with smaller, more insulin sensitive adipocytes seems to be more benign in terms of progression of metabolic dysfunction, than hypertrophic obesity.

Endocrine activity of adipose tissue

Several lines of evidence suggest that the disturbed endocrine function of adipose tissue found in obesity and/or type 2 diabetes represents one of the mechanisms implicated in development of insulin resistance, low-grade inflammation and related abnormalities [39].

Leptin

Leptin, the first adipokine indentified in 1994 [40], is a 16-kDa cytokine-like peptide encoded by *ob* gene that is produced exclusively by differentiated adipocytes. Leptin exerts both central and peripheral actions. At the level of central nervous system (CNS), leptin serves as a satiety-signal regulating food intake and increasing energy expenditure [41]. It crosses the blood-brain barrier by diffusion through capillary junctures in the median eminence and by saturable receptor transport in the choroid plexus [42]. In the hypothalamic feeding-regulating areas, such as the arcuate, dorsomedial and ventromedial nuclei, leptin stimulates release of anorexigenic peptides (i.e. proopiomelanocortin) and inhibits the orexigenic peptides (neuropeptide Y or agouti gene-related protein). After binding to the leptin receptor, cellular signal transduction cascades of Janus kinase (JAK), activators of transcription (STATs) and IRS/PI-3 kinase are activated and lead to subsequent specific changes in gene expression [43].

Besides its central signalling role, leptin also regulates peripheral metabolism and insulin sensitivity both directly, since almost all tissues express leptin receptor, and indirectly via stimulation of α-adrenergic signalling [44]. In skeletal muscle, liver, beta cells and also locally within adipose tissue,

leptin stimulates fatty acid oxidation, increases lipolysis of triglycerides and inhibit lipogenesis. Thus it contributes to prevention of ectopic lipid accumulation and reduction of lipotoxicity [45]. These effects are involved in the insulin-sensitizing action of leptin and are mediated through direct activation of AMP-kinase (adenosin monophosphate-activated protein kinase), activation of Jak/STAT pathway or inhibition of SREBP-1c expression [44-46]. Leptin signalling also activates protein of insulin signalling cascade, such as PI-3 kinase [45, 47]. Based on experimental models, leptin was suggested as an important player in the adipo-insular axis, as it decreases insulin secretion and gene expression and protects beta cells from lipid accumulation [43]. Leptin production is stimulated by insulin or in satiety, while it is decreased during starvation, by catecholamines or TNFα (tumor necrosis factor α).

The first promising results in leptin-deficient rodent models (*ob/ob* mice) showed a decline in IR and reduction of body weight after leptin substitution [48]. In humans, the positive effect of leptin administration on IR and obesity could only be reproduced in states of leptin deficiency, such as in congenital and HIV-associated lipodystrophy [49, 50] or extreme rare cases of obesity based on mutations of *ob* gene or leptin receptor gene [44]. On the contrary, in common human obesity characterised by high levels of circulating leptin without adequate end-organ response, the attempts to treat “typical” obesity with leptin failed [51], suggesting development of leptin resistance [44, 45].

Linear correlation between serum leptin levels and total body fat mass in humans led to the postulation of leptin’s role as a signal of adipose tissue stores [44]. Enlarged adipocytes in obesity secrete up to seven times more leptin than small fat cells in lean subjects [52]. Despite of elevated leptin, obese subjects appear to be insensitive to its action. Several mechanisms underlying leptin resistance have been described in experimental models: impaired leptin transport across the blood-brain barrier as a response to hyperleptinaemia with resulting ”hypothalamic leptin insufficiency” [43], down-regulation of leptin receptor or postreceptor inhibition of leptin signalling via induction of suppressor of cytokine signalling-3 (SOCS-3), protein tyrosine phosphatase 1B (PTP1B) or endoplasmic reticulum stress [44, 45].

Additionally, leptin reveals angiogenic activity, may contribute to thrombus formation through platelet leptin receptor, stimulates production of reactive oxygen species (ROS), activates macrophages and affects production of other cytokines [42, 53]. While increased leptin concentrations found in obesity are not able to regulate energy metabolism any more, they may have negative influence on endothelium and vessel walls and thus contribute to the progression of atherosclerosis.

Adiponectin

Adiponectin is a 30-kDa insulin-sensitizing glycoprotein expressed specifically and abundantly in adipocytes that is released into circulation at high concentrations. Adiponectin forms homotrimers that further associate in larger multimer complexes [54, 55]. In the circulation adiponectin can be detected in form of low-molecular weight oligomers (trimers and hexamers) and high-molecular weight (HMW) multimers (12- to 18- mer).The latter ones are supposed to represent the most active form of adiponectin showing the highest receptor-binding activity and the most potent AMP-kinase activation [56].

Two adiponectin receptors AdipoR1 and AdipoR2 have been identified. AdipoR1 ubiquitously expressed in many tissues including skeletal muscle, primarily up-regulates AMP-kinase pathways

leading to inhibition of gluconeogenesis and increased fatty acid oxidation. AdipoR2, which is also expressed in most tissues, but plays a dominant role in liver, is more tightly linked to PPARα activation that increases fatty acid oxidation and inhibits oxidative stress and inflammation [54, 56]. Adiponectin directly increases hepatic insulin sensitivity, decreases hepatic gluconeogenesis and stimulates muscle glucose uptake, promotes fatty acid oxidation, inhibits lipogenesis and improves lipid profile. In a paracrine manner within adipose tissue, it attenuates TNFα expression, production of reactive oxygen species and inflammation. Its role in vascular protection has been also well explored: adiponectin decreases expression of adhesion molecules, inhibits proliferation of vascular smooth-muscle cells, suppresses transformation of macrophages to foam cells [57]. Additionally, adiponectin has been shown to be involved also in regulation of food intake at the brain level [56]. Its function appears to be complementary to leptin: adiponectin concentrations and AdipoR1 expression increase during fasting, leading to stimulation of AMP-kinase activity in hypothalamus and subsequently to promotion of food intake [58].

Based on experimental and clinical studies, hypoadiponectinaemia is consistently related to insulin resistance, obesity, type 2 diabetes, coronary heart disease, hypertension and atherosclerosis [54, 57]. HMW adiponectin was even suggested as a predictor of IR and type 2 diabetes [56, 59]. Despite of seemingly strong anti-atherosclerotic effects of adiponectin, its association with cardiovascular risk was only moderate or not proved, especially after adjustment for other classical risk marker [60]. The mechanisms underlying the down-regulation of adiponectin production on context of increased fat mass and IR are not clear. They may include altered endocrine activity of hypertrophic adipocytes and the whole adipose tissue, increased oxidative stress or pro-inflammatory state [57]. In conjunction with lower circulating adiponectin, an impaired tissue response to this adipokine has been demonstrated in obese rodent and human muscle [45]. Decreased expression levels of AdipoR1/R2 may contribute to reduced adiponectin sensitivity, at least in rodent models of obesity [56]. In humans, the comparison of AdipoRs mRNA and protein levels in lean and obese and/or diabetic subjects is equivocal [44, 45].

Adiponectin exhibits sexual dimorphism with higher concentrations in females, as well as diurnal variation with a decline at night [55].These variations are lost in obesity and diabetes and restored upon weight loss. Its secretion acutely stimulated by insulin, chronic hyperinsulinaemia results in decline in adiponectin expression. Adiponectin gene expression is known to be reduced by TNFα, glucocorticoids, interleukin 6, β—adrenergic agonists or in specific genetic polymorphisms, whereas insulin sensitizers, PPARγ agonists or weight loss have been shown to increase circulating adiponectin [54, 55, 61]. Increased adiponectin levels, especially its HMW isoform, have been attributed as one of the mechanisms of insulin-sensitizing effects of thiazolidinediones (TZDs). Similar positive effect on adiponectin concentrations was observed following administration of angiotensin II receptor blockers (ARBs) or angiotensin-converting enzyme inhibitors (ACEI) [54]. Beside the therapeutic strategies to increase adiponectin concentrations (i.e. PPARγ agonists), interventions improving adiponectin action via increase in AdipoRs are tested. Here, PPARα agonists up-regulate expression of AdipoRs in adipose tissue [62].

Despite the well characterized insulin-sensitizing and antiatherosclerotic effect of adiponectin, several recent findings brought controversy in the ”puzzle” [61]. Adiponectin is increased in patients with high risk of cardiovascular death and myocardial infarction, in chronic heart failure, while simvastatin treatment reduced adiponectin. Moreover, circulating adiponectin was actually

increased in chronic inflammatory state not associated with obesity, such as lupus erythematodes, rheumatoid arthritis, inflammatory bowel disease and type 1 diabetes. These findings await further clarification.

Resistin

Resistin is a 12-kDa peptide that represented after its discovery in 2001 another candidate for link between IR and obesity [63].The causal role of resistin in insulin resistance was postulated based on rodent models with altered circulating levels of resistin [64-66]. Both gain- and loss-of-function studies demonstrated the role of resistin in induction hepatic and skeletal muscle IR, mediated primarily by increased hepatic glucose production, but also by decrease in fatty acid uptake and oxidation in skeletal muscle, inhibition of adipocyte differentiation and stimulation of lipolysis in adipocytes. In rodent models of obesity, circulating resistin was increased [65, 67]. At the molecular level, resistin effects are mediated by up-regulation of SOCS-3 protein that interferes with insulin signalling cascade [66].

However, the role of resistin in humans is controversial so far [68, 69]. The results from rodent models could not be repeated in human studies that do not show a consistent association between resistin and either obesity or IR. Human resistin is only 64% homologous with murine resistin [63] and also the source differs between species: rodent resistin is primarily secreted by differentiating adipocytes, whereas in humans, its major source is represented by mononuclear-macrophage cells [64, 66, 70]. Expression of resistin in other tissues, including adipose tissue, is likely the result of macrophage infiltration.

Although the results regarding the effect of resistin in human glucose metabolism are contradictory, there are more explicit data proving its role in inflammation. Resistin is up-regulated by several cytokines, such as TNFα, IL-6, IL-1β, its secretion from macrophages is stimulated by lipopolysaccharide endotoxin [66]. Resistin concentrations are increased in coronary artery disease [71], but also in inflammatory bowel disease or rheumatoid arthritis [43, 66]. The NFκB pathway is activated by resistin and may mediate the role of resistin in inflammation [72]. With regard to the development of atherosclerosis, resistin was demonstrated to promote foam cell formation and migration of endothelial and smooth muscle cells, as well as to stimulate production of different pro-inflammatory factors (plasminogen activator inhibitor-1, endothelin-1, monocyte chemoattractant protein-1) [66]. Even if several molecular mechanisms of resistin function have been recently defined, the resistin receptor has not been identified yet.

In humans, resistin appears to be involved mainly in low-grade inflammation and development of atherosclerosis. An indirect effect on development of IR through promotion of inflammation cannot be excluded [66].

Tumor necrosis factor α (TNFα)

TNFα is a multifunctional regulatory cytokine, which is synthesised as a 26-kDa transmembrane protein and released into the circulation as a 17-kDa soluble protein. It plays a role in inflammation, apoptosis, cytotoxicity, regulates production of other cytokines (IL-1, IL-6) and also induces IR [73]. Adipocytes are able to produce TNFα per se, but macrophages of M1 phenotype are the main source of adipose TNFα [44, 74]. Adipose tissue expresses both type I and type II of TNFα receptors. Their soluble forms are released into circulation mirroring the TNFα activation. Circulating concentrations of TNFα and its adipose tissue expressions are elevated in obesity and decrease after weight loss,

TNFα reduces insulin-stimulated glucose uptake [57, 75]. Again, initial experimental evidence demonstrated promising results regarding causal role of TNFα in the pathophysiology of IR: TNFα-neutralising antibodies restored insulin sensitivity, mice with targeted gene deletion of TNFα or its receptors are protected from IR [76]. The underlying molecular mechanisms include activation of pro-inflammatory NFκB and c-Jun NH2-terminal kinase (JNK) pathways that leads to serine phosphorylation of IRS-1 and inhibits normal downstream insulin signalling [57]. Furthermore, TNFα also affects lipid metabolism [33, 46]. In adipose tissue, it stimulates lipolysis leading to elevation of circulating fatty acids, inhibits transcription factors of adipogenesis and lipogenesis. On the contrary in liver, TNFα increases the expression of genes essential for de novo lipogenesis, while it decreases expression of those involved in fatty acid oxidation. Additionally, TNFα alters expression of other cytokines from adipose tissue (reduces adiponectin and stimulates IL-1 and IL-6) and thus, it is suggested as a crucial and proximal contributor to adipokine dysregulation in obesity [57].

Despite of the clear linkage between TNFα and whole-body IR shown in rodent models, the role of TNFα in humans is still a matter of debates. In humans serum concentrations of TNFα are much lower than tissue concentrations, systemic administration of TNFα neutralising antibodies did not improve insulin sensitivity [43, 57]. TNFα seems to act predominantly in an autocrine/paracrine manner in the respective tissues, modulating local cytokine and NEFA release [21, 33, 57]. Its endocrine effects appear to be less important in humans.

Interleukin 6 (IL-6)

IL-6 is a pleiotropic cytokine, circulating as a glycosylated protein at high concentrations. It is secreted by several cell types, including immune cells, endothelial cells, fibroblasts, myocytes and adipocytes [42]. 15-35% of systemic IL-6 is attributed to the release from adipose tissue [77] with the stromal vascular fraction representing the major source. IL-6 production in visceral adipose tissue is 3-fold higher than in subcutaneous depot.

IL-6 plasma concentrations and adipose tissue expression correlate with fat mass, IR and NEFA concentrations, weight loss leads to reduction of IL-6 levels [42, 44]. IL-6 administration results in elevated blood glucose and NEFAs and induces hepatic IR in experimental and clinical studies. In hepatocytes and adipocytes, IL-6 has been shown to impair insulin signalling through up-regulation of SOCS-3 and consequent inhibition of IRS-1 phosphorylation [44, 46]. IL-6 has been also demonstrated to stimulate adipose tissue lipolysis and de novo lipogenesis in liver and to suppress activity of lipoprotein lipase in adipose tissue, which are responsible for IL-6 induced NEFA elevation [46].

In contrast, several studies in rodent models and humans focused on IL-6 function in skeletal muscle brought different information, suggesting possible anti-inflammatory role of IL-6 in skeletal muscle [44, 57]. Acute IL-6 infusion increased skeletal muscle glucose uptake [78] and exercise associated with enhanced insulin action in skeletal muscle also increased local and circulating IL-6 concentrations. It is hypothesised that in vivo chronic, but not acute IL-6 elevation has a weak or no effect in muscle, whereas its IR-inducing action in liver and adipose tissue may contribute to whole-body IR [79].

Visfatin

Visfatin was originally isolated as a protein enhancing immune B-cell maturation (pre-B colony enhancing factor) that also displays nicotinamide phosphoribosytransferase activity [43, 57]. In 2005, visfatin was reported to be highly expressed in visceral adipose tissue, to correlate with obesity but

on the other hand, also to show insulin-mimetic action and lower plasma glucose in mice [80]. Subsequent studies failed to confirm the depot difference in visfatin expression in humans [81-83]. The postulated associations between visfatin and obesity, IR or type 2 diabetes could be reproduced only in some but not all clinical studies [43, 57, 83], which might be partly attributed to differences in visfatin immunoassays. Furthermore, methodological concerns appeared about experiments demonstrating insulin-mimicking action and the initial paper was partly retracted [84].

Nevertheless, visfatin was identified as a rate-limiting enzyme essential for glucose-stimulated insulin secretion from β-cell [85]. Based on the beneficial visfatin effect on glucose homeostasis that was shown in experimental studies [86], visfatin has been speculated to provide a compensatory mechanism in response to hyperglycaemia in conditions of IR. Visfatin also plays a role in regulation of immune responses [87], being secreted predominantly from macrophages rather than from adipocytes. As a pro-inflammatory cytokine, visfatin may be implicated in the pathogenesis of acute and chronic inflammatory states, including atherosclerosis.

Retinol binding protein-4 (RBP4)

Circulating RBP4 is the main transport protein for retinol (vitamin A) that is secreted by adipose tissue and liver [88]. Studies in gain- and loss-of-function animal models suggested the causal role of RBP4 in development of IR with several underlying mechanisms [44, 61]: via serine phosphorylation of IRS-1, RBP4 impairs insulin signalling in muscle and adipocytes and by enhancing the expression of phosphoenolpyruvate carboxykinase, it stimulates hepatic gluconeogenesis [89, 90]. RBP4 was also suggested to impair β-cell function [91].

In humans, increased RBP4 levels were reported in obesity, IR, type 2 diabetes, metabolic syndrome or NAFLD, as well as in lean normoglycaemic offspring of type 2 diabetic parents [43, 92, 93]. RBP4 concentrations correlate with visceral fat mass and in accordance, increased mRNA expression was found in visceral adipose tissue compared to subcutaneous one [61]. Significant weight loss achieved by bariatric surgery or lifestyle modification, and exercise lead to decline in RBP4 concentrations along with improvement of insulin sensitivity [43, 61]. However, other larger studies did not confirm the above-mentioned associations [43, 94, 95]. RBP4 exhibits sexual dimorphism with higher concentrations in men than in women and increase in RBP4 over the age of 50 years in the latter ones [96]. Additionally, an interesting positive association between iron, plasma retinol and RBP4 has been described [61].

Due to inconsistent findings in clinical studies, the function of RBP4 in glucose metabolism in humans remains unclear and might be restricted to rodent models [97].

Monocyte chemoattractant protein-1 (MCP-1)

MCP-1 is an inducible chemokine responsible for recruitment of monocytes and T cells to sites of injury and infection. It is secreted by various cell types such as endothelial, skeletal muscle, smooth muscle cells, adipocytes and macrophages and its action is mediated by chemokine CC motif receptor (CCR)2 [98]. This potent chemoattractant is required for recruitment of monocytes/macrophages into adipose tissue and its expression correlates with the degree of macrophage accumulation in adipose tissue. In animal models, MCP-1 plasma concentrations and expressions are increased in obesity and diabetes, MCP-1 overexpression in adipose tissue results in IR and macrophage infiltration, whereas MCP-1- or CCR2-deficient mice prevented diet-induced obesity, IR and adipose tissue inflammation [57, 98]. In line with these observations, MCP-1 decreased insulin-stimulated

glucose uptake and expression of adipogenic genes in cell cultures [57]. MCP-1 is up-regulated by insulin, TNFα, IL-6 or growth hormone.

These experimental results have been only partly confirmed in humans. Several studies described increased plasma concentrations and expressions in obesity [98, 99] with higher expression levels in visceral depot, as well as ability of insulin-sensitizing treatments (weight loss, thiazolidinediones) to decrease MCP-1 levels. On the other hand, other authors reported comparable MCP-1 serum concentrations between lean and obese subjects [100-102], comparable [101] or increased [102] adipose tissue expressions in obesity. Additionally, circulating MCP-1 and its expressions are differentially regulated by insulin when comparing insulin-resistant and insulin-sensitive subjects [101].

MCP-1 might represent an important link between adipose tissue inflammation in obesity and pathogenesis of insulin resistance however, its role in humans remains to be confirmed.

Macrophage inflammatory protein 1α (MIP-1α)

MIP-1α is another adipocyte- and macrophage-secreted chemokine that is responsible for chemotactic attraction of mononuclear cells from circulation into tissues. Similarly to MCP-1, it is postulated to play a role in low-grade inflammation seen in obesity or atherosclerosis. In humans its circulating concentrations are low, which is connected with detection difficulties leading to conflicting results: its serum levels were under the detection limit [101] or comparable between type 2 diabetic subjects and controls [103] or between obese and lean subjects [102]. Regarding its adipose tissue expression, this was found to be increased in obesity [102] and comparable between subcutaneous and visceral depots. Other study showed no difference in MIP-1α expression between Insulin-resistant and insulin-sensitive subjects, while insulin stimulated MIP-1α expressions only in insulin-resistant group [101]. The significance of MIP-1α and its potential as a therapeutic target in humans need further clarification.

Interleukin-1β (IL-1β)

As a mononuclear cells-derived pro-inflammatory cytokine, IL-1β is specifically implicated the progression of type 2 diabetes through promotion of pancreatic β-cell apoptosis and destruction. Its concentrations are increased in populations with metabolic risk, such as NGT offspring of parents with type 2 diabetes [104, 105], they correlate with IR indices, HbA1c, lipid profile and high-fat/high-carbohydrate diet in obesity [106, 107]. Furthermore, decreased IL-1β expression in peripheral mononuclear cells was documented after weight loss [108], as well as enhanced IL-1β release from mononuclear cells of obese patients in response to hyperglycaemia [109]. For evaluation of IL-1β biological activity, the ratio IL-1 receptor antagonist / IL-1β seems to be important, being close to 1 in healthy population with minimal variation [110].

Interleukin-1 receptor antagonist (IL-1ra)

IL-1ra competitively antagonizes the inflammatory effects of IL-1β and IL-1α by binding to interleukin-1 receptor without inducing a cellular response [110]. Thus, IL-1ra reflects the inflammatory response and due to its anti-inflammatory properties, it represents a compensatory mechanism for IL-1 induced disease processes. IL-1ra is secreted by immune cells, epithelium, keratinocytes, stromal cells, hepatocytes and adipocytes. Adipose tissue has been reported as an important source of IL-1ra [111].

IL-1ra has protective effect on pancreatic β-cell function and survival [112], its concentrations decrease, when type 2 diabetes develops [113]. Moreover, treatment with IL-1ra led to improved glycaemia, β-cell secretory function and reduced CRP and IL-6 concentrations in patients with type 2 diabetes [114].

In contrast, experimental studies in rodents with altered IL-1ra production and in patients with metabolic syndrome showed an opposite role of IL-1ra in obesity-related abnormalities [110]. Increased IL-1ra concentrations are reported in metabolic syndrome, obesity and prediabetes state in offspring of type 2 diabetic parents, they correlate with number of metabolic syndrome components and insulin resistance [104, 115-117]. Weight loss results in decline in IL-1ra expression in peripheral blood mononuclear cells [108] and lower serum concentrations [110]. In mice with diet-induced obesity, IL-1ra expression in adipose tissue was up-regulated and IL-1ra administration induced IR via decreased muscle glucose uptake [118].

The dual pro- and anti-inflammatory functions of IL-1ra and they role in IR and obesity need further clarification.

CCL5/RANTES (Regulated on Activation, Normal T cell Expressed and Secreted)

RANTES belongs to T-lymphocyte-produced chemokines involved in T-cell recruitment in a positive feedback loop. T-cells, RANTES and its major receptor CCR5 are increased in adipose tissue in human and murine obesity [119] contributing to inflammatory state in an auto- and paracrine manner [120]. Several clinical studies reported increased RANTES plasma concentrations and adipose tissue expressions in obese compared to lean subjects [102, 119], with higher RANTES expression in visceral fat depot. Additionally, progression to type 2 diabetes in Finnish Diabetes Prevention Study was associated with higher RANTES concentrations [121]. KORA S4 Study [122] demonstrated increased RANTES levels in groups of impaired glucose tolerance and type 2 diabetes compared to NGT subjects.

Vascular endothelial growth factor (VEGF)

As an angiogenic factor with pro-inflammatory and atherosclerotic effects, VEGF stimulates intimal hyperplasia, increases vascular permeability and contributes to plaque instability. It has been shown that VEGF interacts with renin-angiotensin system [123, 124], demonstrating VEGF as an essential mediator of angiotensin II induced vascular inflammation and remodelling. Angiotensin II potentiates VEGF induced intimal proliferation. IR stimulates VEGF expression and intimal neoplasia in rats [125] and insulin regulates VEGF expression in cardiomyocytes via insulin receptor and PI3-kinase pathway [126]. In humans, elevated VEGF plasma concentrations were demonstrated in obese and hypertensive subjects [127, 128], antihypertensive or lipid-lowering treatment results in decrease in VEGF levels [88].

Renin-angiotensin system (RAS)

Adipose tissue disposes of all components of renin-angiotensin system, whose role in blood pressure regulation by influencing the salt-fluid homeostasis and vascular tone is well known. Adipose tissue is considered to be the major extrahepatic source of angiotensinogen and thus substantially contributes to its increased levels and development of hypertension in obesity [42, 57]. Differentiating adipocytes, mainly of the visceral fat depot, appear to be quantitatively the most important source of angiotensinogen. Concentrations of renin and angiotensin-converting enzyme activity are also increased in obesity [129]. Both types of angiotensin II receptors (AT1- and AT2-

receptors) can be found on adipocytes. Signal transduction of angiotensin II is mediated by signal proteins shared with insulin signalling cascade (PI3-kinase, Akt kinase), and thus angiotensin II inhibits insulin stimulated glucose uptake [130]. Angiotensin II also inhibits adipocyte differentiation [23, 131]. Moreover, evidence has been accumulated that RAS inhibition may improve insulin sensitivity, decrease incidence of type 2 diabetes [132-134], increase adipocyte differentiation and adiponectin expression [135].

Transcription factors in adipocytes

Fatty acid-binding proteins (FABPs)

An important molecular pathway, which integrates metabolic and inflammatory response involves the fatty acid-binding proteins (FABPs) commonly present in adipocytes and macrophages in two isoforms – adipocyte FABP (A-FABP) and epidermal FABP (E-FABP) coded by FABP4 and FABP5 genes, respectively [136]. As cytoplasmic lipid chaperons FABPs are responsible for cellular trafficking of fatty acids (to the mitochondria and peroxisomes for oxidation, to the endoplasmic reticulum for reesterification, to the lipid droplet for storage, or to the nucleus for regulation of gene expression). Moreover, experimental evidence based on comprehensive research on knock-out mice models, supports the role of A-FABP in systemic regulation of lipid and glucose metabolism as well as inflammation, since A-FABP deficiency prevents the development of obesity, insulin resistance and atherosclerosis [137-139]. In human studies, A-FABP was found to be also present in plasma [140], although its physiological function or mechanisms of its appearance in circulation have not been elucidated until now. A-FABP plasma concentrations are increased in patients with obesity and/or metabolic syndrome [140-142] and it is suggested as a novel risk marker predicting development of metabolic syndrome [141] or type 2 diabetes [143]. On the contrary, clinical studies focused on adipose tissue expression are inconclusive [144] – they report no differences in A-FABP expression or a decrease in E-FABP expression in obese subjects [145-148]. Additionally, no consistent association between A-FABP expression and measures of obesity or insulin resistance has been found [146, 148].

Adipocyte/macrophage FABP clearly links several mechanisms and pathways that are involved in the development of obesity, metabolic syndrome and atherosclerosis. To translate these important data from mice models to humans will require further comprehensive investigations. Whether circulating adipocyte/macrophage FABP represents a biomarker of obesity, metabolic syndrome and atherosclerosis or whether it is a causative factor of metabolic and inflammatory dysregulation, which can be effectively and safely inhibited, remains to be elucidated.

Peroxisome proliferator activated receptor-γ (PPARγ)

PPARγ are nuclear receptors, which serve as ligand-activated transcription factors regulating expression of genes involved in carbohydrate and lipid metabolism, adipocyte differentiation and inflammation. In the nucleus they are found in the complex with retinoid X receptor (RXR). Their natural ligands are fatty acids and lipid-derived substrates [149, 150]. PPARγ are expressed predominantly in adipose tissue, but they are also present in other cell types such as vascular smooth muscle cells, endothelial cells or monocytes. PPARγ mutations in humans are associated with manifest IR, dyslipidaemia and metabolic syndrome [151].

Most of the understanding of PPARγ function is derived from studies with thiazolidinediones (TZDs), which are synthetic PPARγ agonists. PPARγ activation leads to improvement in insulin sensitivity, decreased plasma levels of insulin, NEFAs and triglycerides. Adipose tissue clearly represents the site of TZD action [150]. Among mechanisms of action, NEFAs re-partitioning into white adipose tissue must be mentioned. Stimulated NEFA trapping and storage in adipocytes as well as stimulated adipogenesis ensure the increased lipid clearance. Resulting decrease in circulating NEFAs and triglycerides lowers lipotoxicity in other tissues and prevents ectopic lipid accumulation [150, 152]. Treatment with PPARγ agonists leads to redistribution of adipose tissue with preferential adipogenesis in subcutaneous depot and parallel lack of change or reduction of visceral depot [153]. Another potential mechanism of effect on insulin sensitivity is the alteration of adipokine production: TZDs lower the expression of TNFα, plasminogen activator inhibitor-1 or resistin and stimulate secretion of adiponectin, especially its HMW form [154]. Increased adiponectin concentrations may mediate the effects of TZDs on decrease in hepatic steatosis, increase in fatty acid oxidation, attenuation of inflammation and anti-atherosclerotic effects [150]. However, the full PPARγ agonism is associated with undesired side effects such as fluid retention, oedema, increased risk of heart failure and cardiovascular events that substantially limit their therapeutical use.

Sterol regulatory element- binding protein-1c (SREBP-1c)

SREBP-1c is highly expressed in most tissues, including adipose tissue. It is responsible for activation of genes required for endogenous lipogenesis and adipocyte differentiation and it is activated postprandially by insulin.

Links between obesity, IR, type 2 diabetes

Inflammation

In the past decade, experimental, epidemiological and clinical evidence have clearly shown that chronic inflammation, characterized by abnormal cytokine production, increased acute-phase reactants and activation of networks of inflammatory signalling pathways, is causally linked to the development of obesity, IR and type 2 diabetes [155]. Immune response and metabolic regulation are highly integrated processes ensuring whole-body homeostasis. Due to its secretory functions, adipose tissue represents a critical site for the interaction between metabolic and inflammatory response systems that play a significant role in the pathogenesis of metabolic diseases[40].

Several aspects of the role of inflammation have been discussed in detail above. Here a brief summary concludes this topic. First, adipose tissue contributes to inflammatory status of obesity via production of cytokines, such as TNFα, MCP-1, IL-1, IL-6, resistin etc. Second, infiltration of immune cells (monocytes, macrophages and T-cells) in adipose tissue substantially contributes to initiation and promotion of inflammation in a positive feedback loop. Additionally, adipocytes share a number of commonalities with immune cells (e.g. complement activation, production of inflammatory mediators, lipid-accumulating and phagocytic properties). Third, lipids and particularly fatty acids influence both metabolic and immune processes via specific transcription factors. In this regard, PPARγ represents one of the crucial modulators that inhibit expression of genes involved in inflammatory response, while adipocyte/macrophage FABP seems to have pro-inflammatory and dysmetabolic functions. Inflammatory signals have been demonstrated to impair insulin action and contribute to pathophysiology of IR through multiple molecular mechanisms inducing serine

phosphorylation of IRS-1 which results in impaired insulin signalling. Here, the induction of SOCS-3 protein through several cytokines or activation of JNK, IκB kinase (IKK) and protein kinase C (PKC) should be mentioned. The kinases also affect gene expression through activation of pro-inflammatory NFκB pathway.

Endoplasmic reticulum and adipocyte dysfunction

However, a question remains: Why inflammation develops in obesity and what is the primary cause of activation of inflammatory pathways? Recent data from experimental models suggest that endoplasmic reticulum (ER) stress is the critical initial impulse integrating inflammatory and metabolic pathways in obesity and IR [32, 155]. Nutrient oversupply, excess adipose tissue and increased lipid and glucose concentrations increase ER activity, but the excessive demands on ER can lead to dysfunction of protein folding, lipid-droplet creation and cholesterol sensing. The unfolded protein response (UPR), characterized by aggregation of abnormally folded proteins in the cytosol, partly compensates for ER stress through alteration of regulatory pathways aiming to restore cellular integrity [156]. Among them the principal inflammatory pathways known to impair insulin action, JNK and IKK-NFκB pathways, are activated via UPR during ER stress. UPR can also induce apoptosis. ER stress is a major source of reactive oxygen species (ROS) and together with excess of NEFAs leads to oxidative stress in mitochondrion [32] seen in obesity and IR. Moreover, ROS impair insulin secretion and promote β-cell apoptosis, further exacerbating glucose-insulin homeostasis and the vicious cycle of inflammation.

Blockade of Renin-angiotensin system (RAS) and its metabolic effects

Rationale

In the last decade, a number of experimental and clinical studies indicated that pharmacological RAS blockade by either angiotensin-converting enzyme inhibitors (ACEI) or angiotensin II type 1 receptor blockers (ARBs) might have positive metabolic effects and decrease the incidence of new onset type 2 diabetes [133, 157, 158]. Several independent meta-analysis of interventional randomized controlled trials published till 2006 [132, 133, 159, 160] confirmed the protective effect of treatment with ACEIs or ARBs on the progression to overt diabetes in risk populations (patients with hypertension, cardiovascular risk factor and/or disease or heart failure). RAS inhibition reduced the relative risk of new-onset diabetes by 22-26 %. This effect was comparable for different ACEIs and ARBs tested and it was present regardless of comparator used (placebo or pro-diabetogenic beta-blockers and diuretics or metabolically neutral calcium-channel blockers). The most important studies are summarized in Table 1. However, these results show several limitations: first, a number of trials included in the analysis did not have diabetes as a pre-specified outcome leading to possible substantial differences in diabetes diagnostics; second, the well-known negative metabolic effects of beta-blockers and diuretics may be responsible for the difference seen between therapies [158, 161].

Further placebo-controlled, randomized, diabetes incidence outcome studies were designed to resolve the issue; they yielded however inconsistent results (see Table 1). In the DREAM trial (Diabetes Reduction Assessment with Ramipril and Rosiglitazon Medication), ramipril treatment failed to reduce the incidence of new-onset diabetes and cardiovascular disease compared to placebo in patients with impaired glucose homeostasis [162]. The explanation may lie in several

aspects of study design, such as short trial duration, the low-risk profile of trial population compared to previous studies (resulting in lower degree of baseline RAS activation) or placebo control [163]. However, ramipril treatment led to more frequent regression to fasting normoglycaemia and lower glucose levels 2 hours after OGTT. Similarly, in 56-months ONTARGET (Ongoing Telmisartan Alone and in Combination with Ramipril Global Endpoint Trial) [164] and TRANSCEND (Telmisartan Randomised Assessment Study in ACE Intolerant Subjects with Cardiovascular Disease) [165] trials in high-risk patients with cardiovascular disease, the differences in diabetes incidence between the respective groups were not significant. On the other hand, the most recent NAVIGATOR trial (Nateglinide and Valsartan in Impaired Glucose Tolerance Outcome Research) [166] have shown that 5-year treatment with valsartan led to 14% reduction in diabetes incidence but did not reduce the rate of cardiovascular events among patients with impaired glucose tolerance and cardiovascular disease or risk factors.

Thus, evidence accumulated to date indicates that RAS inhibiting treatments are associated with reduced risk of new-onset diabetes or at least with beneficial metabolic effects compared with other class of antihypertensives, but the prognostic significance of this differential effect regarding cardiovascular disease risk remains controversial [158].

Potential mechanisms underlying metabolic effects of ACEIs and ARBs

Both ACEIs and ARBs are able to counteract the adverse metabolic effects of angiotensin II (AII) on the level of insulin sensitivity and insulin secretion [167, 168] but there is also accumulating evidence on compound-specific insulin-sensitizing mechanisms independent on AII blockade [169, 170].

Effects on insulin sensitivity mediated by interruption of AII signalling

The ACEI- or ARB-mediated vasodilatation and increased skeletal muscle blood flow have been firstly postulated to play role in increased glucose uptake, but these results were not confirmed by later experiments. On the contrary, several groups described even a dissociation of haemodynamic and metabolic effects of AII [171] with AII treatment leading to increase in glucose uptake.

As mentioned above, there is a direct crosstalk between AII and insulin signalling pathways at the level of IRS-1 phosphorylation and PI-3 kinase activation [157, 167]. RAS blockade prevents the inhibition of insulin downstream signalling by AII, resulting in enhanced PI-3 kinase activation, increased production and availability of GLUT-4 and increased glucose uptake as well as restoration of insulin-stimulated nitric oxide (NO) production. Several experimental data suggest that AII-induced oxidative stress additionally contributes to impaired insulin signalling [130, 167].

Vasodilatation reached by ACEI and ARB treatment is not accompanied by increase in sympathetic activity, on the contrary, the circulating catecholamines are reduced [172]. The decreased sympathetic activation can contribute to improvement of insulin sensitivity [173].

Next, angiotensin II type 1 receptor blockade (ARB) has been proved to exert direct effects on adipocytes [157, 167]: it stimulates adipocyte differentiation and promotes recruitment of small insulin-sensitive adipocytes [174-176], which prevents ectopic lipid accumulation and improves insulin sensitivity. In rodent models, ARBs also inhibit activation of inflammatory processes in adipose tissue manifested as decrease in NFκB, MCP-1 and increase in adiponectin [177]. Whether the modulation of adipokine expression and release, which often accompanies treatment with ARBs and ACEI, is mediated primarily by angiotensin II type 1 receptor blockade, remains unclear.

Study	Comparators	Duration (years)*	Patient population	Relative risk (95% CI)	p-Value	Prespecified end-point
ACE inhibitors						
CAPPP	Captopril vs. b-blocker ⁄ diuretic	6.1	10,985 hypertensive patients	0.79 (0.67–0.94)	0.007	Yes
HOPE	Ramipril vs. placebo	5	9297 patients with history of CAD, stroke, PVD, for diabetes and >1 other CVD risk factor	0.66 (0.51–0.85)	< 0.001	Yes
ALLHAT	Lisinopril vs. diuretic	4.9	33,357 hypertensive patients with >1 other CVD risk factor	0.70 (0.56–0.86)	< 0.001	No
PEACE	Trandolapril vs. placebo	4.8 (median)	8290 with stable CAD	0.83 (0.72–0.96)	0.001	No
ASCOT-BPLA	Amlodipine (± perindopril) vs. atenolol (±diuretic)	5.5 (median)	19,257 hypertensive patients with >3 other CVD risk factors	0.70 (0.63–0.78)	< 0.0001	Yes
DREAM	Ramipril vs. placebo	3.0 (median)	5269 patients with IFG and ⁄ or IGT but without CVD or renal disease	0.91 (0.80–1.03)	ns	Yes
ARBs						
LIFE	Losartan vs. Atenolol	4.8	9193 hypertensive patients with LVH	0.75 (0.63–0.86)	0.001	Yes
SCOPE	Candesartan vs. placebo ⁄ other drugs	3.7	4964 hypertensive patients aged 70–89 years	0.81 (0.61–1.02)	0.09	No
CHARM	Candesartan vs. Placebo	3.1	7599 patients with HF	0.78 (0.64–0.96)	0.02	Yes
VALUE	Valsartan vs. amlodipine	4.2	15,245 hypertensive patients with high risk of CVD events	0.77 (0.69–0.86)	< 0.0001	Yes
TRANSCEND	Telmisartan vs. placebo	4.7 (median)	5926 patients intolerant to ACE inhibitors with CAD, PVD, CBVD or diabetes with end-organ damage	0.85 (0.71–1.02)	0.081	Yes
NAVIGATOR	Valsartan vs. placebo	5.0 (median)	9306 patients with IGT and CVD or CVD risk factors	0.86 (0.80-0.92)	< 0.001	Yes
ACE inhibitor ⁄ ARB combination						
ONTARGET	Telmisartan vs. ramipril; telmisartan + ramipril vs. ramipril	4.7 (median)	25,620 patients with CAD, PVD, CBVD or diabetes with end-organ damage	1.12 (0.97–1.29) 0.91 (0.78–1.06)	ns ns	Yes

Table 1 Effects of inhibition of the RAS on risk of new-onset diabetes in selected key randomized controlled trials (adapted from [158]) ****Mean years of follow-up unless indicated. ALLHAT, Antihypertensive and Lipid-Lowering treatment to Prevent Heart Attack Trial; ASCOT-BPLA, Anglo-Scandinavian Cardiac Outcomes Trial-Blood Pressure Lowering Arm; CAD, coronary artery disease; CAPP, Captopril Prevention Project; CBVD, cerebrovascular disease; CHARM, Candesartan in Heart failure – Assessment of Reduction in Morbidity and Mortality; CVD, cardiovascular disease; DREAM, Diabetes Reduction Assessment with Ramipril and Rosiglitazone Medication; HF, heart failure; HOPE, Heart Outcomes Protection Study; LIFE, Losartan Intervention For End-point reduction in hypertension; LVH, left ventricular hypertrophy; ns, not significant; NAVIGATOR, Nateglinide and Valsartan in Impaired Glucose Tolerance Outcome Research; ONTARGET, Ongoing Telmisartan Alone and in Combination with Ramipril Global Endpoint Trial; PEACE, Prevention of Events with Angiotensin-Converting Enzyme Inhibition; PVD, peripheral vascular disease; SCOPE, Study on Cognition and Prognosis in the Elderly; TRANSCEND, Telmisartan Randomised Assessment Study in ACE Intolerant Subjects with Cardiovascular Disease; VALUE, Valsartan Long-term Use Evaluation.***

Effects on insulin sensitivity independent on angiotensin II type 1 receptor (ACEI vs. ARB)

RAS blockade is supposed to dispose of effects that extend beyond the interruption of AII signalling [178] depending on properties of particular compounds. Indeed, ACEIs and ARBs appear to have different metabolic potential.

In case of ACEIs, the positive metabolic effects are at least in part attributed to increased bioavailability of bradykinin leading to stimulation of NO-production [161, 169]. NO mediates vasodilatation and modulates glucose transport in skeletal muscle through enhancement of GLUT-4 (glucose transporter type 4) translocation to plasma membrane. This mechanism seems to be independent of the insulin signalling and as such it is synergistic to the direct effect of bradykinin on insulin signalling via IRS 1-4 phosphorylation and PI3-kinase activation [161].

The specific effects of ARBs on IR and glucose metabolism have been intensively investigated. Some ARBs are able to directly activate the nuclear receptors PPARγ independently on AII signalling [169]. Thanks to its physicochemical properties with high lipophility, telmisartan was identified to act as a partial PPARγ agonist [179, 180], even already at concentrations achieved by conventional oral dosing [181]. Similar effect can be also reached with relatively high concentrations of irbesartan or with one of the active metabolites of losartan – EXP3179 [182]. In experimental models of IR, telmisartan reduced concentrations of glucose, insulin, NEFAs and triglycerides [179] or regulated the expression of PPARγ-dependent genes that are involved in carbohydrate and lipid metabolism [183, 184]. As an example, telmisartan compared to other ARBs in cell culture was effective in stimulation of gene expression of phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme necessary for glycerol synthesis and fatty acid reesterification and with consequence of reduction of NEFA levels.

Compared to TZDs, telmisartan acts only as a partial PPARγ agonist and therefore its insulin-sensitizing action is not associated with increased risk of fluid retention and heart failure [178, 180]. Additionally, further experiments indicated that telmisartan also exerts anti-inflammatory actions (inhibition of chemokine-induced lymphocyte migration) [185] or vascular effects (increase in NO levels, inhibition of vascular smooth cell proliferation) [186, 187], both of them being PPARγ-mediated. Thanks to its pleiotropic effects, telmisartan was postulated to influence not only blood pressure and IR, but also prevent development of diabetes, atherosclerosis and cardiovascular disease [169, 178].

Other potential ARB-specific, AT-1 receptor independent effects may include reduction of serum uric acid [170], inhibition of vascular smooth cell proliferation [187], reduction of platelet activation and vasoconstriction through interference with thromboxane A_2/prostaglandin endoperoxide H_2 (TxA_2/PGH_2) receptors [188] and through activation of NO synthesis [189], reduction of oxidative stress or reduction of β-amyloid protein accumulation [170]. Different ARB compound differ in their potential to affect certain pathways [170], as it is summarized in Table 2. Ideally, these mechanisms should be confirmed in animal models lacking AT1-receptor and also the clinical relevance of these mechanisms remains to be clarified.

Modulation of adipokine production in adipose tissue represents another, potentially AT-1 receptor independent mechanism, how ACEIs and ARBs affect IR. Besides of direct effects of treatment compounds on adipocytes (e.g. via PPARγ), indirect action on systemic glucose metabolism secondary to increased adipocyte capacity possibly accounts for changes in adipokines. Adiponectin

is mostly found to be stimulated by RAS blockade; effects on other adipokines are inconsistent and rather modest.

Inhibition of uric acid uptake	*Los > Tel > control = Can, Val, Olm*
Blockade of TxA2/PGH2 receptors	*Irb > Los > control = Can, Val*
Activation of NO synthesis	*Los > control = Irb*
Inhibition of β-amyloid protein accumulation	*Val > Los > control = Irb, Olm, Tel, Can*
Activation of PPARγ [a]	*Tel > Irb > Los > Can > control = Val, Olm, Epro*
Activation of PPARα	*Tel > control = Irb, Los*
Reduction of oxidative stress caused by factors other than angiotensin II [a]	*Tel > Can = control or Tel > Los = control*
Inhibition of cell proliferation caused by factors other than angiotensin II [a]	*Tel > Irb > control = Can, Val, Epro*

Table 2 Cellular effects of ARBs beyond AT1-receptor blockade (adapted from [170]); ***NO, nitric oxide; Epro, eprosartan; Can, candesartan; Olm, olmesartan; Tel, telmisartan; Val, valsartan.[a]Some of these effects have also been observed in cells lacking AT1 receptor.***

Effects on insulin secretion

Similarly to adipose tissue, all components of RAS system are expressed in pancreas and thus modulate local blood flow and hormone release. Exaggerated islet RAS activation (e.g. hyperglycaemia-induced) is supposed to potentiate oxidative stress, inflammation and increase NEFA concentrations, contributing thus to β-cell dysfunction [190]. Improved islet microcirculation induced by RAS blockade attenuates oxidative stress and β-cell apoptosis and improves first phase insulin secretion [157, 167]. Improved ionic balance of potassium and magnesium ensured by RAS blockade was also postulated to improved insulin secretion [167]. However based on recent experiments, the role of AII in islets seems to be more complex [191]. Opposite to chronic effects, the acute AII-induced AT1-receptor stimulation enhances insulin secretion.

Clinical studies evaluating the ARB effects on insulin sensitivity and metabolic syndrome

The above-described mechanisms of metabolic effects of ARBs have been postulated based on experimental studies in models of IR, diabetes and obesity. The promising experimental conclusions cannot be always confirmed in studies with human cell cultures, in clinical studies *in vivo* and interventional studies. This only mirrors the complexity of metabolic regulations on the level of whole organism. Unfortunately, due to differences in study design and conditions, the comparison of conclusions of available clinical studies has important limitations. The confounding factors include dose, duration and type of ARB treatment, studied population (ethnics, healthy vs. insulin-resistant subjects, patients with impaired glucose homeostasis or diabetes, presence of additional cardiovascular risk factors, age) or method of IR assessment (most studies only evaluate fasting insulinaemia, glycaemia, HOMA-IR or QUICKI-IR, only minority of the results are derived from clamp studies).

Losartan

First evidence for positive effects of losartan on carbohydrate metabolism came from large interventional LIFE trial (Losartan Intervention for Endpoint), where incidence of new-onset type 2 diabetes was reduced by 25% in the losartan arm [192]. However, the potentially pro-diabetogenic

atenolol was used as a comparator in this trial. The promising results of this study subsequently stimulated further clinical research of losartan aiming to confirm the experimental data. However their results are not conclusive. Even the studies that used hyperinsulinaemic- euglycaemic clamp as a gold standard for estimation of IR, have equivocal conclusions: some were able to demonstrate an increase in glucose disposal [172, 193-195] or glucose oxidation [196], whereas others did not find any effects on insulin action in hypertensive subjects [197-199] or in normotensive insulin-sensitive offspring of hypertensive patients [200]. Several studies proved an increase in circulating HMW and/or total adiponectin following losartan treatment [201-204], although the changes in IR were only assessed as HOMA-IR. On the contrary, a recent study that showed increase in glucose disposal with losartan treatment, did not detect any changes in adipokines or inflammatory markers [205]. The plausibility of partial PPARγ agonism of losartan metabolite EXP3179 [182] has been recently supported by a clinical study demonstrating that chronic losartan treatment results in serum levels of the EXP3179 metabolite sufficient for PPARγ activation, which was confirmed by monocytic PPARγ-target gene expression analysis [206].

Telmisartan

With regard to its well-documented PPARγ agonism, telmisartan appears to be the most potent ARB promising the possibility of metabolic treatment of hypertension. Indeed, there are number of studies documenting that long-term administration of telmisartan improves of fasting blood glucose and HbA1c in patients with type 2 diabetes [207-214], reduces serum triglycerides together with improvement of cholesterol profile [207, 208, 215, 216], leads to decrease visceral fat mass [217, 218] or increase in adiponectin [211, 212, 215, 217, 219, 220]. All the studies demonstrate also attenuation of IR associated with telmisartan treatment, which is interestingly documented solely with reduced HOMA-IR index and/or decreased fasting insulin concentrations. There are virtually no clinical studies available using hyperinsulinaemic-euglycaemic clamps for exact evaluation of IR, especially in diabetic population.

Improvement of metabolic profile should be confirmed by clinical studies using clamp technique in different populations and ethnics. Another remaining question is: whether the pleiotropic effects of telmisartan do really translate into clinical benefit and if so, what is the target patient group. The interventional trials ONTARGET and TRANSCEND were supposed to answer this question, but unfortunately they failed to prove the superiority of telmisartan in prevention of new-onset diabetes and cardiovascular events. Therefore, further studies are needed to disclose if telmisartan provides long-term metabolic benefits.

AIMS

The thesis comprises of 4 related studies that investigate potential IR mechanisms in vivo in humans with focus on adipose tissue using the hyperinsulinaemic-euglycaemic clamp or lipid infusion technique. The aim of the work was to extend the knowledge of mechanisms by which adipose tissue contributes to IR.

Study I. To investigate the effect of acutely induced hyperinsulinaemia with and without acute ARB using losartan on plasma concentrations and SAT expressions of selected cytokines and adipokines in patients with type 2 diabetes and healthy control subjects and to relate their changes to metabolic status

Hypothesis I. Acute hyperinsulinaemia leads to significant changes in gene expression of selected cytokines in SAT that are accompanied by changes in their circulating concentrations in healthy subjects, while different reactions will be found in patients with type 2 diabetes. Significant relations between humoral activity of adipose tissue and insulin sensitivity or inflammation will be found.

Study II. To investigate the effect of 3-week telmisartan treatment on insulin resistance, energy metabolism and plasma concentrations and SAT expressions of selected cytokines and adipokines in subjects with metabolic syndrome and impaired fasting glucose: a randomized, double-blind, placebo controlled, cross-over trial

Hypothesis II. Telmisartan but not placebo treatment leads to improvement in Insulin sensitivity, energy metabolism and other metabolic parameters that are accompanied by significant changes in gene expression and circulating concentrations of selected cytokines in subjects with metabolic syndrome.

Study III. To investigate the effect of prolonged hypertriglyceridaemia on plasma concentrations and SAT expressions of selected cytokines and adipokines in patients with type 2 diabetes and healthy control subjects

Hypothesis III. Acute hypertriglyceridaemia leads to significant changes in gene expression of selected cytokines in SAT that are accompanied by changes in their circulating concentrations in healthy subjects, while different reactions will be found in patients with type 2 diabetes.

Study IV. To assess the plasma concentrations and SAT expressions of selected cytokines and adipokines and markers of low-grade inflammation in subjects with different categories of glucose intolerance and to explore their potential relationships with anthropometric and metabolic characteristics.

Hypothesis IV. Gene expression of selected cytokines in SAT and their circulating concentrations differ between subjects with different categories of glucose intolerance and healthy subjects. Significant relations between humoral activity of adipose tissue and dysregulation of glucose metabolism, insulin sensitivity or inflammation will be found.

METHODS

This chapter briefly describes the investigational and analytical methods and used in all studies. The study-specific design and protocols can be found in the particular Results sections and in the publications.

Subjects

4 groups of subjects with different categories of glucose intolerance were recruited for the clinical studies described in this thesis. The detailed study group characteristics can be found in the Appendix as indicated below. Only male subjects participated in the studies in order to exclude variability in insulin sensitivity in women according to menstrual cycle and childbearing potential and with respect to sexual dimorphism of a number of investigated adipokines. Informed consent was obtained from all the individuals prior to any study procedure after explaining the purpose, nature and potential risks of the study. The local Ethics committee approved the study protocols.

First, a group of 8 healthy young men (H) with normal glucose tolerance, normal blood pressure and serum lipids, without concomitant diseases or family history of diabetes was enrolled for Studies I. and III. For detailed characteristics please refer to Appendix 1 and 4.

Next, eleven overweight/obese patients with type 2 diabetes (D) and 12 age-matched healthy controls(C) were enrolled for Studies I., III. and IV. Detailed characteristics of both groups can be found in Appendix 2. The patients with type 2 diabetes were treated with oral agents except of TZDs, or with diet. Their medication was stable over three months prior to study. Subjects treated with insulin, having micro- or macrovascular complications of diabetes, acute or chronic inflammatory or other major organ diseases were excluded from the study. Six of the type 2 diabetes patients were treated with antihypertensive drugs (calcium-channel blockers, diuretics or ACEIs). The ACEIs were replaced with calcium-channel blockers two weeks prior to clamp procedures and maintained for the whole study duration.
Healthy control subjects had normal glucose tolerance confirmed by oral glucose tolerance test, normal blood pressure and serum lipids. They were not taking any drugs and none had a family history of diabetes. All control subjects were euthyroid and none had a concomitant disease.

The last group of 12 patients with impaired fasting glucose (IFG) fulfilling the criteria of metabolic syndrome, defined according to NCEP-ATPIII criteria revised in 2005 [221], was investigated in Studies II and IV. For detailed characteristics please refer to Appendix 3. Patients enrolled in the study were treatment-naive with regard to oral antidiabetic medication. Antihypertensive treatment was adjusted during the 4-week run-in period as follows: beta-blockers, ACEIs and ARBs had been stopped and replaced by metabolic neutral calcium-channel blockers. The dose of concomitant calcium-channel blocker (if required) was stable during the whole the study duration. None of the patients had their dietary intake of salt or protein restricted. Patients with overt diabetes (diagnosed by an oral glucose tolerance test), acute or chronic inflammatory or other major organ diseases were excluded from the study.

Because the groups of patients with type 2 diabetes, impaired glucose homeostasis and the healthy control subjects were all age-matched, their comparison within the Study IV. was possible.

Procedures

All subjects were examined on an outpatient basis, after overnight fasting with only tap water allowed ad libitum. They were instructed to adhere to their ordinary lifestyle, avoid any changes in food intake and alcohol consumption and to refrain from strenuous physical activity for a period of 24 hours before the experiment.

Hyperinsulinaemic-euglycaemic clamp (HEC)

The HEC was conducted as previously described [222]. Briefly, a Teflon cannula (Venflon; Viggo, Helsingborg, Sweden) was inserted into an antecubital vein for the infusion of all test substances. A second cannula was inserted retrogradely into a wrist vein for blood sampling, and the hand was placed in a heated (65^0 C) box in order to achieve venous blood arterialization. A primed-continuous insulin infusion (1 $mU.kg.^{-1}.min^{-1}$ of Actrapid HM; NovoNordisk, Copenhagen, Denmark - dissolved in 0.9% NaCl) was administered to acutely raise and maintain the plasma concentrations of insulin. Plasma glucose concentrations during the clamp were maintained at the 5 $mmol.l^{-1}$ level by continuous infusion of 15% glucose. To prevent hypokalaemia during insulin infusion, potassium chloride was added to the 15% glucose infusion (30 $mmol.l^{-1}$ KCl). Blood samples for analysis of plasma concentrations of selected adipokines and cytokines were obtained at baseline and during the clamp as specified in the respective studies.

Volume control examination - Saline infusion (SAL)

In order to distinguish possible non-specific volume (haemodilution) and time effects during hyperinsulinaemia on plasma concentrations of proteins tested, SAL was performed in the Study I – part B. The total volume of infusions and water equivalent to HEC was given *per os* and intravenously in the course of 4 hours. Blood samples for measurement of selected cytokines and adipokines were taken at 0 min and 240 min. Based on an assumption that adipose tissue expressions are not affected by haemodilution during clamp, the adipose tissue biopsies were not repeated during SAL volume control.

Prolonged hypertriglyceridaemia

A 24-hour infusion of lipid emulsion (Intralipid 20%; Fresenius Kabi AB, Uppsala, Sweden) was administered through Teflon cannula in the antecubital vein. Intralipid 20% contained soya oil 200 g, egg lecithin 12 g and glycerol 22 g per 1 liter. For the first 4 hours, the infusion rate 125 $ml.h^{-1}$ was used and subsequently until the 24th hour a constant rate was calculated to reach a total dose of 3g of fat.kg body $weight^{-1}.d^{-1}$. This two-step infusion was chosen to achieve both maximum effect and to stay within the clinically allowed boundaries. A second cannula was inserted retrogradely into a wrist vein for blood collection. To assess the plasma levels of selected adipokines, arterialized venous blood samples were taken at 0 min, 30 min, 240 min and 24 hours of the infusion. During the interventions, subjects were only allowed to drink water ad libitum. No heparin infusion was used to avoid the confounding effect of intravascular heparin-stimulated lipolysis.

Indirect calorimetry

Substrate utilization and energy expenditure were assessed by indirect calorimetry [223]. Gas exchange measurements were performed during a 45-min basal period prior to starting the insulin infusion and during the last 45-min period of the clamp. A transparent plastic ventilated hood was placed over the subject's head and made airtight around the neck. A slight negative pressure was maintained in the hood to avoid loss of expired air. A constant fraction of air flowing out of the hood was automatically collected for analysis. Air flow and O_2 and CO_2 concentrations in expired and inspired air were measured by a continuous open-circuit system (metabolic monitor VMAX; Sensor Medics**,** Anaheim, CA, USA). Blood samples were taken at 0 and 120 min of the clamp study to assess plasma levels of selected adipokines and blood urea nitrogen. Urine was collected a) during the night before the study (basal) and b) during the clamp study (0-120 min) to measure the urinary nitrogen excretion to be able to calculate protein oxidation.

Adipose tissue needle biopsy

Needle biopsy of abdominal subcutaneous adipose tissue was performed at baseline and during the clamp and lipid infusion as specified in the respective studies. Based on an assumption that adipose tissue expressions are not affected by haemodilution during clamp, the adipose tissue biopsies were not repeated during SAL volume control.

Under local anaesthesia (1% trimecain in a field block pattern) an incision (3-4 mm) was made through the skin at the lower abdomen and a subcutaneous fat specimen (300 mg) was obtained by needle aspiration. The samples were washed in natrium chloride 0.9% solution, immediately frozen by liquid nitrogen and stored at -80°C until used for RNA extraction.

Analytical methods

Plasma glucose concentrations were measured on a Beckman analyzer (Beckman Instruments Inc, Fullerton, CA, USA) using glucose oxidase method. Immunoreactive insulin (IRI) was determined by radioimmunoassay method using an IMMUNOTECH Insulin IRMA kit (IMMUNOTECH as, Prague, Czech Republic) with analytical sensitivity 0.5 $\mu IU.ml^{-1}$, intra-assay and inter-assay coefficient of variation below or equal to 4.3% and 3.4%, respectively. Haemoglobin A1c (HbA1c) was measured by high-performance liquid chromatography using the Variant II HbA1c Program (Bio-Rad Laboratories GmbH, Munich, Germany). Plasma concentrations of non-esterified fatty acids (NEFA) were estimated by Half-micro test Free fatty acids (Roche Diagnostics GmbH, Penzberg, Germany). Triglyceride (TG) plasma concentrations were assessed by enzymatic assay (BIO-LA-TEST; PLIVA-Lachema, Brno, Czech Republic).

Plasma concentrations of adipokines and cytokines were measured by commercially available kits according to manufacturer's instructions.

Human TNFα UltraSensitive RIA kit (BioSource int., Camarillo, CA, USA) with the detection limit <0.09 pg/ml, intra-assay and inter-assay coefficients of variation were 5.3-6.7% and 8.2-9.7%, respectively;

Human Resistin ELISA kit (BioVendor Lab. Med. Inc., Brno, Czech Republic) with the detection limit 0.033 ng/ml, intra-assay and inter-assay coefficients of variation of 2.8 to 3.4% and 5.1 to 6.9%, respectively;

Human Leptin ELISA kit (BioVendor Lab. Med. Inc., Brno, Czech Republic) with the detection limit 0.5 ng/ml, intra-assay and inter-assay coefficients of variation of 3 to 7.5% and 3.2 to 9.2%, respectively;

Human Adiponectin ELISA kit (BioVendor Lab. Med. Inc., Brno, Czech Republic) with the detection limit 210 ng/ml, intra-assay and inter-assay coefficients of variation of 6.4 to 7% and 7.3 to 8.2%, respectively;
Human HMW Adiponectin ELISA kit (Linco Research, Billerica, MA, USA)with the detection limit 0.5 $ng.ml^{-1}$, intra-assay and inter-assay coefficients of variation of 3.0-8.8% and 1.8-6.1%, respectively;
Human A-FABP ELISA kit (BioVendor Lab. Med. Inc., Brno, Czech Republic) with the detection limit 0.1 $ng.ml^{-1}$, intra-assay and inter-assay coefficients of variation of 5.3% and 3.9%, respectively;
Human Visfatin ELISA kit (BioVision Research Products, Mountain View, CA, USA)with the detection limit 0.03 $ng.ml^{-1}$, intra-assay and inter-assay coefficients of variation of 5.3% and 3.9%, respectively;
Human Retinol-binding protein (RBP4) Sandwich ELISA kit (Adipogen, Soul, Korea) with the detection limit 0.38 $ng.ml^{-1}$, intra-assay and inter-assay coefficients of variation of 1.74-3.70% and 5.94-8.83%, respectively.

For analysis of selected cytokines and markers of low-grade inflammation multiplex immunoassay Fluorokine Multianalyte Profiling (MAP) kit (R&D; Minneapolis, MN, USA) was used. The principle of the method is described elsewhere [224]. Briefly, human fluorokine MAP Base Kit, Panel A was used in combination with Fluorokine MAP Bead Set including MCP-1, VEGF, IL-1ra, RANTES, IL-6, IL-8, MIP, IL-1β and IL-10. The results were evaluated using Luminex analyzer (a dual laser, flow-based detection instrument). In the cohorts studied, MCP-1, IL-1ra, RANTES, VEGF were detectable, while IL-6, IL-8, MIP-1α, IL-1β, IL-10 lied under the detection limit in all groups studied using the multiplex ELISA.

Relative expressions of selected genes in SAT specimens were analysed by the real-time polymerase chain reaction (RT-PCR) method using following protocol:
1) The RNA was isolated from the liquid nitrogen frozen biopsy of the human adipose tissue using the RNeasy Lipid Tissue Mini Kit and QIAzol Lysis Reagent (QIAGEN, Valencia, CA, USA). The starting amount of 100 mg tissue was excised from the biopsy, and homogenized in 1 ml of a QIAzol Lysis Reagent (Guanidin Thiocyanate – Phenol solution) for 2 minutes. From the homogenate, the RNA was isolated by extraction on silica-gel- based column, according to the kit handbook. Possible contamination of RNA with genomic DNA remains was taken off by DNase digestion (RNase-free DNase Set; QIAGEN, Valencia, CA, USA). This step is supposed to prevent any later DNA amplification. Beyond this, the denaturating curves were measured during each reaction and there was just one product present in all the measurements.
2) The cDNA was synthesized using a recombinant Omniscript Reverse Transcriptase (QIAGEN, Valencia, CA, USA), Ribonuclease Inhibitor from human placenta (SIGMA, St. Louis, MO, USA), and $(dT)_{16}$ oligonucleotides.
3) RT-PCR procedure itself has been carried out on the DNA Engine Opticon 2 System (MJ Research, Waltham, MA, USA). HotStar Taq DNA polymerase and SYBR Green fluorescent dye (QuantiTec SYBR Green PCR Kit; QIAGEN, Valencia, CA, USA) were used for the RT-PCR procedure. To eliminate the influence of primer dimmers negative controls were used. To account for differences in cDNA loading, the results were expressed relative to the expression of human cyclophilin (used as a reference gene). The primers used for RT-PCR are shown in Table 3.
4) The data were processed by Q-gene 96 software.

Gene	Forward primer	Reverse primer
Cyclophilin	5′-CAA ATG CTG GAC CCA CA-3‘	5‘-TGCCATCCAACCACTCAGTC-3‘
Leptin	5´-CCC TAA GCC TCC TTT TGC T-3´	5´-GCT AAG AGG GGA CAA GAC A-3´
Adiponectin	5´-GGT TCA ATG GCT TGT TTG C-3´	5´-TCA TCC CAA GCT GAT TCT G-3´
AdipoR1	5´-TCG GAC TTT TTC CAA ACT GG-3´	5´ -CCA CAA TGA TGG CAG AAA TG-3´
AdipoR2	5´-CTC CCT TTT TGC GGT GTG TA -3´	5´ -AGA GCA AGC AAG CCT CTG AC-3´
TNFα	5´-CTA TCT GGG AGG GGT CTT C-3´	5´-TTG GGA AGG TTG GAT GTT C-3´
Resistin	5´-ATA AGC AGC ATT GGC CTG G-3´	5´-TGG CAG TGA CAT GTG GTC T-3´
A-FABP (FABP4)	5‘-ATG GCC AAA CCT AAC ATG A-3‘	5‘-ATT CCT GGC CCA GTA TG-3‘
E-FABP (FABP5)	5‘-AAT GGC CAA GCC ACA TTG TA-3‘	5‘-CAC TCC TGA TGC TGA-3‘
PPARγ	5‘-GAG CCC AAG TTT GAG TTT GC-3‘	5‘-CTG TGA GGA CTC AGG GTG GT-3‘
MCP-1 (CCL2)	5‘-AGCATGAAAGTCTCTGCCGCCCTTCTG-3‘	5‘-ATTACTTAAGGCATAATGTTTCACA-3‘

Table 3 ***Primers used for RT-PCR analysis***

Calculations

Insulin action was estimated as the glucose disposal (M) calculated during the last 30 min of the clamp as the rate of glucose infusion after correction for changes in glucose pool size and urinary glucose loss. Additionally in Study II and IV, metabolic clearance rate of glucose (MCR) was estimated as the glucose disposal divided by ambient glucose concentration to account for differences in steady state glucose concentrations.

Insulin sensitivity indices M/I and MCR/I, respectively (glucose disposal and metabolic clearance rate of glucose, respectively, normalized by plasma insulin during steady state period) were calculated to correct for any variations in plasma insulin.

Calculations of substrate oxidation during indirect calorimetry were performed using standard equations [223]. Urinary urea excretion during the clamp was corrected for changes in urea pool size [225]. Non-oxidative glucose disposal was calculated by subtracting the rate of glucose oxidation from M.

Statistical methods

The data are expressed as means ± SEM or mean (95% Confidence Interval) as appropriate. The means of two groups (Studies I. and III.) were compared using Mann-Whitney test, the means of three groups (Study IV.) using one-way ANOVA model with least significant difference (LSD) post-tests to elucidate differences between subgroups. In Study II., the telmisartan and placebo treatment were compared using Wilcoxon's paired test.

The differences between the interventions and between the groups in Studies I, II and III were evaluated by repeated measures ANOVA model. In Studies I and II following factors and interactions were included as appropriate: Effect of Group (D vs. C) as the between factor, Effects of Intervention (HEC vs. SAL and HEC vs. AT-HEC, respectively) and Time (0 min vs. 240 min) as the within-factors, Subject factor (represents the inter-individual variability of subjects) and their interactions (Intervention × Time; Intervention × Group; Time × Group; Group × Intervention × Time). The interactions indicate if the shapes of the time profiles for HEC, AT-HEC and SAL, for D and C, respectively were different or not. In Study II following factors and interactions were included: Effect of telmisartan treatment (placebo vs. telmisartan) and Effect of hyperinsulinaemia (0 min vs. 120 min) as the within factors, subject factor (inter-individual variability of subjects) and telmisartan ×

Hyperinsulinaemia interaction. The differences between subgroups in all studies were evaluated using LSD multiple comparisons. Additionally in Study I., separate ANOVA models for Diabetes and Control groups were performed for comparison of the interventions within the group. The significances of the separate models and LSD are described in the text.
The statistical significance $p<0.05$ was chosen for both ANOVA testing and multiple comparisons. Due to non-Gaussian data distribution in all dependent variables, the data underwent power transformations to attain distributional symmetry and a constant variance in the data as well as in residuals. The non-homogeneities were detected using residual diagnostics. The experimental points with absolute values of Studentized residual (after data transformation) greater than 3, were excluded from the analysis. The fraction of such points never exceeded 5% of the total number.
For correlation analysis Spearman correlation coefficient and multivariate regression analysis using the method of orthogonal projection to latent structures were applied.
Statistical software Statgraphics Centurion v. XV (Statpoint Inc.; Herndorn, Virginia, USA) was used for the data analysis.

RESULTS

Study I. Acute effects of hyperinsulinaemia and losartan on endocrine activity of adipose tissue in type 2 diabetes and healthy subjects

Aim

To investigate the effect of acutely induced hyperinsulinaemia with and without acute ARB using losartan on plasma concentrations and SAT expressions of selected cytokines and adipokines in patients with type 2 diabetes and healthy control subjects and to relate their changes to metabolic status.

Subjects

For part A of the study I., a group of 8 healthy male subjects (H) with normal glucose tolerance and mean age of 25.75±1.29 years was recruited (see Appendix 1).

In part B of the study I., 11 overweight/obese patients with type 2 diabetes (D) and 12 age-matched healthy control subjects (C) were enrolled (see Appendix 2).

Study protocol

Part A. In random order, the healthy subjects (H) underwent two hyperinsulinaemic euglycaemic clamps at least 4 weeks apart, both taking 4 hours to complete: HEC (described above) and an identical clamp study after acute AT-1 receptor blockade (AT-HEC) reached by oral administration of losartan 2x 100mg prior to the study (8 hours and immediately prior to the clamp). Blood samples and adipose tissue biopsies were collected at 0 min, 30 min and 240 min of the clamp to determine plasma concentrations and expressions of selected adipokines.

Part B. The D and C groups underwent three 4-hour examinations at least 3 weeks apart, in random order: HEC, AT-HEC and SAL (described above) as a control examination. Blood samples and adipose tissue biopsies were collected at 0 min and 240 min of the clamp to determine plasma concentrations and gene expressions of selected adipokines.

Results

Study I - Part A

During the steady state periods of HEC vs. AT-HEC the clamps were comparable in terms of the mean plasma glucose concentrations (4.68±0.32 vs. 4.75±0.20 $mmol.l^{-1}$) with coefficient of variation 3.37±0.55 vs. 3.42±1.87 %. Although the mean IRI levels were different comparing both clamps (65.11±7.53 vs. 75.28±6.95 $\mu IU.ml^{-1}$; $p<0.05$), the parameters of insulin sensitivity did not significantly differ between HEC and AT-HEC, being expressed as the glucose disposal (M): (9.55±0.56 vs. 9.15±1.68 $mg.kg^{-1}.min^{-1}$), as well as calculated as the insulin sensitivity index M/I (0.15±0.01 vs. 0.13±0.01 $mg.kg^{-1}.min^{-1}/\mu IU.ml^{-1}$).

Plasma concentrations of resistin during HEC and AT-HEC are shown in Figure 2a. ANOVA model indicated a significant increase in plasma resistin during both clamps (Time effect: $p<0.05$). Despite the multiple comparisons reached significance only for the difference between basal value and 240 min for AT-HEC ($p<0.05$), the time trend did not differ between the clamps.

The time profiles of resistin expression (Figure 2b) differed between HEC and AT-HEC (treatment × time interaction: $p<0.05$): resistin expression increased during HEC (multiple comparisons: 0 min vs.

240 min: p<0.05), while during AT-HEC the expression did not change. Moreover, at 240 min of AT-HEC, resistin expression was lower compared to HEC (p<0.05 by multiple comparisons).

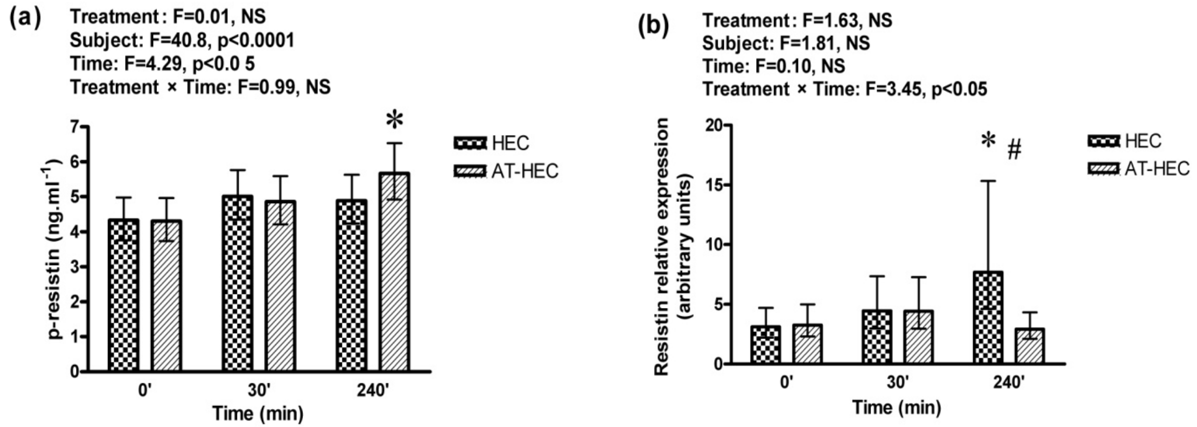

Figure 2 ***Plasma concentrations of resistin (a) and its relative expressions in subcutaneous adipose tissue (b) during HEC and AT-HEC. The bars with error bars represent retransformed mean values with their 95 % confidence intervals. F in the embedded table symbolizes the Fisher's statistics for individual factors and interactions. The asterisks correspond to significant difference between the basal and stimulated values (p<0.05). Hashes denote significant difference between HEC and AT-HEC in individual stages (p<0.05).***

No changes in plasma adiponectin were detected (Figure 3a). Relative expressions of adiponectin (Figure 3b) differed in terms of time profiles between HEC and AT-HEC (treatment × time interaction: p<0.05). Adiponectin expression increased during HEC (multiple comparisons 0 min vs. 240 min: p<0.05), while it did not change during AT-HEC.

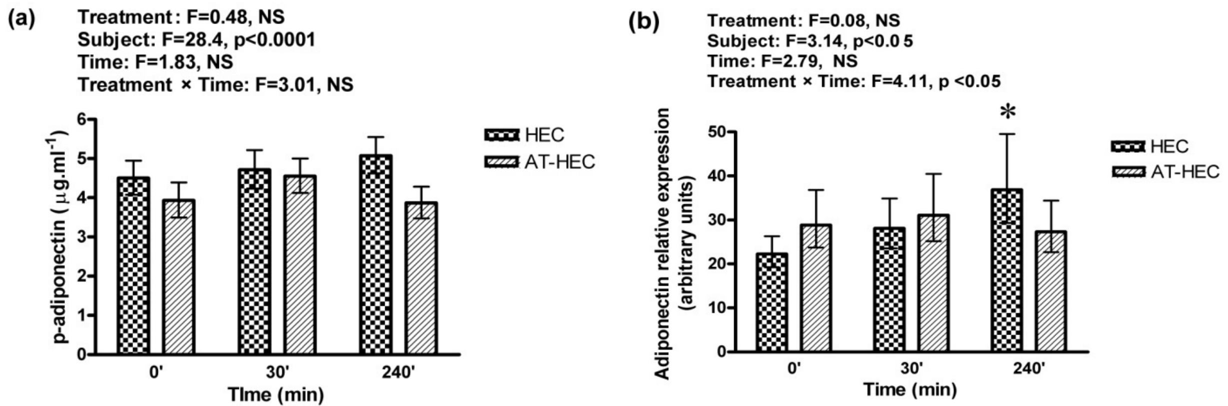

Figure 3 ***Plasma concentrations of adiponectin (a) and its relative expressions in subcutaneous adipose tissue (b) during HEC and AT-HEC. The bars with error bars represent retransformed mean values with their 95 % confidence intervals. F in the embedded table symbolizes the Fisher's statistics for individual factors and interactions. The asterisks correspond to significant difference between the basal and stimulated values (p<0.05).***

Study I - Part B

Characteristics of the steady state periods of interventions

Parameters characterising the steady state periods of clamps are shown in Table 4. The clamps were comparable within as well as between D and C groups in terms of the mean plasma glucose concentrations with coefficients of variation below 3%, the mean IRI and NEFA levels. As expected, the insulin sensitivity expressed as M was significantly higher in C compared to D group (Group effect: $p<0.01$). No differences in M were detected between HEC and AT-HEC in any group.

During SAL control intervention the total fluid volume administered was comparable to HEC and AT-HEC. The steady-state periods of SAL (last 30 min) were characterised by mean plasma glucose 5.72±0.29 and 4.76±0.14 mmol.l^{-1}($p<0.01$); mean IRI concentrations 5.3±1.33 and 2.92±0.28 μU.ml^{-1} (NS); and mean NEFA concentrations 0.48±0.04 and 0.34±0.05 mmol.l^{-1} (NS) in D vs. C, respectively.

Variable	C (n=12)		D (n=11)		*p*-value
	HEC	AT-HEC	HEC	AT-HEC	
Mean plasma glucose (mmol.l^{-1})	5.18±0.12	5.23±0.10	5.35±0.20	5.17±0.13	NS
Coefficient of glucose variation (%)	2.23±0.27	2.65±0.17	2.60±0.32	2.86±0.41	NS
Mean insulin (μU.ml^{-1})	71.87±4.89	77.13±6.25	80.58±5.58	79.56±7.55	NS
NEFA (mmol.l^{-1})	0.059±0.01	0.099±0.04	0.094±0.02	0.083±0.02	NS
M (mg.kg^{-1}.min^{-1})	10.13±0.86	10.68±0.94	6.70±0.44	6.35±0.59	**

Table 4 ***Characteristics of the steady state periods of HEC vs. AT-HEC. Data are means ± SEM. ** p<0.01 for D vs. C***

The effect of metabolic status, hyperinsulinaemia and acute ARB on plasma concentrations and SAT expressions of selected adipokines and cytokines

Leptin

Plasma concentrations of leptin during HEC and SAL are shown in Figure 4a. Basal leptin levels were higher in D group (Group effect $p<0.05$; LSD post-tests $p<0.05$). In C, plasma leptin increased during HEC and reached concentrations comparable to D, while during SAL plasma leptin only tended to decrease (Intervention effect: $p<0.001$; Intervention × Time Interaction: $p<0.05$ in ANOVA model for controls). In D group, the changes in leptin during HEC and SAL were not significant. Leptin expression in SAT (Figure 4c) was higher in D (Group effect: $p<0.001$) and was not affected by hyperinsulinaemia.

The time profile of plasma leptin during HEC and AT-HEC is depicted in Figure 4b. Again, basal leptin concentrations were higher in D ($p<0.05$ in LSD post-tests). In C, acute ARB did not influence the increase in leptin concentrations during hyperinsulinaemia (Time effect: $p<0.01$ in ANOVA model for controls and $p<0.05$ in LSD post-tests, Intervention effect NS). A different regulation was observed in D group (Group × Time Interaction: $p<0.01$ in full ANOVA model): leptin concentrations declined during both HEC and AT-HEC (Time effect: $p<0.05$ in ANOVA model for diabetes and in LSD post-tests). Additionally, leptin concentrations tended to be suppressed after acute ARB both at baseline

and during clamp, which contrasted with comparable time profiles in C group (Group × Intervention Interaction: p=0.05; Intervention effect: p=0.07 in model for diabetes).

SAT expressions of leptin (Figure 4c) did not show any changes during AT-HEC.

In summary, baseline plasma concentrations and SAT expressions of leptin are higher in diabetes. Insulin increases circulating concentrations of leptin in healthy subjects, while in diabetes plasma leptin declines during hyperinsulinaemia. Differential regulation of circulating leptin cannot be explained by its expression in SAT, which implicates the involvement of other fat depots as a leptin source. Acute losartan administration suppresses plasma leptin levels in diabetes but not in healthy subjects, while leptin expression in SAT is not subject of acute losartan regulation.

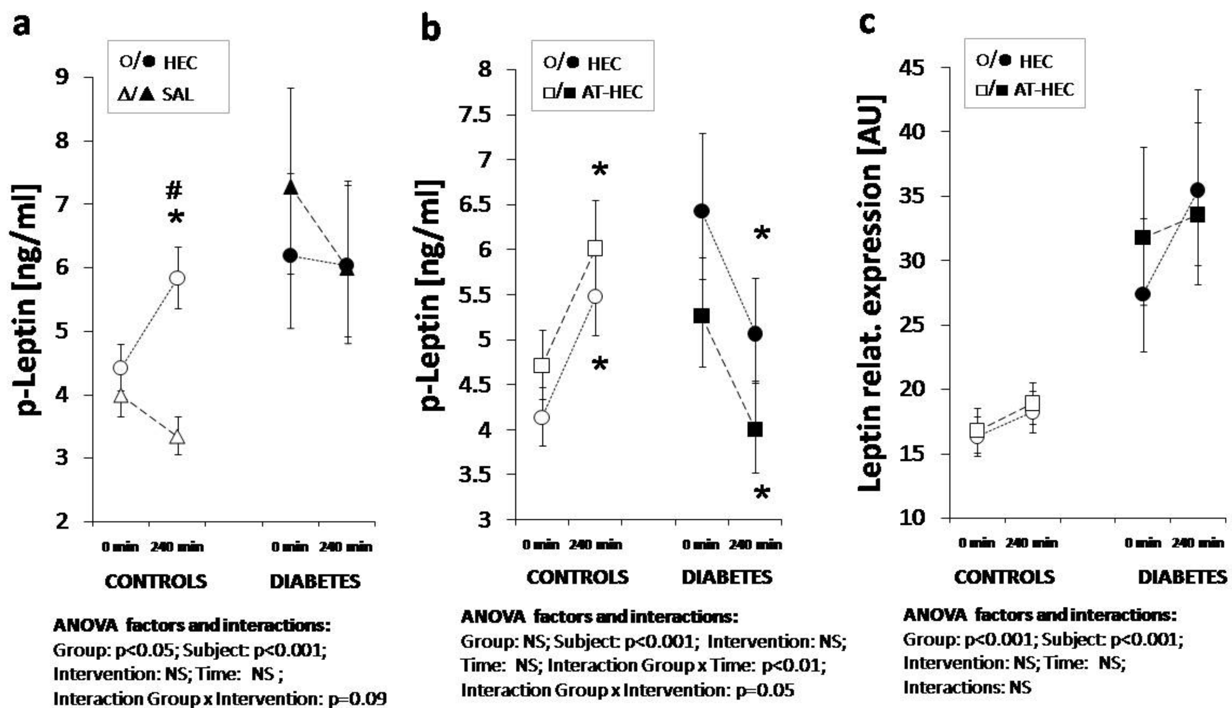

Figure 4 ***Leptin plasma concentrations during interventions: (a) HEC vs. SAL and (b) HEC vs. AT-HEC. Relative expression of leptin (c) during HEC vs. AT-HEC. The empty and full symbols with error bars represent retransformed mean values with their 95 % confidence intervals for C and D groups, respectively. The asterisks correspond to significant difference between the basal and stimulated values (p<0.05). Hashes denote significant difference between interventions (HEC vs. SAL and HEC vs. AT-HEC, respectively) in individual stages (p<0.05). Embedded tables summarize the significant factors of full ANOVA model.***

Adiponectin

Plasma concentrations of total adiponectin during HEC and SAL (depicted in Figure 5a) revealed unexpected differences. During HEC, the concentrations were comparable between groups without any dynamic changes (an observation which is in line with further results). During SAL, basal adiponectin concentrations were higher compared to HEC in C group (Intervention effect: p<0.05 in full ANOVA model and in model for controls, p<0.05 in LSD post-tests). On the contrary in D group, total adiponectin concentrations were decreased both at baseline and 240 min of SAL (Intervention effect: p<0.05 and Diabetes × Intervention interaction: p<0.001 in full ANOVA model; Intervention effect: p<0.001 in model for diabetes). The decreased total adiponectin concentrations during SAL the D group account for the significant Group effect: p<0.001 in full ANOVA model.

Plasma concentrations of HMW adiponectin were comparable between D and C groups (Figure 5c). During both HEC and SAL no significant changes in HMW adiponectin were observed in either group.

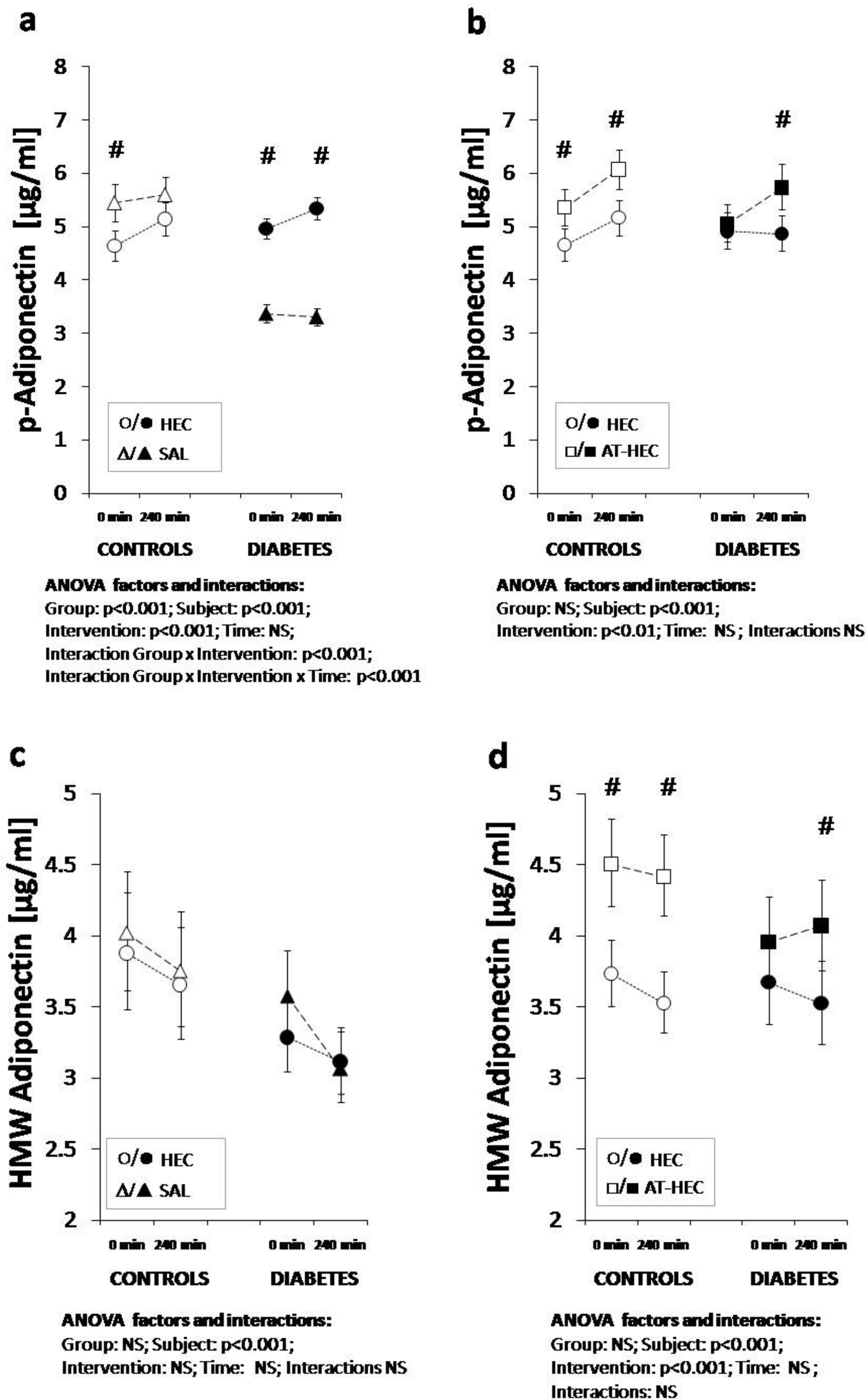

Figure 5 *Plasma concentrations of total and HMW adiponectin during interventions: (a, c) HEC vs. SAL and (b, d) HEC vs. AT-HEC. The empty and full symbols with error bars represent retransformed mean values with their 95 % confidence intervals for C and D groups, respectively. The asterisks correspond to significant difference between the basal and stimulated values (p<0.05). Hashes denote significant difference between interventions (HEC vs. SAL and HEC vs. AT-HEC, respectively) in individual stages (p<0.05). Embedded tables summarize the significant factors of full ANOVA model.*

Surprisingly, adiponectin expression in SAT was even higher in D compared to C (Group effect: $p<0.001$). We also assessed relative expressions of adiponectin receptors AdipoR1 and AdipoR2 in SAT. Both receptors showed higher expressions in C compared to D (Group effect: $p<0.05$ and $p<0.001$, resp. for AdipoR1 and AdipoR2, respectively). AdipoR2 expression rates in SAT were approximately 3 times higher than AdipoR1 mRNA. During hyperinsulinaemia, adiponectin expressions were stable in C group, whereas in D they showed an increase (Group × Time Interaction: $p<0.05$ in full ANOVA model). AdipoR1 and AdipoR2 expressions were not influenced by hyperinsulinaemia in both groups. For expression of adiponectin and its receptors please refer to Table 5.

On the other hand during AT-HEC, a consistently higher total and HMW adiponectin concentrations (Figure 5b and Figure 5d, resp.) (Intervention effect: $p<0.01$ and $p<0.001$, respectively) as well as higher adiponectin mRNA expression (Intervention effect: $p<0.01$) compared to HEC were detected in both groups. The insulin-stimulated increase in adiponectin expression in D group was not further affected by losartan. Expression of adiponectin receptors was not influenced by losartan administration.

In summary, plasma concentrations of total and HMW adiponectin are comparable between D and C groups. However, the total adiponectin concentrations during SAL are not in line with other results and remain unclear. Adiponectin expression in SAT was even higher in D group, while adiponectin receptors were more expressed in C group. Hyperinsulinaemia had no effect on adiponectin concentrations, whereas adiponectin expressions were stimulated by insulin in D but not in C. Acute losartan administration stimulates both total and HMW adiponectin and its expression in SAT, expression of adiponectin receptors is not influenced by losartan.

Variable		C (n=12)		D (n=11)		Group effect (p value)
		0 min	240 min	0 min	240 min	
Adiponectin mRNA / Cyclophilin mRNA	HEC	**17.36** ^{a} (15.78–19.18)	**17.06** ^{a} (15.52–18.83)	**36.35** ^{a} (32.17–41.29)	**41.11** ^{a,b} (36.19–46.99)	<0.001
	AT-HEC	**21.59** ^{a} (19.31–24.29)	**18.0** ^{a} (16.34–19.93)	**45.57** ^{a} (39.93–52.34)	**54.63** ^{a,b} (47.03–64.01)	
AdipoR1 mRNA / Cyclophilin mRNA	HEC	79.71 (68.35–93.32)	71.76 (61.69–83.80)	70.72 (65.41–76.59)	71.3 (65.94–77.23)	<0.05
	AT-HEC	83.28 (70.47–98.88)	73.52 (63.17–85.90)	69.57 (64.37–75.32)	65.55 (60.72–70.87)	
AdipoR2 mRNA/ Cyclophilin mRNA	HEC	233.1 (196.7–276.8)	244.3 (206.1–290.3)	179.2 (165.2–193.8)	195.9 (181.2–211.1)	<0.001
	AT-HEC	275.6 (228.9–332.6)	261.3 (220.2–310.8)	181.6 (167.5–196.2)	191.6 (177.1–206.6)	

Table 5 ***Relative expressions of adiponectin, AdipoR1 and AdipoR2 in SAT. Data are means (5%-95% CI). Statistical significance (in bold): Group effect – difference between groups; ^{a}Intervention effect p<0.05 – difference between interventions (HEC vs. AT-HEC); ^{b} Time effect – difference between 0 min and 240 min. For detailed description of ANOVA model, see text.***

Resistin

Plasma concentrations of resistin were comparable between groups. However, group-specific changes in plasma resistin were found during HEC and SAL (Figure 6a). In D, basal resistin concentrations were increased in SAL compared to HEC ($p<0.05$ in LSD post-tests) and they remained stable at 240 min of SAL. During HEC, the D group showed a clear increase in plasma resistin that reached concentrations comparable to 240 min of SAL (Time effect: $p<0.01$ in full ANOVA model; $p<0.05$ in LSD post-tests). In C, a general increase in plasma resistin was found during both HEC and SAL (Time effect: $p<0.01$ in full ANOVA model), but there was also a significant difference between the interventions in this group. Both at baseline and 240 min of HEC, resistin concentrations were lower than in SAL (Intervention effect: $p<0.05$ and $p=0.08$ in ANOVA model for Controls and in full model, respectively; $p<0.05$ in LSD post-tests). These observations suggest that in diabetes, insulin increases plasma resistin, whereas in healthy subjects an increase in resistin is also seen in SAL and as such cannot be attributed to hyperinsulinaemia. However, plasma resistin was suppressed during HEC in control subjects. Relative expression of resistin in SAT was found to be higher in D; hyperinsulinaemia did not affect resistin expression in either group (Figure 6c).

Accordingly, comparing HEC and AT-HEC clamps (Figure 6b), a significant increase in plasma resistin was detected in D group (Time effect: $p<0.001$ in ANOVA model for Diabetes, which accounted for Time effect: $p<0.001$ in full ANOVA model). Basal resistin concentrations in AT-HEC were reduced in D ($p<0.05$ in LSD post-tests), but an increase during clamp compensated for the initial lower levels. On the other hand in controls, only a non-significant trend for increase in resistin was demonstrated (Time effect: $p=0.096$ in ANOVA model for controls) that did not differ between HEC and AT-HEC. Resistin expression in SAT did not show any changes after losartan administration in either group.

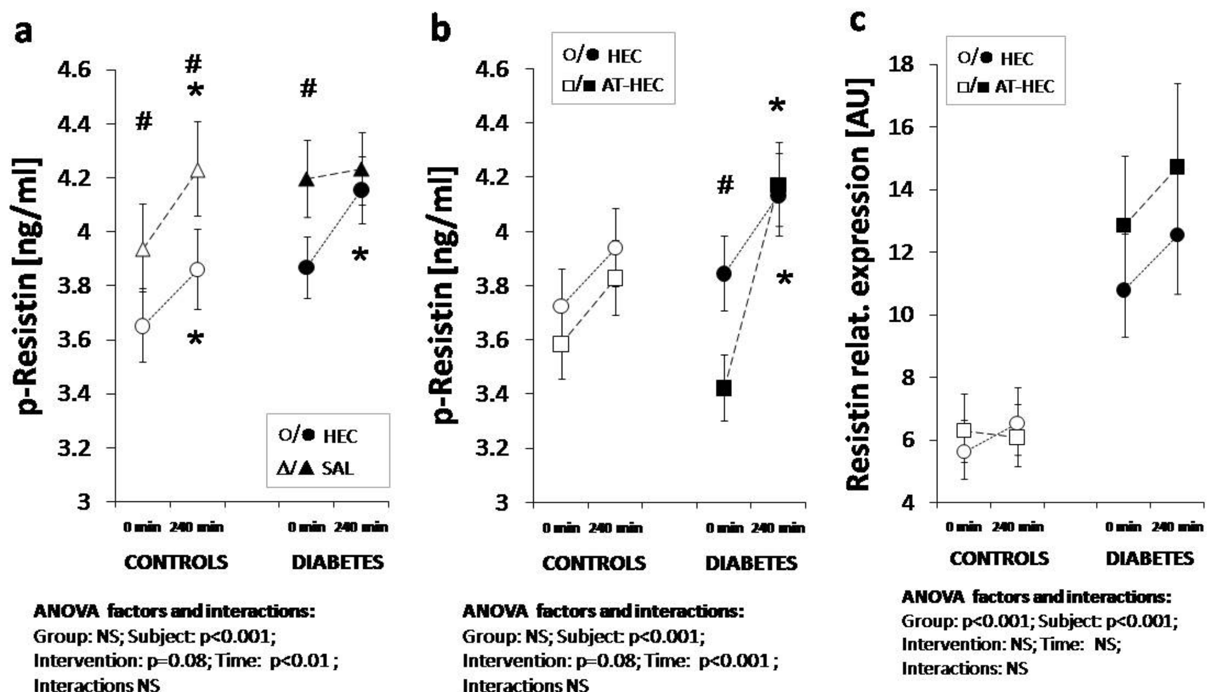

Figure 6 ***Resistin plasma concentrations during interventions: (a) HEC vs. SAL and (b) HEC vs. AT-HEC; relative expression of resistin (c) during HEC vs. AT-HEC. The empty and full symbols with error bars represent retransformed mean values with their 95 % confidence intervals for C and D groups, respectively. The asterisks correspond to significant difference between the basal and stimulated values (p<0.05). Hashes denote significant difference between interventions (HEC vs. SAL and HEC vs. AT-HEC, respectively) in individual stages (p<0.05). Embedded tables summarize the significant factors of full ANOVA model.***

In summary, plasma concentrations of resistin are not different between D and C groups, whereas resistin expression in SAT is increased in diabetes. In diabetes, insulin increases plasma resistin. On the contrary, in controls the increase in resistin is observed also during SAL and thus it is not caused by insulin. However, during hyperinsulinaemia plasma resistin was suppressed in C. Losartan reduces basal resistin concentrations in D but not in C. Resistin expression in SAT is not affected by hyperinsulinaemia or losartan.

$TNF\alpha$

Plasma concentrations of TNFα (Figure 7) were higher in D compared to C group (Group effect: $p<0.001$ in full ANOVA model). In C, TNFα levels were comparable within and between HEC and SAL, whereas in D an unexpected difference between HEC and SAL was measured. In SAL, TNFα concentrations were substantially lower both at baseline and 240 min in the D group (Intervention effect: $p<0.01$ and $p<0.001$ in full ANOVA model and in model for Diabetes, respectively; Group × Intervention Interaction: $p<0.001$ in full model). No dynamic changes during HEC or SAL were detected in D. TNFα relative expressions in SAT (Figure 7c) were also higher in D compared to C (Group effect: $p<0.001$ in full ANOVA model). Acute hyperinsulinaemia resulted in an increase in TNFα expressions in D (Time effect: $p<0.01$ and $p<0.05$ in full ANOVA model and in model for Diabetes, respectively), while in C only a trend for increase was observed.

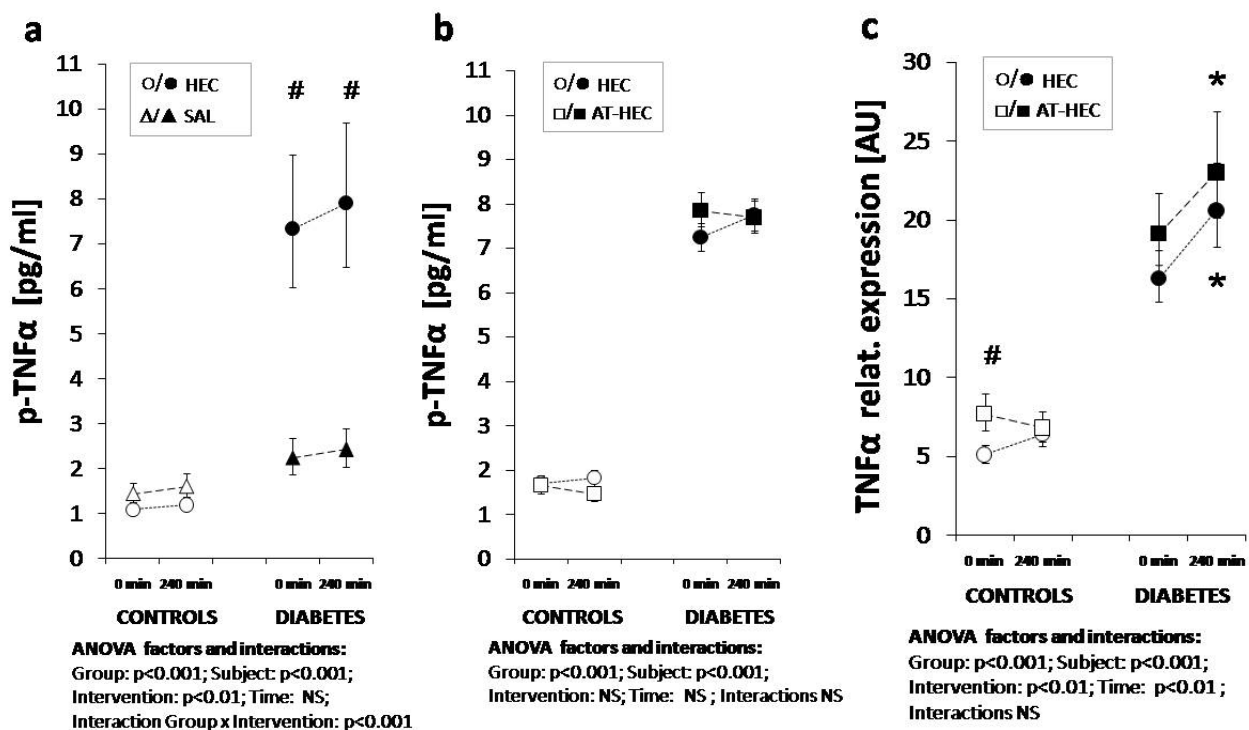

Figure 7 ***TNFα plasma concentrations during interventions: (a) HEC vs. SAL and (b) HEC vs. AT-HEC; relative expression of TNFα (c) during HEC vs. AT-HEC. The empty and full symbols with error bars represent retransformed mean values with their 95 % confidence intervals for C and D groups, respectively. The asterisks correspond to significant difference between the basal and stimulated values (p<0.05). Hashes denote significant difference between interventions (HEC vs. SAL and HEC vs. AT-HEC, respectively) in individual stages (p<0.05). Embedded tables summarize the significant factors of full ANOVA model.***

During HEC and AT-HEC, plasma TNFα was comparable within and between clamps and showed the above described difference between groups (Figure 7b). Relative expressions of TNFα were increased during AT-HEC in C (Intervention effect: $p<0.01$ and $p<0.05$ in full ANOVA model and in model for

Controls, respectively). In the post-hoc analysis of TNFα expression in C, there was a clear increase at baseline of AT-HEC compared to HEC (*p*<0.05 in LSD post-tests), while the insulin-stimulated TNFα expressions were comparable between clamps (Intervention × Time Interaction: *p*=0.056; LSD post-test NS). In D, the increase in TNFα expression during hyperinsulinaemia was not influenced by losartan.

In summary, TNFα circulating concentrations and expressions are increased in diabetes. However, the low TNFα concentrations in SAL are controversial and remain unclear. Plasma TNFα is not affected by insulin or losartan. In diabetes, TNFα expression is stimulated by insulin independently on AT-1 receptor blockade, while in controls losartan increases basal TNFα expression.

Visfatin

Plasma levels of visfatin (Figure 8) were surprisingly lower in D compared to C group (Group effect: *p*<0.01 in full ANOVA model). During SAL, visfatin concentrations were increased both at baseline and 240 min compared to HEC in both groups (Intervention effect: *p*<0.001 in full ANOVA model and model for Controls; Intervention effect: *p*<0.01 in model for Diabetes). No dynamic changes during HEC and SAL were observed in either group. Comparing HEC and AT-HEC, no differences within and between clamps were detected. The measurement of visfatin expressions in SAT samples was not possible.

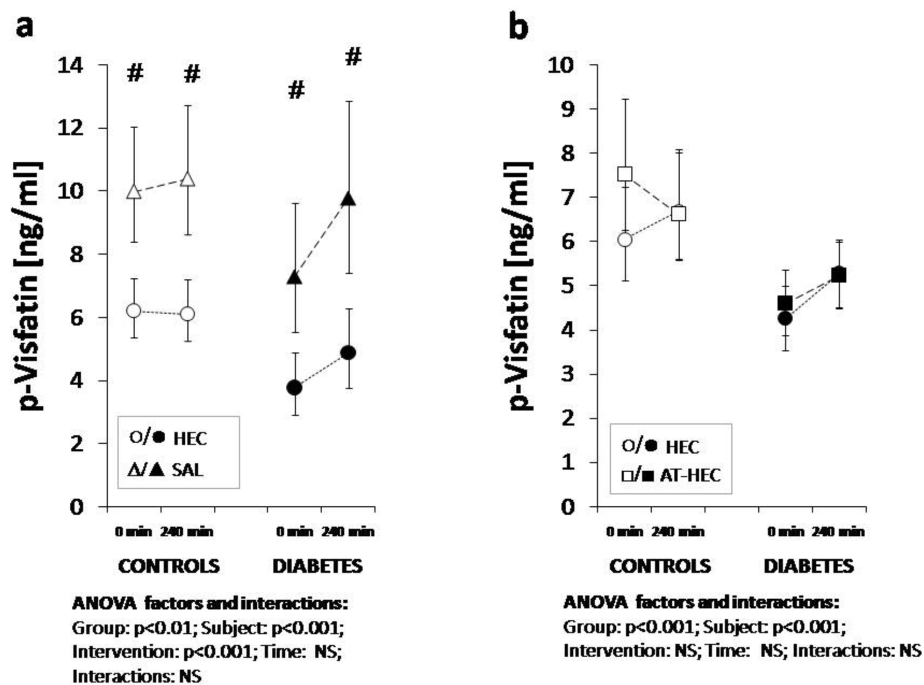

Figure 8 ***Visfatin plasma concentrations during interventions: (a) HEC vs. SAL and (b) HEC vs. AT-HEC. The empty and full symbols with error bars represent retransformed mean values with their 95 % confidence intervals for C and D groups, respectively. The asterisks correspond to significant difference between the basal and stimulated values (p<0.05). Hashes denote significant difference between interventions (HEC vs. SAL and HEC vs. AT-HEC, respectively) in individual stages (p<0.05). Embedded tables summarize the significant factors of full ANOVA model.***

RBP4

Baseline plasma concentrations of RBP4 were comparable between groups (Figure 9). However, different responses to interventions were demonstrated. While during SAL no changes in RBP4 were

measured in both groups, during HEC a decline in RBP4 was detected in C but not in D (Intervention effect: $p<0.05$ in model for Controls, Group × Intervention Interaction: $p<0.01$ in full ANOVA model). The decreased RBP4 concentrations at 240 min of HEC accounted for the difference between groups (Group effect: $p<0.01$) despite of comparable baseline values. The decrease in RBP4 in controls during hyperinsulinaemia was confirmed in the analysis of HEC vs. AT-HEC (Time effect: $p<0.05$ in model for Controls), while no effect of losartan was shown. On the other hand in diabetes, RBP4 time profile differed between clamps: RBP4 was stable during HEC, while during AT-HEC decreased basal concentrations reversed 240 min (Intervention effect: $p<0.05$ in full ANOVA model and model for Diabetes; Group × Time Interaction: $p<0.01$). The measurement of RBP4 expressions in SAT samples was not possible.

Although RBP4 plasma concentrations are comparable between diabetes and controls, they are differentially regulated. In controls but not in diabetes, RBP4 is suppressed by insulin. Losartan has no effect on RBP4 in controls but it reduced basal RBP4 levels in diabetes.

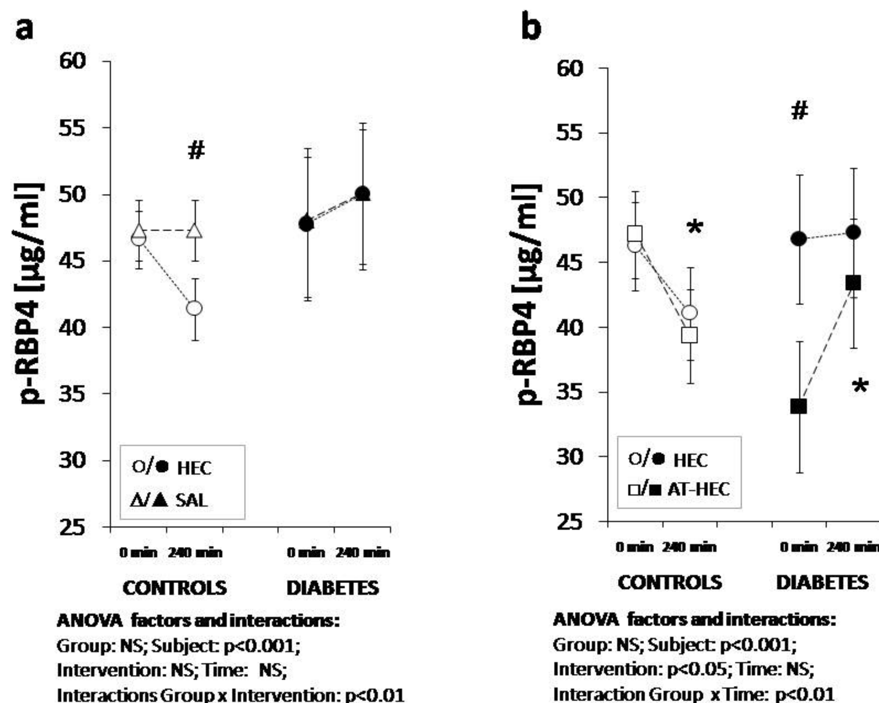

Figure 9 ***RBP4 plasma concentrations during interventions: (a) HEC vs. SAL and (b) HEC vs. AT-HEC. The empty and full symbols with error bars represent retransformed mean values with their 95 % confidence intervals for C and D groups, respectively. The asterisks correspond to significant difference between the basal and stimulated values (p<0.05). Hashes denote significant difference between interventions (HEC vs. SAL and HEC vs. AT-HEC, respectively) in individual stages (p<0.05). Embedded tables summarize the significant factors of full ANOVA model.***

Other cytokines

Both MCP-1 plasma concentrations and SAT expressions (Figure 10) were increased in diabetes (Group effect: $p<0.001$ in both full ANOVA models). In both groups, plasma MCP-1 concentrations were stable and lower during SAL compared to HEC (Intervention effect: $p<0.001$ in full ANOVA, in model for Controls and for Diabetes). While hyperinsulinaemia did not change MCP-1 levels in C, it

increased MCP-1 in D group (Group × Time Interaction: $p<0.05$; Time effect: $p<0.01$ in model for Diabetes). MCP-1 expression did not show any differences during HEC in both groups. Losartan did not change MCP-1 time profile in C and prevented the increase in MCP-1 in D (Group × Intervention × Time Interaction: $p<0.05$). MCP-1 expression in SAT was stable and comparable between HEC and AT-HEC in both groups.

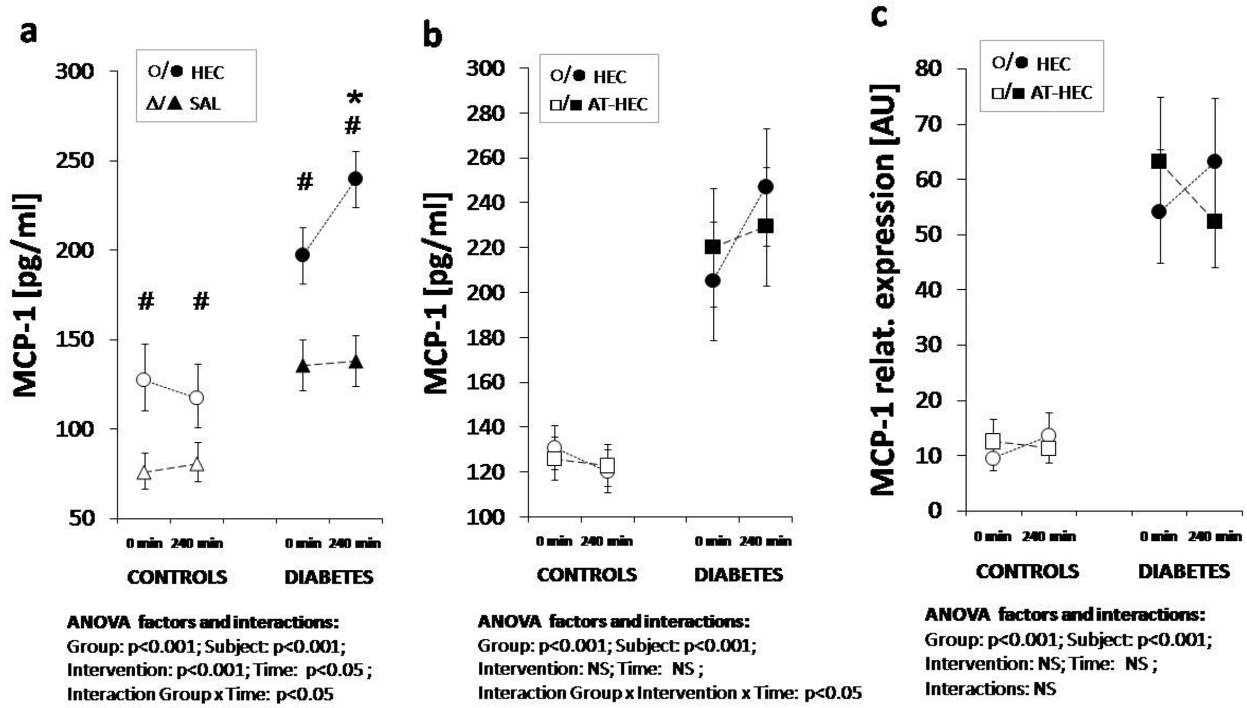

Figure 10 ***MCP-1 plasma concentrations during interventions: (a) HEC vs. SAL and (b) HEC vs. AT-HEC. Relative expression of MCP-1 (c) during HEC vs. AT-HEC. The empty and full symbols with error bars represent retransformed mean values with their 95 % confidence intervals for C and D groups, respectively. The asterisks correspond to significant difference between the basal and stimulated values (p<0.05). Hashes denote significant difference between interventions (HEC vs. SAL and HEC vs. AT-HEC, respectively) in individual stages (p<0.05). Embedded tables summarize the significant factors of full ANOVA model.***

IL-1ra plasma (Table 6) concentrations were higher in diabetes compared to controls (Group effect: $p<0.001$ in full ANOVA model). Changes in IL-1ra during and between all interventions were not significant in either group.

Plasma concentrations of RANTES (Table 6) were paradoxically decreased in diabetes compared to controls (Group effect: $p<0.001$ in full ANOVA model). In both groups, RANTES concentrations were also lower during HEC compared to SAL (Intervention effect: $p<0.001$ in full ANOVA, in model for Controls and for Diabetes). In C, RANTES additionally displayed a parallel decline during both HEC and SAL, whereas in D no significant dynamic changes were detected (Group × Time Interaction: $p<0.05$; Time effect: $p<0.05$ in model for Controls). Comparing HEC vs. AT-HEC, the decline in RANTES during clamp was not influenced by losartan in C group (Time effect: $p<0.05$ and $p=0.07$ in full ANOVA model and in model for controls, respectively). In D group, changes in RANTES levels during HEC and AT-HEC were not significant. In summary, RANTES concentrations are unexpectedly lower in diabetes. Insulin suppresses RANTES in controls but not in diabetic patients. RANTES is not acutely regulated by losartan.

VEGF plasma concentrations were comparable between groups, their changes during and between all interventions were not significant (Table 6).

Variable		C (n=12)		D (n=11)		Group effect (p value)
		0 min	240 min	0 min	240 min	
IL-1ra [pg/ml]	HEC	372.2 (319.1–436.1)	353.2 (303.4–413.2)	499.8 (412.1–615.0)	492.6 (406.6–605.5)	<0.001
	SAL	273.8 (236.8–317.8)	326.2 (278.4–384.2)	399.1 (338.1–476.1)	478.0 (400.6–577.3)	
	AT-HEC	315.8 (277.1–362.9)	289.5 (255.3–330.8)	554.1 (477.1–649.5)	513.3 (448.6–591.8)	
RANTES [ng/ml]	HEC	**8.87** [a] (7.58–10.21)	**7.32** [a,b] (6.10–8.60)	**4.36** [a] (2.92–5.99)	**4.40** [a] (2.95–6.04)	<0.001
	SAL	**13.52** [a] (11.81–14.73)	**10.77** [a,b] (9.41–12.17)	**7.92** [a] (6.22–9.76)	**10.76** [a] (8.85–12.80)	
	AT-HEC	**7.49** (6.51–8.56)	**6.25** [b] (5.39–7.21)	5.51 (4.77–6.24)	4.75 (4.12–5.38)	
VEGF [pg/ml]	HEC	5.15 (3.09–7.75)	2.67 (1.26–4.61)	2.61 (1.19–5.02)	4.75 (2.44–8.39)	NS
	SAL	5.50 (3.36–8.17)	5.09 (3.04–7.67)	4.05 (1.84–7.79)	6.94 (3.53–12.33)	
	AT-HEC	3.85 (2.29–5.79)	6.99 (5.01–9.30)	3.51 (1.49–7.34)	3.01 (1.24–6.42)	

Table 6 ***Plasma concentrations of IL-1ra, RANTEs and VEGF during interventions. Data are means (5%-95% CI). Statistical significance (in bold): Group effect – difference between groups; [a] Intervention effect p<0.001 – difference between interventions (HEC vs. SAL); [b] Time effect – difference between 0 min and 240 min. For detailed description of ANOVA model, see text.***

A-FABP and related gene expression

Plasma concentrations of A-FABP during HEC and SAL are displayed in Figure 11a. They were 1.6-fold higher in D group compared to C group (Group effect: $p<0.001$). In D, a significant decrease in A-FABP was detected during HEC (Time effect: $p<0.05$ in full ANOVA and in LSD post-test: $p<0.05$), while during SAL no difference in A-FABP concentrations could be shown. This implicates that the decline in plasma A-FABP during clamp is independent on haemodilution and is attributed to hyperinsulinaemia. In C, no changes in plasma A-FABP were demonstrated during HEC. During SAL, the basal A-FABP was comparable to HEC, while at 240 min higher concentrations compared to HEC were measured (Intervention effect: $p<0.05$ in ANOVA for Controls and in LSD).

Relative expressions of A-FABP and related genes in SAT during HEC and AT-HEC are summarized in Table 7. Similarly to plasma concentrations, the relative expressions of A-FABP in SAT were 3.0-fold higher in D group (Group effect: $p<0.001$), while no dynamic changes in the course of HEC were observed (Time and Intervention effects and all interactions were not significant).
The relative expressions of E-FABP were opposite to A-FABP. Higher E-FABP expressions were detected in C compared to D (Group effect: $p<0.01$). During HEC no changes were measured.

The A-FABP/E-FABP mRNA ratio reflecting the relative contribution of both FABP isoforms secreted by adipose tissue was higher in D compared to C (Group effect: $p<0.001$) and showed no differences during HEC (Time, Intervention effect and all interactions not significant).
Relative expression of PPARγ was higher in C compared to D (Group effect: $p<0.01$), no dynamic changes during HEC were detected in either group (Time, Intervention effect and all interactions not significant).

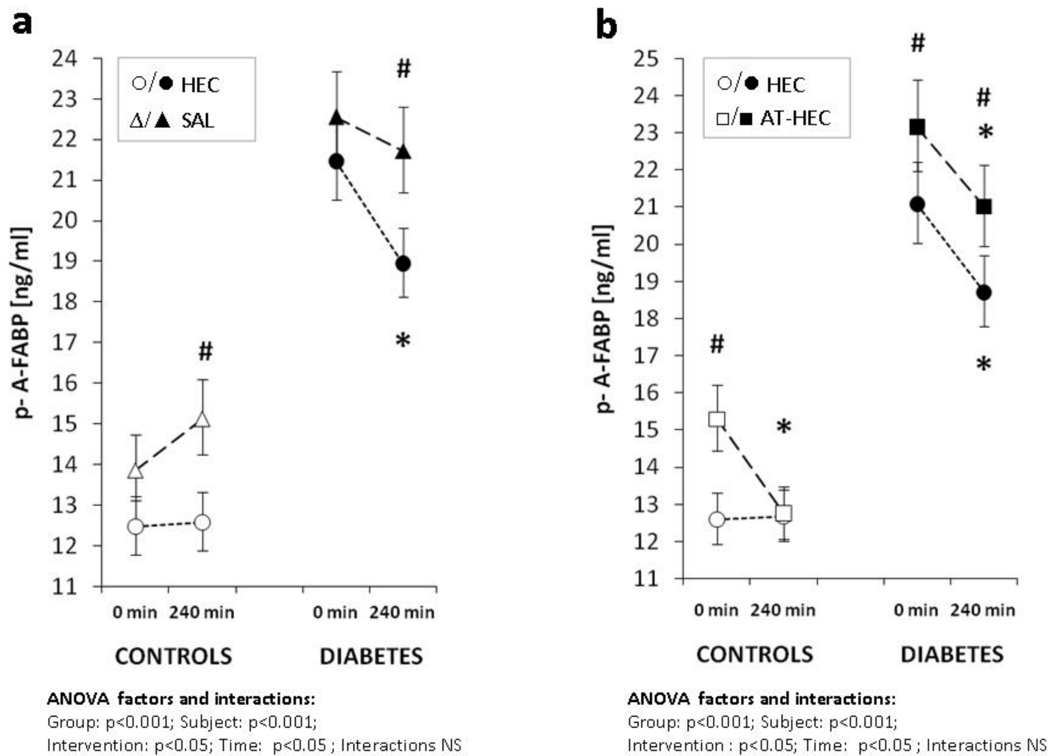

Figure 11 ***Plasma concentrations of A-FABP during interventions:*** *a* ***HEC (/) and SAL (Δ/▲);*** *b* ***HEC (/) and AT-HEC (/■).The empty and full symbols with error bars represent retransformed mean values with their 95 % confidence intervals for C and D groups, respectively. The asterisks correspond to significant difference between the basal and stimulated values (p<0.05). Hashes denote significant difference between interventions (HEC vs. SAL and HEC vs. AT-HEC, respectively) in individual stages (p<0.05). Embedded tables summarize the significant factors of full ANOVA model.***

A-FABP concentrations during HEC and AT-HEC are shown in Figure 11b. In D, A-FABP concentrations were increased during AT-HEC (Intervention effect: $p<0.05$ in Full ANOVA model and in model for Diabetes). A parallel decrease in plasma A-FABP was detected in D during both HEC and AT-HEC (Time effect: $p<0.05$ in ANOVA model for Diabetes and LSD post-test: $p<0.05$). In C, plasma A-FABP was stable during HEC, whereas during AT-HEC increased basal A-FABP reversed at 240 min (Intervention effect: $p<0.05$ in Full ANOVA and LSD post-test: $p<0.05$).

Regarding the effect of ARB on A-FABP expressions, A-FABP mRNA levels were stable during both clamps in both groups. Higher E-FABP expressions during AT-HEC in C but not in D could be demonstrated (Intervention effect: $p<0.05$), while the Time factor and all interactions were not significant. The A-FABP/E-FABP mRNA ratio did not differ between HEC and AT-HEC in either group. Similarly, PPARγ mRNA expression was comparable between clamps in both groups.

Variable		C (n=12)		D (n=11)		Group effect (*p* value)
		0 min	240 min	0 min	240 min	
A-FABP mRNA / Cyclophilin mRNA	HEC	718.4 (647.6–808.6)	650.3 (592.8–721.8)	1937 (1686–2314)	1971 (1695–2401)	<0.001
	AT-HEC	758.3 (679.2–860.9)	675.6 (610.0–758.9)	2004 (1732–2422)	1940 (1688–2318)	
E-FABP mRNA / Cyclophilin mRNA	HEC	**257.8** [a] (210.7–322.7)	**238.5** [a] (196.3–295.9)	196.5 (181.0–214.3)	184.1 (170.2–200.0)	<0.001
	AT-HEC	**292.9** [a] (232.5–380.1)	**259.8** [a] (212.2–325.6)	198.1 (182.4–216.1)	184.7 (169.8–201.8)	
A-FABP mRNA/ E-FABP mRNA	HEC	2.88 (2.44–3.42)	2.75 (2.33–3.26)	10.25 (8.43–12.83)	9.56 (8.10–11.52)	<0.001
	AT-HEC	3.14 (2.58–3.82)	2.06 (1.71–2.47)	10.3 (8.66–12.53)	11.1 (9.26–13.65)	
PPARγ mRNA / Cyclophilin mRNA	HEC	169.8 (116.3–243.5)	168.1 (115.1–241.2)	139.6 (112.4–166.3)	158.9 (132.1–185.3)	<0.01
	AT-HEC	167.5 (111.1–247.3)	202.5 (140.0–288.2)	137.0 (109.8–163.8)	158.9 (132.1–185.2)	

Table 7 Relative expressions of *A-FABP, E-FABP* and *PPARγ mRNA* in subcutaneous adipose tissue. ***Data are means (5%-95% CI). Statistical significance (in bold): Group effect – difference between groups; [a]Intervention effect p<0.05 – difference between interventions (HEC vs. AT-HEC). For detailed description of ANOVA model, see text.***

Relationship of A-FABP to anthropometric and metabolic parameters

A-FABP plasma concentrations were positively correlated with its expression in SAT (r = 0.59; p<0.01). The correlation analysis of A-FABP and E-FABP combined for both groups is summarized in Table 8. Both A-FABP plasma concentrations and expressions correlated positively with BMI, waist circumference, fasting plasma glucose, fasting insulin and negatively with M; additionally, positive correlation of A-FABP mRNA and HbA1c was found. The A-FABP/E-FABP mRNA ratio correlated with fasting plasma glucose and HbA1c. E-FABP mRNA expressions were negatively correlated with HbA1c; relations to other parameters of metabolic syndrome were not significant.

In multivariate regression, which reflects the weight of individual factors without contribution of other factors included in the model, BMI, waist, fasting glycaemia, insulinaemia and M remained significant predictors (p<0.05) of both A-FABP plasma concentrations and expression (detailed analysis in Supplementary file of Appendix 2). This confirms that A-FABP is closely linked to dysregulation of glucose metabolism and insulin resistance independently of obesity per se.

Analyzing the relation between PPARγ mRNA and FABPs, we were able to detect significant positive correlation between PPARγ mRNA and E-FABP mRNA (r = 0.80; p<0.001). Neither plasma A-FABP, nor its expressions were related to PPARγ mRNA in SAT.

	plasma A-FABP (ng/ml)	*A-FABP* mRNA	*E-FABP* mRNA	*A-FABP* / *E-FABP* mRNA
BMI	0.85***	0.65***	0.03	0.40
Waist circumference	0.86***	0.56**	-0.07	0.34
Fasting glucose	0.46*	0.73***	-0.35	0.74***
Fasting insulin	0.50*	0.48*	-0.11	0.39
Fasting NEFA	0.13	0.17	-0.09	-0.33
Fasting TG	0.19	0.24	-0.05	0.23
Total cholesterol	-0.05	0.21	-0.08	-0.21
HDL-cholesterol	-0.06	-0.28	0.04	-0.10
LDL-cholesterol	-0.10	0.17	-0.18	0.22
HbA1c	0.08	0.46*	-0.56*	0.65**
M	-0.64**	-0.53**	+0.18	-0.35

Table 8 ***The relationships (Spearman's correlation coefficients) between A-FABP, E-FABP and selected metabolic parameters.* p<0.05, ** p<0.01, *** p<0.001***

Study II. Effect of 3-week telmisartan treatment on insulin resistance, energy metabolism and endocrine activity of adipose tissue in subjects with impaired fasting glucose

Aim

To investigate the effect of 3-week telmisartan treatment on insulin resistance, energy metabolism and plasma concentrations and SAT expressions of selected cytokines and adipokines in subjects with metabolic syndrome and impaired fasting glucose: a randomized, double-blind, placebo controlled, cross-over trial

Subjects

12 subjects with impaired fasting glucose as an obligate criterion of metabolic syndrome were recruited for the study (see Appendix 3).

Study protocol

This randomized, double-blind, placebo controlled, cross-over trial consisted of two treatment periods. After a 4-week run-in period, the subjects were randomly assigned to receive telmisartan 160 mg (T) daily (Micardis 80 mg; Boehringer Ingelheim Pharma GmbH, Germany) or placebo (P) for 3 weeks. After a 2-week wash-out period, the medication was changed and continued for other 3 weeks. Randomization was performed by a standard procedure. Drug compliance was assessed by the effect on blood pressure as well as by the study drug accountability. Patients were instructed to adhere to their ordinary lifestyle and avoid changes in food intake, alcohol consumption and exercise during the whole study duration. At the end of both 3-week treatment periods all patients underwent a two-hour euglycaemic hyperinsulinaemic clamp (HEC) combined with indirect calorimetry and adipose tissue biopsies. These procedures are described above.

Results

T compared to P treatment induced no differences in body weight (89.8±19 vs. 89.8±18 kg) but resulted in lower blood pressure, both systolic (126±10 vs. 135±12 mmHg; $p<0.03$) and diastolic (81±10 vs. 90±10 mmHg; $p<0.01$), lower fasting plasma glucose ($p<0.05$) and higher fasting IRI ($p<0.05$) (See Appendix 3, Figure 1).

The clamps performed after treatment with T versus P were comparable in terms of the mean plasma glucose concentrations (5.29±1.9 vs. 5.34±0.3 mmol/l), coefficient of variation of glucose (2.89±1.9 vs. 2.56±1.2 %) and mean IRI levels. Insulin action was comparable after T and P: MCR (4.15±1.0 vs. 4.08±1.9 $ml.kg^{-1}.min^{-1}$), M (4.4±1.8 vs. 3.9±1.7 $mg.kg^{-1}.min^{-1}$) and non-oxidative glucose disposal (4.1±1.9 vs. 3.0 ±2.3 $mg.kg^{-1}.min^{-1}$) did not differ between T and P. Similarly, energy expenditure, glucose and fat oxidations have not been statistically different comparing T and P (See Appendix 3, Table 3).

No significant effect of telmisartan on basal plasma concentrations of selected adipokines has been detected, except of basal plasma leptin that has significantly increased after T treatment.

Time profiles of adipokines during clamp experiments. For detailed description and Figures see Appendix 3, Figures 2-5).

Plasma concentrations of TNFα were decreased in T as compared to P (Telmisartan effect: $p<0.05$), while no significant Hyperinsulinaemia effect or interaction were detected. However, the plasma changes after T do not correspond with relative expression of TNFα in SAT. Here the effect of clamp

induced Hyperinsulinaemia has been found to be significant ($p<0.001$) and no Telmisartan effect was detected, implicating the suppression of TNFα expression by insulin which is not influenced by telmisartan.

Plasma adiponectin concentrations have shown a different time course during insulin-stimulated conditions in T and P, documented by the significant Telmisartan × Hyperinsulinaemia interaction ($p<0.05$). A significant increase in plasma adiponectin was detected by LSD multiple comparisons in 120 min of HEC (0 min vs. 120 min: $p<0.05$) after telmisartan treatment while no changes were seen after P. Relative expressions of adiponectin were comparable during both treatment periods and clamps, both Telmisartan and Hyperinsulinaemia factors were not significant.

Both basal and insulin-stimulated plasma leptin concentrations were significantly increased after T treatment compared to P (Telmisartan effect: $p<0.001$), while telmisartan did not alter the relative expressions of leptin in SAT. There was a significant decrease in leptin expressions during insulin-stimulated conditions (Hyperinsulinaemia factor: $p<0.001$).

The telmisartan treatment resulted in an increase in plasma resistin concentrations, as documented by the significant Telmisartan factor ($p<0.01$). During insulin-stimulated conditions an increase in resistin plasma concentrations was detected (Hyperinsulinaemia factor: $p<0.001$), while the effect of insulin on resistin expression was opposite. There is a significant decrease in resistin expressions during HEC both in T and P (Hyperinsulinaemia factor: $p< 0.01$) without differences between T and P.

Study III. Effect of prolonged hypertriglyceridaemia on endocrine activity of adipose tissue in patients with type 2 diabetes and healthy subjects

Aim

To investigate the effect of prolonged hypertriglyceridaemia on plasma concentrations and SAT expressions of selected cytokines and adipokines in patients with type 2 diabetes and healthy control subjects

Subjects

For part A of the study III, a group of 8 healthy male subjects (H) with normal glucose tolerance and mean age of 25.75±1.29 years was recruited (see Appendix 4).

In part B of the study III, 11 overweight/obese patients with type 2 diabetes (D) and 12 age-matched healthy control subjects (C) were enrolled (see Appendix 2).

Study protocol

In both parts A and B, the subjects underwent a 24-h infusion of lipid emulsion (Intralipid 20%; Fresenius Kabi AB, Uppsala, Sweden) as it was described previously. Blood samples for estimation plasma concentrations of selected adipokines were collected at 0 min, 30 min, 240 min and 24 hours of the infusion. In the Part B of the study, plasma concentrations of visfatin, RBP4, HMW adiponectin and A-FABP were measured at time points 0 min, 240 min and 24 hours. To assess relative expressions of adipokines, needle biopsies of abdominal subcutaneous adipose tissue were performed at 0 min, 4 hours and 24 hours of the infusion.

Results

Study III – Part A

Characteristics of the steady state periods of the intervention

To validate the effectiveness of lipid infusion plasma levels of TG, NEFA, glucose and IRI were monitored. TG increased almost 8-fold and NEFA about 3.5-fold in the 4th hour and they remained elevated until the end of infusion at more than 3-fold and 2-fold, respectively. Blood glucose was stable throughout the 24 h. After initial modest rise, IRI concentrations were also stable (for Figures see Appendix 4, Figure 3).

The effect of lipid infusion on selected adipokines

TNFα plasma concentration did not change during the first 4 h of hypertriglyceridaemia but a significant increase after 24 h was detected ($p<0.001$; Figure 12a). The expression of TNFα in subcutaneous adipose tissue did not change (see Appendix 4, Figure 2). Plasma concentration of resistin significantly increased at 30 min of infusion and remained elevated throughout the 24 h ($p<0.01$; Figure 12b). The expression of resistin in the subcutaneous adipose tissue tended to increase, but the change was not significant (see Appendix 4, Figure 2). Plasma concentrations of leptin and adiponectin (Figure 12c-d) did not show any significant changes and their expressions were not significantly altered. Cytokine plasma concentrations did not correlate with IRI concentrations (data not shown).

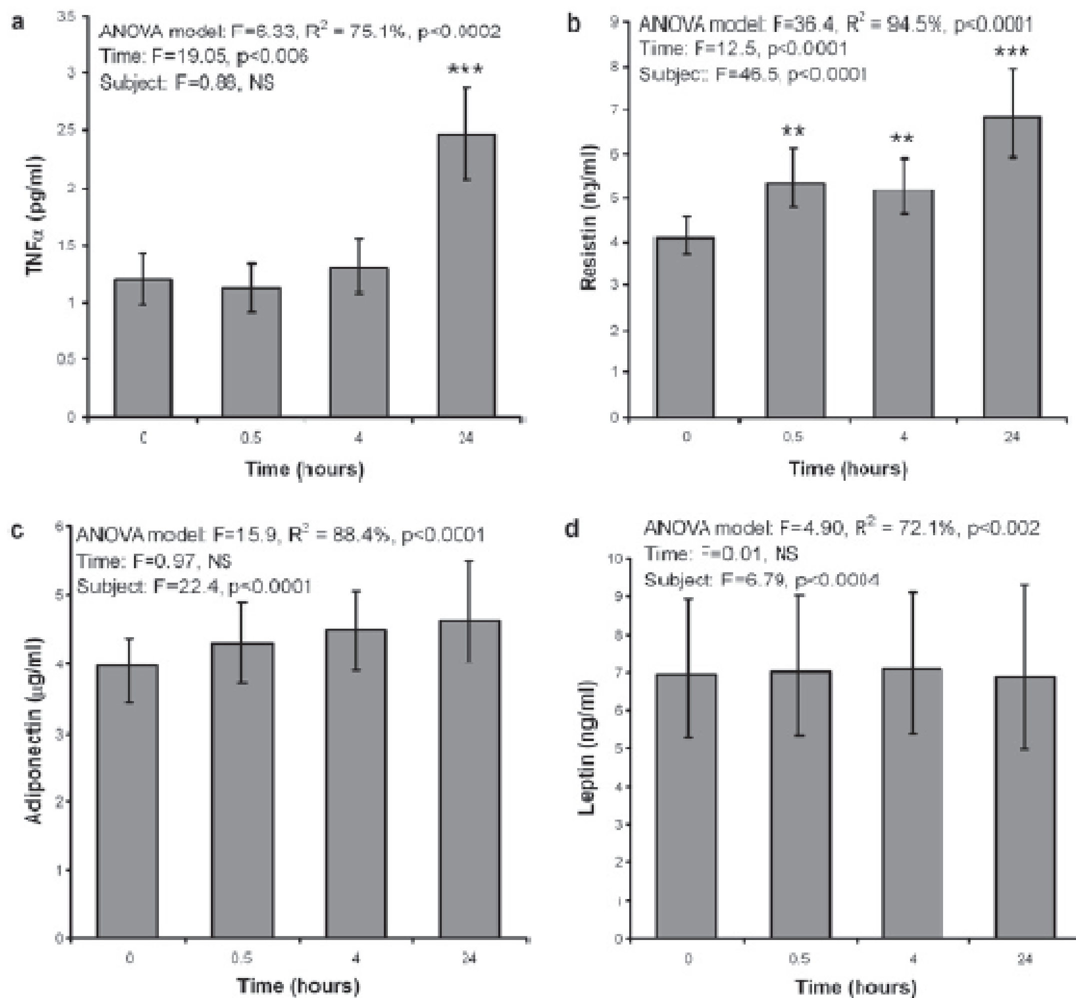

Figure 12 ***Plasma levels of selected adipokines during 24-hour lipid infusion. The bars with error bars represent re-transformed means with their 95 % confidence intervals. F in the embedded tables represents Fisher's statistics for individual factors. R2 (squared correlation coefficient of the linear model) symbolize the proportion of the total variability in the dependent variable, which is explained by the ANOVA model. Asterisks symbolize significant differences between individual stages of the time profiles and basal values (** for p<0.01, *** for p<0.001) as detected by least significant difference multiple comparisons.***

Study III - Part B

Characteristics of the steady state periods of the intervention

Figure 13 shows the time profiles of triglycerides (a), NEFA (b), glucose (c) and IRI (d) during the 24 hours of lipid infusion. The Intralipid-induced triglyceride concentrations were comparable between groups except of the final 2 hours (Group x Time Interaction: p<0.001, LSD post-test: p<0.05 at 24h). Towards the end of experiment (starting at 16. hour) the triglycerides concentrations remained elevated in D group, while in the control group a decline was observed. The NEFA concentrations were comparable between groups with a 4-fold increase at 240 min and a sustained 2-fold elevation until the 24 hour (Time effect: p<0.001). The blood glucose levels were higher in D group compared to controls (Group effect: p<0.001). Blood glucose was stable during the 24 hours in C group, whereas in D group, blood glucose decreased between 3rd and 6th hour to a plateau of approximately 5.7 mmol/l and raised again from 20th till 24th hour to the baseline values (Time effect: p<0.001; Group x Time interaction: p<0.001, LSD post-tests: p<0.05). The IRI concentrations were higher in D

group with borderline significance (Group effect: p=0.053), both as peak concentrations during the first 4 hours and as steady state concentrations in the second half of experiment (Time effect: p<0.001).

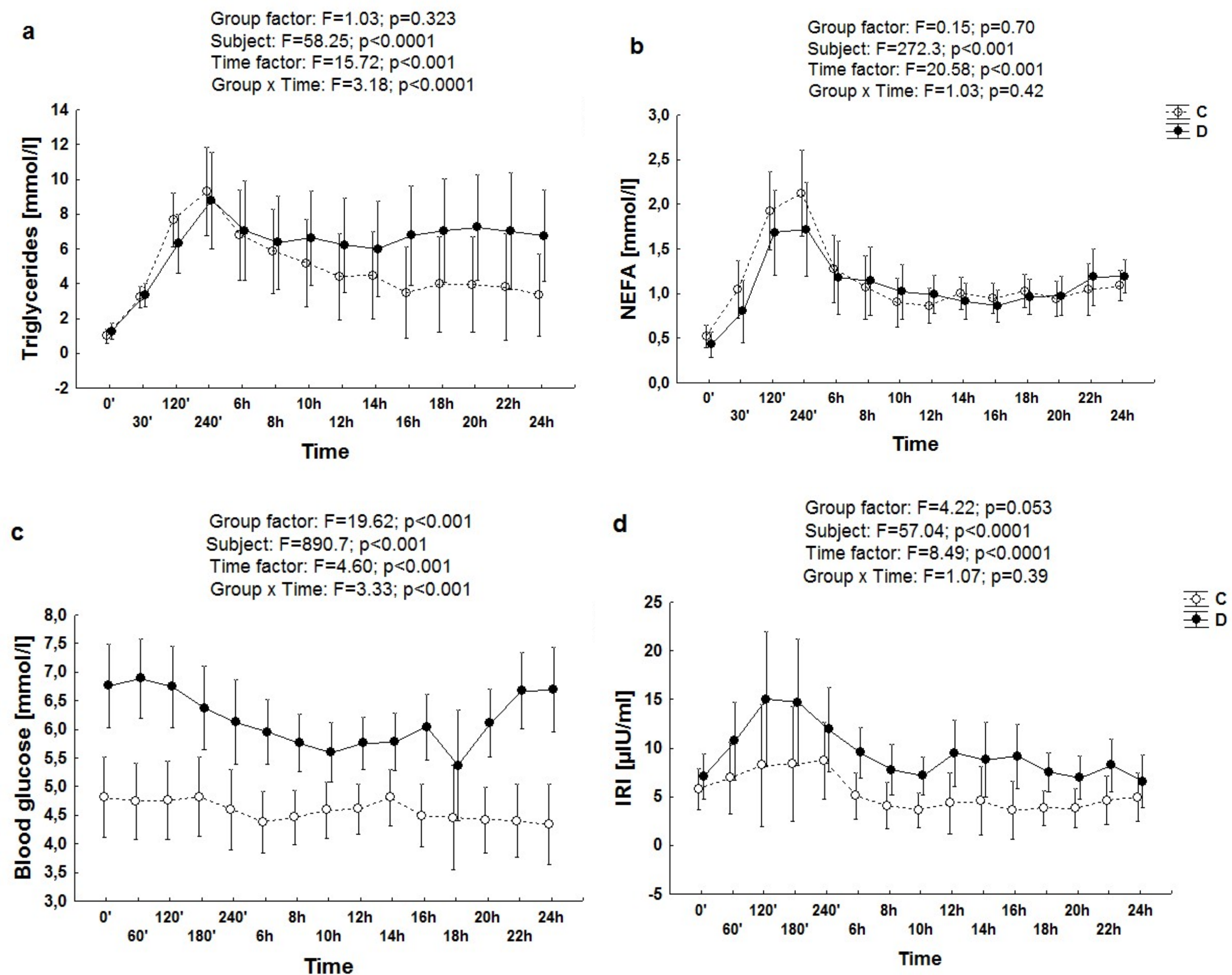

Figure 13 ***Concentrations of serum triglycerides (a), non-esterified free fatty acids (NEFA, b), blood glucose (c) and Immunoreactive insulin (IRI, d) during the 24-hour lipid infusion. Data are shown as means with their 95 % confidence intervals. Full circles represent mean values of diabetes group (D), open circles mean values of control group (C).***

The effect of lipid infusion on selected adipokines

TNFα

Both TNFα plasma concentrations and SAT expressions (displayed in Figure 14) were significantly higher in D (Group effect: p<0.001 for both plasma concentrations and expressions). During hypertriglyceridaemia plasma TNFα remained stable in D group, whereas in C group TNFα levels increased over 24 hours (Time effect: p<0.01; Group x Time Interaction: p<0.01). Changes in TNFα expression in SAT were not significant in any group.

Resistin

Resistin plasma concentrations and its expression in SAT is shown in Figure 15. Plasma resistin did not differ between groups. An increase in resistin concentrations during hypertriglyceridaemia was detected in both groups (Time effect: p<0.05; Group x Time Interaction ns). On the other hand,

relative expression of resistin in SAT were higher in D group compared to C (Group effect: p<0.001), but no dynamic changes in resistin expression were demonstrated in any group.

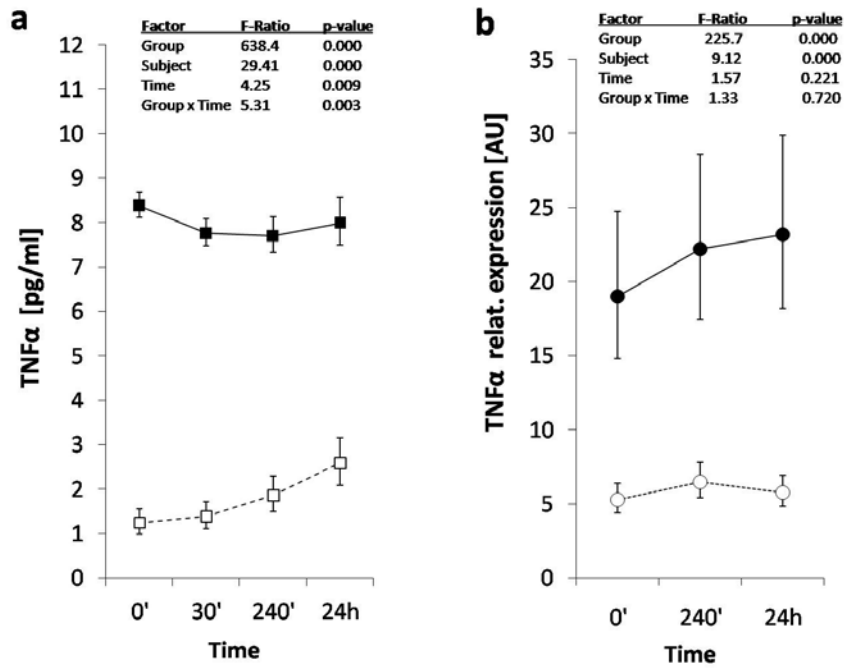

Figure 14 ***TNFα plasma concentrations (a) and SAT expressions (b) during hyperlipidaemia. The empty and full symbols with error bars represent retransformed mean values with their 95 % confidence intervals for C and D groups, respectively. Embedded tables summarize the significant factors of ANOVA model.***

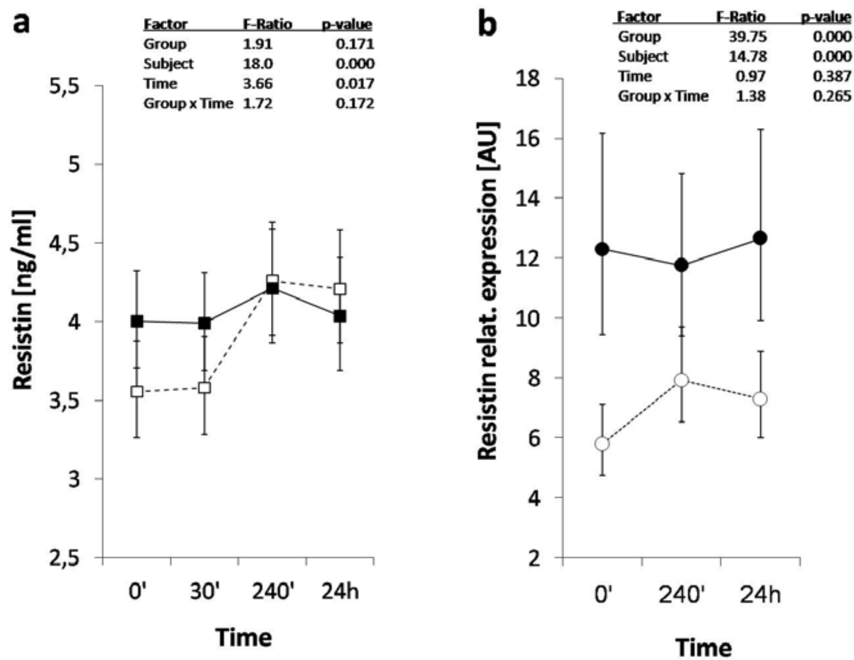

Figure 15 ***Resistin plasma concentrations and SAT expressions during hyperlipidaemia. The empty and full symbols with error bars represent retransformed mean values with their 95 % confidence intervals for C and D groups, respectively. Embedded tables summarize the significant factors of ANOVA model.***

Leptin

Plasma concentrations of leptin (Figure 16a) were higher in D group (Group effect: p<0.001). The plasma leptin time profiles during hypertriglyceridaemia are displayed in Figure 16a. They differed between groups (Group x Time Interaction: p<0.001; Time effect: p<0.01) as follows: in D group, leptin rapidly declined in 30 min and 240 min of infusion but it reversed to the basal concentrations after 24 hours of hypertriglyceridaemia. On the contrary in C group, leptin concentrations were stable during the first 4 hours and did not decrease until 24 hours of Intralipid infusion. *Leptin* expressions are depicted in Figure 16b. Similarly to plasma concentrations, higher leptin expression was detected in D group (Group effect: p<0.001). However, no dynamic changes in leptin expression in SAT were observed in any group.

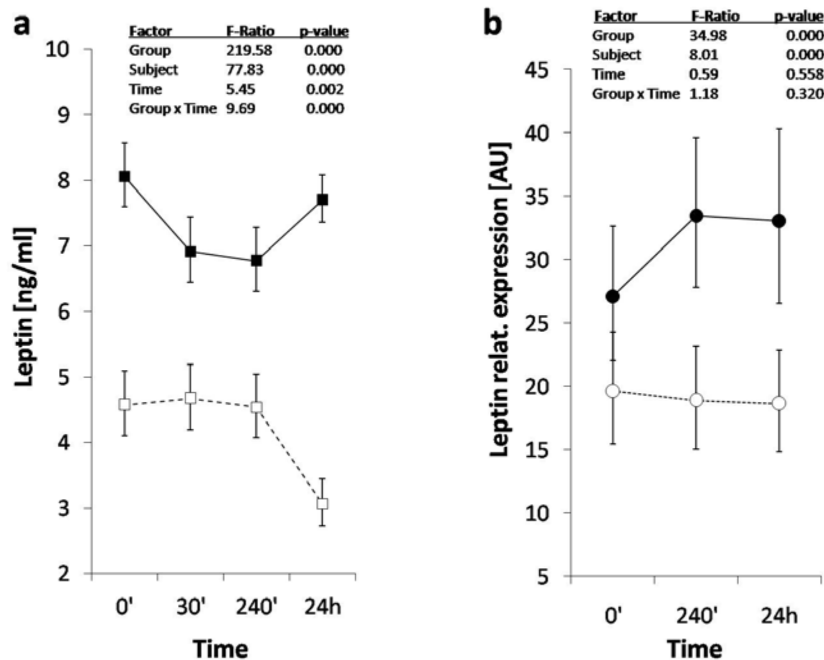

Figure 16 ***Leptin plasma concentrations (a) and SAT expressions (b) during hyperlipidaemia. The empty and full symbols with error bars represent retransformed mean values with their 95 % confidence intervals for C and D groups, respectively. Embedded tables summarize the significant factors of ANOVA model.***

Adiponectin

Plasma concentrations of total adiponectin (Figure 17) were unexpectedly found to be higher in D group compared to C group (Group effect: p<0.001). No changes in total adiponectin levels during hypertriglyceridaemia were detected in either group. On the other hand, concentrations of HMW adiponectin were comparable between groups as well as during intervention. Again, adiponectin expression in SAT was higher in D group compared to C group (Group effect: p<0.001) without any dynamic changes during Intralipid infusion.

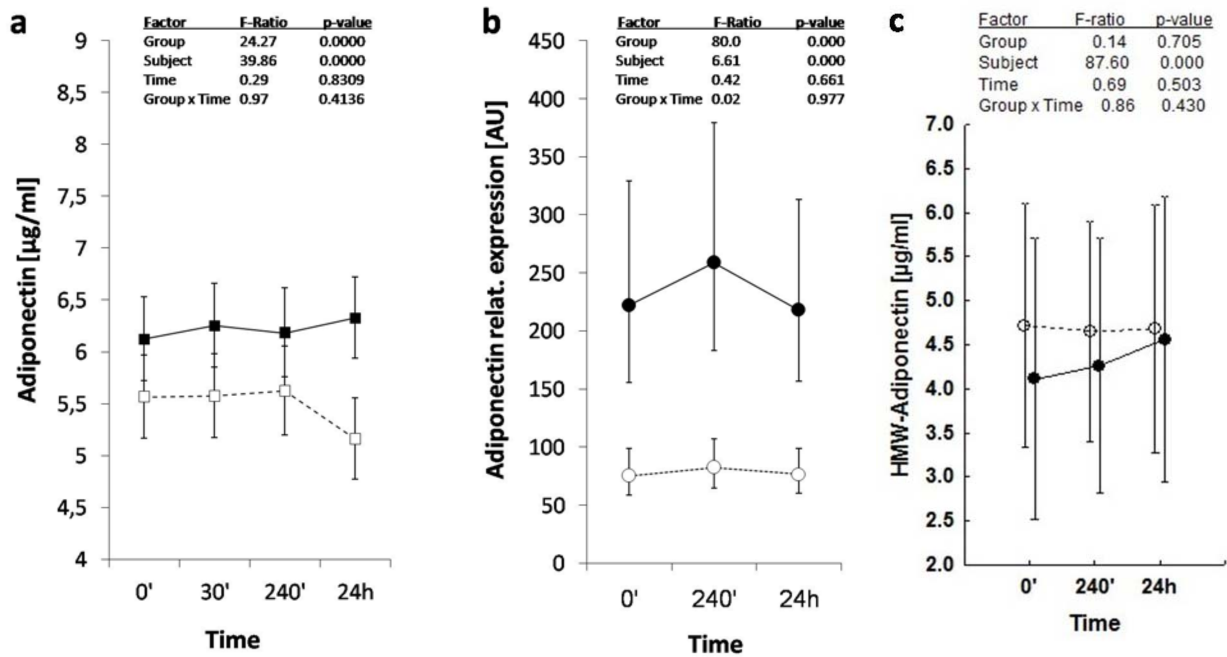

Figure 17 ***Adiponectin plasma concentrations (a) and SAT expressions (b) and HMW Adiponectin concentrations (c) during hyperlipidaemia. The empty and full symbols with error bars represent retransformed mean values with their 95 % confidence intervals for C and D groups, respectively. Embedded tables summarize the significant factors of ANOVA model.***

Visfatin and RBP4

Plasma concentrations of both visfatin and RBP4 (Table 9) were comparable between groups. Similarly, for both adipokines no dynamic changes during hypertriglyceridaemia were found in any group.

Variable	D (n=11)			C (n=12)		
	0 h	4 h	24 h	0 h	4 h	24 h
Visfatin [ng/ml]	5.31±0.75	5.52±0.66	5.82±0.85	8.68±2.71	7.52±1.03	7.67±0.65
RBP4 [µg/ml]	47.38±5.20	50.76±8.20	45.68±5.38	39.12±5.82	50.23±3.74	46.99±2.32

Table 9 ***Plasma concentrations of visfatin and RBP4 during hypertriglyceridaemia. Data expressed as mean ± SEM***

A-FABP

A-FABP plasma concentrations as well as its expressions in SAT (Figure 18) were increased in D group compared to controls (Group effect: $p<0.01$ and $p<0.001$ for plasma concentrations and expressions, respectively). During hypertriglyceridaemia, a parallel decline in plasma A-FABP was detected in both groups (Time effect: $p<0.01$; Group x Time Interaction: ns). The A-FABP expressions in SAT showed no significant changes during intervention in any group.

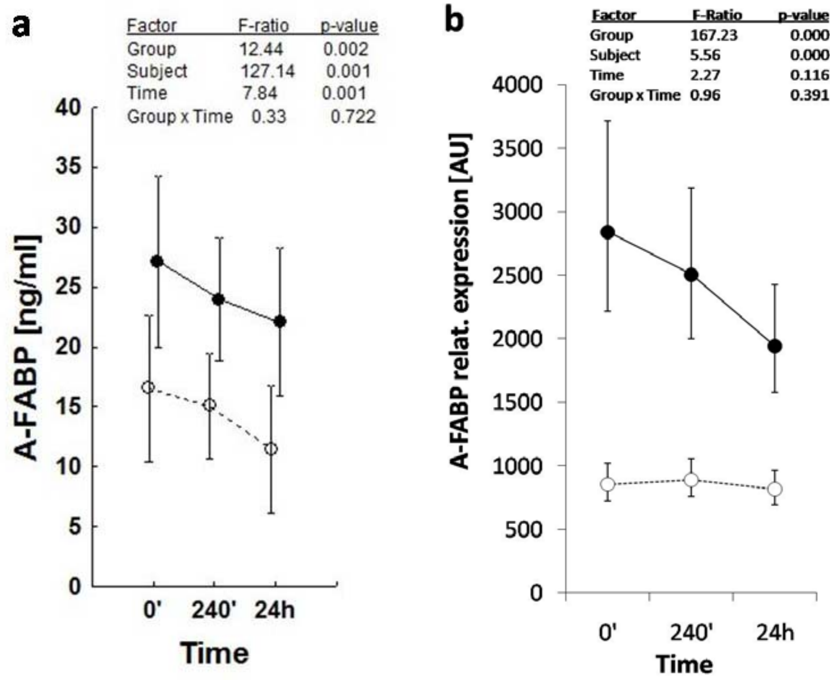

Figure 18 ***A-FABP plasma concentrations (a) and SAT expressions (b) during hyperlipidaemia. The empty and full symbols with error bars represent retransformed mean values with their 95 % confidence intervals for C and D groups, respectively. Embedded tables summarize the significant factors of ANOVA model.***

Study IV. Endocrine activity of adipose tissue in subjects with different categories of glucose intolerance

Aim

To assess the plasma concentrations and SAT expressions of selected cytokines and adipokines and markers of low-grade inflammation in subjects with different categories of glucose intolerance and to explore their potential relationships with anthropometric and metabolic characteristics.

Subjects

Three age-matched study groups with different categories of glucose intolerance were investigated: 1) 11 overweight/obese patients with type 2 diabetes (D); 2) 12 overweight/obese patients with metabolic syndrome with impaired fasting glucose (IFG) as an obligate criterion and 3) 11 age-matched healthy control subjects (C). For confirmation of their glucose tolerance status and exclusion of overt diabetes, C and IFG groups underwent an oral glucose tolerance test with 75 g glucose. Diabetes was confirmed based on medical records in the D group. The baseline characteristics and differences between the study groups are summarized in Table 10.

Variable	C (n=11)	IFG (n=12)	D (n=11)	*p-value C vs.IFG*	*p-value C vs. D*	*p-value IFG vs.D*
Age (years)	48.05 ± 1.38	50.5 ± 1.74	50.27 ± 1.38	0.260	0.317	0.916
Weight (kg)	77.63 ± 3.85	90.42 ± 5.28	93.72 ± 2.48	**0.035**	**0.011**	0.572
Body mass index ($kg.m^{-2}$)	24.63 ± 0.98	29.19 ± 1.21	30.02 ± 0.58	**0.002**	**0.001**	0.553
Waist circumference (cm)	89.09 ± 2.61	103.83 ± 2.17	101.8 ± 2.37	**0.000**	**0.000**	0.551
Fasting blood glucose ($mmol.l^{-1}$)	5.2 ± 0.06	6.08 ± 0.10	7.49 ± 0.43	**0.024**	**0.000**	**0.000**
Blood glucose at 120 min of OGTT ($mmol.l^{-1}$)	5.56 ± 0.34	6.98 ± 0.58	-	**0.025**	-	-
Fasting insulin ($\mu U.ml^{-1}$)	5.06 ± 0.59	12.67 ± 2.00	8.42 ± 1.05	**0.000**	0.087	**0.034**
Fasting C-peptide (	0.61 ± 0.09	1.27 ± 0.18	1.06 ± 0.17	**0.004**	**0.036**	0.324
HbA1c (%)	5.47 ± 0.10	5.69 ± 0.11	6.48 ± 0.41	0.529	**0.008**	**0.030**
Total cholesterol ($mmol.l^{-1}$)	4.86 ± 0.22	5.48 ± 0.30	5.58 ± 0.27	0.110	0.072	0.796
Triglyceride ($mmol.l^{-1}$)	1.15 ± 0.17	2.60 ± 0.41	2.61 ± 0.49	**0.011**	**0.012**	0.999
HDL-cholesterol ($mmol.l^{-1}$)	1.20 ± 0.05	1.06 ± 0.05	1.10 ± 0.04	0.056	0.171	0.583
LDL-cholesterol ($mmol.l^{-1}$)	3.25 ± 0.20	3.20 ± 0.37	3.57 ± 0.17	0.906	0.413	0.342
Fasting NEFA ($mmol.l^{-1}$)	0.458 ± 0.05	0.444 ± 0.05	0.502 ± 0.12	0.838	0.093	0.213

Table 10 ***Clinical and biochemical characteristics of the study groups. Data shown as mean ± SEM***

IFG and D group were comparable in terms of weight, BMI and waist circumference, whereas C group was leaner. Regarding glucose metabolism, fasting blood glucose differed between all groups as defined, HbA1c was comparable between C and IFG groups and higher in D group. Insulin and C-

peptide were lowest in C group, IFG had highest insulinaemia, C-peptide was comparable between IFG and C groups. Except of triglycerides that were lower in C compared to D and IFG, the lipid profile did not show any differences between groups.

Concomitant medication. D group comprised of patients with well-controlled type 2 diabetes, who were treated with oral agents (4 patients with metformin monotherapy, 1 patient with sulphonylurea monotherapy, 3 patients with combination metformin/sulphonylurea) or diet alone (3 patients). Six patients were treated with stable dose of statin. Six patients received antihypertensive treatment (calcium-channel blockers, diuretics or ACEIs). ACEIs were replaced with calcium-channel blockers prior to study procedures. IFG group was treatment-naive with regard to oral antidiabetic medication or statins. Antihypertensive treatment was adjusted during the run-in period as it is described in Study II. C group had no concomitant medication.

Study protocol

All subjects underwent a 2-hour hyperinsulinaemic-euglycaemic clamp to quantify insulin sensitivity. At baseline, blood samples for assessment of selected adipokines and cytokines were taken and a needle biopsy of SAT was performed. Relative expressions of selected adipokines and cytokines were analyzed in adipose tissue samples using RT-PCR.

Results

Parameters characterising insulin sensitivity

Steady state periods characteristics of 2-hour hyperinsulinaemic euglycaemic clamp and the differences between groups are shown in Table 11. The mean plasma glucose concentrations were higher in D compared to C and IFG, while the mean IRI levels were higher in IFG compared to C. NEFA levels were comparable between groups. Nevertheless, the groups clearly differed in insulin sensitivity expressed as M, MCR (accounts for blood glucose variability) or M/I and MCR/I (account for variability in IRI concentrations): C group demonstrated the highest glucose disposal, while there were no differences between IFG and D group.

Variable	C (n=11)	IFG (n=12)	D (n=11)	*p-value C vs.IFG*	*p-value C vs. D*	*p-value IFG vs.D*
Mean plasma glucose ($mmol.l^{-1}$)	5.18±0.05	5.29±0.10	5.64±0.16	0.493	**0.006**	**0.031**
Coefficient of glucose variation (%)	2.07±0.24	2.90±0.59	2.38±0.34	0.170	0.599	0.388
Mean IRI ($\mu IU.ml^{-1}$)	68.09±5.25	95.43±10.86	75.30±6.77	**0.018**	0.492	0.076
NEFA ($mmol.l^{-1}$)	0.10±0.03	0.08±0.02	0.09±0.01	0.437	0.750	0.650
M ($mg.kg^{-1}.min^{-1}$)	9.04±0.92	3.89±0.49	4.92±0.48	**0.000**	**0.000**	0.270
MCR ($ml. kg^{-1}.min^{-1}$)	9.64±0.94	4.47±0.66	4.88±0.49	**0.000**	**0.000**	0.686
M/I ($mg.kg^{-1}.min^{-1}$ per $\mu IU.ml^{-1}$)	0.145±0.021	0.044±0.009	0.073±0.010	**0.000**	**0.002**	0.196
MCR/I ($ml. kg^{-1}.min^{-1}$ per $\mu IU.ml^{-1}$)	0.154±0.021	0.049±0.012	0.072±0.010	**0.000**	**0.000**	0.323

Table 11 ***Characteristics of the steady state periods of HEC. Data shown as mean ± SEM***

The effect of metabolic status on endocrine activity of adipose tissue

Adipokines Plasma concentrations of leptin were increased in IFG compared to both other groups (p<0.001 vs. C and p<0.01 vs. D). Leptin levels tended to be higher in D compared to C group, however the difference was not statistically significant (Figure 19a). On the other hand, relative expressions of leptin in SAT were lower in C compared to other groups (p<0.01 vs. IFG and p<0.05 vs. D) and did not differ between IFG and D (Figure 19b).

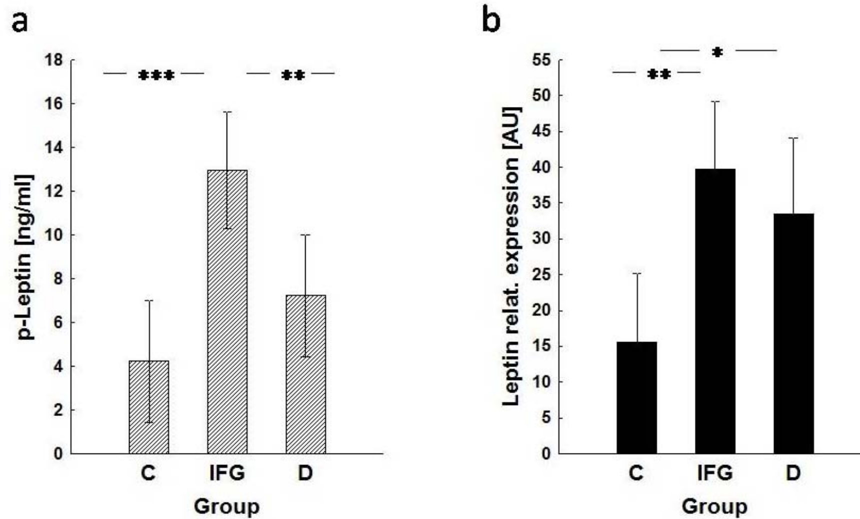

Figure 19 ***Leptin plasma concentrations (a) and mRNA expressions in SAT (b). Data shown as mean ± 95% confidence interval. *p<0.05; **p<0.01; ***p<0.001***

Unexpectedly, plasma concentrations of both total and HMW adiponectin did not differ between groups (Table 12 shows adiponectin concentrations and expressions of the protein and its receptors). Relative expressions of adiponectin were comparable between C and IFG and even higher in D group (p<0.01 vs. C and p<0.05 vs. D). Expression of adiponectin receptor AdipoR1 was comparable between groups, whereas AdipoR2 showed higher expression rates in C compared to other groups (p<0.01 vs. IFG and p<0.05 vs. D). AdipoR2 gene expression was higher than that of AdipoR1 (p<0.05) in all groups.

Variable	C (n=11)	IFG (n=12)	D (n=11)	*p-value C vs.IFG*	*p-value C vs. D*	*p-value IFG vs.D*
Total adiponectin [µg/ml]	4.77±0.52	6.42±0.67	5.61±0.86	0.103	0.408	0.416
HMW adiponectin [µg/ml]	4.18±0.44	3.59±0.75	4.11±0.89	0.542	0.946	0.611
Adiponectin mRNA [AU]	18.86±2.41	28.50±2.39	46.97±8.30	0.198	**0.002**	**0.043**
AdipoR1 mRNA [AU]	81.79±7.51	69.76±4.38	74.82±7.75	0.227	0.481	0.576
AdipoR2 mRNA [AU]	238.74±24.49	145.43±8.34	184.64±21.79	**0.002**	**0.048**	0.130

Table 12 ***Plasma concentrations of total and HMW adiponectin and expression of adiponectin and its receptors in SAT. Data shown as mean ± SEM***

Surprisingly lower concentrations of plasma resistin were found in IFG compared to both other groups (p<0.05 vs. C and D), that displayed comparable resistin concentrations (Figure 20a). On the contrary, resistin expressions in SAT (Figure 20b) were comparable between C and IFG, but they were increased in D group (p<0.01 vs. C and p<0.01 vs. IFG).

TNFα plasma concentrations (Figure 20c) were lowest in C (p<0.01 vs. IFG and p<0.05 vs. D) and comparable between IFG and D. However, the relative expressions of TNFα in SAT (Figure 20d) differed among all groups with lowest expressions in C, higher in IFG and highest rates in D group.

Plasma visfatin was found to be lower in D group compared to C group (p<0.05 for C vs. D). The differences between D and IFG, as well as between C and IFG were not significant (Table 13). Plasma concentrations of RBP4 were comparable between all three groups (Table 13).

A-FABP and related gene expression Figure 20e-f shows A-FABP plasma concentrations and expressions. Plasma concentrations of A-FABP were lowest in C (p<0.01 vs. IFG and p<0.05 vs. D), no differences between IFG and D were detected. A-FABP relative expressions in SAT displayed highest rates in D group compared to C and IFG (p<0.001 vs. C and p<0.001 vs. IFG), while the expressions did not differ between C and IFG. Expression of E-FABP (Table 13), a minor adipose tissue isoform of FABP, was higher in C compared to IFG (p<0.05) and tended to be higher in C compared to D group (p=0.08). No differences between IFG and D group were detected. Expressions of PPARγ (Table 13) were comparable among groups.

Markers of inflammation (summarized in Table 13) Plasma concentrations of MCP-1 were decreased in C compared to IFG and D (p<0.01 vs. IFG and p<0.05 vs. D), without any differences between IFG and D groups. IL-1ra and VEGF concentrations were comparable among groups. Decreased RANTES concentrations were measured in D group (p<0.05 vs. C and p=0.053 vs. IFG), while no differences between C and IFG were observed.

Variable	C (n=11)	IFG (n=12)	D (n=11)	*p-value C vs.IFG*	*p-value C vs. D*	*p-value IFG vs.D*
Visfatin [ng/ml]	8.10±1.70	6.24±0.44	4.33±0.64	0.222	**0.025**	0.235
RBP4 [ng/ml]	46.37±3.73	44.37±5.16	46.79±3.80	0.743	0.949	0.707
E-FABP mRNA [AU]	269.97±25.25	186.64±16.28	212.91±22.98	**0.012**	0.081	0.361
PPARγ mRNA [AU]	176.02±19.23	161.23±13.63	137.29±24.46	0.611	0.196	0.370
MCP-1 [pg/ml]	136.68±14.81	229.05±20.86	203.98±30.75	**0.005**	**0.043**	0.437
IL-1ra [pg/ml]	436.45±103.4	973.54±208.7	959.31±391.9	0.118	0.147	0.968
VEGF [pg/ml]	5.91±1.26	9.22±2.09	7.27±3.780	0.308	0.705	0.573
RANTES [pg/ml]	9723±1636	9615±1062	5629±1302	0.954	**0.048**	0.053

Table 13 ***Plasma concentrations of selected adipokines and inflammatory markers and expressions of selected genes. Data shown as mean ± SEM***

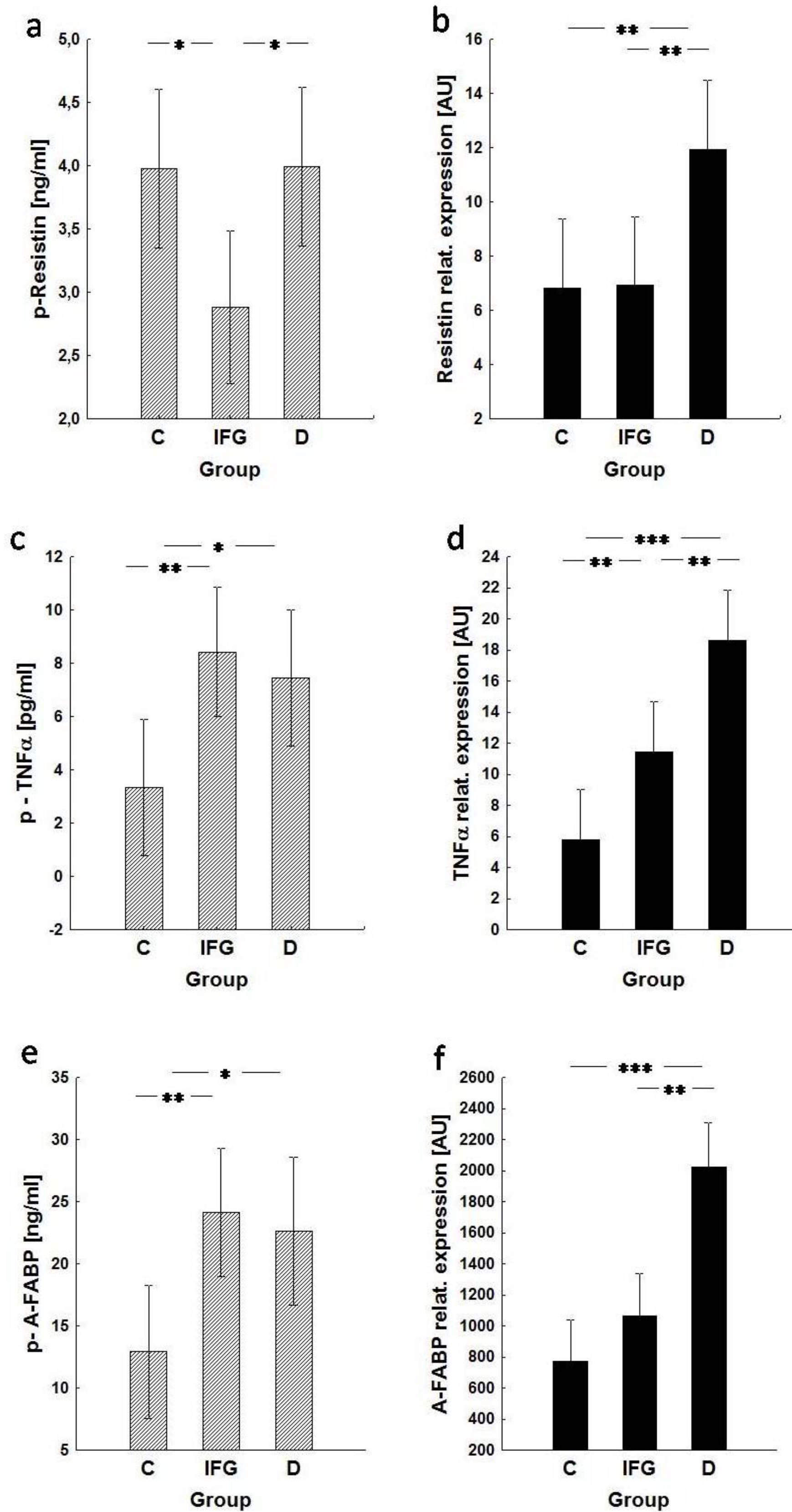

Figure 20 *Resistin, TNFα and A-FABP plasma concentrations (a, c, e) and mRNA expressions in SAT (b, d, f), respectively. Data shown as mean ± 95% confidence interval.*p<0.05; **p<0.01; ***p<0.001*

Relationship of endocrine activity of adipose tissue with anthropometric and metabolic parameters

For correlation analysis of potential relationships (summarized in Table 14), all three groups were combined in order to increase the number of subjects and to achieve a broad spectrum of anthropometric and metabolic parameters. Leptin plasma concentrations were found to be positively related to obesity (BMI and waist circumference) and negatively to MCR and HDL-cholesterol. Leptin expressions in SAT revealed additional positive correlation with fasting glucose and insulin. Neither total and HMW adiponectin plasma concentrations nor its expressions were found to be related to the selected metabolic parameters. Plasma resistin was not related to any of the parameters, but its expression was positively related to BMI and triglycerides. Plasma TNFα was negatively related to insulin sensitivity (MCR) but did not display any relation to parameters of obesity. In contrast, TNFα expressions were positively correlated with BMI, waist circumference, triglycerides, fasting plasma glucose, HbA1c and negatively with MCR. Plasma visfatin was surprisingly positively related to insulin sensitivity (MCR) and negatively to fasting plasma glucose.

Analyzing possible relationships among adipokines, we found plasma TNFα to be negatively correlated with expression of adiponectin receptors AdipoR1 and AdipoR2 (for both $r=-0.46$; $p<0.05$). Total adiponectin concentrations were related to HMW adiponectin ($r=0.41$; $p<0.05$), to adiponectin expressions ($r=0.43$; $p<0.05$), while negative correlation between total adiponectin and AdipoR1 ($r=-0.37$; $p<0.05$) and AdipoR2 ($r=-0.36$; $p<0.05$) were demonstrated. The expressions of AdipoR1 and AdipoR2 were also highly interrelated ($r=0.88$; $p<0.05$). In addition, TNFα expressions were associated with expressions of leptin ($r=0.66$; $p<0.05$) and resistin ($r=0.76$; $p<0.05$), while no relations between circulating adipokines could detected. We did not find any association between plasma levels and SAT expressions of leptin, resistin and TNFα.

A-FABP plasma concentrations showed positive relation to obesity (BMI, waist circumference) and hyperinsulinaemia and negative correlation with MCR. A-FABP expressions were positively related to BMI and additionally to fasting plasma glucose and HbA1c but the relation to MCR was not significant. Additional positive correlations between A-FABP expression and expressions of TNFα ($r=0.77$; $p<0.05$), resistin ($r=0.62$; $p<0.05$), leptin ($r=0.54$; $p<0.05$) and also adiponectin ($r=0.54$; $p<0.05$) were found. No significant relation between A-FABP expressions in SAT and its plasma concentrations or expression of E-FABP and PPARγ could be demonstrated.

PPARγ expression was not related to any of the anthropometric or metabolic parameters, but its negative correlations with plasma TNFα ($r=-0.59$; $p<0.05$) and plasma resistin ($r=-0.45$; $p<0.05$) and positive correlations with expressions of adiponectin receptors AdipoR1 ($r=0.72$; $p<0.05$) and AdipoR2 ($r=0.67$; $p<0.05$) were demonstrated.

MCP-1 was positively related to waist circumference and negatively to MCR. IL-1ra showed also positive correlations with obesity, fasting plasma glucose and insulin. In contrast, RANTES was unexpectedly found to be positively associated with MCR and negatively with BMI. Exploring the relations of inflammatory markers to adipokines, positive correlations between MCP-1 and plasma leptin ($r=0.45$; $p<0.05$) and plasma A-FABP ($r=0.46$; $p<0.05$), as well as between IL-1ra and expressions of TNFα ($r=0.62$; $p<0.05$) and leptin ($r=0.78$; $p<0.05$) were demonstrated.

Variable	BMI	Waist circumference	HDL-cholesterol	Triglycerides	Fasting glucose	Fasting IRI	HbA1c	MCR
p-Leptin	**0.49**	**0.56**	**-0.47**	0.25	0.04	0.34	-0.10	**-0.53**
Leptin mRNA	**0.52**	**0.60**	-0.11	0.27	**0.49**	**0.46**	0.31	**-0.44**
Resistin mRNA	**0.38**	**0.34**	-0.16	**0.42**	0.33	0.09	0.27	-0.13
p-TNFα	0.24	0.26	-0.11	0.16	0.24	0.12	0.18	**-0.57**
TNFα mRNA	**0.51**	**0.44**	-0.16	**0.38**	**0.61**	0.10	**0.43**	**-0.36**
p-Visfatin	-0.19	-0.19	0.07	-0.22	**-0.39**	0.01	-0.17	**0.42**
p- A-FABP	**0.39**	**0.47**	-0.12	0.15	0.15	**0.40**	-0.10	**-0.43**
A-FABP mRNA	**0.38**	**0.39**	-0.11	0.28	**0.77**	0.09	**0.51**	-0.31
MCP-1	0.24	**0.41**	-0.29	0.25	0.10	0.13	-0.03	**-0.40**
IL-1ra	**0.44**	**0.40**	-0.12	0.05	**0.43**	**0.48**	0.24	-0.32
RANTES	**-0.41**	-0.32	0.28	-0.22	-0.22	-0.09	-0.07	**0.36**

Table 14 ***The relationships (Spearman's correlation coefficients) between adipokines and cytokines and selected metabolic parameters; $p<0.05$ in cases highlighted in bold; Units in text***

DISCUSSION

Study I. Acute effects of hyperinsulinaemia and losartan on endocrine activity of adipose tissue in type 2 diabetes and healthy subjects

Study design

In part A of the study I, only hyperinsulinaemic clamps with and without losartan administration were compared. However, for a comprehensive interpretation of the results an additional volume control examination is of importance, in order to distinguish eventual non-specific volume (haemodilution) and time effects during clamp and thus to justify the interpretation of the observed changes as an insulin effect. Therefore, saline infusion was performed in part B of the study I. Because of the effort, only minority of clinical clamp studies in the literature included a volume control examination in the study protocol [101, 226-230], or instead of cross-over design, the authors submitted an additional matched group to a saline infusion [231]. To our knowledge, a complex evaluation of adipose tissue endocrine activity during hyperinsulinaemia including volume control examination and adipose tissue biopsies has not been performed so far.

Changes in endocrine activity of adipose tissue - Effects of metabolic status and hyperinsulinaemia

Several adipokines demonstrated changes in their circulating concentrations and/or expressions during hyperinsulinaemia. The interpretation of the findings regarding resistin and adiponectin might differ between parts A and B of the study, because the additional information from saline infusion is available only for part B.

Resistin. In young healthy subjects (Part A) acute hyperinsulinaemia induces an increase in resistin plasma concentration and stimulates the expression of resistin in subcutaneous adipose tissue. This finding is consistent with several studies in rodent models [64] or in vitro studies [64, 232]. On the contrary, other groups showed the opposite in mice and in 3T3-L1 adipocytes [64]. Only few studies have investigated the effect of insulin in vivo in humans; Heilbronn [233] observed an increase in serum resistin concentrations in response to supraphysiological doses of insulin (164±5 $mIU.l^{-1}$) in obese subjects with and without diabetes. However, clinical studies in humans do not show a consistent link between serum resistin and either insulin resistance or obesity [21, 46, 68, 234].

Comparing D and C groups in part B of the study, plasma concentrations of resistin were not different between groups, whereas resistin expression in SAT was increased in diabetes. The comparable circulating concentrations of resistin are in line with most other studies [66, 233, 235], although several reports were able to confirm a difference between diabetic and non-diabetic subjects [66]. Similarly, the available human data on resistin expression in diabetes are controversial [66]. Our finding of increased resistin expression in SAT that is not accompanied with difference in circulating levels implicates an involvement of other source of plasma resistin, such as other fat depot or mononuclear cells. Since the macrophages are recognized as a primary source of resistin in humans, the differences in expression might relate more to different degree of macrophage infiltration in adipose tissue than to diabetes per se.

In part B of the study, group-specific regulation of plasma resistin in response to hyperinsulinaemia was demonstrated. In diabetes, insulin stimulates an increase in plasma resistin, because during volume control examination resistin concentrations did not change. On the contrary, in controls the

increase in resistin is observed also during SAL and as such it cannot be attributed to insulin. We might speculate on possible effects of prolonged fasting or circadian rhythm on resistin. Resistin expression in SAT is not affected by hyperinsulinaemia in any group. As mentioned above, literature evidence on resistin regulation by insulin is controversial and not exhaustive. Even if insulin does not appear to play a significant direct regulatory role in resistin expression in humans [66], the group-specific changes in circulating resistin demonstrated in our study might be indirect based on different activation of inflammatory pathways during hyperinsulinaemia between groups.

Another unclear observation is the lower baseline resistin concentration in both groups in HEC compared to SAL that remained decreased during clamp in controls but increased in diabetes as mentioned above. The baseline difference cannot be explained by the following procedure (except of potentially confounding subjects' perception of less stress prior to SAL where no SAT biopsies were awaited). As a potential explanation intraindividual variability in resistin concentrations could be also mentioned.

Adiponectin. In young healthy subjects (part A), adiponectin plasma concentrations were not affected by hyperinsulinaemia, whereas its expression was stimulated by insulin. These findings are in agreement with the evidence that circulating adiponectin *per se* is not a subject to acute regulation, but its expression seems to be regulated acutely [55], preceding thereby systemic changes. Similar results in vivo were shown in some studies [236-238], but other groups reported a 7-8% decline of circulating adiponectin during hyperinsulinaemia in healthy men [239, 240], that is more pronounced (16-20% decrease), when 2-fold higher insulin infusion rate is used [241, 242]. The source of such discrepancies might partly lie in diverse analytics that was used or different ethnics that were studied (there is a broad range of reported absolute values for serum adiponectin: 2 - 20 $\mu g.ml^{-1}$).

In accordance with previously mentioned study [236], we report an increase in adiponectin mRNA in the subcutaneous adipose tissue in response to insulin in vivo. 24-hour insulin treatment induced an increase in adiponectin mRNA in isolated human visceral adipose tissue [243], but a reduction of adiponectin expression was detected in 3T3-L1 adipocytes [244]. Although the secretion of adiponectin from visceral and subcutaneous depots appears comparable [74], the regulations may exhibit some depot specificity [55].

The comparison of D and C groups in the part B of the study yielded unexpected results. Plasma concentrations of both total and HMW adiponectin were not different between patients with well controlled type 2 diabetes and healthy control subjects. This finding is in strong contrast with all published data [54, 56], including Caucasian population [43] that report a consistent association of hypoadiponectinaemia with metabolic syndrome, IR, obesity and type 2 diabetes. In our study group, adiponectin concentrations in healthy subjects were lower than reported in literature and moreover, the absolute values are similarly low also in young healthy subjects in part A. Even if small sample size has to be considered, one might speculate on potential regional differences in circulating adiponectin. Unfortunately, the total adiponectin concentrations during SAL are not in line with previously described results and remain unclear.

Additionally, adiponectin expression in SAT appears even increased in diabetic patients, which implicates different contribution of particular fat depots to the comparable circulating adiponectin concentrations in diabetes. In parallel we have shown that expression of both adiponectin receptors is decreased in diabetes. Thus, the comparable circulating adiponectin and increased SAT expression

in well-controlled diabetes may be partly explained as a compensatory mechanism for the diminished cellular response to adiponectin demonstrated by down-regulation of receptors.

In accordance with Study I - part A and the literature discussed above, hyperinsulinaemia had no effect on adiponectin concentrations in both D and C group. On the other hand, adiponectin expressions were stimulated by insulin in diabetes but surprisingly not in healthy controls. This finding is opposite to the previously discussed study [236], however other authors did not find any changes in adiponectin expression during hyperinsulinaemia in healthy subjects [245, 246]. The insulin-induced increase in adiponectin expression may represent a protective mechanism to hyperinsulinaemia in early phase of type 2 diabetes, similarly to the report that higher insulin-induced up-regulation of adiponectin expression in adipose tissue explants is associated with IR [247]. Expression of adiponectin receptors in SAT was not affected by acute hyperinsulinaemia.

Leptin. In accordance with general agreement [44, 52], baseline plasma concentrations and SAT expressions of leptin were higher in diabetes, suggesting the presence of leptin resistance in the group of overweight/obese type 2 diabetic patients. Insulin increases circulating concentrations of leptin in healthy subjects, while in diabetes plasma leptin declines during hyperinsulinaemia. Even if most reports document an insulin-stimulated increase in plasma leptin in lean, obese and diabetic subjects [228, 229], other authors did not detect any changes in circulating leptin during clamp [248-250] or described differences in leptin regulation in diabetes depending on fasting or previous treatment [229, 251]. Therefore the decline in plasma leptin in response to acute hyperinsulinaemia in well-controlled type 2 diabetes may represent an adaptive mechanism.

Leptin expression appears not to be acutely regulated by insulin in the presented study. Despite of the stimulatory effect of leptin expression in vitro [248], no changes in leptin expression after 3-hour hyperinsulinaemia in vivo are reported [252]. In line with published data [251], differential regulation of circulating leptin cannot be explained by its mRNA expression in SAT, which implicates the involvement of other fat depots as a leptin source or specific regulation at the level of posttranslational modification or secretion.

TNFα. In line with the literature evidence [57], TNFα circulating concentrations and expressions were increased in diabetes. However, the low TNFα concentrations in SAL in diabetic subjects are controversial and remain unclear. Plasma TNFα were not affected by insulin, whereas TNFα expression was stimulated by hyperinsulinaemia to a greater extent in diabetic patients. Analogous regulations were reported in healthy subjects [253]. However, other groups using hyperinsulinaemic clamp technique did not demonstrate any insulin-dependent changes in TNFα mRNA expressions [242] or interstitial concentrations [230]. Circulating TNFα does not seem to be acutely regulated by insulin [230, 253, 254] which corresponds with the more important role of TNFα on the local paracrine level.

Visfatin. Plasma concentrations of visfatin were unexpectedly found to be lower in diabetic patients compared to healthy subjects. The physiological function of visfatin and its relation to IR and obesity in humans is controversial and thus there are several studies confirming increased circulating visfatin in obesity [226, 255], while other groups showed analogous to us reduced concentrations in obesity [256] or diabetes [257]. Visfatin appears not to be regulated by acute hyperinsulinaemia in any group, which is a novel observation in vivo. However, the increased visfatin concentrations in SAL both at baseline and 240 min in both groups remain unclear. Since the difference cannot be attributed to the interventions per se, the potential intraindividual variability in plasma visfatin, as

well as confounding interindividual variability or assay difficulties should be taken into account. The assessment of visfatin mRNA expressions in SAT specimen was unfortunately not possible.

RBP 4. Among the equivocal data on RBP4 association with obesity and type 2 diabetes [43], our results correspond to those who did not prove any difference in circulating RBP4 between obese, non-obese and diabetic patients [94, 95, 97, 258]. Although RBP4 concentrations were comparable between groups, they were differentially regulated. For the first time we show that in healthy subjects but not in diabetic patients, RBP4 is suppressed by insulin. This finding suggests a protective regulation in healthy subjects which is not present in type 2 diabetes. Unfortunately, from technical reasons we were not able to disclose the potential changes in RBP4 expression in SAT.

MCP-1. Consistent with several recent studies [98, 259, 260], both MCP-1 plasma concentrations and SAT expressions were increased in overweight/obese diabetic patients. On the other hand, other groups were not able to reproduce this difference in circulating MCP-1 [100-102] or adipose tissue expressions [101]. This controversy may be at least in part explained by the differences in the groups examined, since some studies included obese non-diabetic patients [101] and increased MCP-1 concentrations were found specifically in patients with diabetes [260].

In both groups, stable and lower MCP-1 concentrations were demonstrated during SAL compared to HEC. This observation remains unclear. However, the finding is consistent in both groups and throughout the interventions. Hyperinsulinaemia did not affect MCP-1 levels in healthy subjects, whereas it stimulated MCP-1 in diabetes. MCP-1 expression was not changed by hyperinsulinaemia in any group. It was already shown that insulin regulation of MCP-1 in vivo differs between insulin-sensitive and insulin-resistant women [101], although it was in a different manner than our results: serum MCP-1 declined in insulin-sensitive but not in insulin-resistant subjects and insulin-stimulated MCP-1 expression was pronounced in insulin-resistant subjects. Two other studies showed a decline in MCP-1 in response to mild hyperinsulinaemia (28-50 µU/ml) [231, 261]. Differences in study design (6-hour vs. our 4-hour hyperinsulinaemic clamp vs. insulin infusion 2IU/h) and/or study groups (non-diabetic obese women were not age-matched to the insulin-sensitive group) may account for the discrepant results. Since the changes in plasma MCP-1 are not accompanied with changes in MCP-1 expression, we suggest that SAT is not the major determinant of circulating MCP-1 in humans.

IL-1ra. In agreement with recent literature [115-117], plasma concentrations of IL-1ra were increased in overweight/obese diabetic patients. Circulating IL-1ra does not appear to be acutely regulated by insulin. Unfortunately, concentrations of IL-1β were under detection limit using the multiplex ELISA. Even though we cannot assess the IL-1ra / IL-1β ratio [110] we conclude that IL-1ra exerts pro-inflammatory functions in IR and obesity.

RANTES. Paradoxically, overweight/obese diabetic subjects displayed lower RANTES concentrations compared to controls. This observation is in contrast with data in both obese and diabetic cohorts [102, 122, 262, 263] and thus needs further clarification. Insulin suppressed RANTES in controls but not in diabetic patients. Recently, low-dose insulin infusion (2 IU/h) with final insulin concentrations approximately 50 µU/ml has been shown to decrease RANTES concentrations in diabetic patients [231]. Using standardized hyperinsulinaemic clamp technique, we confirmed this suppressive effect of insulin only in healthy subjects, suggesting differential regulations of RANTES between groups.

VEGF. In contrast with previous studies in hypertensive or obese patients [127, 128], VEGF concentrations were comparable between groups. We first described that acute hyperinsulinaemia did not affect VEGF levels.

Effect of acute losartan administration on insulin sensitivity

Acute ARB did not influence the parameters on insulin sensitivity in any of the three groups. Based on the pharmacokinetics of losartan and its active metabolite (biological period of 2 and 6-9 hours respectively), effective circulating concentrations during the clamp period should have been achieved. However the plasma levels may not mirror effective tissue concentrations, which are crucial for potential metabolic effects. Since the active losartan metabolite EXP3179 shows a partial PPARγ agonism [182], the absence of acute changes in insulin sensitivity do not exclude positive metabolic effects of losartan in long-term administration. Supportive for this hypothesis is a recent finding that chronic losartan treatment results in serum levels of metabolite EXP3179 sufficient for PPARγ activation that was demonstrated by up-regulation of PPARγ genes in monocytes [206].

The effect of acute ARB on insulin sensitivity has not been studied in humans so far. Clinical studies using long-term administration of ARBs with hyperinsulinaemic-euglycaemic clamp technique reported both increase [135, 194, 195] and no changes [199, 264] in glucose disposal in insulin resistant subjects.

Changes in endocrine activity of adipose tissue - Effect of acute losartan administration

Resistin. In young healthy subjects (Study I - part A), plasma concentrations of resistin increased during hyperinsulinaemia independently on ARB. On the contrary, the insulin-stimulated expressions of resistin in SAT were attenuated by losartan. The finding of increased plasma resistin in spite of decreased expression in subcutaneous fat implicates a role of another source of circulating resistin – a different fat depot or potential non-adipose sources (e.g. stromal vascular fraction of adipose tissue, macrophages or endothelium). Other studies in rodents and humans [21, 64] found increased expression of resistin in visceral fat depots compared to subcutaneous at basal conditions, although the source of resistin was thought to be non-adipocytes. In part B of the study, the promising losartan effect on resistin expression has not been reproduced. Losartan reduced basal resistin concentrations only in D but not in C group. Resistin expression in SAT was neither affected by hyperinsulinaemia nor by losartan. Nevertheless the suggested changes in resistin concentrations or expressions following acute ARB may intensify with long-term losartan treatment.

Adiponectin. An increase in circulating adiponectin after 2-month treatment with losartan in hypertensive patients has been reported [204] and there are other experimental data showing enhanced adiponectin expression in response to long-term ARB [174, 181], possibly via PPARγ activation. All the experiments and clinical studies tested different ARB substances in different models of insulin resistance and there is no evidence about the regulations in healthy subjects. In part A of the study, we have observed that acute losartan administration does not influence circulating adiponectin but it attenuates the insulin-induced expression of adiponectin in SAT. This unexpected trend in young healthy subjects was not confirmed in part B in middle-aged man. In both diabetic patients and healthy controls, acute losartan administration led to increase in both total and HMW adiponectin in plasma, as well as in its expression in SAT, whereas it did not influence expression of adiponectin receptors. Thus stimulation of adiponectin might represent one of the underlying mechanisms of positive metabolic effects of sartans.

Leptin. Acute losartan administration suppressed plasma leptin levels in diabetes but not in healthy subjects, while leptin expression in SAT was not subject of acute losartan regulation. This novel

finding suggests a potential adaptive mechanism in well-controlled type 2 diabetes that may together with suppression of leptin concentrations in response to acute hyperinsulinaemia, help to overcome the present leptin resistance. To our knowledge, the effect of losartan treatment on plasma leptin was tested only in hypertensive subjects so far [205, 265] in whom no changes in leptin were found. Therefore, further studies with chronic losartan administration in different populations are needed, ideally also in diabetic patients with different levels of blood glucose control and broad range of adiposity.

TNFα. In agreement with studies in hypertensive subjects with long-term losartan treatment [205, 266], plasma TNFα was not acutely regulated by losartan in any group. There are virtually no data on effect of losartan on TNFα expression in SAT. In diabetes, TNFα expression was stimulated by insulin independently on AT-1 receptor blockade, while in controls losartan increased basal TNFα expression. The relevance of this modest but significant increase within overall low expression rates remains to be clarified.

Visfatin plasma concentrations are apparently not regulated by acute losartan administration.

RBP4. Acute losartan administration had no effect on insulin-mediated suppression of RBP4 during clamp in controls. On the other hand in diabetes, losartan reduced baseline RBP4 levels that were reversed by insulin to the concentrations observed during HEC. Since there is no literature available regarding losartan effect on RBP4, our finding is novel.

MCP-1. In animal models [267] and cell cultures [268, 269] losartan has been shown to effectively reduce MCP-1 production. Accordingly, 6-8 weeks of losartan treatment reduced circulating MCP-1 in hypertensive patients [204, 270]. In our study, acute losartan administration did not affect the low plasma MCP-1 in healthy subjects, but in diabetes it attenuated the insulin-stimulated increase in MCP-1. MCP-1 expression in SAT was not regulated by acute losartan administration in any group. Our results suggest that positive effects of losartan may be mediated at least in part by suppression of chemokine MCP-1.

Plasma concentrations of IL-1ra, RANTES and VEGF (in line with published data [271, 272]) were not affected by acute losartan administration.

Recently it was proved that the beneficial metabolic effect of ARB goes beyond simple interruption of RAS [273]. Our results suggest potential suppressive effect of losartan on plasma concentrations of resistin, leptin, RBP4 and MCP-1 that were demonstrated in diabetic patients already following acute treatment, while no acute regulation of the adipokines' expression was detected. The presence of the losartan effect only in diabetic group might be related to the degree of RAS activation, which is assumed to be higher in patients with diabetes and treated hypertension. Importantly, losartan-induced increase in both total and HMW adiponectin as well as in adiponectin expressions suggests another potential mechanism for metabolic effects of losartan. This hypothesis needs to be confirmed in further studies with chronic losartan administration in different populations.

A-FABP and related gene expression

To our knowledge, this is the first clinical study analyzing A-FABP plasma concentrations in parallel with its expressions in SAT. Showing higher plasma concentrations and expression of A-FABP in overweight/obese patients with type 2 diabetes compared to healthy subjects, our findings are in accordance with current evidence on circulating A-FABP [140-143, 274]. In addition to plasma

concentrations, our results confirm an equivalent difference in A-FABP expressions between the groups studied. This fact has not been sufficiently clarified in previous studies [144, 146-148]. The strong positive correlation of plasma A-FABP with its expression in SAT indicates that SAT is a substantial source of circulating A-FABP. The contribution of other fat depots cannot be excluded, however previous studies showed higher or comparable A-FABP expression in SAT in comparison to visceral adipose tissue [275, 276]. Additionally, macrophages might also play a role in whole-body A-FABP balance [277]. Their contribution to circulating A-FABP levels has not been addressed in our study.

The minor adipocyte isoform - E-FABP revealed higher expression rates in healthy subjects. Here, the literature evidence is modest and inconclusive [144]. A-FABP/E-FABP mRNA ratio suggested by Fisher and co-workers [147] as a summarizing measure for FABPs in adipose tissue was 3-fold higher in the overweight/obese diabetic patients. It could be hypothesized that in obesity and type 2 diabetes, the E-FABP expression is down-regulated in order to at least partially compensate for increase in A-FABP. The clarification of FABPs' regulations in obesity and type 2 diabetes awaits definitely further studies.

Additionally, we measured relative expressions of PPARγ, which is involved in regulation of A-FABP gene expression. Conversely A-FABP enhances PPARγ activity [144]. The studies comparing PPARγ expression between lean and obese/type 2 diabetic patients reveal inconsistent data with reports of decreased, unchanged or increased PPARγ expression in obese subjects [236, 278-280]. We report a down-regulation of PPARγ expression in diabetes. We were not able to show any relationship of PPARγ expression to A-FABP plasma concentrations or expressions, while a positive correlation between PPARγ mRNA and E-FABP mRNA suggests that the expressions of PPARγ and the minor FABP isoform are closely related, at least in SAT. Further interventional studies with PPARγ agonists are needed to disclose the possible causal relationship suggested by the finding of Cabre [274], who showed an increase in plasma A-FABP after treatment with rosiglitazone.

Another important finding of our study is the close association of both A-FABP plasma concentration and expression not only with measures of obesity, but also with fasting plasma glucose, insulin and insulin resistance defined by means of hyperinsulinaemic-euglycaemic clamp. Additionally, the A-FABP mRNA and A-FABP/E-FABP mRNA ratio was positively correlated with HbA1c. The association with hyperglycaemia, insulinaemia and glucose disposal remained significant also in multivariate regression analysis suggesting that the relation of A-FABP to insulin resistance and blood glucose is independent of obesity.

In both D and C groups, acute hyperinsulinaemia suppressed circulating A-FABP in D and C, while no direct impact on A-FABP expression in SAT was detected in either group. This discrepancy between changes in plasma and expression in SAT implicates a role of another A-FABP source (macrophages or other fat depot) in the response to insulin.

To our knowledge, the presented study is also the first one using the control volume examination to distinguish non-specific time and volume effect on A-FABP during clamp. In type 2 diabetes, the decline in A-FABP during hyperinsulinaemia is accordance with a recent report [281]. Using the SAL control examination, during which no changes were detected, the direct regulation of plasma A-FABP by insulin in diabetes was confirmed. Importance of volume control examinations can be even better demonstrated in the control group where no changes during hyperinsulinaemia, but an increase in plasma A-FABP during saline infusion were measured. Since neither secretion mechanisms, nor function of circulating A-FABP in vivo in humans are elucidated, the interpretation of the observation during SAL is not clear – we might speculate on effects of prolonged fasting or circadian rhythm on

plasma A-FABP. Moreover, the stable A-FABP concentrations during HEC might be interpreted as an influence of insulin that prevented the increase in A-FABP seen in SAL. Recently, direct regulation of circulating A-FABP by insulin was described [281], showing a decrease in A-FABP also in healthy controls. The difference to our study may be explained by the well-described gender differences in A-FABP plasma concentrations [140-143, 274]. The higher A-FABP levels in women may be also differentially regulated compared to men, which has been already demonstrated by an independent association of A-FABP with carotid intima-media thickness in women but not in men [282]. Expressions of both E-FABP and PPARγ in SAT were not affected by hyperinsulinaemia in our study population.

We could demonstrate distinct effects of ARB on FABPs: in healthy subjects acute ARB stimulated basal A-FABP plasma concentrations, which were reversed by insulin during clamp. In type 2 diabetes, the stimulatory effect of ARB on plasma A-FABP was independent of hyperinsulinaemia. In healthy subject, the expression of E-FABP but not A-FABP in SAT was also stimulated after acute ARB. In type 2 diabetes, regulation A-FABP and E-FABP expressions by acute ARB has not been proven. The different changes in plasma and expression in SAT suggest an involvement of another A-FABP source in the response to ARB.
Considering the potential PPARγ activation by sartans [170, 174], long-term exposure to ARB in vivo, as well as experimental models have to be evaluated in order to confirm the suggested effects of ARB on A-FABP. Plausibility of such a hypothesis has been recently supported by an observation of a cross-sectional study with patients submitted for coronary angiograms [283]. Those patients treated with ACEIs or angiotensin II type 1 receptor blockers revealed significantly higher A-FABP levels.

In conclusion in young healthy subjects, acute hyperinsulinaemia stimulates an increase in plasma concentration and expression of resistin in subcutaneous adipose tissue. While the ARB does not modify the insulin-induced changes in plasma resistin, it attenuates the response of resistin expression in adipose tissue. Acute hyperinsulinaemia is associated with an increase in adiponectin expression, but not in its plasma levels. Losartan reduces the insulin-stimulated expression of adiponectin.

To conclude the findings in overweight/obese patients with well-controlled diabetes, plasma concentrations and/or SAT expressions of resistin, leptin, TNFα, MCP-1 and IL-1ra are increased, whereas plasma levels of adiponectin, RBP4 and VEGF are comparable with healthy subjects. Unexpectedly, increased adiponectin SAT expressions in diabetic subjects are accompanied with lower expression of adiponectin receptors.
Insulin differentially regulates circulating resistin, leptin, RBP4, MCP-1 and RANTES in diabetes and healthy subjects, while plasma adiponectin, TNFα, visfatin, IL-1ra and VEGF do not appear to be acutely regulated by insulin. Stimulatory effect of insulin on SAT expressions was demonstrated only for TNFα and adiponectin, expressions other adipokines were not influenced by hyperinsulinaemia.
Suppressive effect of losartan on plasma concentrations of resistin, leptin, RBP4 and MCP-1 was demonstrated in diabetic patients already following acute treatment, while no acute regulation of the adipokines' expression was detected. Importantly, losartan-induced increase in both total and HMW adiponectin as well as in adiponectin expressions suggests a potential mechanism for metabolic effects of losartan.
Changes in plasma adipokines and cytokines cannot be explained by changes in their SAT expressions. Therefore, other sources such as other fat depots and/or circulating monocytes are implicated in regulation of systemic adipokine balance.

Circulating A-FABP as well as its expressions in subcutaneous adipose tissue are closely and independently related to obesity, insulin resistance and hyperglycaemia. Hyperinsulinaemia suppresses plasma A-FABP but does not influence its expression. Acute ARB stimulates basal A-FABP plasma concentrations without any effect on its expression.

Study II. Effect of 3-week telmisartan treatment on insulin resistance, energy metabolism and endocrine activity of adipose tissue in subjects with impaired fasting glucose

This short term placebo-controlled cross-over study demonstrates in accordance with other studies [284] that telmisartan decreases the fasting plasma glucose and blood pressure, while the insulin sensitivity assessed by hyperinsulinaemic clamp technique did not change after telmisartan treatment in our study population. Thus, the decrease in blood glucose cannot be explained by the improvement in insulin sensitivity, but the observed increase in basal plasma IRI could partly account for the lower plasma glucose. In addition, we have not found any significant effect of telmisartan on the substrate utilization.

There are still controversial results regarding the effect of telmisartan or other ARBs on insulin sensitivity in vivo in humans [212, 219, 284, 285]. Telmisartan is considered to be a partial PPARγ agonist and there is experimental evidence that it has superior metabolic advantages compared to other sartans [179]. However, a recent study evaluating the long-term effect of telmisartan on insulin sensitivity suggests that the magnitude of PPARγ stimulation by telmisartan may be modest compared with TZDs [219]. On the other hand, some metabolic effects of telmisartan could be mediated independently of PPARγ [286]. In addition in a rat model of metabolic syndrome, it increases energy expenditure and protects against dietary induced obesity [287]. Although telmisartan has gained much attention as one of most metabolically potent ARB compounds, there are virtually no clinical studies evaluating the effect of telmisartan on insulin sensitivity and substrate utilization using the clamp technique combined with indirect calorimetry. The homeostasis model assessment–index (HOMA) has been used in most of them [209, 284] and thus, our study may be the first of its kind.

In our study we have failed to show a statistically significant effect of telmisartan on insulin sensitivity and substrate utilization but our results might be influenced by the short period of the study and the small number of patients included. Insulin secretion has not been carefully evaluated in our study, but the improvement in blood glucose level after telmisartan could be related to the improvement in β-cell function. It has been shown in experimental animals that treatment with telmisartan reduces oxidative stress and protects against islet β-cell damage and dysfunction [203].

The main finding of the present study is the significant short-term effect of telmisartan on adipokine production during insulin-stimulated conditions. Adiponectin, leptin and resistin plasma concentrations increased while a decrease in TNFα had been found after telmisartan treatment. We also found an increase in basal leptin concentrations. The changes in plasma adipokines could not be directly explained by changes in their expressions in SAT. The short-term effect of telmisartan on adipokines during clamp-induced hyperinsulinaemia has not been investigated up to now; only the fasting plasma concentrations or adipose tissue expressions have been reported in the literature so far.

TNFα. In the present study, the telmisartan treatment was followed by a decrease in plasma TNFα concentrations during insulin-stimulated conditions. The expressions of TNFα in subcutaneous fat declined during hyperinsulinaemia but were not affected by telmisartan treatment. The results are in agreement with the decrease in fasting TNFα in plasma reported after 8 month telmisartan treatment in hypertensive patients after stent implantation [288] and after 6 and 12 month

administration of telmisartan in patients with metabolic syndrome [209]. In addition, proinflammatory properties of TNFα could be attenuated by telmisartan. Inhibition of TNFα-induced interleukin**-6** expression in vascular smooth muscle cells has been reported after telmisartan treatment [289].

Adiponectin. In our study telmisartan increased the plasma adiponectin concentrations during insulin-stimulated conditions while the expressions of adiponectin in subcutaneous fat were not changed. In contrast to some [124, 212, 220, 288, 290] but not all [284] previous studies we failed to observe any stimulatory effect of telmisartan on basal plasma adiponectin concentrations. Only the trend of an increase has been found in our insulin resistant subjects. The results could implicate the stronger metabolic effect of telmisartan during hyperinsulinaemia compared to basal (pre-prandial) conditions. ARBs induced activation of PPARγ is advocated [181]. In addition, it has been shown in 3T3-L1 adipocytes that telmisartan may stimulate adiponectin gene transcription independent on PPARγ [286].

Leptin. An overall increase in circulating leptin following the telmisartan treatment was documented in the present study. Telmisartan had no significant effect on leptin expressions in SAT, but the expression declined during hyperinsulinaemia. The decline was independent on telmisartan treatment**.** In the literature, both increase [284] and decrease [209] or no changes [219] in fasting leptin concentrations have been reported after long-term administration of telmisartan in hypertensive and type 2 diabetic patients. Telmisartan-induced increase in plasma concentrations of leptin might attenuate body weight gain by reduced food intake with HOMA-IR improvement [284]**.** The effect of telmisartan on leptin increase could not be clarified within the context of metabolic processes in this short time study [284]**.** However, in accordance with some authors we have found a telmisartan-induced increase in leptin and this finding remains to be explained.

Resistin. To our surprise, an unexpected increase in plasma resistin concentrations was found during hyperinsulinaemia after telmisartan administration in our study, which is a novel observation in vivo. In contrast, Derosa et al [290] demonstrated a decrease in plasma resistin following the telmisartan treatment in type 2 diabetic patients. However, only fasting concentrations were assessed, the treatment period was much longer (6 and 12 months) and the subjects were treated also by rosiglitazone [290]. In our study, resistin expression decreased during the hyperinsulinaemia independently on telmisartan treatment.

The changes in the plasma concentrations and tissue expressions of adiponectin, TNFα, leptin and resistin during hyperinsulinaemia are discordant and the expressions in SAT cannot explain the changes in circulating adipokines. This finding implicates the role of other sources of circulating adipokines - other fat depots or non-adipose sources (e.g. stromal vascular fraction of adipose tissue, macrophages or endothelium), which have not been addressed in the present study. However, the differences in the timing of blood sampling and biopsies should be considered as well. Body weight and exercise had been kept stable throughout our study and the patients were not treated with oral hypoglycaemic agents. Thus, we can exclude the interference of these confounding factors with effect of telmisartan.

The present study also allowed us to roughly examine the in vivo effect of insulin on adipokines and their expressions comparing the fasting values with insulin-stimulated conditions during clamp. Only a few studies have investigated the effect of insulin in vivo in humans. It has been shown by Westerbacka et al. [236] that insulin per se could increase the expression of number of cytokines

involved in insulin sensitivity in adipose tissue, including adiponectin or TNFα. We did not find any significant effect of acute in vivo hyperinsulinaemia on plasma adiponectin, leptin or TNFα. On the other hand, the increase in plasma resistin concentrations has been confirmed. An increase in plasma resistin during clamp-induced hyperinsulinaemia was reported in our previous study in healthy subjects [183]. Similarly, Heilbronn et al. [233] found an increase in serum resistin concentrations in response to supraphysiological doses of insulin (164±5 mIU.l^{-1}) in obese subjects with and without diabetes. Contrary to the result of Westerbacka [236], the expressions of adiponectin did not change and expressions of TNFα, leptin and resistin were even suppressed by acute hyperinsulinaemia. However, the present cross-over study was not designed to assess the acute effect of insulin on adipokines. The control infusion to match the volume expansion during clamp was not included. Furthermore, the biopsies were taken at 30 min of clamp – very early after starting the insulin infusion, which is also important limitation factor of our study.

The major limitations of our study are small number of subjects and/or short study duration. Since we did not measure peripheral blood flow, we could not exclude that the effect of telmisartan has been mediated through the blood flow improvement as well as blood pressure improvement both directly or indirectly. Moreover, we were not able to measure tissue protein levels of adipokines and thus we could not exclude that telmisartan controls post-transcriptional rather than transcriptional regulation. However, we found the effect of telmisartan on the parameters mentioned above in spite of the short treatment period. The timing of biopsy at 30 min before equilibrium status had been achieved, might have an impact on the results.

In conclusion, we firstly describe that in patients with impaired fasting glucose a short-term telmisartan treatment increases plasma adiponectin, leptin and resistin concentrations and decreases plasma TNFα levels. These effects appear to be important during hyperinsulinaemia. The changes in plasma concentrations of adipokines cannot be explained by their expressions in subcutaneous adipose tissue. The results support the hypothesis that the changes in selected plasma adipokines might be involved in the beneficial metabolic effects of telmisartan in patients with metabolic syndrome.

Study III. Effect of prolonged hypertriglyceridaemia on endocrine activity of adipose tissue in patients with type 2 diabetes and healthy subjects

In this study the plasma levels of adipose tissue cytokines and their expression in adipose tissue during pharmacologically-induced hyperlipidaemia were characterized. The effectiveness of the intravenous lipid infusion to raise plasma TG and NEFA was verified. No heparin co-infusion was used to prevent the potential confounding effect of heparin as a co-factor of a lipoprotein lipase leading to decrease in plasma TG and increase in NEFA concentrations. Since heparin was used in some further cited experiments [291-293], this sole fact could account for differences in results. Additionally, the mentioned studies also vary in Intralipid infusion rates and duration of intervention. In part B of the study, comparable conditions between D and C groups were achieved in terms NEFAs and TG time profile. The differences in blood glucose, IRI and TG in last 2 hours of the infusion are related to the natural difference between groups (IR, lower clearance of TG and accelerated hepatic gluconeogenesis in diabetic subjects). The between-group comparison of baseline plasma concentrations and expressions of the adipokines is discussed in the Study I.

TNFα. Consistently in both parts A and B of the study, an increase in TNFα plasma concentrations during lipid infusion was demonstrated in healthy subjects, while in diabetes the overall increased TNFα concentrations were not influenced by hypertriglyceridaemia. The changes in circulating TNFα were not accompanied with adequate changes in SAT expression which were stable in all groups and elevated in diabetes. This finding implicates another source of plasma TNFα, such as other fat depot or circulating monocytes. Previously, Nisoli [291] reported an increase in TNFα gene expression in subcutaneous gluteal fat tissue after a 5-hour Intralipid/heparin infusion in healthy subjects. The discrepancy to our results may lie in fat depot-specific expressions and their regulation, since higher TNFα expression in visceral fat has been shown [74, 141].

Resistin. In both parts A and B (in all groups), lipid infusion stimulated an increase in plasma concentrations of resistin, which did not differ between C and D. This is in accordance with reports from animal models [294, 295] where Intralipid/heparin infusion induced hepatic and peripheral IR and was associated with elevated plasma resistin levels. In humans, only one available shorter study did not show any changes in resistin following 5-hour lipid/heparin infusion [293]. Since changes in resistin mRNA in subcutaneous adipose tissue were not significant in any group, we can conclude that this fat depot is not responsible for the regulation of plasma resistin in response to lipid load. Similarly to TNFα, circulating monocytes appear to be an important source of resistin [70].

Leptin. In young healthy subjects (part A), plasma concentration and expressions of leptin did not show any changes during hypertriglyceridaemia. On the other hand in part B, lipid infusion resulted in group-specific time profiles of circulating leptin: the initial decrease in leptin was followed by reversal to initial levels at 24 hours in diabetic patients, whereas in healthy controls a late decline at 24 hours was detected. Leptin expressions were not affected by lipid infusion. Both plasma concentrations and expressions of leptin were increased in diabetes. The different response between the groups of healthy subjects might be related to age. Most previous studies in healthy subjects have not shown any changes in leptin in response to lipid infusion [296-299], even if one have to note that the interventions were shorter (3-5 hours). Other studies reported increased leptin concentrations [293] or stable plasma levels with increase in leptin RNA expression in adipocytes in gluteal region during 5-hour lipid/heparin infusion [291], while 7-hour lipid/heparin infusion already induced a decline plasma leptin [292]. Here we first demonstrate a direct comparison of patients

with type 2 diabetes and healthy subjects, who display differential regulation of circulating leptin. Since the changes mentioned are not mirrored in the leptin expressions in SAT, another fat depot or different level of regulation (post-translational modification or secretion) may play an important role.

Adiponectin. In both parts A and B (all groups), adiponectin appears not to be regulated acute lipid load, neither at the level of HMW or total plasma adiponectin nor its expression. Analogous result of unchanged plasma concentrations was observed during 7-hour Intralipid/heparin infusion [300] [293]. Paradoxically higher plasma levels of total adiponectin and higher adiponectin expression rates and comparable HMW adiponectin concentrations were found in diabetes. For discussion of this unclear finding please refer to Study I.

Visfatin and RBP4. In agreement with a previous study in healthy subjects [256], circulating visfatin is not affected by lipid load in any group. Due to interindividual variability plasma visfatin levels tended to be lower in diabetes (similarly to previous results [256]) but the difference did not reach statistical significance. Plasma RBP4 was not influenced by lipid infusion either, which is a novel finding in vivo.

A-FABP. In line with study I, plasma concentrations and expressions of A-FABP were increased in diabetes. Since there are no data available on A-FABP regulation by hyperlipidaemia, we first report a suppressive effect of lipid load on circulating A-FABP in both diabetic and healthy subjects that was not accompanied by adequate changes in A-FABP expression in SAT.

Lipid infusion resulting in a subsequent increase in NEFA plasma concentrations is a well known method used for inducing IR [301-303]. Therefore, although we have not measured insulin sensitivity, we assume that changes we have observed might be implicated in the pathogenesis of lipid-induced IR. For TNFα, our results are in agreement with a theory that it is implicated in NEFA induced IR as shown in vitro [304]. For resistin it is an unclear result as it is often found not to be associated with IR [305], although clinical studies are often limited to correlations between resistin and various measures of metabolic syndrome. The decrease in leptin in healthy subjects may represent an adaptive reaction to prolonged hypertriglyceridaemia that was initiated but reversed in diabetes. The impact of decline in A-FABP concentrations on IR in response to lipid load remains to be clarified.

There are several limitations of this study. Increase in plasma lipid concentrations by intravenous infusion certainly differs from dietary and lifestyle induced hyperlipidaemia observed in real life. Small number of subjects examined and the lack of placebo control are other factors that should be considered. Placebo control was not included because of the confounding effect of a 24-hour long fasting that induces IR per se. Circadian variations were partly assessed by 24-hour duration. Glycerol (a component of Intralipid emulsion) does not affect insulin secretion [301, 306], glucose effectiveness [154] or insulin sensitivity [307]. Effect of glycerol on resistin, TNFα, leptin or A-FABP is however not excluded. The confounding effect of hyperinsulinaemia is assumed to be negligible due to only modest increase in IRI during the experiment.

In summary, we report that prolonged hyperlipidaemia stimulates an increase in secretion of TNFα and resistin, while it results in decline in plasma leptin and A-FABP. Expressions of adipokines in SAT are not affected by hyperlipidaemia and thus, other sources such as other fat depot or circulating monocytes are of importance in the lipid-induced regulation of circulating adipokines. The findings support the hypothesis that the adipokines could be involved in the pathogenesis of lipid-induced IR.

Study IV. Endocrine activity of adipose tissue in subjects with different categories of glucose intolerance

Adipokines

Leptin. In line with recent agreement [44], leptin concentrations as well as SAT expressions were lowest in healthy subjects compared to both other groups. Both plasma levels and expression of leptin were also positively related to adiposity and negatively to glucose disposal, which suggest the presence of leptin resistance connected with obesity and IR. Supportive to this hypothesis, an additional association of plasma leptin with HDL-cholesterol and leptin expressions with fasting glucose and insulin were demonstrated. However, an unexpected elevation of plasma leptin in IFG compared to both other groups cannot be explained by leptin expression in SAT that was comparable between IFG and D. Thus, other fat depots have to be involved as a source of circulating leptin. Moreover, other factors may contribute to the observed difference in plasma leptin, such as differential regulatory mechanisms of expression and secretion during the progression of glucose intolerance (demonstrated e.g. by association of plasma leptin but not its expressions with MCP-1), or concomitant medication in D group.

Adiponectin. In contrast with the recent knowledge [44, 56, 57] we were not able to reproduce the association of low adiponectin levels with metabolic syndrome, obesity, IR or type 2 diabetes. In our cohorts, both total and HMW adiponectin concentrations were comparable between groups with markedly low concentrations in healthy subjects. Relative expression of adiponectin in SAT was even increased in patients with type 2 diabetes. The SAT expressions of adiponectin receptor AdipoR1 was comparable between groups, whereas the more abundant adipose tissue receptor AdipoR2 revealed decreased expressions in IFG and D groups, which is in accordance with several published data [308-310]. HMW adiponectin does not appear to be major determinant of adiponectin action in our study groups, as total adiponectin showed more associations within adiponectin system, but total and HMW adiponectin levels were related. Expression of both AdipoR1 and AdipoR2 was highly interrelated and seems to depend on circulating adiponectin (inverse relation) but not on its expression. Similar association has been recently found demonstrated for hepatic adiponectin receptors [311]. Further regulations of AdipoRs expression are suggested by association with PPARγ expression and inverse relation to circulating TNFα. We speculate that the comparable circulating adiponectin in IFG and diabetes and increased SAT expression in well-controlled diabetes may be partly explained as a compensatory mechanism for the diminished cellular response to adiponectin demonstrated by down-regulation of the dominant AdipoR2 receptor. The group-specific adiponectin mRNA levels in SAT implicate different contribution of particular fat depots to the comparable circulating adiponectin concentrations in IFG and diabetes.

Resistin. IFG group displayed surprisingly decreased resistin plasma concentrations compared to both C and D groups where plasma resistin was equal. This IFG-specific difference in circulating protein is not mirrored in resistin expressions in SAT that showed an increase in diabetes but was comparable between IFG and C. Resistin expression was associated with adiposity and triglycerides. The human data regarding relation of resistin to IR, obesity and diabetes are controversial [66] showing either comparable [232, 233] or increased [66] plasma resistin in obesity, IFG and/or type 2 diabetes. Since mononuclear cells and macrophages are found to be more important source of circulating resistin in humans [66], resistin secretion from monocytes reflecting inflammatory status may be responsible for the observed difference in plasma resistin among groups. Accordingly, the

enhanced expression of resistin in SAT of diabetic patients might be a consequence of increased macrophage infiltration and adipose tissue inflammation connected to overt diabetes which is supported by association between resistin and TNFα expressions.

TNFα. In accordance with literature [57], TNFα plasma concentrations as well as SAT expressions were lowest in healthy subjects. Interestingly, diabetic subjects were characterized by higher TNFα expression in SAT compared to IFG despite of comparable TNFα plasma levels. In the literature, comparison of subjects with different stages of glucose intolerance reveals equivocal results, showing comparable plasma levels and expressions [312], increased plasma TNFα just in diabetic subjects [313] or subsequent increase in plasma TNFα during progression to overt diabetes [314]. The ethnicity of studied population and differences in basal characteristic may partly account for differences in results. Showing plasma concentrations in parallel to SAT mRNA expression, our results implicate different contribution of fat depots and/or other TNFα sources (such as monocytes) to the comparable circulating TNFα concentrations in IFG and diabetes. We can speculate that regulation of TNFα expression and secretion is altered during the progression of glucose intolerance. The importance of TNFα on the local/paracrine level is supported by substantial associations of TNFα mRNA expressions with measures of obesity, insulin resistance, hyperglycaemia and triglycerides, whereas plasma TNFα was only related to insulin resistance. In addition, the association of TNFα expressions with plasma IL-1ra and expressions of leptin and resistin suggests the potential involvement of TNFα in endocrine and inflammatory activity of SAT.

Visfatin and RBP4. Plasma concentrations of visfatin were unexpectedly found to be lower in diabetes compared to both other groups and were inversely associated with IR and hyperglycaemia. Even if visfatin was initially declared as an IR factor with increased concentrations and expression in obesity and/or diabetes [226, 255, 315], there are several reports showing opposite results comparable to the ours [256, 295], including the positive correlation of visfatin expression with insulin sensitivity [82]. Thus, visfatin involvement in IR and glucose intolerance is more complex and needs further clarification.

RBP4 plasma concentrations did not differ between groups and we did not find any associations with metabolic parameters or other cytokines, similarly to other groups who did not confirm the postulated association of RBP4 with IR and diabetes [94, 95, 97, 258]. On the other hand, other authors do report the relation of RBP4 to severity of glucose intolerance, mostly in Asian [96, 316, 317] or Mexican [318] populations, but also in Caucasians [319, 320]. The controversial human data on RBP4 role in pathogenesis of IR and glucose intolerance await further clinical studies.

A-FABP and related gene expression

We first demonstrate A-FABP plasma concentrations and mRNA expressions in parallel in subjects with different categories of glucose intolerance. A-FABP expression and secretion appear to be differentially regulated in different source tissues and depots during the progression of glucose intolerance: while A-FABP plasma concentrations were comparable in IFG and D, the A-FABP expressions in SAT were higher in D, which suggests the involvement of other important source of circulating A-FABP in IFG than SAT. Here, other fat depots or circulating monocytes may substantially contribute to the whole-body A-FABP balance. E-FABP, the minor adipose tissue FABP isoform displayed higher expression in healthy subjects compared to both other groups. This finding is in line with the suggested down-regulation of E-FABP production as a partial compensation for increased A-FABP in obesity and type 2 diabetes [147]. PPARγ expressions did not significantly differ between

groups. Due to interindividual variability in PPARγ expression rates the small number of subjects may be the reason for failure to prove a significant difference.

Our results confirm the close association of both A-FABP plasma concentrations and expression with parameters of obesity [144]. Insulin resistance appears to be more related to circulating A-FABP, whereas hyperglycaemia is an important determinant of A-FABP expressions in SAT. Additionally, A-FABP expressions were found to be associated with expressions of leptin, resistin, TNFα, but also adiponectin, suggesting an involvement of A-FABP pathways in endocrine activity of SAT. E-FABP expressions do not seem to be associated with selected metabolic parameters in our cohorts, while PPARγ expression was found to be associated inversely with plasma concentrations of TNFα and resistin and positively with expression of adiponectin receptors. These findings implicate an important role of PPARγ in humoral functions of adipose tissue.

Markers of inflammation

Healthy subjects displayed lower plasma concentrations of MCP-1 compared to both other groups, which is in line with several published studies [98, 259, 260], although other authors did not confirm this difference [100-102]. MCP-1 levels are enhanced already in IFG and overt diabetes is not associated with further increase in circulating MCP-1, similarly to a recent report [260]. The association of plasma MCP-1 with waist circumference and MCR also supports the hypothesis that circulating MCP-1 is related to obesity and IR, but not to dysregulation of glucose metabolism. Moreover, the association of MCP-1 with plasma leptin and A-FABP suggests that inflammatory mediators may affect other adipose tissue pathways and endocrine activity.

Although IL-1ra concentrations did not differ between groups (possibly due to small number of subjects), its circulating levels were positively related to parameters of obesity, fasting plasma glucose and insulinaemia, suggesting increased activation of IL-1 system in obesity and glucose intolerance. Additionally, showing positive association with expressions of TNFα and leptin, IL-1ra may also play a role in endocrine activity of adipose tissue.

In contrast to previous studies in hypertensive and obese subjects [127, 128], VEGF displayed comparable concentrations in all groups and no associations with metabolic characteristics or other cytokines were found.

RANTES was surprisingly lowest in diabetes and as such it was positively related to insulin sensitivity and inversely to BMI. This unexpected finding is opposite to previous literature data [100, 102, 122, 263] showing increased RANTES levels in obese, impaired glucose tolerant and diabetic patients and therefore it remains unclear.

Baseline metabolic characteristic of the groups

The IFG and D groups actually differed only in parameters of glucose metabolism (fasting plasma glucose and HbA1c). IFG displayed more pronounced fasting hyperinsulinaemia compared to D group, which may be related to differences in concomitant medications between the groups. Patients with type 2 diabetes received antidiabetic (mostly insulin-sensitizing), antihypertensive and statin therapy, whereas IFG group was treatment-naive except of antihypertensive medication. Potential confounding effect of concomitant medication on observed differences or similarities in adipokines between groups cannot be excluded. Nevertheless, IFG and D groups were well comparable in terms of adiposity, lipid profile and insulin resistance (MCR and insulin sensitivity indices). However, in order to completely disclose the effects of different categories of glucose intolerance on endocrine

activity of adipose tissue, an additional obese normal glucose tolerant group would be necessary. Unfortunately we did not include this group, which limits the interpretation of the results.

In conclusion, the selected adipokines including A-FABP display differential regulations on the level of circulating concentrations and SAT expressions during the progression of glucose intolerance. The between-group differences in plasma concentrations are not mirrored in SAT expressions and they are also not related to each other, which implicates involvement of other sources of circulating proteins than SAT, such as other fat depots or monocytes in some cases. Apparently, the particular source tissue may contribute with different intensity to the whole-body adipokine balance during the progression to overt diabetes.

General comments and limitations

The studies presented in this thesis show that changes in circulating adipokines induced by different interventions are not accompanied with parallel changes in SAT expressions. Thus, other sources such as other fat depots or circulating monocytes importantly contribute to the systemic balance of these adipokines and cytokines. Alternatively, regulatory mechanisms on other than transcriptional level may be involved, such as posttranscriptional, posttranslational modifications or regulation of adipokine secretion. Since we only addressed the transcriptional level, the interpretation of the changes found cannot be exhaustive. A parallel analysis of tissue proteins or concentrations of proteins in interstitial fluid may be useful to get more complex insight into the in vivo regulations. Additionally, characterising the SAT infiltration with macrophages (by means of analysis of CD68 expression or SAT histology) or separate analysis of isolated adipocytes and stromal-vascular fraction could bring further information on the inflammatory status and activity of SAT in different groups and interventions.

The protocol used was quite complex which also demanded a complex statistical analysis. Thus, the interpretation of results with respect to other discussed studies has several limitations. The between-group comparison may be different, if there are only basal values compared or if one analyses also the effect of intervention using repeated measures.

The importance of volume control examination could be demonstrated in study I – part B, since the interpretation of several findings during clamp would substantially differ without the knowledge of the adipokine time profile during saline infusion. Here, the effect of circadian rhythm or prolonged fasting might be postulated as an underlying mechanism for changes in proteins during saline infusion. On the other hand, in several cytokines we also detected different baseline concentrations at the occasion of clamp and saline infusion. These differences remain unclear, since they cannot be explained by the following procedure (except of potentially confounding subjects' perception of less stress prior to saline infusion where no SAT biopsies were awaited). As a potential explanation intraindividual variability in cytokine concentrations should be also mentioned.

Another unclear observation was the comparable levels of total and HMW adiponectin in patients with well-controlled type 2 diabetes and healthy subjects. We cannot exclude a potential bias induced by selection of healthy subjects who even if did not fulfil the criteria of metabolic syndrome, were partly overweight and had e.g. comparable total and LDL-cholesterol levels with the diabetic group, while the diabetic patients had very well-controlled diabetes and were treated with protective concomitant medication. These factors might have attenuated the difference between the groups. The potential bias induced during pre-analytical and analytical procedures (sample collection and processing, kit selection, etc.) has to be mentioned, despite it is not likely, since the procedures were standardized and there were no analytical difficulties with adiponectin estimation.

Despite of the limitations mentioned, the presented studies bring new knowledge on endocrine and inflammatory activity of adipose tissue in vivo in humans under different conditions. Further studies in larger populations as well as experimental studies with cell and tissue cultures are needed to confirm the presented findings and to elaborate in detail the suggested hypotheses.

SUMMARY OF MAIN OUTCOMES

Study I – part A

In young healthy subjects, acute hyperinsulinaemia stimulates an increase in plasma concentration and expression of resistin in subcutaneous adipose tissue. While the ARB does not modify the insulin-induced changes in plasma resistin, it attenuates the response of resistin expression in adipose tissue. Acute hyperinsulinaemia is associated with an increase in adiponectin expression, but not in its plasma levels. Losartan reduces the insulin-stimulated expression of adiponectin.

Study I – part B

In obese patients with well-controlled diabetes, plasma concentrations and/or SAT expressions of resistin, leptin, TNFα, MCP-1 and IL-1ra are increased, whereas plasma levels of adiponectin, RBP4 and VEGF are comparable with healthy subjects. Unexpectedly, increased adiponectin SAT expressions in diabetic subjects are accompanied with lower expression of adiponectin receptors.

Insulin differentially regulates circulating resistin, leptin, RBP4, MCP-1 and RANTES in diabetes and healthy subjects, while plasma adiponectin, TNFα, visfatin, IL-1ra and VEGF do not appear to be acutely regulated by insulin. Stimulatory effect of insulin on SAT expressions was demonstrated only for TNFα and adiponectin, expressions other adipokines were not influenced by hyperinsulinaemia.

Suppressive effect of losartan on plasma concentrations of resistin, leptin, RBP4 and MCP-1 was demonstrated in diabetic patients already following acute treatment, while no acute regulation of the adipokines' expression was detected. Importantly, losartan-induced increase in both total and HMW adiponectin as well as in adiponectin expressions suggests a potential mechanism for metabolic effects of losartan.

Changes in plasma adipokines and cytokines cannot be explained by changes in their SAT expressions. Therefore, other sources such as other fat depots and/or circulating monocytes are implicated in regulation of systemic adipokine balance.

Circulating A-FABP as well as its expressions in subcutaneous adipose tissue are closely and independently related to obesity, insulin resistance and hyperglycaemia. Hyperinsulinaemia suppresses plasma A-FABP but does not influence its expression. Acute ARB stimulates basal A-FABP plasma concentrations without any effect on its expression.

Study II

We firstly describe that in patients with impaired fasting glucose a short-term telmisartan treatment increases plasma adiponectin, leptin and resistin concentrations and decreases plasma TNFα levels. These effects appear to be important during hyperinsulinaemia. The changes in plasma concentrations of adipokines cannot be explained by their expressions in subcutaneous adipose tissue. The results support the hypothesis that the changes in selected plasma adipokines might be involved in the beneficial metabolic effects of telmisartan in patients with metabolic syndrome.

Study III

Prolonged hyperlipidaemia stimulates an increase in secretion of TNFα and resistin, while it results in decline in plasma leptin and A-FABP. Expressions of adipokines in SAT are not affected by hyperlipidaemia and thus, other sources such as other fat depot or circulating monocytes are of importance in the lipid-induced regulation of circulating adipokines. The findings support the hypothesis that the adipokines could be involved in the pathogenesis of lipid-induced IR.

Study IV

The selected adipokines including A-FABP display differential regulations on the level of circulating concentrations and SAT expressions during the progression of glucose intolerance. The between-group differences in plasma concentrations are not mirrored in SAT expressions and they are also not related to each other, which implicates involvement of other sources of circulating proteins than SAT, such as other fat depots or monocytes. Apparently, the particular source tissue may contribute with different intensity to the whole-body adipokine balance during the progression to overt diabetes.

CONCLUSIONS

Providing a comprehensive evaluation of adipose tissue endocrine activity in vivo under different experimental conditions, the presented studies broaden the recent knowledge on the role of adipose tissue in pathophysiology of insulin resistance in humans. Type 2 diabetic patients, healthy subjects and also patients with IFG differed in terms of baseline plasma concentrations and SAT expressions of selected adipokines and cytokines. We have also demonstrated differential group-specific regulations of adipokines' concentrations and expressions in response to hyperinsulinaemia and hypertriglyceridaemia as well as to treatment with losartan or telmisartan. The changes in adipokines' plasma concentrations are not accompanied by parallel changes in SAT expressions and thus other sources of circulating adipokines have to be involved in the regulation of the systemic adipokine balance. Nevertheless, the presented results support the role of adipokines in the pathogenesis of IR. Even though we brought some light in this topic, there are still shadows remaining that need to be cleared to make the field of insulin resistance as blue as a sky.

ABBREVIATIONS

- AII: angiotensin II
- ACE: angiotensin-converting enzyme
- ACEI: angiotensin-converting enzyme inhibitor
- AdipoR: adiponectin receptor
- A-FABP: adipocyte fatty acid-binding protein
- AMP: adenosin monophosphate-activated protein
- ANOVA: analysis of variance
- ARB: angiotensin II type 1 receptor blockade
- ARBs: angiotensin II type 1 receptor blockers
- AT-1 receptor: angiotensin II type 1 receptor
- AT-2 receptor: angiotensin II type 2 receptor
- AT-HEC: hyperinsulinaemic euglycaemic clamp after ARB
- ATP: adenosin triphoshate
- BMI: body mass index
- C: healthy control subjects
- CCR: chemokine CC motif receptor
- CI: confidence interval
- CNS: central nervous system
- D: type 2 diabetic subjects
- E-FABP: epidermal fatty acid-binding protein
- ER: endoplasmic reticulum
- FABPs: fatty acid-binding proteins
- GLUT-4: glucose transporter type 4
- HbA1c: haemoglobin A1c
- HEC: hyperinsulinaemic-euglycaemic clamp
- HMW adiponectin: high-molecular weight adiponectin
- HOMA: homeostasis model assessment
- IFG: impaired fasting glucose
- IGT: impaired glucose tolerance
- IKK: IκB kinase
- IR: insulin resistance
- IRI: immunoreactive insulin
- IRS: insulin receptor substrate
- IL: interleukin
- IL-1ra: interleukin 1 receptor antagonist
- Jak: Janus kinase
- JNK: c-Jun NH2-terminal kinase
- KCl: potassium chloride
- LSD: least significant difference
- MAP-kinase: mitogen activated protein kinase
- MAP kit: Multianalyte profiling kit
- M: glucose disposal
- MCR: metabolic clearance rate of glucose
- MCP-1: monocyte chemoattractant protein 1
- M/I and MCR/I: insulin sensitivity indices
- MIP-1α: macrophage inflammatory protein 1α
- NAFLD: non-alcoholic fatty liver disease
- NaCl: natrium chloride
- NEFA: non-esterified fatty acids
- NF-κB: nuclear factor kappa B
- NGT: normal glucose tolerance
- NO: nitric oxide
- NS: not significant
- OGTT: oral glucose tolerance test
- PEPCK: phosphoenolpyruvate carboxy-kinase
- PI3-kinase: phosphatidylinositol-3 kinase
- PKC: protein kinase C
- PPAR: peroxisome proliferator-activated receptor
- PTP1B: protein tyrosine phosphatase 1B
- RAS: renin –angiotensin system
- RANTES: Regulated on Activation, Normal T cell Expressed and Secreted
- RBP4: retinol-binding protein 4
- ROS: reactive oxygen species
- RT-PCR: real-time polymerase chain reaction
- SAL: infusion of natrium chloride 0.9% solution
- SAT: subcutaneous adipose tissue
- SEM: standard error of the mean
- SOCS-3: suppressor of cytokine signalling-3
- SREBP-1c: sterol response element binding protein 1c
- TG: triglycerides
- TxA_2/PGH_2: thromboxane A_2/prostaglandin endoperoxide H_2
- TNFα: tumor necrosis factor α
- TZDs: thiazolidinediones
- UPR: unfolded protein response
- VEGF: vascular endothelial growth factor

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APPENDIX

Appendix 1

Krušinová E, Klementová M, Kopecký jr. J, Wohl P, Kazdová L, Mlejnek P, Pravenec M, Hill M, Pelikánová T: Effect of acute hyperinsulinaemia with and without angiotensin II type 1 receptor blockade on resistin and adiponectin concentrations and expressions in healthy subjects. *Eur J Endocrinol*. 2007 Oct; 157(4): 443-9 **(IF 3.145)**

Appendix 2

Švehlíková E, Klementová M, Kratochvílová S, Wohl P, Kazdová L, Mlejnek P, Pravenec M, Hill M, Pelikánová T: Adipocyte fatty acid binding protein in type 2 diabetes mellitus – effects of hyperinsulinaemia and acute angiotensin II type 1 receptor blockade. *Diabetologia* **(IF 6.41)** - *submitted*

Appendix 3

Wohl P, Krušinová E, Hill M, Kratochvílová S, Zídková K, Kopecky J, Neškudla T, Pravenec M, Klementová M, Vrbíková J, Wohl P, Mlejnek P, Pelikánová T: Effect of telmisartan on selected adipokines, insulin sensitivity and substrate utilization during insulin-stimulated conditions in patients with metabolic syndrome and impaired fasting glucose. *Eur J Endocrinol*. 2010 Oct; 163(4): 573-83. Epub 2010 Jul 14. **(IF 3.539)**

Appendix 4

Kopecký jr J, Krušinová E, Klementová M, Kazdová L, Mlejnek P, Pravenec M, Hill M, Pelikánová T: Selected adipokines - plasma concentrations and adipose tissue expressions during 24-hour lipid infusion in healthy men. *Physiol Res.* 2010; 59(1):89-96. Epub 2009 Feb 27. **(IF 1.505)**

Appendix 5

Krušinová E, Pelikánová T: Fatty acid binding proteins in adipose tissue: a promising link between metabolic syndrome and atherosclerosis? *Diabetes Res Clin Pract*. 2008 Dec 15; 82 Suppl 2:S127-34. Epub 2008 Oct 31. Review. **(IF 1.823)**

Appendix 1

Effect of acute hyperinsulinaemia with and without angiotensin II type 1 receptor blockade on resistin and adiponectin concentrations and expressions in healthy subjects

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CLINICAL STUDY

Effect of acute hyperinsulinaemia with and without angiotensin II type 1 receptor blockade on resistin and adiponectin concentrations and expressions in healthy subjects

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Abstract

Objective: The potential insulin-sensitizing function of angiotensin II type 1 receptor blockade (ARB) with regard to selected adipokines is not fully explained so far. Our study aimed to explore the influence of acute hyperinsulinaemia and acutely induced ARB on resistin and adiponectin concentrations and expressions in healthy subjects.

Design and methods: Plasma adipokines were measured: 1) at 0, 30 and 240 min of hyperinsulinaemic (1 mU/kg per min) euglycaemic (5 mmol/l) clamp (HEC), and 2) during HEC after acute ARB (losartan 200 mg; AT-HEC) using the same protocol, in eight healthy subjects. Needle biopsy of abdominal s.c. fat was performed at 0, 30 and 240 min of both clamps to assess the adipokines' expressions.

Results: Comparing the glucose disposals of HEC and AT-HEC, no difference in insulin sensitivity was found. Plasma resistin increased equally during HEC and AT-HEC ($P<0.05$). The expression of resistin in s.c. fat increased during HEC ($P<0.05$), while no significant changes in expression were observed during AT-HEC. Plasma levels of adiponectin did not change during both clamps. Adiponectin expression increased during HEC ($P<0.05$), while it did not change during AT-HEC.

Conclusions: In healthy subjects, acute hyperinsulinaemia is associated with an increase in plasma resistin independently of ARB, while plasma adiponectin is not influenced by insulin or ARB. The expressions of both resistin and adiponectin in s.c. adipose tissue are stimulated by acute hyperinsulinaemia, whereas losartan attenuates their insulin-stimulated expressions. This suggests a potential effect of losartanon adipokines' expression.

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Introduction

Resistin is an adipocyte-secreted peptide hormone that has been proposed to link obesity with insulin resistance. It was identified as a factor impairing glucose homeostasis and inducing hepatic insulin resistance in mice (1). Recent studies in rodent models and humans show contradictory results (2–6). Mouse resistin is predominantly expressed in white adipose tissue, whereas human resistin expression in adipocytes is significantly lower and there are other important sources of resistin, e.g. mononuclear cells, endothelial, vascular smooth cells (2) or islets of Langerhans (7). The receptor for resistin, its signalling pathway, target tissues and its biological effect and regulation in human physiology have not been clearly identified up to now.

Adiponectin is an insulin-sensitizing adipokine specifically and abundantly expressed in adipose tissue (8, 9). It is present in the circulation at high concentrations in the form of different multimeric complexes, among which the high-molecular weight (HMW) multimers exert the predominant action. Two adiponectin receptors, AdipoR1 and AdipoR2, have been identified that up-regulate AMP-activated protein kinase activity. Adiponectin directly increases hepatic insulin sensitivity, promotes fuel oxidation in skeletal muscle and decreases vascular inflammation. Hypoadiponectinaemia is known to be consistently related to insulin resistance, obesity, type 2 diabetes, coronary heart disease, hypertension and atherosclerosis based on both experimental and clinical studies (10, 11). Up-regulation of adiponectin is a partial cause of the insulin-sensitizing action of thiazolidinediones.

Evidence has accumulated that insulin resistance may be improved by interrupting renin–angiotensin system (RAS). Angiotensin-converting enzyme (ACE) inhibitors and angiotensin II type 1 receptor antagonists are able to interfere with the adverse metabolic effects of angiotensin II on insulin signalling, tissue blood flow, oxidative stress, sympathetic activity or

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adipogenesis (12, 13). The antidiabetic properties of ACE inhibitors may be largely mediated through increases in bradykinin levels, nitric oxide and the GLUT4 glucose transporter (13).

The effect of angiotensin II type 1 receptor blockade (ARB) on glucose metabolism and insulin resistance remains controversial, and the mechanisms are not fully understood. Experimental and *in vitro* studies showed that ARB may improve insulin sensitivity via decreasing adipocyte size (14), promotion of adipocyte differentiation and preventing ectopic lipid deposition (15). Several randomized, placebo-controlled studies using the HEC technique have found little positive or no effect of ARB on insulin action in rodents (16, 17) and humans (18, 19). Clinical trials using ARB have provided indirect support for the possibility that ARB *per se* might improve insulin sensitivity and decrease incidence of type 2 diabetes (LIFE, CHARM and VALUE) (20).

It has been shown that treatment with losartan does not influence plasma leptin levels (21) and that RAS blockade (22) or losartan (23) increases adiponectin levels, but the effect of losartan on expressions of adipokines in human adipose tissue under conditions of hyperinsulinaemia has not been studied yet.

The aims of our study were twofold: to determine resistin and adiponectin plasma concentrations together with their expressions in abdominal s.c. adipose tissue and also to test their responses to acutely induced hyperinsulinaemia with and without acute ARB in healthy subjects.

Subjects and methods

Subjects

We examined eight healthy men who had normal glucose tolerance (confirmed by an oral glucose tolerance test), blood pressure and serum lipids. Only male subjects participated because of the variable insulin sensitivity in women according to the menstrual cycle and with respect to sexual dimorphism of plasma adiponectin levels. Clinical and physiological characteristics of the study group are summarized in Table 1. The subjects were not taking any drugs and none had a family history of diabetes. They were euthyroid and none had a concomitant disease. Informed consent was obtained from all the individuals after the purpose, nature and potential risks of the study had been explained. The study was approved by the local ethics committee.

Study protocol

The subjects were examined on an outpatient basis, after overnight fasting with only tap water allowed *ad libitum*. They were instructed to adhere to their ordinary lifestyle, avoid any changes in food intake or alcohol consumption and to refrain from strenuous physical activity for a period of 24 h before the experiment. The subjects underwent two HECs at least 4 weeks apart, both taking 4 h to complete, in random order:

Table 1 Clinical and physiological characteristics of the study group.

$n=8$	Mean ± S.E.M.
Age (years)	25.75 ± 1.29
Body mass index (kg/m^2)	24.96 ± 0.60
Waist circumference (cm)	88.50 ± 1.78
Fasting blood glucose (mmol/l)	4.51 ± 0.23
Blood glucose at 120 min of OGTT (mmol/l)	4.55 ± 0.24
Fasting IRI (mIU/l)	4.15 ± 0.91
HbA1c (%), according to IFCC	2.58 ± 0.22
Albuminuria (μg/min)	2.52 ± 0.91
Creatinine (μmol/l)	86.95 ± 3.71
Total cholesterol (mmol/l)	4.24 ± 0.26
Triglycerides (mmol/l)	0.87 ± 0.11
HDL cholesterol (mmol/l)	1.45 ± 0.12
LDL cholesterol (mmol/l)	2.48 ± 0.20

Study 1 The HEC was conducted as previously described (24). Briefly, a Teflon cannula (Venflon; Viggo, Helsingborg, Sweden) was inserted into an antecubital vein for the infusion of all test substances. A second cannula was inserted retrogradely into a wrist vein for blood sampling and the hand was placed in a heated (65 °C) box in order to achieve venous blood arterialization. A primed continuous insulin infusion (1 mU/kg per min of Actrapid HM (NovoNordisk, Copenhagen, Denmark) dissolved in 0.9% NaCl) was administered to acutely raise and maintain the plasma concentrations of insulin. Plasma glucose concentrations during the clamp were maintained at the 5 mmol/l level by continuous infusion of 15% glucose. To prevent hypokalaemia during insulin infusion, potassium chloride was added to the 15% glucose infusion (30 mmol/l KCl). To assess plasma levels of selected adipokines, blood samples were taken at 0, 30 and 240 min of the clamp study.

Needle biopsy of abdominal s.c. adipose tissue was performed at 0, 30 and 240 min of the clamp. Under local anaesthesia (1% trimecain in a field block pattern), an incision (3–4 mm) was made through the skin at the lower abdomen and an s.c. fat specimen (300 mg) was obtained by needle aspiration. The samples were washed in 0.9% natrium chloride solution, immediately frozen by liquid nitrogen and stored at −80 °C until used for RNA extraction.

Study 2 An identical clamp study was performed after acute ARB (AT-HEC). Losartan 2×100 mg was given *per os* prior to the study (8 h and immediately prior to the clamp) and a hyperinsulinaemic (1 mU/kg per min) euglycaemic (5 mmol/l) clamp was conducted as described previously. Blood samples and adipose tissue biopsies were collected at 0, 30 and 240 min of the

clamp to determine plasma concentrations and expressions of selected adipokines.

Analytical methods

Plasma concentrations of glucose were measured using the Beckman analyzer (Beckman Instruments Inc., Fullerton, CA, USA) with the glucose oxidase method. Immunoreactive insulin (IRI) was determined by the RIA method using an IMMUNOTECH Insulin IRMA kit (IMMUNOTECH AS, Prague, Czech Republic) with an analytical sensitivity 0.5 μIU/ml, and intra-assay and inter-assay coefficients of variation below or equal to 4.3 and 3.4% respectively. Glycosylated haemoglobin was measured by HPLC method using the Variant II HbA1c Program (Bio-Rad Laboratories GmbH), and for calibration, a method approved by International Federation of Clinical Chemistry and Laboratory Medicine was used (25). Plasma concentrations of resistin were measured according to manufacturer's instructions by Human Resistin ELISA kit (BioVendor Lab. Med. Inc., Brno, Czech Republic; the detection limit was 0.033 ng/ml and intra-assay and inter-assay coefficients of variation are 2.8–3.4 and 5.1–6.9% respectively). For analysis of total adiponectin plasma levels, Human Adiponectin ELISA kit was used (BioVendor Lab. Med. Inc.; the detection limit was 210 ng/ml and intra-assay and inter-assay coefficients of variation are 6.4–7.0 and 7.3–8.2% respectively).

Expression of adipokines was analysed by the real-time PCR (RT-PCR) using the following protocol:

(1) The RNA was isolated from the liquid nitrogen frozen biopsy of the human fat tissue using the RNeasy Lipid Tissue Mini Kit and QIAzol Lysis Reagent (Qiagen). The starting amount of 100 mg tissue was excised from the biopsy, and homogenized in 1 ml QIAzol Lysis Reagent (guanidine thiocyanate–phenol solution) for 2 min. From the homogenate, the RNA was isolated by extraction on silica gel-based column, according to the kit handbook. Possible contamination of RNA with genomic DNA remains was taken off by DNase digestion (RNase-free DNase Set; Qiagen). This step is supposed to prevent any later DNA amplification. Beyond this, the denaturation curves were measured during each reaction and there was just one product present in all the measurements.

(2) The cDNA was synthesized using a recombinant Omniscript Reverse Transcriptase (Qiagen), RNase inhibitor from human placenta (Sigma) and $(dT)_{16}$ oligonucleotides.

(3) RT-PCR procedure itself was carried out on the DNA Engine Opticon 2 System (MJ Research, Waltham, MA, USA). HotStar Taq DNA polymerase and SYBR Green fluorescent dye (QuantiTec SYBR Green PCR Kit; Qiagen) were used for the RT-PCR procedure. To eliminate the influence of primer dimers, negative controls were used. To account for differences in cDNA loading, the results were expressed relative to the expression of human cyclophilin (used as a reference gene). There is no evidence of insulin or ARB influence on cyclophilin, which is a housekeeping gene encoding a cytoskeleton protein. Primers used for RT-PCR are given in Table 2.

(4) The data were processed by Q-gene 96 software.

Calculations

Insulin action was estimated as the glucose disposal (M) calculated during the last 30 min of the clamp as the rate of glucose infusion after correction for changes in glucose pool size and urinary glucose loss. Insulin sensitivity index M/I (glucose disposal normalized by plasma insulin during steady-state period) was calculated to correct for any variations in plasma insulin.

Statistical analysis

The data are expressed as mean ± S.E.M., unless provided otherwise. Steady-state periods of both clamps were compared using Wilcoxon's paired test. The differences between time courses of both clamps were evaluated by repeated measures ANOVA model, including following factors and interactions: effects of treatment (HEC versus AT-HEC) and time (the effect of hyperinsulinaemia) as the within factors, subject factor (represents the interindividual variability of subjects) and treatment × time interaction. The last term indicated if the shapes of the time profiles for HEC and AT-HEC were different or not. The differences between subgroups were evaluated

Table 2 Primers used for RT-PCR.

Gene	Accession number	Forward primer	Reverse primer
Resistin	AY207314	HRETN-F: 5′-ATA AGC AGC ATT GGC CTG G-3′	HRETN-R: 5′-TGG CAG TGA CAT GTG GTC T-3′
Adiponectin	XM_290602	HACRP30-F: 5′-GGT TCA ATG GCT TGT TTG C-3′	HACRP30-R: 5′-TCA TCC CAA GCT GAT TCT G-3′
Cyclophilin	XM_090070	HCLPNa-F: 5′-CAA ATG CTG GAC CCA ACA CA-3′	HCLPNa-R: 5′-TGC CAT CCA ACC ACT CAG TC-3′

using least significant difference multiple comparisons. A statistical significance of $P<0.05$ was chosen for both ANOVA testing and multiple comparisons. Due to non-Gaussian data distribution in all dependent variables, the data underwent power transformations to attain distributional symmetry and a constant variance in the data as well as in residuals. The non-homogeneities were detected using residual diagnostics. The experimental points with absolute values of studentized residual (after data transformation) >3 were excluded from the analysis. The fraction of such points never exceeded 5% of the total number. Statistical software Statgraphics Plus v. 5.1 (Manugistics; Rockville, MD, USA) was used for the data analysis.

Results

Acute ARB had no impact on diuresis, systolic and diastolic blood pressure in our study group (data not shown).

During the steady-state periods of HEC versus AT-HEC, the clamps were comparable in terms of the mean plasma glucose concentrations (4. 68±0.32 vs 4.75±0.20 mmol/l) with coefficient of variation 3.37±0.55 vs 3.42±1.87%. Although the mean IRI levels were different when comparing both the clamps (65.11±7.53 vs 75.28±6.95 μIU/ml; $P<0.05$), the parameters of insulin sensitivity did not significantly differ between HEC and AT-HEC, being expressed as the glucose disposal (*M*; 9.55±0.56 vs 9.15±1.68 mg/kg per min), as well as calculated as the insulin sensitivity index *M*/*I* (0.15±0.01 vs 0.13±0.01 mg/kg per min/μIU per ml).

Plasma concentrations of resistin during HEC and AT-HEC are shown in Fig. 1a. The ANOVA model indicated a significant increase in plasma resistin during both clamps. The time factor was significant ($P<0.05$), while the treatment factor as well as the interaction between the factors time and treatment were not. Despite the multiple comparisons reaching significance only for the difference between basal value and 240 min for AT-HEC ($P<0.05$), the time trend did not significantly differ between the clamps.

Figure 1b shows relative expressions of resistin. Here, the time effect, as well as the treatment effect, was not significant. On the other hand, the shapes of the expressions' time profiles differed significantly between HEC and AT-HEC (treatment×time interaction; $P<0.05$): resistin expression increased during HEC, as also documented by multiple comparisons (0 vs 240 min; $P<0.05$), while during AT-HEC the expression did not change. Moreover, at 240 min of AT-HEC, resistin expression was significantly lower when compared with HEC ($P<0.05$ by multiple comparisons).

In plasma adiponectin, no factor or interaction reached significance (Fig. 2a). In addition, the multiple comparisons did not show any significant differences

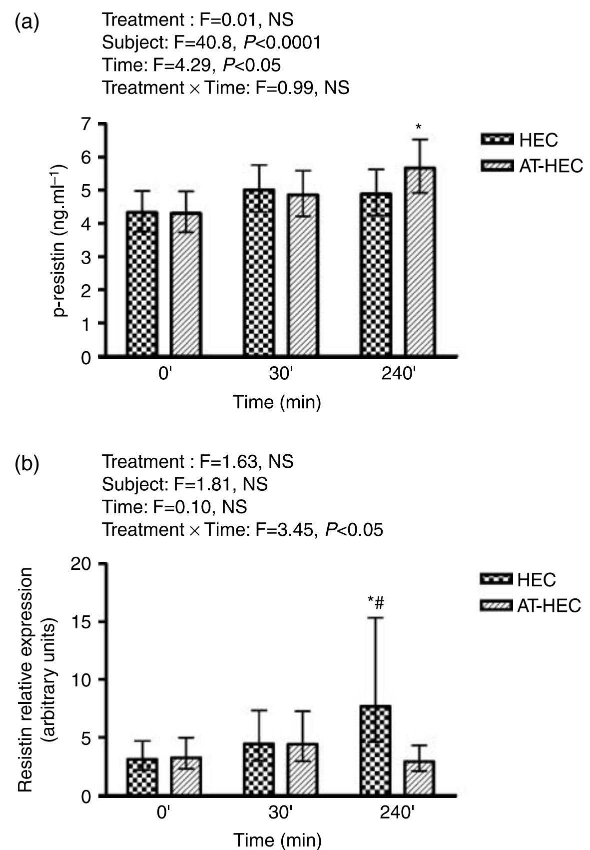

Figure 1 Resistin during HEC and AT-HEC as (a) plasma concentration and (b) relative expression levels. Data shown as retransformed means with 95% confidence intervals. F, Fisher's statistics for individual factors and interactions. *$P<0.05$ between basal and stimulated values, #$P<0.05$ between HEC and AT-HEC in individual stages.

between clamps for basal (0 min) or stimulated (240 min) values.

Figure 2b represents relative expressions of adiponectin. In this variable, both time and treatment effects were not significant. However, the shapes of the time profiles differed between HEC and AT-HEC (as documented by significant treatment×time interaction; $P<0.05$). Adiponectin expression increased during HEC, as also confirmed by multiple comparisons (0 vs 240 min; $P<0.05$), while it did not change during AT-HEC. Multiple comparisons found no significant differences between clamps for basal (0 min), as well as stimulated (240 min) values.

Discussion

Our study shows that in healthy subjects, acute hyperinsulinaemia induces an increase in resistin plasma concentration and stimulates the expression of resistin in s.c. adipose tissue, which is a novel observation *in vivo*. Our finding is consistent with

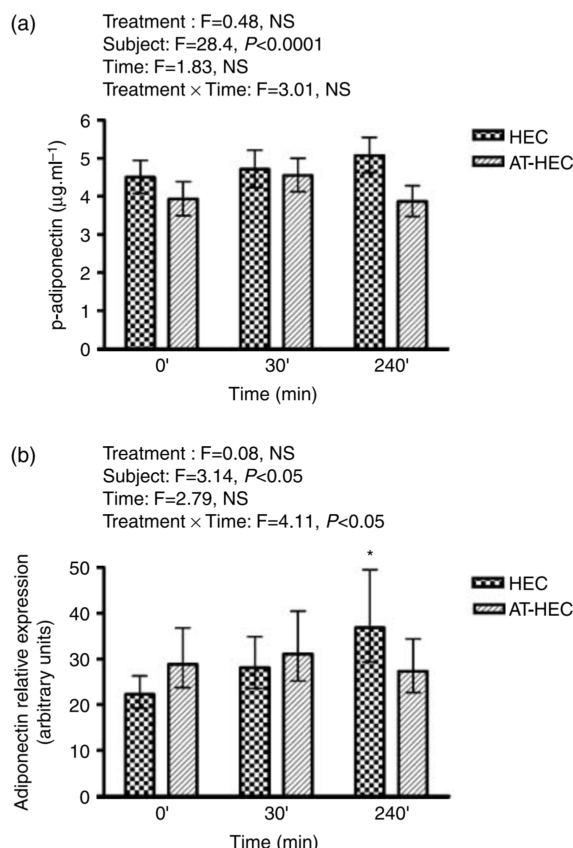

Figure 2 Adiponectin during HEC and AT-HEC as (a) plasma concentration and (b) relative expression levels. Data shown as retransformed means values with 95% confidence intervals. F, Fisher's statistics for individual factors and interactions. $^*P < 0.05$ between the basal and stimulated values.

several studies in rodent models (2) or *in vitro* studies (26, 2). To the contrary, other groups showed the opposite in mice and 3T3-L1 adipocytes (2). Few studies have investigated the effect of insulin *in vivo* in humans; Heilbronn (27) observed an increase in serum resistin concentrations in response to supraphysiological doses of insulin (164 ± 5 mIU/l) in obese subjects with and without diabetes. However, clinical studies in humans do not show a consistent link between serum resistin and either insulin resistance or obesity (3–6).

In our study group, adiponectin plasma concentrations were not affected by hyperinsulinaemia, whereas its expression was stimulated by insulin. These findings are in agreement with recent evidence that circulating adiponectin *per se* is not a subject for acute regulation, but its expression seems to be regulated acutely (9), preceding thereby systemic changes. Consistent results *in vivo* were shown by Westerbacka *et al.* (28), who found an increased adiponectin expression in s.c. fat after 6 h of hyperinsulinaemia in insulin-sensitive subjects, which was not accompanied by any changes in serum levels of adiponectin. However, in the literature, a 7–8% decline of circulating adiponectin during hyperinsulineamia in healthy men (29–31) is reported, which is more pronounced (16–20% decrease) when a twofold higher insulin infusion rate is used (32, 33). To the contrary, other groups did not find any changes in plasma adiponectin in response to insulin (34, 35), which is in agreement with our results. The source of such discrepancies might lie partly in diverse analyses that were used or different ethnic groups that were studied (there is a broad range of reported absolute values for serum adiponectin: 2–20 μg/ml). Another point that should be specified accurately is a potential confounding factor of haemodilution during clamp. To further distinguish the regulations *in vivo*, it will be necessary to measure the adiponectin isoforms selectively, since the HMW form is known to be the most active one (8) and it correlates better with glucose disposal (36).

In accordance with previously mentioned study (28), we report an increase in adiponectin mRNA in s.c. adipose tissue in response to insulin *in vivo*. A 24-hour insulin treatment induced an increase in adiponectin mRNA in isolated human visceral adipose tissue (37), but a reduction of adiponectin expression was detected in 3T3-L1 adipocytes (38). Although the secretion of adiponectin from visceral and s.c. depots appears comparable (39), the regulations may exhibit some depot specificity (9). It will be important to further elucidate the effect of insulin on adiponectin production in different fat depots and in different stages of insulin resistance.

Acute ARB did not change the parameters of insulin sensitivity in our study group, that can be defined as insulin sensitive. Interruption of angiotensin II signalling was reached by administration of losartan $2\times$ 100 mg total, which ensured linear pharmacokinetics within the biological period of the drug (2 h) and its active metabolite (6–9 h). However, the plasma levels may not mirror effective tissue concentrations, which are crucial for potential metabolic effects. Recently, it was proved that the active losartan metabolite EXP3179 also shows a partial PPARγ agonistic effect (40).

The effect of acute ARB on insulin sensitivity has not been studied in humans thus far; clinical trials using long-term administration of ARBs (other than telmisartan) with the HEC technique reported both increases (41, 22) and no effect (42, 43) on glucose disposal in insulin-resistant subjects. Hence, it is necessary to compare our findings in healthy controls with insulin-resistant subjects.

Independent of ARB, plasma concentrations of resistin increased during hyperinsulinaemia, and adiponectin plasma levels were not influenced. To the contrary, the insulin-stimulated expressions of resistin and adiponectin in s.c. adipose tissue were attenuated by losartan.

Our finding of increased plasma resistin in spite of decreased expression in s.c. fat implicates a role of another source of circulating resistin – a different fat depot or potential non-adipose sources (e.g. stromal vascular fraction of adipose tissue, macrophages or endothelium). Other studies in rodents and humans

(2, 5) found increased expression of resistin in visceral fat depots when compared with s.c. at basal conditions, although the source of resistin was thought to be non-adipocytes. Nevertheless, the attenuation of insulin-stimulated resistin expression following acute ARB implies a positive role of losartan in endocrine activity of adipose tissue.

An increase in circulating adiponectin after 2-month treatment with losartan in hypertensive patients has been reported (23), and there are other experimental data showing enhanced adiponectin expression in response to long-term ARB (44, 45), possibly via PPARγ activation. All the experiments and clinical studies tested different ARB substances in different models of insulin resistance, and there is no evidence about the regulations in healthy subjects. We have observed that acute losartan administration attenuates the insulin-induced expression of adiponectin. This unexpected trend in adiponectin expression should be proven in long-term treatment to distinguish acute and long-term regulations *in vivo*. However, the limited sample size of our study should be considered as well. Further studies are needed to support the hypothesis that losartan exerts its positive effects on insulin sensitivity through other mechanisms than stimulation of adiponectin.

Recently, it was proven that the beneficial metabolic effect of ARB goes beyond simple interruption of RAS (12). Our results suggest potential effects of losartan on resistin and adiponectin expression that might influence further metabolic or proinflammatory factors.

Paradoxically, higher insulin plasma levels were detected in the steady-state period of AT-HEC when compared with HEC. There were no changes in body weight between the clamps, and therefore, the insulin infusion rates were comparable for both clamps. Higher insulin levels after losartan administration cannot be explained by involvement of insulin secretion, because angiotensin II receptor activation, not inhibition, was shown to stimulate insulin secretion (46). Impairment of insulin clearance in liver by ARB cannot be excluded, but there is no corresponding data available. However, the beneficial effects of losartan in our study were seen despite higher insulin levels.

We can conclude that in healthy subjects, acute hyperinsulinaemia stimulates an increase in plasma concentration and expression of resistin in s.c. adipose tissue. While the ARB does not modify the insulin-induced changes in plasma resistin, it attenuates the response of resistin expression in adipose tissue. Acute hyperinsulinaemia is associated with an increase in adiponectin expression, but not in its plasma levels. Losartan reduces the insulin-stimulated expression of adiponectin.

These findings have to be further investigated in larger cohorts in comparison with insulin-resistant subjects, by short- or long-term ARB administration, together with analysis of non-adipose sources of resistin.

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Appendix 2

Adipocyte fatty acid binding protein in type 2 diabetes mellitus – effects of hyperinsulinaemia and acute angiotensin II type 1 receptor blockade

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Abstract

***Aims/hypothesis*:** To explore the role of adipocyte fatty acid-binding protein (A-FABP, coded by *FABP4* gene) in insulin resistance, we investigated its plasma concentrations and expressions in subcutaneous adipose tissue (SAT) in response to hyperinsulinaemia and acute angiotensin type 1 receptor blockade (ARB) in type 2 diabetes.

Methods: Eleven type 2 diabetic patients and 12 healthy age-matched controls underwent: 1) hyperinsulinaemic-euglycaemic clamp (HEC); 2) HEC after acute losartan treatment (AT-HEC); 3) saline infusion (SAL) as a control examination. At baseline and 240 min of the interventions, plasma A-FABP was measured and biopsies of abdominal SAT were performed to analyse gene expression.

Results: In diabetes, A-FABP concentrations were higher compared to controls ($p<0.001$), they showed a parallel decrease during both clamps, but no changes during SAL; during AT-HEC plasma A-FABP was increased. In controls, hyperinsulinaemia prevented the increase in plasma A-FABP detected during SAL; in AT-HEC increased A-FABP basal concentrations were reversed at 240 min. *A-FABP* expressions were higher in diabetes ($p<0.001$) without any dynamic changes in either group. Plasma A-FABP correlated with its expression ($p<0.01$) and both correlated with BMI, waist circumference, glycaemia, insulinaemia and glucose disposal. *E-FABP* (epidermal-FABP, coded by *FABP5* gene) showed higher expressions in controls ($p<0.01$).

Conclusions/interpretation: Both A-FABP plasma concentrations and expressions are increased in type 2 diabetes. They are independently associated with parameters of obesity, insulin resistance and hyperglycaemia. Acute hyperinsulinaemia suppresses plasma A-FABP but does not influence its expression in SAT. Acute ARB stimulates basal A-FABP plasma concentrations without any effect on its expression.

Trial registration: ClinicalTrials.gov NCT01011062

Keywords: Adipocyte fatty acid binding protein, adipocyte, type 2 diabetes, insulin resistance, glucose clamp

Abbreviations:

ACEI: inhibitors of angiotensin-converting enzyme
A-FABP: adipocyte fatty acid-binding protein
ARB: angiotensin type 1 receptor blockade
AT-HEC: hyperinsulinaemic euglycaemic clamp after ARB
C: healthy control subjects
D: type 2 diabetic subjects
E-FABP: epidermal fatty acid-binding protein
FABPs: fatty acid-binding proteins
HEC: hyperinsulinaemic-euglycaemic clamp
IRI: immunoreactive insulin
KCl: potassium chloride
LSD: least significant difference
M: glucose disposal
NaCl: natrium chloride
OPLS: orthogonal projections to latent structures
PPAR-γ: peroxisome proliferator-activated receptor gamma
RT-PCR: real-time polymerase chain reaction
SAL: infusion of natrium chloride 0.9% solution
SAT: subcutaneous adipose tissue
TG: triacylglycerol

Introduction

Low-grade inflammation is considered to be a key feature of obesity and type 2 diabetes, playing a significant role in the pathogenesis of metabolic abnormalities related to insulin resistance [1]. Due to its secretory functions, adipose tissue represents a critical site for metabolic-inflammatory interactions. Indeed, the disturbed endocrine activity of adipose tissue is consistently found in obesity and type 2 diabetes [2].

An important molecular pathway, which integrates metabolic and inflammatory response involves the fatty acid-binding proteins (FABPs) commonly present in adipocytes and macrophages in two isoforms – adipocyte FABP (A-FABP) and epidermal FABP (E-FABP) coded by *FABP4* and *FABP5* genes, respectively [3]. As cytoplasmic lipid chaperons FABPs are responsible for cellular trafficking of fatty acids. Moreover, experimental evidence, based on comprehensive research on knock-out mice models, supports the role of A-FABP in systemic regulation of lipid and glucose metabolism and inflammation, since A-FABP deficiency prevents the development of obesity, insulin resistance and atherosclerosis [4-6]. In human studies, A-FABP was found to be also present in plasma [7], although its physiological function or mechanisms of its appearance in circulation have not been elucidated until now. A-FABP plasma concentrations are increased in patients with obesity and/or metabolic syndrome [7-9] and it is suggested as a novel risk marker predicting development of metabolic syndrome [8] or type 2 diabetes [10]. On the contrary, clinical studies focused on adipose tissue expression are inconclusive [11] – they report no differences in A-FABP expression or a decrease in E-FABP expression in obese subjects [12-14]. Additionally, no consistent association between A-FABP expression and measures of obesity or insulin resistance has been found [13, 15]. Plasma concentrations and adipose tissue expression of A-FABP have never been shown in parallel so far. Similarly, there is little evidence about their regulation by insulin in vivo in humans or about possible differences in these regulations between healthy subjects and patients with type 2 diabetes.

A number of experimental and clinical studies indicate that angiotensin type 1 receptor blockade (ARB) might improve insulin resistance and thereby decrease incidence of new onset type 2 diabetes [16-18]. The underlying mechanisms are not fully clarified. Apart from inhibition of adverse metabolic effects of angiotensin II on insulin signalling, tissue blood flow, oxidative stress, sympathetic activity, pancreatic beta cell dysfunction or adipogenesis [16, 19], ARB is supposed to dispose of effects that extend beyond the angiotensin-receptor blockade [20], depending on properties of particular ARB compounds, e.g. activation of peroxisome proliferator-activated receptor gamma (PPAR-γ). Experimental studies showed that ARB influences lipid metabolism in adipose tissue by promoting adipose tissue rearrangement, decreasing adipocyte size and modulating adipokine expression and release [21-23].

We hypothesized that ARB would influence A-FABP plasma concentrations or its expressions due to the potential effects of ARB in adipose tissue metabolism. Therefore the aims of this study were: 1) to assess A-FABP plasma concentrations in parallel with expressions of *A-FABP* and selected related genes (*E-FABP, PPAR-γ*) in abdominal subcutaneous adipose tissue (SAT); and 2) to investigate their responses to acutely induced hyperinsulinaemia with and without acute ARB in patients with type 2 diabetes and healthy control subjects.

Subjects and methods

Subjects

Eleven overweight/obese male patients with type 2 diabetes and 12 age-matched healthy male subjects were enrolled in the study. Clinical characteristics of both groups are summarized in Table 1. The type 2 diabetic patients (D) were treated with diet or oral agents (except of thiazolidinediones) in stable dosing over three months prior to the study. Subjects treated with insulin, having micro- or macrovascular complications of diabetes, acute or chronic inflammatory or other major organ diseases were excluded from the study. Six of the diabetic patients were treated with antihypertensive drugs (calcium-channel blockers, diuretics or angiotensin-converting enzyme inhibitors- ACEI). The ACEIs were replaced with calcium-channel blockers two weeks prior to clamp procedures and maintained for the whole study duration.

Healthy control subjects (C) had normal glucose tolerance confirmed by OGTT, normal blood pressure and serum lipids. None had a family history of diabetes. All control subjects were euthyroid without any concomitant disease or medication. Informed consent was obtained from all the individuals after explaining the purpose, nature, and potential risks of the study. The local Ethics committee approved the study protocol.

Variable	C (n=12)	D (n=11)	*p* value
Age (years)	48.75 ± 1.45	50.27 ± 1.38	NS
Weight (kg)	93.73 ± 2.48	79.0 ± 3.77	**
Body mass index ($kg.m^{-2}$)	25.65 ± 1.16	30.23 ± 0.60	**
Waist circumference (cm)	90.91 ± 3.00	100.91 ± 2.71	*
Fasting blood glucose ($mmol.l^{-1}$)	5.2 ± 0.08	7.56 ± 0.53	**
Blood glucose at 120 min of OGTT ($mmol.l^{-1}$)	5.56 ± 0.34	-	
Fasting insulin ($\mu U.ml^{-1}$)	5.50 ± 0.70	8.42 ± 1.05	*
HbA1c (%)	5.46 ± 0.09	6.48 ± 0.41	*
Albuminuria ($\mu g.min^{-1}$)	3.66 ± 0.84	5.54 ± 1.54	NS
Creatinine ($\mu mol.l^{-1}$)	87.37 ± 1.88	80.83 ± 2.30	NS
Total cholesterol ($mmol.l^{-1}$)	4.81 ± 0.20	5.60 ± 0.27	*
Triacylglycerol ($mmol.l^{-1}$)	1.14 ± 0.15	2.61 ± 0.49	*
HDL-cholesterol ($mmol.l^{-1}$)	1.19 ± 0.04	1.09 ± 0.04	NS
LDL-cholesterol ($mmol.l^{-1}$)	3.22 ± 0.19	3.59 ± 0.17	NS
Fasting NEFA ($mmol.l^{-1}$)	0.284 ± 0.04	0.502 ± 0.12	NS

Table 1. Clinical and physiological characteristic of the study groups. Data are means ± SEM.
* $p<0.05$, ** $p<0.01$;

Study protocol

The subjects were examined on an outpatient basis, after overnight fast with only tap water allowed ad libitum. They were instructed to adhere to their ordinary lifestyle, avoid changes in food intake, alcohol consumption and to refrain from strenuous physical activity for 24 hours before the experiment. In random order the subjects underwent three examinations at least 3 weeks apart, all taking 4 hours to complete:

Study 1. The hyperinsulinaemic euglycaemic clamp (HEC) was conducted as previously described [24]. Briefly, a Teflon cannula (Venflon; Viggo, Helsingborg, Sweden) was inserted into an antecubital vein for administration of infusions. A second cannula was inserted retrogradely into a wrist vein for blood sampling and the hand was placed in a heated (65^0 C) box in order to achieve venous blood arterialization. A primed-continuous insulin infusion (1 $mU.kg^{-1}.min^{-1}$ of Actrapid HM; NovoNordisk, Copenhagen, Denmark - dissolved in 0.9% natrium chloride [NaCl]) was administered to acutely raise and maintain the plasma concentrations of insulin. Plasma glucose concentrations during the clamp were maintained at the 5 $mmol.l^{-1}$ level by continuous infusion of 15% glucose. To prevent hypokalaemia during insulin infusion, potassium chloride (KCl) was added to the 15% glucose infusion (30 $mmol.l^{-1}$ KCl). To assess plasma levels of A-FABP, blood samples were obtained at 0 min and 240 min of the clamp study.

Needle biopsy of abdominal SAT was performed at 0 min and 240 min of the clamp. Under local anaesthesia (1% trimecain in a field block pattern) an incision (3-4 mm) was made through the skin at the lower abdomen and a subcutaneous fat specimen (300 mg) was obtained by needle aspiration. The samples were washed in 0.9% NaCl solution, immediately frozen by liquid nitrogen and stored at -80ºC until used for RNA extraction.

*Study 2.*An identical clamp study was performed after acute ARB (AT-HEC). Losartan 2x 100mg was given *per os* prior to the study (8 hours and immediately prior to the clamp) and a hyperinsulinaemic (1 $mU.kg^{-1}.min^{-1}$) euglycaemic (5 $mmol.l^{-1}$) clamp was conducted as it is described above. Using the mentioned dosing, the interruption of angiotensin II signalling was reached due to losartan linear pharmacokinetics within the active biological period of the drug (2 hours) and its active metabolite (6-9 hours). Blood samples and adipose tissue biopsies were collected at 0 min and 240 min of the clamp to determine plasma concentrations of A-FABP and expressions of selected genes.

Study 3. A 0.9% NaCl infusion (SAL) was performed as a control examination in order to distinguish possible non-specific volume (haemodilution) and time effects during above described interventions

on plasma concentrations of proteins tested. The total volume of infusions and water equivalent to HEC was given *per os* and intravenously in the course of 4 hours. Blood samples for measurement of plasma A-FABP were taken at 0 min and 240 min. Based on an assumption that adipose tissue expressions are not affected by haemodilution during clamp, the adipose tissue biopsies were not repeated during SAL volume control.

Analytical Methods

Plasma glucose concentrations were measured on a Beckman analyzer (Beckman Instruments Inc, Fullerton, CA, USA) using glucose oxidase method. Immunoreactive insulin (IRI) was determined by radioimmunoassay method using an IMMUNOTECH Insulin IRMA kit (IMMUNOTECH as, Prague, Czech Republic) with analytical sensitivity 0.5 $\mu IU.ml^{-1}$, intra-assay and inter-assay coefficient of variation below or equal to 4.3% and 3.4%, respectively.

Plasma concentrations of A-FABP were measured according to manufacturer's instructions using Human A-FABP ELISA kit (BioVendor Lab. Med. Inc., Brno, Czech Republic), the detection limit was 0.1 $ng.ml^{-1}$, intra-assay and inter-assay coefficients of variation of 5.3% and 3.9%, respectively.

Plasma concentrations of NEFA were estimated by Half-micro test Free fatty acids (Roche Diagnostics GmbH, Penzberg, Germany). Triacylglycerol (TG) plasma concentrations were assessed by enzymatic assay (BIO-LA-TEST; PLIVA-Lachema, Brno, Czech Republic).

Relative expression of *A-FABP (FABP4)*, *E-FABP (FABP5)* and *PPAR-γ* was analysed by the real-time polymerase chain reaction (RT-PCR) method using following protocol:

1) The RNA was isolated from the liquid nitrogen frozen biopsy of the human fat tissue using the RNeasy Lipid Tissue Mini Kit and QIAzol Lysis Reagent (QIAGEN, Valencia, CA, USA). The starting amount of 100 mg tissue was excised from the biopsy and homogenized in 1 ml of a QIAzol Lysis Reagent (Guanidin Thiocyanate – Phenol solution) for 2 minutes. From the homogenate, the RNA was isolated by extraction on silica-gel based column, according to the kit handbook. Possible contamination of RNA with genomic DNA remains was taken off by DNase digestion (RNase-free DNase Set; QIAGEN, Valencia, CA, USA). This step is supposed to prevent any later DNA amplification.

2) The cDNA was synthesized using a recombinant Omniscript Reverse Transcriptase (QIAGEN, Valencia, CA, USA), Ribonuclease Inhibitor from human placenta (SIGMA, St. Louis, MO, USA), and $(dT)_{16}$ oligonucleotides.

3) RT-PCR procedure itself has been carried out on the DNA Engine Opticon 2 System (MJ Research, Waltham, MA, USA). HotStar Taq DNA polymerase and SYBR Green fluorescent dye (QuantiTec SYBR Green PCR Kit; QIAGEN, Valencia, CA, USA) were used for the RT-PCR procedure. To eliminate the influence of primer dimmers negative controls were used. To account for differences in cDNA loading, the results were expressed relative to the expression of human cyclophilin (used as a reference gene).Following primers were used for RT-PCR:

A-FABP:
forward primer 5'-ATGGCCAAACCTAACATGA-3'
reverse primer 5'-CAAATTCCTGGCCCAGTATG-3'

E-FABP:
forward primer 5'-AATGGCCAAGCCACATTGTA-3'
reverse primer 5'-CACTCCTGATGCTGA-3'

PPAR-γ:
forward primer 5'-GAGCCCAAGTTTGAGTTTGC-3'
reverse primer 5'-CTGTGAGGACTCAGGGTGGT-3'

Cyclophilin:
forward primer 5'-CAAATGCTGGACCCACA-3'
reverse primer 5'-TGCCATCCAACCACTCAGTC-3'

PPAR-γ primers were designed to cover all splicing variants of *PPAR-γ* mRNA.

4) The data were processed by Q-gene 96 software.

Calculations

Insulin action was estimated as the glucose disposal (M) calculated during the last 30 min of the clamp as the rate of glucose infusion after correction for changes in glucose pool size and urinary glucose loss.

Statistical analysis

The data are expressed as means ± SEM or mean (5% - 95% CI). The groups were compared using Mann-Whitney test. The differences between the interventions and between the groups were evaluated by repeated measures ANOVA model, including following factors and interactions: Effect of Group (D vs. C) as the between factor, Effects of Intervention (HEC vs. SAL and HEC vs. AT-HEC, respectively) and Time (0 min vs. 240 min) as the within-factors, Subject factor (represents the interindividual variability of subjects) and their interactions(Intervention × Time; Intervention × Group; Time × Group; Group × Intervention × Time). The interactions indicate if the shapes of the time profiles for HECvs. SAL or HEC vs. AT-HEC and for D and C, respectively were different or not.The differences between subgroups were evaluated using least significant difference (LSD) multiple comparisons. Additionally, separate ANOVA models for Diabetes and Control groups were

performed for comparison of the interventions within the group. The significances of the separate models and LSD are described in the text. The statistical significance of $p<0.05$ was chosen for both ANOVA testing and multiple comparisons. Due to non-Gaussian data distribution in all dependent variables, the data underwent power transformations to attain distributional symmetry and a constant variance in the data as well as in residuals. The non-homogeneities were detected using residual diagnostics. The experimental points with absolute values of Studentized residual (after data transformation) greater than 3, were excluded from the analysis. The fraction of such points never exceeded 5% of the total number. To evaluate relationships between the variables, Spearman correlations and multivariate regression with reduction of dimensionality using the method of bidirectional orthogonal projections to latent structure (O2PLS) were applied. For details of O2PLS model see Supplementary file.

Statistical software SIMCA v. 12.0.1.0. (Umetrics, Umeå, Sweden) and Statgraphics Centurion v. XV Statpoint Inc.; Herndorn, Virginia, USA) were used for the data analysis.

Results

Characteristics of the steady state periods of interventions

Parameters characterising the steady state periods of clamps are shown in Table 2. The clamps were comparable within as well as between groups in terms of the mean plasma glucose concentrations with coefficients of variation below 3%, the mean IRI and NEFA levels. As expected, the insulin sensitivity expressed as M was significantly higher in C compared to D group (Group effect: $p<0.01$). No differences in M were detected between HEC and AT-HEC in both groups.

During SAL control intervention the total fluid volume administered was comparable to HEC and AT-HEC. The steady-state periods of SAL (last 30 min) were characterised by mean plasma glucose 5.72±0.29 and 4.76±0.14 $mmol.l^{-1}$($p<0.01$); mean IRI concentrations 5.3±1.33 and 2.92±0.28 $\mu U.ml^{-1}$ (NS); and mean NEFA concentrations 0.48±0.04 and 0.34±0.05 $mmol.l^{-1}$ (NS) in D vs. C, respectively.

Variable	C (n=12)		D (n=11)		*p* value
	HEC	**AT-HEC**	**HEC**	**AT-HEC**	
Mean plasma glucose ($mmol.l^{-1}$)	5.18±0.12	5.23±0.10	5.35±0.20	5.17±0.13	NS
Coefficient of glucose variation (%)	2.23±0.27	2.65±0.17	2.60±0.32	2.86±0.41	NS
Mean insulin ($\mu U.ml^{-1}$)	71.87±4.89	77.13±6.25	80.58±5.58	79.56±7.55	NS
NEFA ($mmol.l^{-1}$)	0.059±0.01	0.099±0.04	0.094±0.02	0.083±0.02	NS
M ($mg.kg^{-1}.min^{-1}$)	10.13±0.86	10.68±0.94	6.70±0.44	6.35±0.59	**

Table 2. Characteristics of the steady state periods of HEC vs. AT-HEC. Data are means ± SEM.
** $p<0.01$ for D vs. C.

The effect of metabolic status and insulin on A-FABP plasma concentrations and SAT expressions

Plasma concentrations of A-FABP during HEC and SAL are displayed in Figure 1a. They were 1.6-fold higher in D group compared to C group (Group effect: $p<0.001$). In D, a significant decrease in A-FABP was detected during HEC (Time effect: $p<0.05$ in full ANOVA and in LSD post-test: $p<0.05$), while during SAL no difference in A-FABP concentrations could be shown. This implicates that the decline in plasma A-FABP during clamp is independent on haemodilution and is attributed to hyperinsulinaemia. In C, no changes in plasma A-FABP were demonstrated during HEC. During SAL, the basal A-FABP was comparable to HEC, while at 240 min higher concentrations compared to HEC were measured (Intervention effect: $p<0.05$ in ANOVA for Controls and in LSD).

Relative expressions of selected genes in SAT are summarized in Table 3. Similarly to plasma concentrations, the relative expressions of *A-FABP* in SAT were 3.0-fold higher in D group (Group effect: $p<0.001$), while no dynamic changes in the course of HEC were observed (Time and Intervention effects and all interactions were not significant).

The relative expressions of *E-FABP* were opposite to *A-FABP*. Higher *E-FABP* expressions were

detected in C compared to D (Group effect: $p<0.01$). During HEC no changes were measured.

The *A-FABP/E-FABP mRNA* ratio reflecting the relative contribution of both FABP isoforms secreted by adipose tissue was higher in D compared to C (Group effect: $p<0.001$) and showed no differences during HEC (Time, Intervention effect and all interactions not significant).

Relative expression of *PPAR-γ* was higher in C compared to D (Group effect: $p<0.01$), no dynamic changes during HEC were detected in either group (Time, Intervention effect and all interactions not significant).

The effect of ARB on A-FABP plasma concentrations and SAT expressions

A-FABP plasma concentrations during HEC and AT-HEC are shown in Figure 1b. In D, A-FABP concentrations were increased during AT-HEC (Intervention effect: $p<0.05$ in Full ANOVA model and in model for Diabetes). A parallel decrease in plasma A-FABP was detected in D during both HEC and AT-HEC (Time effect: $p<0.05$ in ANOVA model for Diabetes and LSD post-test: $p<0.05$).

In C, plasma A-FABP was stable during HEC, whereas during AT-HEC increased basal A-FABP reversed at 240 min (Intervention effect: $p<0.05$ in Full ANOVA and LSD post-test: $p<0.05$).

For mRNA expressions of selected genes during HEC and AT-HEC see Table 3. The *A-FABP* expressions were stable during both clamps in both groups. Higher *E-FABP* expressions during AT-HEC in C but not in D could be demonstrated (Intervention effect: $p<0.05$), while the Time factor and all interactions were not significant. The *A-FABP/E-FABP mRNA* ratio did not differ between HEC and AT-HEC in either group. Similarly, *PPAR-γ mRNA* expression was comparable between clamps in both groups.

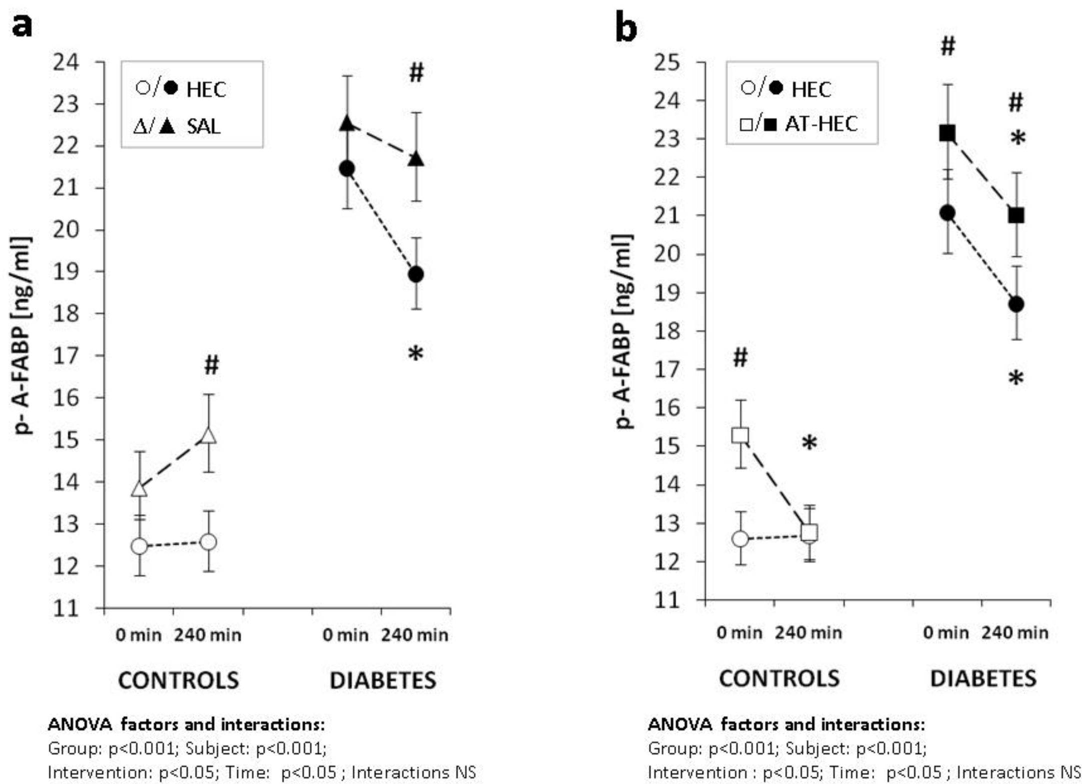

Figure 1. Plasma concentrations of A-FABP during interventions: **a** HEC (○/●) and SAL (Δ/▲); **b** HEC (○/●) and AT-HEC (□/■). The empty and full symbols with error bars represent retransformed mean values with their 95 % confidence intervals for C and D groups, respectively. The asterisks correspond to significant difference between the basal and stimulated values ($p<0.05$). Hashes denote significant difference between interventions (HEC vs. SAL and HEC vs. AT-HEC, respectively) in individual stages ($p<0.05$). Embedded tables summarize the significant factors of full ANOVA model.

Variable		C (n=12)		D (n=11)		Group effect (*p* value)
		0 min	240 min	0 min	240 min	
A-FABP mRNA / Cyclophilin mRNA	HEC	718.4 (647.6–808.6)	650.3 (592.8–721.8)	1937 (1686–2314)	1971 (1695–2401)	<0.001
	AT-HEC	758.3 (679.2–860.9)	675.6 (610.0–758.9)	2004 (1732–2422)	1940 (1688–2318)	
E-FABP mRNA / Cyclophilin mRNA	HEC	**257.8** [a] (210.7–322.7)	**238.5** [a] (196.3–295.9)	196.5 (181.0–214.3)	184.1 (170.2–200.0)	<0.001
	AT-HEC	**292.9** [a] (232.5–380.1)	**259.8** [a] (212.2–325.6)	198.1 (182.4–216.1)	184.7 (169.8–201.8)	
A-FABP mRNA/ E-FABP mRNA	HEC	2.88 (2.44–3.42)	2.75 (2.33–3.26)	10.25 (8.43–12.83)	9.56 (8.10–11.52)	<0.001
	AT-HEC	3.14 (2.58–3.82)	2.06 (1.71–2.47)	10.3 (8.66–12.53)	11.1 (9.26–13.65)	
PPAR-γ mRNA / Cyclophilin mRNA	HEC	169.8 (116.3–243.5)	168.1 (115.1–241.2)	139.6 (112.4–166.3)	158.9 (132.1–185.3)	<0.01
	AT-HEC	167.5 (111.1–247.3)	202.5 (140.0–288.2)	137.0 (109.8–163.8)	158.9 (132.1–185.2)	

Table 3. Relative expressions of *A-FABP, E-FABP* and *PPAR-γ mRNA* in subcutaneous adipose tissue. Data are means (5%-95% CI). Statistical significance (in bold): Group effect – difference between groups; [a]Intervention effect $p<0.05$ – difference between interventions (HEC vs. AT-HEC). For detailed description of ANOVA model, see text.

Relationship of A-FABP to anthropometric and metabolic parameters

A-FABP plasma concentrations were positively correlated with its expression in SAT ($r = 0.59$; $p<0.01$). The correlation analysis of A-FABP and E-FABP combined for both groups is summarized in Table 4. Both A-FABP plasma concentrations and expressions correlated positively with BMI, waist circumference, fasting plasma glucose, fasting insulin and negatively with M; additionally, positive correlation of *A-FABP mRNA* and HbA1c was found. The *A-FABP/E-FABP mRNA* ratio correlated with fasting plasma glucose and HbA1c. *E-FABP mRNA* expressions were negatively correlated with HbA1c, relations to other parameters of metabolic syndrome were not significant.

In multivariate regression, which reflects the weight of individual factors without contribution of other factors included in the model, BMI, waist, fasting glycaemia, insulinaemia and M remained significant predictors ($p<0.05$) of both A-FABP plasma concentrations and expression (detailed analysis in Supplementary file). This confirms that A-FABP is closely linked to dysregulation of glucose metabolism and insulin resistance independently of obesity per se.

Analyzing the relation between *PPAR-γ mRNA* and FABPs, we were able to detect significant positive correlation between *PPAR-γ mRNA* and *E-FABP mRNA* ($r = 0.80$; $p<0.001$). Neither plasma A-FABP, nor its expressions were related to *PPAR-γ mRNA* in SAT.

Discussion

To our knowledge, this is the first clinical study analyzing A-FABP plasma concentrations in parallel with its expressions in SAT. Showing higher plasma concentrations and expression of A-FABP in overweight/obese patients with type 2 diabetes compared to healthy subjects, our findings are in accordance with current evidence on circulating A-FABP [7-10, 25]. In addition to plasma concentrations, our results confirm an equivalent difference in *A-FABP* expressions between the groups studied. This fact has not been sufficiently clarified in previous studies [11, 13-15]. The strong positive correlation of plasma A-FABP with its expression in SAT indicates that SAT is a substantial source of circulating A-FABP. The contribution of other fat depots cannot be excluded, however previous studies showed higher or comparable *A-FABP* expression in SAT in comparison to visceral

adipose tissue [26, 27]. Additionally, macrophages might also play a role in whole-body A-FABP balance [28]. Their contribution to circulating A-FABP levels has not been addressed in our study.
The minor adipocyte isoform - *E-FABP* revealed higher expression rates in healthy subjects. Here, the literature evidence is modest and inconclusive [11]. *A-FABP/E-FABP mRNA* ratio suggested by Fisher and co-workers [14] as a summarizing measure for FABPs in adipose tissue was 3-fold higher in the overweight/obese diabetic patients. It could be hypothesized that in obesity and type 2 diabetes, the *E-FABP expression* is down-regulated in order to at least partially compensate for increase in A-FABP. The clarification of FABPs' regulations in obesity and type 2 diabetes awaits definitely further studies.
Additionally, we measured relative expressions of *PPAR-γ*, which is involved in regulation of *A-FABP* gene expression. Conversely A-FABP enhances PPAR-γ activity [11].The studies comparing *PPAR-γ* expression between lean and obese/type 2 diabetic patients reveal inconsistent data with reports of decreased, unchanged or increased *PPAR-γ* expression in obese subjects[29-32].We report a down-regulation of *PPAR-γ* expression in diabetes. We were not able to show any relationship of *PPAR-γ* expression to A-FABP plasma concentrations or expressions, while a positive correlation between *PPAR-γ mRNA* and *E-FABP mRNA* suggests that the expressions of *PPAR-γ* and the minor FABP isoform are closely related, at least in SAT. Further interventional studies with PPAR-γ agonists are needed to disclose the possible causal relationship suggested by the finding of Cabre[25], who showed an increase in plasma A-FABP after treatment with rosiglitazone.

Another important finding of our study is the close association of both A-FABP plasma concentration and expression not only with measures of obesity, but also with fasting plasma glucose, insulin and insulin resistance defined by means of hyperinsulinaemic-euglycaemic clamp. Additionally, the *A-FABP mRNA* and *A-FABP/E-FABP mRNA* ratio was positively correlated with HbA1c. The association with hyperglycaemia, insulinaemia and glucose disposal remained significant also in multivariate regression analysis suggesting that the relation of A-FABP to insulin resistance and blood glucose is independent of obesity.

In our study groups, acute hyperinsulinaemia suppressed circulating A-FABP in D and C, while no direct impact on *A-FABP* expression in SAT was detected in either group. This discrepancy between changes in plasma and expression in SAT implicates a role of another A-FABP source (macrophages or other fat depot) in the response to insulin.
To our knowledge, the presented study is also the first one using the control volume examination to distinguish non-specific time and volume effect on A-FABP during clamp. In type 2 diabetes, the decline in A-FABP during hyperinsulinaemia is accordance with a recent report [33]. Using the SAL control examination, during which no changes were detected, the direct regulation of plasma A-FABP by insulin in diabetes was confirmed.
Importance of volume control examinations can be even better demonstrated in the control group where no changes during hyperinsulinaemia, but an increase in plasma A-FABP during saline infusion were measured. Since neither secretion mechanisms, nor function of circulating A-FABP in vivo in humans are elucidated, the interpretation of the observation during SAL is not clear – we might speculate on effects of prolonged fasting or circadian rhythm on plasma A-FABP. Moreover, the stable A-FABP concentrations during HEC might be interpreted as an influence of insulin that prevented the increase in A-FABP seen in SAL.
Recently, direct regulation of circulating A-FABP by insulin was described [33], showing a decrease in A-FABP also in healthy controls. The difference to our study may be explained by the well-described gender differences in A-FABP plasma concentrations [7-10, 25]. The higher A-FABP levels in women may be also differentially regulated compared to men, which has been already demonstrated by an independent association of A-FABP with carotid intima-media thickness in women but not in men [34].
Expressions of both *E-FABP* and *PPAR-γ* in SAT were not affected by hyperinsulinaemia in our study population.

Acute ARB did not influence the parameters on insulin sensitivity in either group. Based on the pharmacokinetics of losartan and its active metabolite, effective circulating concentrations during the clamp period should have been achieved. However the plasma levels may not mirror effective tissue concentrations, which are crucial for potential metabolic effects. The acute ARB had no effect on glucose disposal in healthy subjects [35]. Clinical studies using long-term administration of ARBs with hyperinsulinaemic-euglycaemic clamp technique reported both increase [36, 37] and no changes [38, 39] in insulin sensitivity in insulin resistant subjects.
We could demonstrate distinct effects of ARB on FABPs: in healthy subjects acute ARB stimulated basal A-FABP plasma concentrations, which were

reversed by insulin during clamp. In type 2 diabetes, the stimulatory effect of ARB on plasma A-FABP was independent of hyperinsulinaemia. In healthy subject, the expression of *E-FABP* but not *A-FABP* in SAT was also stimulated after acute ARB. In type 2 diabetes, regulation *A-FABP* and *E-FABP* expressions by acute ARB has not been proven. The different changes in plasma and expression in SAT suggest an involvement of another A-FABP source in the response to ARB.
Considering the potential PPAR-γ activation by sartans [20, 21], long-term exposure to ARB in vivo, as well as experimental models have to be evaluated in order to confirm the suggested effects of ARB on A-FABP. Plausibility of such a hypothesis has been recently supported by an observation of a cross-sectional study with patients submitted for coronary angiograms [40]. Those patients treated with ACEIs or angiotensin II type 1 receptor blockers revealed significantly higher A-FABP levels.

Even if the limited sample size of our study has to be considered by interpretation, our results in a cohort of overweight/obese men with type 2 diabetes and healthy controls bring new knowledge on in vivo regulations of plasma A-FABP and expressions of *A-FABP* and *E-FABP* in SAT.

In summary, our study has documented that circulating A-FABP as well as its expression in subcutaneous adipose tissue are closely and independently related to obesity, insulin resistance and hyperglycaemia and suggested the subcutaneous adipose tissue as a substantial source of circulating A-FABP in humans. Hyperinsulinaemia suppresses plasma A-FABP but does not influence its expression. Acute ARB stimulates basal A-FABP plasma concentrations without any effect on its expression. Despite of the close associations found in the present study, a causal relationship between A-FABP and development of insulin resistance cannot be drawn. Therefore further studies are needed to determine whether A-FABP only represents a biomarker of the developing insulin resistance and/or type 2 diabetes or whether it is one of their causal factors, indeed.

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	p-A-FABP (ng/ml)	***A-FABP* mRNA**	***E-FABP* mRNA**	***A-FABP* / *E-FABP* mRNA**
BMI	0.85***	0.65***	0.03	0.40
Waist circumference	0.86***	0.56**	-0.07	0.34
Fasting glucose	0.46*	0.73***	-0.35	0.74***
Fasting insulin	0.50*	0.48*	-0.11	0.39
Fasting NEFA	0.13	0.17	-0.09	-0.33
Fasting TG	0.19	0.24	-0.05	0.23
Total cholesterol	-0.05	0.21	-0.08	-0.21
HDL-cholesterol	-0.06	-0.28	0.04	-0.10
LDL-cholesterol	-0.10	0.17	-0.18	0.22
HbA1c	0.08	0.46*	-0.56*	0.65**
M	-0.64**	-0.53**	0.18	-0.35

Table 4. The relationships (Spearman`s correlation coefficients) between A-FABP, E-FABP and selected metabolic parameters. For units, see Table 1 and Table 2. * $p<0.05$, ** $p<0.01$, *** $p<0.001$;

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Appendix 3

Effect of telmisartan on selected adipokines, insulin sensitivity and substrate utilization during insulin-stimulated conditions in patients with metabolic syndrome and impaired fasting glucose

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CLINICAL STUDY

Effect of telmisartan on selected adipokines, insulin sensitivity, and substrate utilization during insulin-stimulated conditions in patients with metabolic syndrome and impaired fasting glucose

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Abstract

Objective: Telmisartan improves glucose and lipid metabolism in rodents. This study evaluated the effect of telmisartan on insulin sensitivity, substrate utilization, selected plasma adipokines and their expressions in subcutaneous adipose tissue (SAT) in metabolic syndrome.

Design and methods: Twelve patients with impaired fasting glucose completed the double-blind, randomized, crossover trial. Patients received telmisartan (160 mg/day) or placebo for 3 weeks and *vice versa* with a 2-week washout period. At the end of each period, a hyperinsulinemic euglycemic clamp (HEC) combined with indirect calorimetry was performed. During HEC (0, 30, and 120 min), plasma levels of adipokines were measured and a needle biopsy (0 and 30 min) of SAT was performed.

Results: Fasting plasma glucose was lower after telmisartan compared with placebo ($P<0.05$). There were no differences in insulin sensitivity and substrate utilization. We found no differences in basal plasma adiponectin, resistin and tumour necrosis factor α (TNFα), but an increase was found in basal leptin, after telmisartan treatment. Insulin-stimulated plasma adiponectin ($P<0.05$), leptin and resistin ($P<0.001$) were increased, whereas TNFα was decreased ($P<0.05$) after telmisartan treatment. Expression of resistin, but not adiponectin, TNFα and leptin was increased after telmisartan treatment.

Conclusions: Despite the decrease in fasting plasma glucose, telmisartan does not improve insulin sensitivity and substrate utilization. Telmisartan increases plasma leptin as well as insulin-stimulated plasma adiponectin, leptin and resistin, and decreases plasma TNFα during HEC. Changes in plasma adipokines cannot be explained by their expressions in SAT. The changes in plasma adipokines might be involved in the metabolic effects of telmisartan in metabolic syndrome.

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Introduction

Several large intervention trials have demonstrated that angiotensin II type 1 receptor antagonists (ARBs) reduce the incidence of new-onset diabetes by 20–25% (1, 2). The protective effects of ARBs during the development of diabetes are probably independent of their antihypertensive properties (3, 4).

However, the mechanisms underlying the effect of ARBs on glucose metabolism are not fully understood. Experimental evidence has been accumulated that insulin resistance (IR) may be improved by interrupting renin–angiotensin system (5). All ARBs have been shown to improve insulin sensitivity in experimental and *in vitro* studies through multiple mechanisms, including increase in muscle blood flow, decreased sympathetic nervous activity, enhanced insulin signalling and adipose-tissue remodelling (5, 6). Angiotensin II inhibits pre-adipocyte differentiation into mature adipocytes, which impairs the fat cells' ability to store fat (6). This in turn results in accumulation of fats in the liver, the skeletal muscle, and the pancreas, which worsens IR. Impaired fat cells produce excessive amounts of pro-inflammatory cytokines (tumour necrosis factor α (TNFα), interleukin (IL) 6, resistin, and others), whereas the insulin-sensitizing cytokines such as adiponectin are reduced (6). Blocking the angiotensin II receptor with ARBs decreases the adipocyte size (7), promotes differentiation of pre-adipocytes to mature adipocytes, and prevents the ectopic fat accumulation (8). In addition, ARBs may alter the adipokine profile.

It has been shown that ARBs increase the adiponectin protein content in isolated adipocytes (9). Increases in plasma adiponectin concentrations after

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ARB treatment have been found also in human subjects by some (10–12), but not all, groups (13). The actual knowledge of the physiological roles of resistin and TNFα in altering muscle glucose and lipid metabolism is more controversial, but each has been shown to directly impair insulin signalling and consequently insulin-stimulated glucose uptake in muscles (14). The role of resistin is less understood. It is implicated in IR in rats but probably not in human subjects. Moreover, other adipokines (such as adiponectin and leptin) in physiological concentrations are insulin sparing as they stimulate β oxidation of fatty acids in skeletal muscle. The effect of ARBs on adipokines *in vivo* in human subjects has not been systematically evaluated until now.

Human studies investigating the effect of ARBs on insulin sensitivity are not conclusive. Most of the randomized, placebo-controlled or head-to-head studies using the hyperinsulinemic euglycemic clamp (HEC) technique for estimation of insulin sensitivity have found no significant effect of ARBs on insulin action in human subjects (5, 15, 16). However, there is evidence that certain ARBs have a further effect on insulin sensitivity, which is mediated through peroxisome proliferator-activated receptor γ (PPARG). PPARG is a nuclear transcription factor which triggers the expression of multiple genes involved in glucose and lipid metabolism (17). Not all ARBs have been shown to bind to PPARG; the strongest activity has been seen with telmisartan (4, 17, 18). Based on rodent experiments, it can be hypothesized that the activation of PPARG may be involved in the ability of telmisartan to increase muscle fatty acid oxidation and energy expenditure (EE) (19). The *in vivo* effects of telmisartan on insulin sensitivity and substrate oxidation estimated by HEC combined with indirect calorimetry have not been studied in human subjects so far.

The aim of our study was to evaluate *in vivo* effects of telmisartan on insulin sensitivity, substrate utilization, basal and insulin-stimulated plasma concentrations of selected adipokines and their expressions in subcutaneous adipose tissue (SAT) in patients with impaired fasting glucose and metabolic syndrome.

Subjects and methods

Subjects

We enrolled 12 male patients with metabolic syndrome defined according to the NCEP-ATPIII criteria, revised in 2005 (20). Impaired fasting glucose was an obligate criterion for enrolment in the study. One patient had both impaired fasting glucose and impaired glucose tolerance (confirmed by an oral glucose tolerance test). Only male subjects participated in the study in order to exclude variability in insulin sensitivity in women according to menstrual cycle and childbearing potential and with respect to sexual dimorphism of adipokines. Characteristics of study group are shown in Table 1.

Table 1 Characteristics of study group.

n	12
Age (years)	50.0±6.02
BMI (kg/m^2)	29.0±4.37
Waist (cm)	104.6±7.8
Systolic blood pressure (mmHg)	133.0±12.0
Diastolic blood pressure (mmHg)	91.3±10.0
HbAlc (%)	3.86±0.44
Total cholesterol (mmol/l)	5.51±1.1
HDL cholesterol (mmol/l)	0.99±0.12
LDL cholesterol (mmol/l)	3.2±1.22
Serum triglycerides (mmol/l)	2.71±1.4
Plasma glucose 120 min, OGTT (mmol/l)	7.6±1.8
Fasting plasma glucose (mmol/l)	6.1±0.43

OGTT, oral glucose tolerance test.

Patients enrolled in the study were treatment-naive with regard to oral antidiabetic medication. Antihypertensive treatment was adjusted during the 4-week run-in period as follows: angiotensin-converting enzyme and ARB treatment had been stopped and replaced by metabolic neutral calcium channel blockers. The dose of concomitant calcium channel blocker (if required) was stable during the whole study duration. None of the patients had their dietary intake of salt or protein restricted. Patients with overt diabetes (diagnosed by an oral glucose tolerance test), acute or chronic inflammatory, or other major organ diseases were excluded from the study.

All subjects gave their informed consent with the study protocol that had been reviewed and approved by the local ethics committee. The study was performed in accordance with the Helsinki Declaration and Title 45, Code of Federal Regulations, Part 46, Protection of Human Subjects. The EudraCT number 2006-000490-29 had been issued for our Sponsor's Protocol Code No. 1, 1.1.2006.

Study protocol

The study was a randomized, placebo-controlled, double-blind, crossover trial consisting of two treatment periods. After a 4-week run-in period, the subjects were randomly assigned to receive telmisartan 160 mg daily (Micardis 80 mg; Boehringer Ingelheim Pharma GmbH, Ingelheim am Rhein, Germany) or placebo for 3 weeks. After a 2-week washout period, the medication was changed and continued for another 3 weeks. Randomization was performed by standard procedure. The code was not broken until all data were entered into a database, which was locked for editing. Drug compliance was assessed by the effect on blood pressure as well as by the study drug accountability. Patients were instructed to adhere to their ordinary lifestyle and avoid changes in food intake, alcohol consumption and exercise during the whole study duration.

At the end of both 3-week periods of treatment with telmisartan or placebo, all patients underwent a 2 h HEC combined with indirect calorimetry and adipose tissue biopsies.

Hyperinsulinemic euglycemic clamp The subjects were examined on an outpatient basis after an 8–10 h overnight fast with only tap water allowed *ad libitum*. The HEC, lasting 2 h, was conducted as described earlier (21). Briefly, a Teflon cannula (VenflonViggo, Helsingborg, Sweden) was inserted into left antecubital vein for infusion of all test substances. A second cannula was inserted in a retrograde manner into a wrist vein of the same hand for blood sampling, and a hand was placed in a heated (65 °C) box to achieve venous blood arterialization. A stepwise primed-continuous infusion (1 mU/kg per min of Actrapid HM; NovoNordisk, Copenhagen, Denmark) was administered to acutely raise and maintain the plasma concentration of insulin at 75 μU/ml. Plasma glucose concentrations during the clamp were maintained at 5.0 mmol/l by continuous infusion of 15% glucose. To prevent hypokalemia during insulin infusion, potassium chloride was added to 15% glucose infusion (30 mmol KCl/l of glucose). Arterialized blood plasma glucose concentration was determined every 5–10 min. Before the clamp, fasting plasma glucose levels had been checked, and at concentration lower than 6.0 mmol/l, the clamp procedure was started. No glucose was infused until plasma glucose had declined to the clamp-target level.

Needle biopsy of abdominal SAT Needle biopsy of abdominal SAT was performed before (0 min) and 30 min into the clamp. Under local anaesthesia (1% trimecain in a field block pattern), an incision (3–4 mm) was made through the skin at the lower abdomen, and a subcutaneous fat specimen (300 mg) was obtained by needle aspiration. The samples were washed in 0.9% natrium chloride solution, immediately frozen by liquid nitrogen, and stored at −80 °C until used for RNA extraction.

Indirect calorimetry Substrate utilization and EE were assessed by indirect calorimetry (22). Gas exchange measurements were performed during a 45 min basal period before starting the insulin infusion and during the last 45 min period of the clamp. A transparent plastic ventilated hood was placed over the subject's head and made airtight around the neck. A slight negative pressure was maintained in the hood to avoid loss of expired air. A constant fraction of air flowing out of the hood was automatically collected for analysis. Airflow and O_2 and CO_2 concentrations in expired and inspired air were measured by a continuous open-circuit system (metabolic monitor VMAX; Sensor Medics, Anaheim, CA, USA). Blood samples were taken at 0 and 120 min of the clamp study to assess plasma levels of selected adipokines and blood urea nitrogen. Urine was collected i) during the night before the study (basal) and ii) during the clamp study (0–120 min) to measure the urinary nitrogen excretion to be able to calculate protein oxidation.

Measurements of blood pressure were performed as three time readings at the beginning of the clamp, after 30 min resting position and during the clamp.

Analytical methods

Plasma concentrations of glucose were measured using the Beckman analyzer (Beckman Instruments Inc., Fullerton, CA, USA) by glucose oxidase method. Immunoreactive insulin (IRI) was determined by RIA method using an IMMUNOTECH Insulin IRMA kit (IMMUNOTECH a.s, Prague, Czech Republic) with analytical sensitivity of 0.5 μIU/ml; intra- and inter-assay coefficients of variation (CV) were below or equal to 4.3 and 3.4% respectively. HbAlc was measured by HPLC method (Tosoh HLC-723 G7; Tosoh Corporation, Tokyo, Japan). This analyzer uses a non-porous ion exchanger that separates HbAlc from other fractions. The method was calibrated to IFCC reference procedure (23).

Plasma concentrations of TNFα were measured by immunoassay (Human TNFα UltraSensitive RIA kit; BioSource International, Camarillo, CA, USA; the detection limit was <0.09 pg/ml, and intra- and inter-assay CV were 5.3–6.7 and 8.2–9.7% respectively); plasma concentrations of resistin were measured by Human Resistin ELISA kit (BioVendor Laboratory Medicine Inc., Brno, Czech Republic; the detection limit

Table 2 Primers used for RT-PCR of the subcutaneous adipose tissue samples.

Gene	Accession number	Forward primer	Reverse primer
Adiponectin	XM_290602	HACRP30-F: 5′-GGT TCA ATG GCT TGT TTG C-3′	HACRP30-R: 5′-TCA TCC CAA GCT GAT TCT G-3′
Leptin	NM_000230	Hleptin-F: 5′-CCC TAA GCC TCC TTT TGC T-3′	Hleptin-R: 5′-GCT AAG AGG GGA CAA GAC A-3′
TNFα	X02910 X02159	HTNFa-F: 5′-CTA TCT GGG AGG GGT CTT C-3′	HTNFa-R: 5′-TTG GGA AGG TTG GAT GTT C-3′
Resistin	AY207314	HRETN-F: 5′-ATA AGC AGC ATT GGC CTG G-3′	HRETN-R: 5′-TGG CAG TGA CAT GTG GTC T-3′
Cyclophilin	XM_090070	HCLPNa-F: 5′-CAA ATG CTG GAC CCA ACA CA-3′	HCLPNa-R: 5′-TGC CAT CCA ACC ACT CAG TC-3′

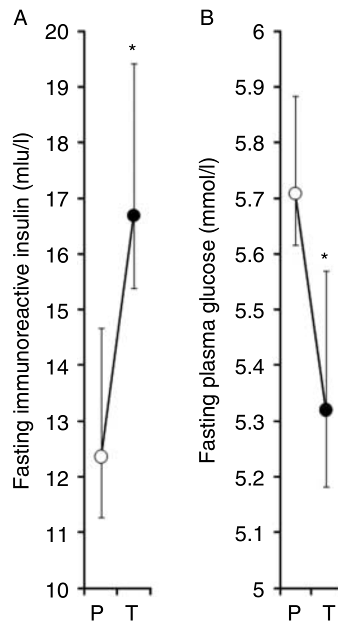

Figure 1 Effect of telmisartan treatment on fasting immunoreactive insulin and plasma glucose. The circles with error bars symbolize the group means with their 95% confidence intervals: telmisartan (T; full circles) and placebo (P; open circles). For the fasting immunoreactive insulin (A), the significance of the factors was as follows: telmisartan: $F=7.6$, $P=0.0224$; subject (inter-individual variability): $F=7.6$, $P=0.0029$. For the fasting plasma glucose (B), the significance of the factors was as follows: telmisartan: $F=8.8$, $P=0.0129$; subject (inter-individual variability): $F=3$, $P=0.0392$. Statistical significance: $^*P<0.05$.

was 0.033 ng/ml, and intra- and inter-assay CV were 2.8–3.4 and 5.1–6.9% respectively); plasma concentrations of leptin were measured by Human Leptin ELISA kit (BioVendor Laboratory Medicine Inc.; the detection limit was 0.5 ng/ml, and intra- and inter-assay CV were 3–7.5 and 3.2–9.2% respectively); plasma concentrations of adiponectin were measured by Human Adiponectin ELISA kit (BioVendor Laboratory Medicine Inc.; the detection limit was 210 ng/ml, and intra- and inter-assay CV were 6.4–7 and 7.3–8.2% respectively), all according to the manufacturer's instructions.

Expression of adipokines was analysed by the real-time PCR method using the following protocol:

i) The RNA was isolated from the liquid nitrogen frozen biopsy of the human fat tissue using the RNeasy Lipid Tissue Mini kit and QIAzolLysis Reagent (Qiagen). The starting amount of 100 mg tissue was excised from the biopsy and homogenized in 1 ml of QIAzolLysis Reagent (guanidine thiocyanate–phenol solution) for 2 min. From the homogenate, the RNA was isolated by extraction on silica gel-based column, according to the kit handbook. Possible contamination of RNA with genomic DNA remains was taken out by DNase digestion (RNase-free DNase Set; Qiagen). This step is supposed to prevent any significant DNA amplification, e.g. by gel electrophoresis.
ii) The cDNA was synthesized using a recombinant Omniscript Reverse Transcriptase (Qiagen), ribonuclease inhibitor from human placenta (Sigma) and $(dT)_{16}$oligonucleotides.
iii) The real-time PCR procedure itself had been carried out on the DNA Engine Opticon 2 System (MJ Research, Waltham, MA, USA). HotStarTaq DNA polymerase and SYBR Green fluorescent dye (QuantiTec SYBR Green PCR kit; Qiagen) were used for the RT-PCR. To eliminate the influence of primer dimers, negative controls were used. As the reference gene, human cyclophilin was used; there is no evidence of insulin or ARB influence on cyclophilin. Primers used for RT-PCR are given in Table 2.
iv) The data were processed by Q-gene 96 software (Institute of Biochemistry and Genetics, University of Basel, Basel, Switzerland).

Calculations

Insulin action was estimated as the glucose disposal (M) and metabolic clearance rate (MCR) of glucose calculated during the last 20 min of the clamp after correction for changes in glucose pool size (21). Calculations of substrate oxidation were made using standard equations (22). Urinary urea excretion during the clamp was corrected for changes in urea pool size (24). Non-oxidative glucose disposal (NEOX) was calculated by subtracting the rate of glucose oxidation from M.

Table 3 Substrate utilization before (basal) and during hyperinsulinemic euglycemic clamp (insulin) after 3-week treatment with telmisartan or placebo.

	Placebo		Telmisartan	
	Basal	Insulin	Basal	Insulin
REE (kcal/24 h)	1729±386	1823±337	1706±370	1728±355
RQ	0.79±0.03*	0.87±0.05	0.77±0.04*	0.86±0.03
Chox (mg/kg per min)	0.69±0.42*	1.36±0.8	0.63±0.42*	1.47±0.39
Fox (mg/kg per min)	0.77±0.2*	0.48±0.2	0.71±0.32*	0.58±0.30
Prox (mg/kg per min)	0.87±0.3*	0.40±0.14	0.88±0.39*	0.43±0.15

REE, resting energy expenditure; RQ, respiratory quotient; Chox, glucose oxidation; Fox, lipid oxidation; Prox, protein oxidation. Differences between telmisartan and placebo were not statistically significant. $^*P<0.02$ versus insulin.

Statistical analysis

The data are expressed as means ± S.E.M. unless indicated otherwise. Steady-state periods of both clamps were compared using Wilcoxon's paired test. The differences between time courses of both clamps were evaluated by a repeated-measures ANOVA model, including the following factors and interactions: effect of telmisartan treatment (placebo versus telmisartan) and effect of hyperinsulinemia (clamp induced) as the within factors, subject factor (represents the inter-individual variability of subjects) and telmisartan × hyperinsulinemia interaction. The last term indicated whether the shapes of the time profiles for telmisartan and placebo were different or not. The differences between subgroups were evaluated using least significant difference (LSD) multiple comparisons. The statistical significance $P<0.05$ was chosen for both ANOVA testing and multiple comparisons. Owing to a non-Gaussian data distribution in all dependent variables, the data underwent power transformations (directly in the ANOVA model) to attain distributional symmetry and a constant variance in the data as well as in residuals. The non-homogeneities were detected using residual diagnostics. The experimental points with absolute values of studentized residual (after data transformation) >3 were excluded from the analysis. The fraction of such points never exceeded 5% of the total number. Statgraphics Centurion v. XV statistical software (Stadtpoint Inc., Herndon, VA, USA) was used for the data analysis.

Results

Telmisartan compared to placebo treatment induced no differences in body weight (89.8 ± 19 vs 89.8 ± 18 kg) but resulted in lower blood pressure, both systolic (126 ± 10 vs 135 ± 12 mmHg; $P<0.03$) and diastolic (81 ± 10 vs 90 ± 10 mmHg; $P<0.01$), lower fasting plasma glucose ($P<0.05$) and higher fasting IRI ($P<0.05$). Fasting plasma glucose and IRI are shown in Fig. 1. Insulin action was estimated by HEC combined with indirect calorimetry. The clamps performed after treatment with telmisartan versus placebo were comparable in terms of the mean plasma glucose concentrations (5.29 ± 1.9 vs 5.34 ± 0.3 mmol/l), CV of glucose (2.89 ± 1.9 vs 2.56 ± 1.2%) and mean IRI levels. Insulin action was comparable after telmisartan and placebo. MCR (4.15 ± 1.0 vs 4.08 ± 1.9 ml/kg per min), *M* (4.4 ± 1.8 vs 3.9 ± 1.7 mg/kg per min) and NEOX (4.1 ± 1.9 vs 3.0 ± 2.3 mg/kg per min) did not differ between telmisartan and placebo. Similarly, EE, glucose and fat oxidations have not been statistically different comparing telmisartan and placebo (Table 3).

No significant effect of telmisartan on basal plasma concentrations of selected adipokines has been detected, except of basal plasma leptin that has significantly increased after telmisartan treatment (Fig. 2C–5C).

Plasma concentrations of TNFα during HEC are shown in Fig. 2A–C. The ANOVA model indicated (Fig. 2A) a significant decrease in plasma TNFα in telmisartan as compared to placebo (telmisartan; $P<0.05$), whereas no significant hyperinsulinemia effect (Fig. 2B) or interaction was detected (Fig. 2C). However, the plasma changes

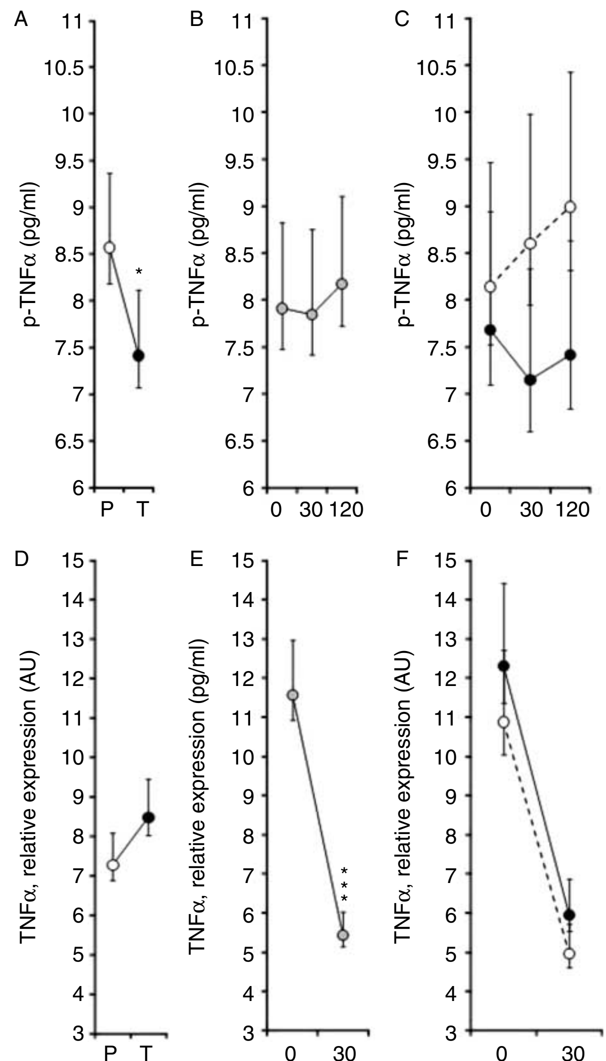

Figure 2 Effects of telmisartan treatment and the clamp-induced hyperinsulinemia on TNFα. The circles with error bars symbolize the group means with their 95% confidence intervals: telmisartan (T; full circles) and placebo (P; open circles). (A and D) The effect of telmisartan is illustrated; (B and E) the effect of clamp-induced hyperinsulinemia is demonstrated; (C and F) the interactions between telmisartan and hyperinsulinemia are shown. For the PTNFα (A–C), the significance of the factors and interactions was as follows: telmisartan: $F=4.8$, $P=0.0328$; subject (inter-individual variability): $F=2.7$, $P=0.0085$; hyperinsulinemia: $F=0.1$, $P=0.8698$; telmisartan × hyperinsulinemia: $F=0.4$, $P=0.6489$. For the ExTNFα (D–F), the significance of the factors and interactions was as follows: telmisartan: $F=3.9$, $P=0.0591$; subject (inter-individual variability): $F=1.8$, $P=0.1122$; hyperinsulinemia: $F=92$, $P<0.0001$; telmisartan × hyperinsulinemia: $F=0.2$, $P=0.6406$. Statistical significance: * $P<0.05$, *** $P<0.001$.

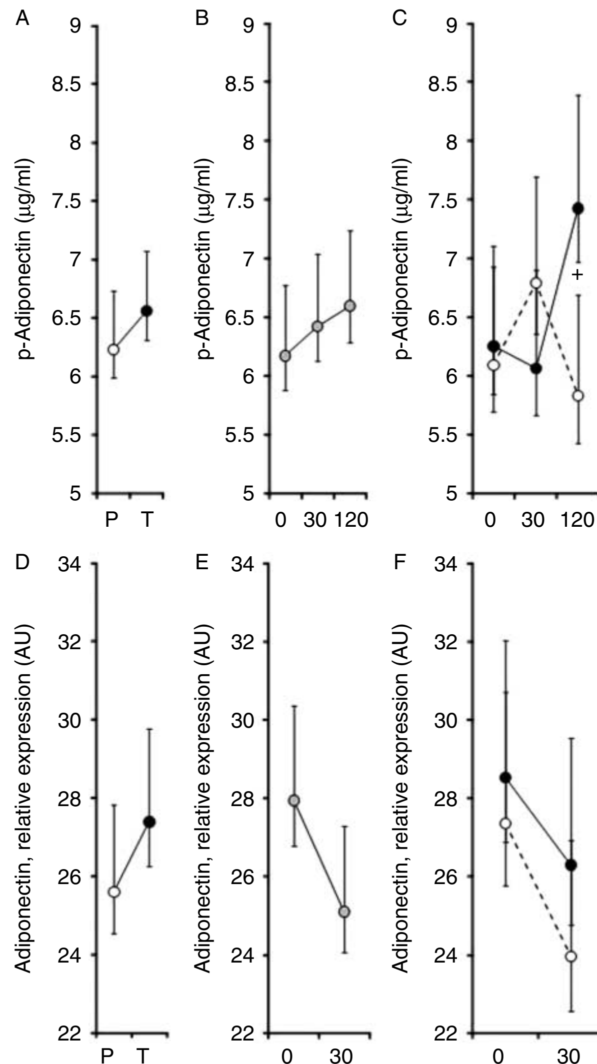

Figure 3 Effects of telmisartan treatment and the clamp-induced hyperinsulinemia on adiponectin. The circles with error bars symbolize the group means with their 95% confidence intervals: telmisartan (T; full circles) and placebo (P; open circles). (A and D) The effect of telmisartan is illustrated; (B and E) the effect of clamp-induced hyperinsulinemia is demonstrated; (C and F) the interactions between telmisartan and hyperinsulinemia are shown. For the plasma adiponectin (A–C), the significance of the factors and interactions was as follows: telmisartan: $F=0.9$, $P=0.3618$; subject (inter-individual variability): $F=14.9$, $P<0.0001$; hyperinsulinemia: $F=0.5$, $P=0.6317$; telmisartan × hyperinsulinemia: $F=3.3$, $P=0.0429$. For the adiponectin expression (D–F), the significance of the factors and interactions was as follows: telmisartan: $F=1.3$, $P=0.2709$; subject (inter-individual variability): $F=6.4$, $P<0.0001$; hyperinsulinemia: $F=3.2$, $P=0.0846$; telmisartan × hyperinsulinemia: $F=0.2$, $P=0.6756$. Statistical significance: $^{+}P<0.05$ for differences between telmisartan and placebo in individual stages of HEC.

after telmisartan do not correspond with relative expression of TNFα in subcutaneous adipose abdominal tissue. In this study, the effect of clamp-induced hyperinsulinemia (Fig. 2E) has been found to be significant ($P<0.001$) and no telmisartan effect was detected (Fig. 2D), implicating the suppression of TNFα expression by insulin, which is not influenced by telmisartan.

Plasma adiponectin concentrations are shown in Fig. 3A–C. Plasma adiponectin concentrations have shown a different time course during insulin-stimulated conditions in telmisartan and placebo (Fig. 3C), documented by the significant telmisartan × hyperinsulinemia interaction ($P<0.05$). A significant increase in plasma adiponectin was detected by LSD multiple comparisons in 120 min of HEC (0 vs 120 min; $P<0.05$) after telmisartan treatment, whereas no changes were seen after placebo treatment. Relative expressions of adiponectin were comparable during both treatment periods and clamps, and both telmisartan and hyperinsulinemia factors were not significant (Fig. 3D–F).

Figure 4A–C shows plasma leptin concentrations. Both basal and insulin-stimulated plasma leptin concentrations were significantly increased after telmisartan treatment compared to placebo treatment (telmisartan effect; $P<0.001$), while telmisartan did not alter the relative expressions of leptin in SAT. There was a significant decrease in leptin expressions during insulin-stimulated conditions (HEC), as documented by the significant hyperinsulinemia factor ($P<0.001$; Fig. 4E).

Plasma concentrations and tissue expressions of resistin are shown in Fig. 5. The telmisartan treatment resulted in an increase in plasma resistin concentrations, as documented by a significant telmisartan factor ($P<0.01$; Fig. 5A). The multiple comparisons tests were in accordance with ANOVA testing. Figure 5B shows the increasing plasma concentrations of resistin during insulin-stimulated conditions (HEC), as indicated by significant hyperinsulinemia factor ($P<0.001$), whereas the effect of insulin on resistin expression was opposite (Fig. 5E). There is a significant decrease in resistin expressions during HEC in both telmisartan and placebo (hyperinsulinemia factor $P<0.01$; Fig. 5E and F) without differences between telmisartan and placebo.

Discussion

This short-term placebo-controlled crossover study demonstrate, in accordance with other studies (25), that telmisartan decreases the fasting plasma glucose and blood pressure, whereas the insulin sensitivity assessed by hyperinsulinemic clamp technique did not change after telmisartan treatment in our study population. Thus, the decrease in blood glucose cannot be explained by the improvement in insulin sensitivity. However, we have found an increase in basal plasma IRI and this finding could partly account for the decrease in plasma glucose concentrations. Additionally, we have not found any significant effect of telmisartan on the substrate utilization.

There are still controversial results dealing with the effect of telmisartan or other ARBs on insulin sensitivity

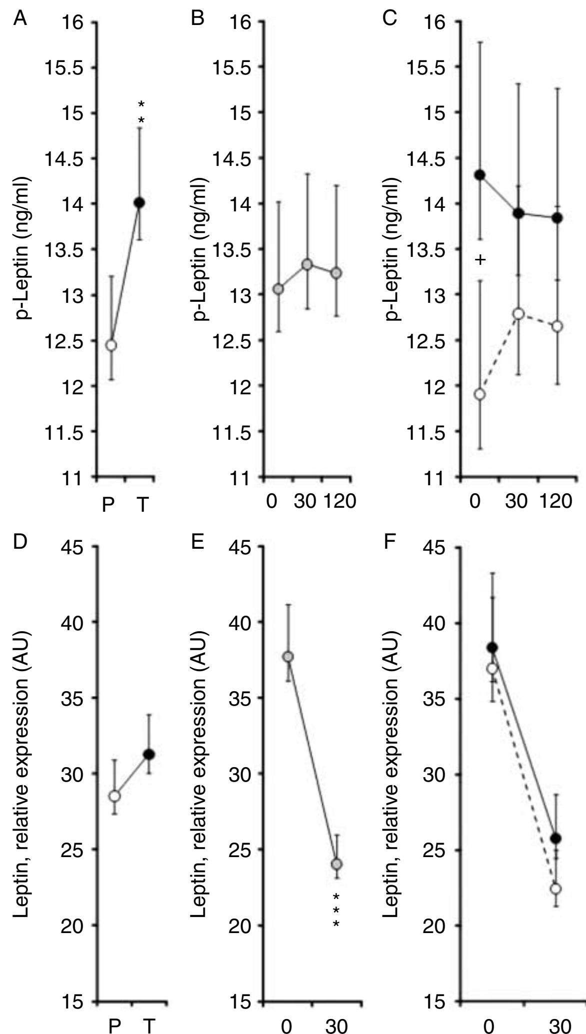

Figure 4 Effects of telmisartan treatment and the clamp-induced hyperinsulinemia on leptin. The circles with error bars symbolize the group means with their 95% confidence intervals: telmisartan (T; full circles) and placebo (P; open circles). (A and D) The effect of telmisartan is illustrated; (B and E) the effect of clamp-induced hyperinsulinemia is demonstrated; (C and F) the interactions between telmisartan and hyperinsulinemia are shown. For the plasma leptin (A–C), the significance of the factors and interactions was as follows: telmisartan: $F=7.6$, $P=0.0079$; subject (inter-individual variability): $F=35.4$, $P<0.0001$; hyperinsulinemia: $F=0.1$, $P=0.9256$; telmisartan×hyperinsulinemia: $F=0.6$, $P=0.5664$. For the leptin expressions (D–F), the significance of the factors and interactions was as follows: telmisartan: $F=2.4$, $P=0.133$; subject (inter-individual variability): $F=4.2$, $P=0.0012$; hyperinsulinemia: $F=56.8$, $P<0.0001$; telmisartan×hyperinsulinemia: $F=1$, $P=0.3357$. Statistical significance: $^{**}P<0.01$ for differences between initial and current state; $^{***}P<0.001$ for differences between 0 and 30 min. $^{+}P<0.05$ for differences between telmisartan and placebo in individual stages of HEC.

in vivo in human subjects (13, 25–27). In our previous study, we did not find any significant changes in insulin sensitivity after acute administration of losartan in healthy subjects (28). Clinical trials with long-term administration of ARBs (other than telmisartan) using the HEC technique for estimation of insulin sensitivity reported both an increase (29, 30) and no effect (5, 15, 16, 31, 32) of ARBs on glucose disposal in insulin-resistant subjects. Telmisartan is considered to be a partial PPARG agonist, and there is experimental evidence that it has specific metabolic advantages (18). Although telmisartan has gained much attention as one of the most metabolically potent ARB compounds, there are virtually no clinical studies evaluating the effect of telmisartan on insulin sensitivity and substrate utilization using the clamp technique combined with indirect calorimetry. However, a recent study evaluating the long-term effect of telmisartan on insulin sensitivity suggests that the magnitude of PPARG stimulation by telmisartan may be modest compared with thiazolidindiones (26). On the other hand, some metabolic effects of telmisartan could be mediated independently of PPARG (33). In addition, in a rat model of metabolic syndrome, it increases EE and protects against dietary-induced obesity (19).

In our study, we have failed to show a statistically significant effect of telmisartan on insulin sensitivity and substrate utilization, but our results might be influenced by the short period of the study and the small number of patients included. To the best of our knowledge, no previous study has carefully evaluated the effect of telmisartan on insulin sensitivity and substrate utilization using the clamp technique combined with indirect calorimetry in human subjects.

The homeostasis model assessment (HOMA) index has been used in most of them (25, 34) and, thus, our study may be the first of its kind.

Insulin secretion has not been carefully evaluated in our study, but the improvement in blood glucose level after telmisartan treatment could be related to the improvement of β-cell function. It has been shown in experimental animals that treatment with telmisartan reduces oxidative stress and protects against islet β-cell damage and dysfunction (35).

The main finding of the present study is the significant short-term effect of telmisartan on adipokine production during insulin-stimulated conditions. Adiponectin, leptin and resistin plasma concentrations increased, whereas a decrease in TNFα had been found after telmisartan treatment. We also found an increase in basal leptin concentrations. The changes in plasma adipokines could not be directly explained by changes in their expressions in SAT. The short-term effect of telmisartan on adipokines during clamp-induced hyperinsulinemia has not been investigated up to this time; only the fasting plasma concentrations or adipose tissue expressions have been reported in the literature so far.

Tumour necrosis factor α

In the present study, the telmisartan treatment was followed by a decrease in plasma TNFα concentrations during insulin-stimulated conditions.

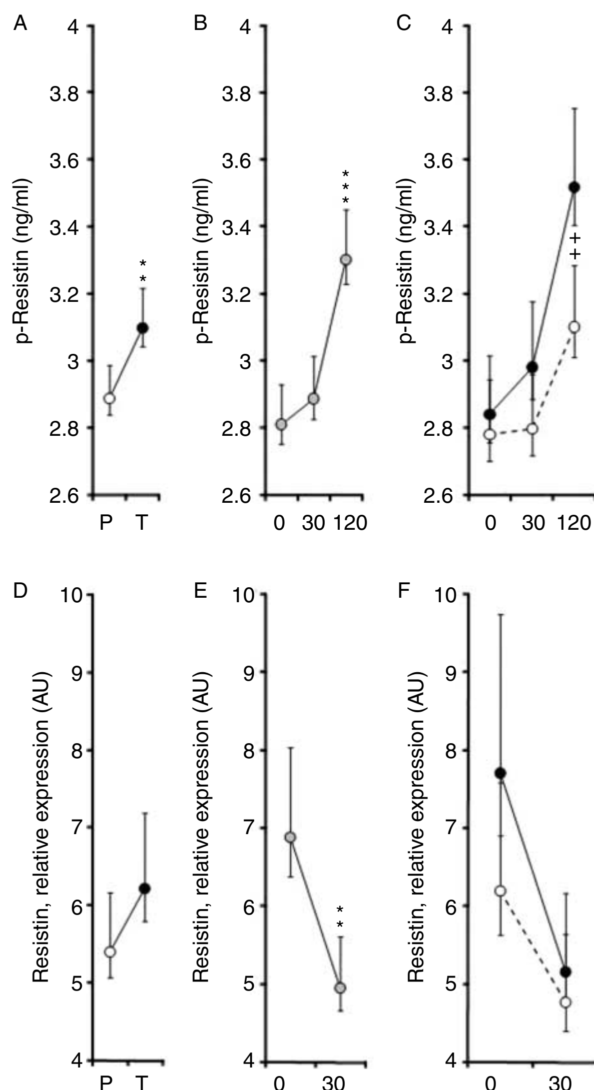

Figure 5 Effects of telmisartan treatment and the clamp-induced hyperinsulinemia on resistin. The circles with error bars symbolize the group means with their 95% confidence intervals: telmisartan (T; full circles) and placebo (P; open circles). (A and D) The effect of telmisartan is illustrated; (B and E) the effect of clamp-induced hyperinsulinemia is demonstrated; (C and F) the interactions between telmisartan and hyperinsulinemia are shown. For the p-resistin (A–C), the significance of the factors and interactions was as follows: telmisartan: $F=9$, $P=0.0043$; subject (inter-individual variability): $F=40.9$, $P<0.0001$; hyperinsulinemia: $F=18.1$, $P<0.0001$; telmisartan × hyperinsulinemia: $F=1.6$, $P=0.2033$. For the Ex-resistin (D–F), the significance of the factors and interactions was as follows: telmisartan: $F=1.9$, $P=0.1753$; subject (inter-individual variability): $F=2.3$, $P=0.0382$; hyperinsulinemia: $F=10.5$, $P=0.0029$; telmisartan × hyperinsulinemia: $F=0.3$, $P=0.6116$. Statistical significances: $^{**}P<0.01$, $^{***}P<0.001$ for differences between initial and current state; $^{++}P<0.01$ for differences between telmisartan and placebo in individual stages of HEC.

The expressions of TNFα in subcutaneous fat declined during hyperinsulinemia but were not affected by telmisartan treatment. The results are in agreement with the decrease in fasting TNFα in plasma reported after 8-month telmisartan treatment in hypertensive patients after stent implantation (36) and after 6- and 12-month administration of telmisartan in patients with metabolic syndrome (34). In addition, pro-inflammatory properties of TNFα could be attenuated by telmisartan. Inhibition of TNFα-induced IL6 expression in vascular smooth muscle cells has been reported after telmisartan treatment (37).

Adiponectin

In our study, telmisartan increased the plasma adiponectin concentrations during insulin-stimulated conditions, whereas the expressions of adiponectin in subcutaneous fat were not changed. In contrast to some (13, 27, 36, 38–40), but not all (25), previous studies, we failed to observe any stimulatory effect of telmisartan on basal plasma adiponectin concentrations. Only the trend of an increase has been found in our insulin-resistant subjects. The results could implicate the stronger metabolic effect of telmisartan during hyperinsulinemia compared to basal (pre-prandial) conditions. ARBs-induced activation of PPARG is advocated (9). However, recently, it has been showed in 3T3-L1 adipocytes that telmisartan may stimulate adiponectin gene transcription independent of PPARG (33).

Leptin

An overall increase in circulating leptin after the telmisartan treatment was documented in the present study. Telmisartan had no significant effect on leptin expressions in SAT, but the expression declined during hyperinsulinemia. The decline was independent of telmisartan treatment. In the literature, both increase (25) and decrease (34) in fasting leptin concentrations have been reported after long-term administration of telmisartan in hypertensive and type 2 diabetic patients. Moreover, the lack of effect of telmisartan on circulating leptin has been found as well (26). Telmisartan-induced increase in plasma concentrations of leptin might attenuate body weight gain by reduced food intake with HOMA-IR improvement (25). The effect of telmisartan on leptin increase could not be clarified within the context of metabolic processes in this short time study (25). However, in accordance with some authors, we have found a telmisartan-induced increase in leptin and this finding remains to be explained.

Resistin

To our surprise, in this study, an unexpected increase in plasma resistin concentration was found during hyperinsulinemia after telmisartan administration, which is a novel observation *in vivo*. In contrast, Derosa *et al.* (40) demonstrated a decrease in plasma resistin after the telmisartan treatment in type 2 diabetic patients.

However, only fasting concentrations were assessed, the treatment period was much longer (6 and 12 months), and the subjects were treated also by rosiglitazone (40). In our study, resistin expression decreased during the hyperinsulinemia independent of telmisartan treatment.

The changes in the plasma concentrations and tissue expressions of adiponectin, TNFα, leptin and resistin during hyperinsulinemia are discordant, and the expressions in SAT cannot explain the changes in circulating adipokines. This finding implicates the role of other sources of circulating adipokines – other fat depots or non-adipose sources (e.g. stromal vascular fraction of adipose tissue, macrophages or endothelium), which have not been addressed in the present study. However, the differences in the timing of blood sampling and biopsies should be considered as well.

In addition, the reduction in adipose tissue mass through weight loss in association with exercise can increase adiponectin concentrations and lower TNFα and IL6 levels in plasma, whereas drugs such as thiazolinediones increase endogenous adiponectin production (34, 41). Body weight and exercise had been kept stable throughout our study, and the patients were not treated with oral hypoglycemic agents. Thus, we can exclude the interference of these confounding factors with effect of telmisartan.

The present study also allowed us to roughly examine the *in vivo* effect of insulin on adipokines and their expressions comparing the fasting values with insulin-stimulated conditions during clamp (hyperinsulinemia factor). Only a few studies have investigated the effect of insulin *in vivo* in human subjects. It has been shown by Westerbacka *et al*. (42) that insulin *per se* could increase the expression of a number of cytokines involved in insulin sensitivity in adipose tissue, including adiponectin and TNFα. We did not find any significant effect of acute *in vivo* hyperinsulinemia induced by clamp on plasma adiponectin, leptin or TNFα (the hyperinsulinemia factor was not significant). On the other hand, the increase in plasma resistin concentrations has been confirmed. An increase in plasma resistin during clamp-induced hyperinsulinemia was reported in our previous study in healthy subjects (28). Similarly, Heilbronn *et al*. (43) found an increase in serum resistin concentrations in response to supraphysiological doses of insulin (164 ± 5 mIU/l) in obese subjects with and without diabetes. Contrary to the result of Westerbacka *et al*. (42), the expressions of adiponectin did not change, and expressions of TNFα, leptin and resistin were even suppressed by acute hyperinsulinemia. However, the present crossover study was not designed to assess the acute effect of insulin on adipokines. The control infusion to match the volume expansion during clamp was not included. Furthermore, the biopsies were taken at 30 min of clamp – very early after starting the insulin infusion, which is also an important limitation factor of our study.

The major limitations of our study are small number of subjects and/or short study duration. We did not measure peripheral blood flow. We could not exclude that the effect of telmisartan has been mediated through the blood flow improvement as well as blood pressure improvement both directly and indirectly. Moreover, we were not able to measure tissue protein levels of adipokines, and thus we could not exclude that telmisartan controls post-transcriptional rather than transcriptional regulation. However, we found the effect of telmisartan on the parameters mentioned earlier in spite of the short treatment period. The timing of biopsy at 30 min before equilibrium status had been achieved might have an impact on the results while assessing the acute effect of insulin when euglycemia had not been reached. The biopsy should have been more appropriately performed during the last part of the clamp after 90 min.

We can conclude that, in patients with metabolic syndrome with impaired fasting glucose, a short-term treatment with telmisartan surprisingly increases plasma adiponectin, leptin and resistin concentrations, and decreases plasma TNFα levels. These results also implicate that the effect of telmisartan could be important during hyperinsulinemia, and this is the first study dealing with positive effect of telmisartan on plasma adipokines during hyperinsulinemia in patients with impaired fasting glucose. The changes in plasma concentrations of adipokines cannot be explained by their expressions in SAT. The results support the hypothesis that the changes in selected plasma adipokines might be involved in the beneficial metabolic effects of telmisartan in patients with metabolic syndrome.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Appendix 4

Selected adipokines - plasma concentrations and adipose tissue expressions during 24-hour lipid infusion in healthy men

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Selected Adipokines - Plasma Concentrations and Adipose Tissue Expressions during 24-Hour Lipid Infusion in Healthy Men

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Summary

Our aim was to assess the reaction of TNFα, resistin, leptin and adiponectin to lipid infusion. Eight healthy subjects underwent a 24-hour lasting infusion of lipid emulsion. Plasma concentrations and expressions of selected cytokines in subcutaneous fat were measured. TNFα plasma concentration did not change during the first 4 hours of hypertriglyceridemia, but a significant increase after 24 hours was detected ($p<0.001$ for 0; 30; 240 min vs. 24 h). Plasma concentration of resistin significantly increased at 30 min of infusion and remained elevated ($p<0.01$ for 0 min vs. 30; 240 min; $p<0.001$ for 0 min vs. 24 h). Plasma concentrations of leptin and adiponectin did not show any significant changes. Although the expression of resistin in the subcutaneous adipose tissue tended to increase, the change was not significant. Expressions of TNFα, leptin and adiponectin were unaffected. In conclusions, our results indicate that acutely induced hyperlipidemia could influence the secretion of TNFα and resistin.

Key words

Resistin • TNFα • Leptin • Adiponectin • Insulin resistance • NEFA • Triglycerides • Lipotoxicity

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Introduction

Hyperlipidemia of either dietary origin or caused by lipid infusion promotes insulin resistance (IR) in rodents and humans (Boden and Chen 1995, Griffin *et al.* 1999, Leung *et al.* 2004). Pathophysiological mechanisms underlying the association between the elevation of plasma non-esterified fatty acids (NEFA) and IR have not been fully determined – for the evidence of interplay between glucose and NEFA see review by Cahová *et al.* (2007). Some of the pathways that lead to IR might include following cytokines produced by adipose tissue: TNFα, resistin, leptin and adiponectin.

TNFα is a promising candidate for mediating IR, although most of it comes from other sources than adipose tissue (Fain *et al.* 2003, Gabriely *et al.* 2002). Plasma concentrations of TNFα are related to obesity and IR (Hotamisligil *et al.* 1993). TNFα knock-out mice are more insulin sensitive than controls and there is the evidence that TNFα may directly interfere with insulin signaling by inhibiting phosphorylation of the insulin receptor (Hotamisligil *et al.* 1994). It impairs human preadipocyte differentiation, in mature adipocytes it decreases the activity of markers of differentiation, (Petruschke and Hauner 1993) and increases their apoptosis (Prins *et al.* 1997). TNFα has been shown to mediate NEFA-induced IR in 3T3-L1 adipocytes *in vitro* (Nguyen *et al.* 2005)

Resistin was named for its putative role in mediating IR in obesity. In mice, it is a product of an adipocyte-specific gene whose expression is down-regulated by rosiglitazone (Arner 2005). Serum levels of resistin were found to be elevated in rodent models of obesity and diabetes implicating a dysregulation of resistin in these disease states (Arner 2005). In humans,

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however, the role of resistin is unclear. The human homologue of resistin is only expressed at very low levels in adipose tissue and in circulating monocytes, and the reports on its function are controversial (Janke *et al.* 2002, Savage *et al.* 2001).

Leptin is a cytokine secreted mainly by adipose tissue, its circulating levels are in direct proportion to adipose tissue mass and nutritional status (Maffei *et al.* 1995). Therefore, it is a likely candidate for mediating insulin sensitivity. It influences the human energy balance by altering energy intake and expenditure (Hukshorn and Saris 2004). It was shown in experimental animals that leptin infusion could prevent IR induced by acute lipid infusion (Dube *et al.* 2007).

Consistent inverse association between adiponectin and IR as well as the pro-inflammatory state has been established (Nedvídková *et al.* 2005). Several mechanisms of its metabolic effects have been described (Kadowaki *et al.* 2006, Whitehead *et al.* 2006): induction of glucose uptake and NEFA oxidation in muscle, increased insulin sensitivity and NEFA oxidation and reduced glucose output and NEFA influx in liver.

Our aim was to assess the reaction of TNFα, resistin, leptin and adiponectin to lipid infusion. We have evaluated both the plasma concentrations and expression of their genes in the abdominal subcutaneous adipose tissue in healthy humans.

Subjects and methods

Subjects

We examined eight young healthy males. All of them were euthyroid and none had a concomitant disease. They were not taking any drugs, and none had a family history of diabetes. Clinical characteristics are in Table 1. The study was conducted after approval by local ethics committee. Informed consent was obtained from all the individuals after the purpose, nature, and potential risks of the study had been explained.

Experimental protocol

The subjects were instructed to adhere to their ordinary lifestyle, to avoid changes in food intake, alcohol consumption, and vigorous exercise on the day before examination. They were examined after overnight fasting with only tap water allowed *ad libitum*. The study always started between 7:45-8:00 AM. Subjects were excluded from the study if the weight change was more than 3 kg in 6 months.

Table 1. Characteristics of the study group.

n	8
Age (years)	25.8±3.7
Weight (kg)	83.4±4.7
Body mass index (kg.m^{-2})	25±1.7
Waist circumference (cm)	88.5±5.0
Fasting blood glucose (mmol.l^{-1})	4.35±0.7
Blood glucose at 120 min of OGTT (mmol.l^{-1})	4.55±0.7
Fasting IRI (mIU.l^{-1})	4.50±2.85
HbA1c (%) - according to IFCC	2.58±0.61
Albuminuria (µg.min^{-1})	2.52±2.6
Serum creatinine (µmol.l^{-1})	87±10.5
Total cholesterol (mmol.l^{-1})	4.24±0.73
Triglycerides (mmol.l^{-1})	0.87±0.30
HDL-cholesterol (mmol.l^{-1})	1.45±0.34
LDL-cholesterol (mmol.l^{-1})	2.48±0.55

Data are expressed as mean ± SD (IFCC, International Federation of Clinical Chemistry and Laboratory Medicine).

A 24-h infusion of lipid emulsion (Intralipid 20 %; Fresenius Kabi AB, Uppsala, Sweden) was administered through teflon cannula in the antecubital vein. Intralipid 20 % contained soya oil 200 g, egg lecithin 12 g and glycerol 22 g per 1 liter. The speed was set to 125 ml.h^{-1} for the first 4 h, and then until the 24th hour to a constant rate that was calculated to achieve a total dose of 3 g of fat.kg body weight^{-1}.d^{-1} This two-step setting of infusion was used to achieve both maximum effect and to stay within the clinically allowed boundaries. Thirty minutes before blood sampling a second cannula was inserted retrogradely into a wrist vein for blood collection. To assess the plasma levels of selected adipokines, blood samples were taken at 0 min, 30 min, 240 min and 24 h of the infusion. Thirty minutes before taking each sample the hand was placed in a heated (65 $^{\circ}$C) box in order to achieve venous blood arterialization. Needle biopsy of abdominal subcutaneous adipose tissue was performed before (0 min), and at the 4th and 24th hour of the lipid infusion. Under local anesthesia (1 % trimecain in a field block pattern) an incision (3-4 mm) was made through the skin at the lower abdomen and a subcutaneous fat specimen (200 mg) was obtained by needle (Braunüle MT, no.4; B. Braun, Melsungen, Germany) aspiration. Different place and incision was used each time, but the same depth in the adipose tissue mass when performing the needle aspiration was attempted. The samples were washed in

NaCl 0.9 % solution, immediately frozen by liquid nitrogen and stored at –80 ºC until used for RNA extraction.

Analytical procedures

Infusion monitoring

Plasma glucose concentrations were measured on a Beckman analyzer (Beckman Instruments Inc, Fullerton, CA, USA) using the glucose oxidase method. Hemoglobin A1c was measured by fully automated analyzer Tosoh HLC-723 G7 (Tosoh Corporation, Tokyo, Japan). Method was calibrated to IFCC reference procedure (Mosca *et al.* 2007). Immunoreactive insulin (IRI) was determined by radioimmunoassay (Insulin IRMA kit, IMMUNOTECH as, Prague, Czech Republic). Plasma concentrations of NEFA were estimated by Free fatty acids, Half-micro test (Roche Diagnostics GmbH, Penzberg, Germany), whereas plasma concentrations of triglycerides (TG) by enzymatic assay (BIO-LA-TEST, PLIVA-Lachema, Brno, Czech Republic).

Adipokine assessment in plasma

Plasma concentrations of TNFα were measured by immunoassay (Human TNF-α UltraSensitive; BioSource Int., Camarillo, CA, USA) and the detection limit was <0.09 pg/ml, intra-assay and inter-assay coefficients of variation (CV) of 5.3 to 6.7 % and 8.2 to 9.7 %, respectively). Plasma concentrations of resistin were determined using a Human Resistin ELISA kit (BioVendor Lab. Med. Inc., Brno, Czech Republic) the detection limit was 0.033 ng/ml, intra-assay and inter-assay CV of 2.8 to 3.4 % and 5.1 to 6.9 %, respectively. Pplasma concentrations of leptin were measured by a Human Leptin ELISA kit (BioVendor Lab. Med. Inc., Brno, Czech Republic), the detection limit was 0.5 ng/ml, intra-assay and inter-assay CV of 3 to 7.5 % and 3.2 to 9.2 %, respectively. Plasma concentrations of adiponectin were determined using a Human Adiponectin ELISA kit (BioVendor Lab. Med. Inc., Brno, Czech Republic) the detection limit was 210 ng/ml, intra-assay and inter-assay CV of 6.4 to 7 % and 7.3 to 8.2 %, respectively (all according to manufacturers instructions).

Assessment of expressions in adipose tissue

Expression of each adipokine was analyzed by the real-time PCR method. Subcutaneous adipose tissue (100 mg) was excised from the biopsy, and homogenized in 1 ml of a QIAzol Lysis Reagent (Guanidin Thiocyanate – Phenol solution) for 2 min. The RNA was isolated from the liquid nitrogen frozen biopsy using the RNeasy Lipid Tissue Mini Kit (QIAGEN, Valencia, CA, USA) and QIAzol Lysis Reagent (QIAGEN, Valencia, CA, USA). Possible contamination of RNA with genomic DNA remains was taken off by DNase digestion (RNase-free DNase Set; QIAGEN, Valencia, CA, USA). The cDNA was synthesized using a recombinant Omniscript Reverse Transcriptase (QIAGEN, Valencia, CA, USA), Ribonuclease Inhibitor from human placenta (SIGMA, St. Louis, MO, USA), and $(dT)_{16}$ oligonucleotides.

The real-time PCR procedure itself has been carried out on the DNA Engine Opticon 2 System (MJ Research, Waltham, MA, USA). HotStar Taq DNA polymerase and SYBR Green fluorescent dye (QuantiTec SYBR Green PCR Kit, QIAGEN, Valencia, CA, USA) were used for the RT-PCR reaction. To eliminate the influence of primer dimmers, negative controls were used. The human gene cyclophilin was used as a reference. Primers used are shown in Table 2. The data were processed by Q-gene 96 software.

Statistical analysis

The time profile was evaluated using a repeated measures ANOVA model consisting of the time and subject factors. To evaluate the differences between basal values and individual stages of the time profile, the ANOVA testing was followed by least significant difference multiple comparisons. A probability level of $p<0.05$ was considered as statistically significant in all statistical tests. Due to non-Gaussian data distribution in most of the dependent variables, these data underwent a power transformation to attain distributional symmetry and constant variance. Non-homogeneities were detected using residual diagnostics. The experimental points showing absolute values of studentized residuals greater than 3 were excluded from the analysis. With the exception of serum leptin levels (6.3 %), the proportions of such data never exceed 5 % of the total number. Statistical software Statgraphics Plus v. 5.1 from Manugistics (Rockville, MD, USA) was used for calculations.

Results

Our primary goal was to assess the reaction of selected adipokines to acute lipid infusion and the following results were obtained. TNFα plasma concentration did not change during the first 4 h of hypertriglyceridaemia, but a significant increase after 24 h

Table 2. Primers used for RT-PCR of the subcutaneous adipose tissue samples taken during the lipid infusion.

Gene	accession number	forward primer	reverse primer
Adiponectin	XM_290602	HACRP30-F: 5´-GGT TCA ATG GCT TGT TTG C -3´	HACRP30-R: 5´-TCA TCC CAA GCT GAT TCT G-3´
Leptin	NM_000230	Hleptin-F: 5´-CCC TAA GCC TCC TTT TGC T-3´	Hleptin-R: 5´-GCT AAG AGG GGA CAA GAC A-3´
TNFα	X02910 X02159	HTNFa-F: 5´-CTA TCT GGG AGG GGT CTT C-3´	HTNFa-R: 5´-TTG GGA AGG TTG GAT GTT C -3´
Resistin	AY207314	HRETN-F: 5´-ATA AGC AGC ATT GGC CTG G-3´	HRETN-R: 5´-TGG CAG TGA CAT GTG GTC T-3´
Cyclophilin	XM_090070	HCLPNa-F: 5´-CAA ATG CTG GAC CCA ACA CA -3´	HCLPNa-R: 5´-TGC CAT CCA ACC ACT CAG TC-3´

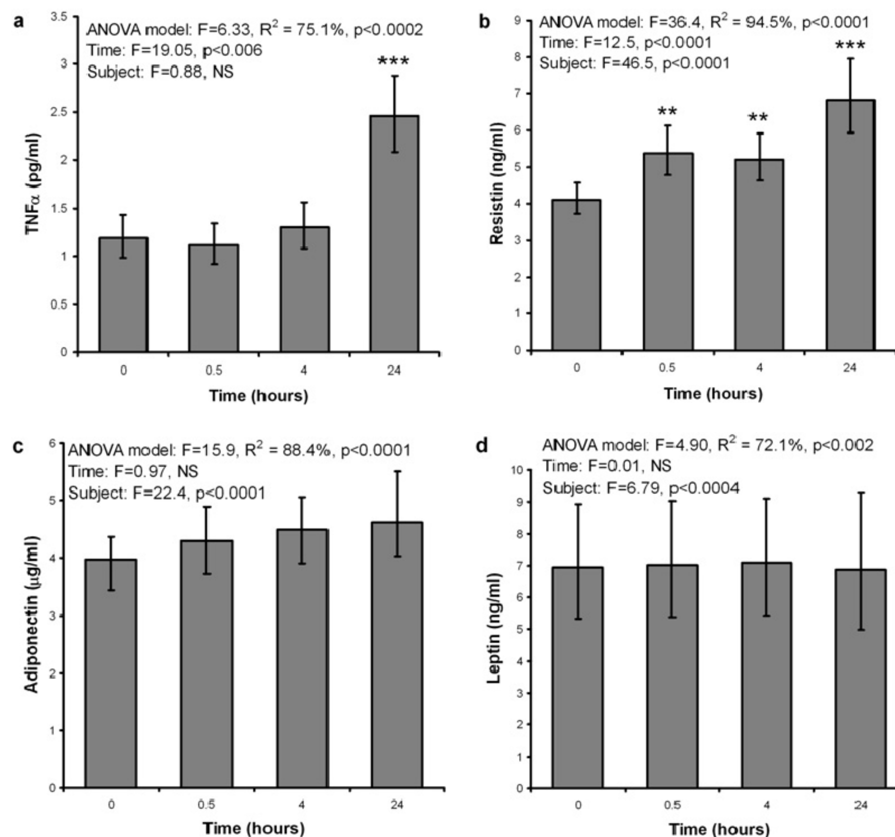

Fig. 1a-d. Plasma levels of selected adipokines during 24-hour lipid infusion. The bars with error bars represent re-transformed means with their 95 % confidence intervals. F in the embedded tables represents Fisher's statistics for individual factors. R^2 (squared correlation coefficient of the linear model) symbolize the proportion of the total variability in the dependent variable, which is explained by the ANOVA model. Asterisks symbolize significant differences between individual stages of the time profiles and basal values (** for p<0.01, *** for p<0.001) as detected by least significant difference multiple comparisons.

was detected (p<0.001 for 0; 30; 240 min vs. 24 h; Fig. 1a). The expression of TNFα in subcutaneous adipose tissue did not change (Fig. 2a). Plasma concentration of resistin significantly increased at 30 min of infusion and remained elevated throughout the 24 h (p<0.01 for 0 min vs. 30; 240 min; p<0.001 for 0 min vs. 24 h; Fig. 1b). The expression of resistin in the subcutaneous adipose tissue tended to increase, but the change was not significant (Fig. 2b). Plasma concentrations of leptin and adiponectin (Fig. 1c-d) did not show any significant changes and their expressions were not significantly altered.

To validate the effectiveness of lipid infusion,

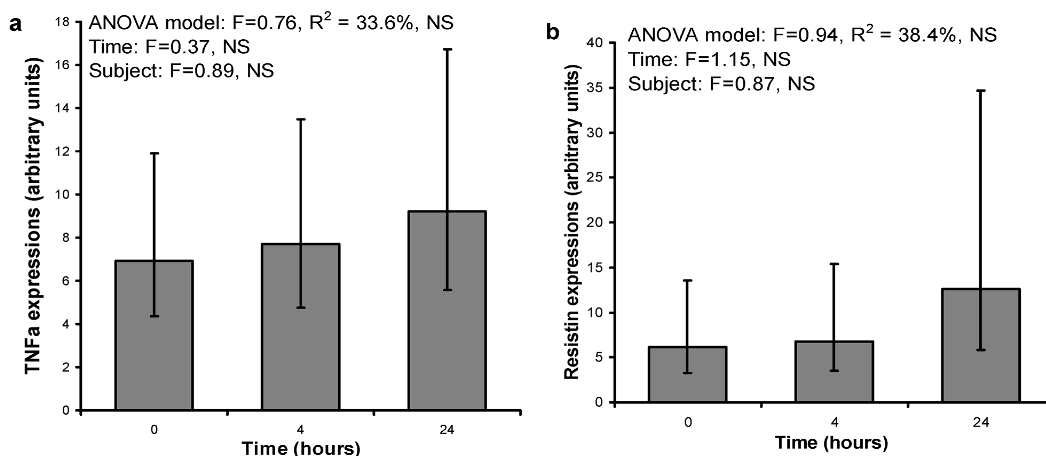

Fig. 2a,b. Expressions of selected adipokines in subcutaneous abdominal adipose tissue during 24-hour lipid infusion. The bars with error bars represent re-transformed means with their 95 % confidence intervals (expression of selected cytokine related to expression of cyclophilin). The changes in time were assessed by ANOVA and are not statistically significant.

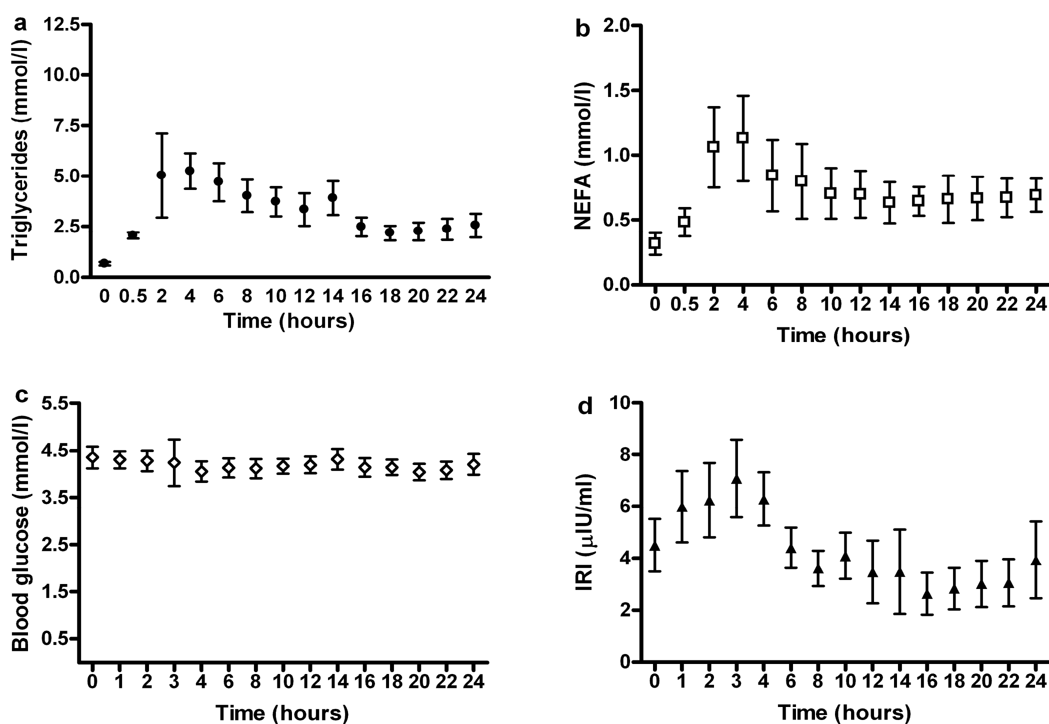

Fig. 3a-d. Concentrations of serum triglycerides (●), non-esterified free fatty acids (NEFA, □), blood glucose (◇) and immunoreactive insulin (IRI, ▲) during the 24-hour lipid infusion. Data are shown as means with their 95 % confidence intervals.

plasma levels of TG, NEFA, glucose and IRI were monitored. TG increased almost 8-fold and NEFA about 3.5-fold in the 4th hour and they remained elevated until the end of infusion at more than 3-fold and 2-fold, respectively (Figs 3a and 3b). Blood glucose was stable throughout the 24 h (Fig. 3c). After initial modest rise, IRI concentrations were also stable (Fig. 3d). Cytokine plasma concentrations did not correlate with IRI concentrations (data not shown).

Discussion

We have characterized the plasma levels of adipose tissue cytokines and their expression in adipose tissue during pharmacologically-induced hyperlipidemia. This was achieved by an intravenous lipid infusion, in which the effectiveness in rise of plasma TG and NEFA was verified. We have decided not to use heparin infusion alongside because heparin as a co-factor of a lipoprotein lipase would decrease plasma TG concentrations and

increase plasma NEFA concentrations, which was not our aim. It could also add further bias through possible unknown effects. Since heparin was used in some further cited experiments (Garcia-Lorda *et al.* 2003, Nisoli *et al.* 2000, Yang *et al.* 2005), this sole fact could be the reason for differences in results obtained.

Our finding of an increase in TNFα plasma concentration during lipid infusion is novel. It is partly in accordance with the report of Nisoli *et al.* (2000) who found the increase in TNFα gene expression in subcutaneous gluteal fat tissue after a 5-h intralipid/heparin infusion. In our results, the expressions in adipose tissue showed only a tendency to increase but not a significant rise. This can be explained by the differences in expressions in different fat depots. Both in human (Fain *et al.* 2003) and rodent (Gabriely *et al.* 2002) studies, it has been reported that production of both of resistin and TNFα is much higher in visceral than in subcutaneous fat. Moreover, most of the free circulating TNFα comes from circulating monocytes and significant amounts of resistin, although originally reported as adipocyte-specific (Kim *et al.* 2001), might be of the same source (Savage *et al.* 2001).

During lipid infusion, plasma concentrations of resistin have increased significantly. This is in accordance with the reports from animal models (Yang *et al.* 2005) where intralipid/heparin infusion induced hepatic and peripheral IR and was associated with elevated plasma resistin levels. In human, it is a novel observation. Since the increase in expression of resistin mRNA in subcutaneous adipose tissue was not significant, we can conclude that this adipocyte depot is not responsible for its increased plasma concentration.

Plasma concentration of leptin did not show any significant changes and its expression was not significantly altered. Although most studies have not shown direct effect of lipid infusion on leptin, as when consistent release from adipose tissue during lipid without heparin infusion was reported (Samra *et al.* 1998), some other results were also obtained: no change in plasma levels together with increase in leptin RNA expression in adipocytes in gluteal region during lipid and heparin infusion (Nisoli *et al.* 2000) and modest decline of its plasma levels during lipid and heparin infusion (Garcia-Lorda *et al.* 2003).

Response of adiponectin to acute lipid load was also not statistically significant. The same result of unchanged plasma concentrations was observed during intralipid/heparin infusion (Krzyzanowska *et al.* 2007).

Lipid infusion resulting in a subsequent increase in NEFA plasma concentrations is a well-known method used for inducing IR (Boden and Chen 1995, Griffin *et al.* 1999, Leung *et al.* 2004). Therefore, although we have not measured insulin sensitivity, we suggest that the changes we have observed might be implicated in the pathogenesis of lipid-induced IR. Our results are in agreement with a theory that TNFα is implicated in NEFA-induced IR as shown *in vitro* (Nguyen *et al.* 2005). For resistin it is an unclear result because it is often found not to be associated with IR (Utzschneider *et al.* 2005), although the studies are usually limited to correlations between resistin and various measures of metabolic syndrome. Rather exception is a report of Sheng *et al.* (2008) where overexpression of resistin in human hepatocytes induced IR most likely by blocking insulin signal transduction pathways of PI-3K/Akt and of CAP/c-cbl.

We are aware that there are limits to our study. Increase in plasma lipid concentrations by intravenous infusion certainly differs from dietary and lifestyle induced hyperlipidemia observed in real life. Small number of subjects examined might have prevented us from seeing some other possibly significant results and therefore the value of “negative” results is limited. In addition, the lack of placebo control, which was not done due to the potential troublesome interpretation of a 24-h long fasting, to some extent limits the reliability of “positive” results. Circadian variations were partly assessed by 24-h duration. Glycerol (a component of intralipid emulsion) fortunately does not affect insulin secretion (Boden and Chen 1999, Pelkonen *et al.* 1968), glucose effectiveness (Hawkins *et al.* 2003) or insulin sensitivity (Ferrannini *et al.* 1983). However, the effect of glycerol on resistin and TNFα can not be excluded. Resistin could also be affected by hyperinsulinemia, although this observation was made at several-fold higher insulin concentrations (Krušinová *et al.* 2007) than observed during our lipid infusion. We have also found significant inter-individual and intra-individual differences of the mRNA content in adipose tissue samples for each adipokine. We must, however, note that this could partly be due to different adipocytes/stroma-vascular cells ratio in each sample. In future studies, this can be assessed by separation of these fractions by collagenase tissue digestion (Rodbell 1963).

In conclusion, our results indicate that acutely induced hyperlipidemia could increase the secretion of TNFα and resistin. This finding supports the hypothesis

that these adipokines could be involved in the pathogenesis of lipid-induced IR.

Conflict of Interest

There is no conflict of interest.

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Appendix 5

Fatty acid binding proteins in adipose tissue: a promising link between metabolic syndrome and atherosclerosis?

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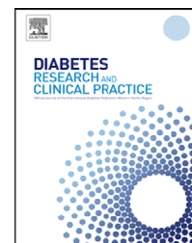

Fatty acid binding proteins in adipose tissue: A promising link between metabolic syndrome and atherosclerosis?

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ABSTRACT

Adipocyte/macrophage fatty acid binding protein (A-FABP) has been shown to be closely associated with metabolic syndrome, obesity and development of atherosclerosis. Moreover, A-FABP has been recently suggested as a potential therapeutic target of these abnormalities in animal models. The present review aims to summarize current knowledge on A-FABP functions and regulations both in animal models and humans, since the role of A-FABP in human physiology and disease has not been presently clarified.

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1. Introduction

Insulin resistance, hypertension, dyslipidaemia and impairment of glucose homeostasis are closely linked to obesity, forming a cluster of abnormalities well known as metabolic syndrome [1] that is associated with accelerated atherosclerosis and cardiovascular disease.

Recently, molecular pathways linking obesity with metabolic and atherosclerotic defects are being intensively explored. Obesity is being considered as a chronic inflammatory state, in which the excess accumulation of adipose tissue plays a central role [2,3]. Increasing evidence shows that adipose tissue is not only an inert energy depot, but also exerts important endocrine functions, secreting multiple cytokines, adipokines, hormones and free fatty acids (FFA) and thus participates in regulation of energy metabolism, insulin sensitivity and inflammation and contributes to the development of metabolic syndrome [4].

Dysregulation of lipid metabolism with increased FFA plasma concentrations is consistently associated with insulin resistance and type 2 diabetes [5]. Elevated FFA not only modify glucose and lipid metabolism, but also influence cell signaling cascades and gene expression [6]. These hydrophobic compounds are transported in the circulation bound to albumin, their cellular uptake occurs by passive diffusion or by protein-mediated binding and translocation mechanisms, while intra-cellularly, fatty acid binding proteins (FABPs) represent the important carriers for FFA.

Specifically, adipocyte-specific FABPs have gained much attention in recent years, since their role in insulin resistance, type 2 diabetes and atherosclerosis has been suggested based on studies in FABP-deficient mice models [7]. Although comprehensive research in knock-out mice models has brought evidence of the specific impact and mechanisms of action of FABPs in metabolism and inflammation, the definitive biology and function of FABPs in human physiology and disease remain not fully clarified.

2. FABP family

FABPs are small (14–15 kDa) cytoplasmic proteins that bind reversibly with high affinity to hydrophobic ligands, such as saturated and unsaturated long chain fatty acids, eicosanoids and other lipids. FABPs can be found across all species, demonstrating strong evolutionary conservation

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[8]. At least 9 tissue-specific cytoplasmic FABPs have been identified at present (Table 1). The overall gene structure conserved between all family members, consists of 4 exons separated by 3 introns and contains canonical TATA box upstream of the transcription site [9]. Chromosomal mapping of the FABP genes shows that they are dispersed in the genome, but some loci create a synteny group (A-FABP, E-FABP, and M-FABP). Within different species the genes are located on different but comparable chromosomes [8]. The three-dimensional protein structure also shows similar features in all FABP types despite the differences in amino acid sequence (22–73% similarity of the protein structure). They are composed of ten antiparallel β strands that form a β barrel. The single bound ligand can be found within the barrel in internal water filled cavity [8,10].

The functions of cytoplasmic FABPs include enhancement of FFA solubility and transport of FFA to specific enzymes and cellular compartments (to the mitochondria and peroxisomes for oxidation, to the endoplasmic reticulum for reesterification, to the lipid droplet for storage, or to the nucleus for regulation of gene expression) [8,10,11]. Over-expression and anti-sense studies have proposed roles of FABP in FFA import, storage and export as well as cholesterol and phospholipid metabolism [12].

By modulation of FFA availability, FABPs have an indirect regulatory effect on various cellular processes in which FFA are involved, such as signal transduction. FFAs transmit a stress response through activation of protein kinase C θ, inhibitor of kappa kinase (IKK) or c-jun NH_2-terminal kinase (JNK) that have been linked to insulin resistance and metabolic syndrome [7]. Additionally, the role of FFAs in regulation of gene transcription has been well characterized, especially for those genes involved in lipid metabolism, e.g. acyl-CoA synthase, acyl-CoA oxidase, stearoyl-CoA desaturase, carinitine–palmitoyl transferase or A-FABP itself [8]. Several mechanisms of FFA-mediated regulation of gene transcription have been proposed [13]: binding to and activation of a transcription factor, modification of the mRNA stability or regulation of the transcription factor expression. It has been proven that FABPs cooperate with transcription factors of PPAR family (peroxisome proliferator-activated receptor) by means of induction of ligand-dependent transactivation of PPARs [11].

Following detailed review of recent knowledge on the function and regulation will focus on adipocyte-specific isoforms—A-FABP and E-FABP.

3. Adipocyte-specific FABPs

3.1. *Adipocytes*

Two types of FABPs are expressed in adipocytes: A-FABP (mouse homologue aP2) – a predominant cytosolic protein of mature adipocytes, accounting for ~6% of total cellular protein [14], and E-FABP (mouse homologue mal1) – a minor isoform found in fat cells.

3.1.1. *Knock-out models*

Their functions have been demonstrated in the following knock-out mice models: $aP2^{-/-}$ mice, $mal1^{-/-}$ mice and aP2-$mal1^{-/-}$ mice. The phenotype of $aP2^{-/-}$ mice is unremarkable at baseline with normal growth, reproduction and no defects in adipose tissue [15]. This is explained by compensatory up-regulation of mal1 protein. However, in the context of dietary or genetic obesity [15,16] the aP2 deficient mice are protected from development of insulin resistance and type 2 diabetes in spite of slightly higher body weight and elevated FFA plasma concentrations. $aP2^{-/-}$ mice also failed to express tumor necrosis factor alpha in adipose tissue. Adipocytes from $aP2^{-/-}$ mice exhibited ~40% decrease in basal lipolysis in vivo and in vitro [14,17] and 2- to 3-fold decrease in FA release [18] and thus higher cellular as well as plasma FFA levels, suggesting that aP2 mediates FA efflux in normal physiology. The reduced lipolysis in $aP2^{-/-}$ mice was initially attributed to the ability of A-FABP to bind and activate the hormone-sensitive lipase (HSL) [14,19] forming 1:1 complexes with the lipase. However the activation of HSL by A-FABP is rather dependent upon FA binding properties of FABP, because in absence of FA no FABP-HSL association could be demonstrated [20]. E-FABP has been also shown to interact with HSL.

Additionally in the absence of aP2, the acute insulin secretory response to β-adrenergic stimulation was markedly suppressed [16,17]. Furthermore an increase in muscle glucose oxidation and de novo lipogenesis in adipocytes, reduced expression of HSL in adipose tissue, together with increased serum and tissue adiponectin, but unaltered adiponectin expression, were detected in aP2 knock-out model maintained on a high-fat diet [21].

The following mice model – $mal1^{-/-}$ mice – also exhibits a small increase in insulin sensitivity, slightly decreased body weight without changes of total fat mass on the background of dietary induced and genetic obesity [22]. Transgenic mice overexpressing mal1 did not show any changes in body

Table 1 – FABP types, their tissue occurrence.

FABP type	Gene name	Alternative names	Tissue
Liver FABP	FABP1	L-FABP	Liver, intestine, stomach
Intestinal FABP	FABP2	I-FABP	Intestine, stomach
Heart FABP	FABP3	H-FABP	Heart, kidney, skeletal muscle, aorta, adrenals, placenta, brain, testes, ovary, lung, mammary gland
Adipocyte FABP	FABP4	A-FABP; aP2; ALBP	Adipose tissue, macrophages
Epidermal FABP	FABP5	E-FABP; mal1; KLBP	Skin, brain, lens, retina, endothelium, adipose tissue, kidney, liver
Ileal FABP	FABP6	Il-FABP	Ileum, ovary
Brain FABP	FABP7	B-FABP	Brain
Myelin FABP	FABP8	MP2	Peripheral nervous system
Testis FABP	FABP9	T-FABP	Testis

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weight, FFA and lipid concentrations during dietary induced obesity but a decrease in glucose clearance was observed [22]. Additionally, increased lipolysis and increased expression but lower serum levels of resistin were detected in this transgenic model [21].

To overcome the compensatory increase of mal1 production in $aP2^{-/-}$ mice and to examine the impact of both FABPs on adipocyte function and systemic metabolic homeostasis, mice lacking both aP2 and mal1 were developed [23]. They exhibit a striking phenotype: in contrast to individual aP2 or mal1 deficiency, they do not gain weight on high fat diet, show increased O_2 consumption and CO_2 production. The observed increase in insulin sensitivity and improvement of lipid profile in $aP2\text{-}mal1^{-/-}$ mice exceed those observed in individual FABP deficiency and the combined knock-out model is also protected from hepatosteatosis. At the cellular level, molecular mechanisms have been identified that underlie the observed phenotypic changes: enhanced insulin receptor signaling and AMP-activated kinase activity in skeletal muscle, and reduced stearoyl-CoA desaturase-1 activity and decreased SREBP1c expression in liver [23].

3.1.2. *Regulation of A-FABP expression in adipocytes*

A-FABP expression is enhanced during adipocyte differentiation [24] and is mostly regulated at the transcriptional level [10]. In vitro studies in adipocytes showed that A-FABP expression is positively regulated by fatty acids, PPARγ [25] and insulin [26,27], as well as by PPARγ agonists such as thiazolidinediones (TZD) [28–30]. The latter was also proven in obese Zucker rats [31]. Recently, Cabré and co-workers have demonstrated that TZD induced human adipocyte differentiation in vitro is associated with increase in A-FABP mRNA and cellular protein levels as well as with increase of A-FABP secreted to culture media [30]

PPARγ regulates the expression of A-FABP through PPRE (peroxisome proliferator responsive element) present in the A-FABP gene promoter [32]. On the other hand, an interaction of FFA with FABP is essential for relocation of the lipid ligand to the nucleus, where the entire FABP/FFA complex activates PPARγ in a ligand dependent fashion. The interaction between FABP and PPAR proceeds in receptor and ligand selective manner: A-FABP activates PPARγ, E-FABP interacts with PPARβ [11].

In obesity, both A-FABP [33,34] and E-FABP [35] expression is increased in animal models.

3.1.3. *A-FABP in human adipose tissue*

The evidence in humans is not that uniform as in the animal models.

In obesity, A-FABP expression in subcutaneous adipose tissue (SAT) was comparable to lean subjects [36,37], neither correlated with measures of adiposity [36], insulin resistance or FFA composition [38]. On the contrary, E-FABP was shown to be more expressed in SAT of lean subjects [37], whereas other studies demonstrated correlation of E-FABP expression in SAT with intra-abdominal fat volume, triglycerides [36] or glucose [38].

Fisher and coworkers [37] suggested the A-FABP/E-FABP ratio to be a summarizing measure of FABP changes. They have shown a lower ratio in lean subjects compared to obese (comparable A-FABP expression, higher E-FABP expression in lean versus obese), as well as in obese patients after substantial weight loss following the gastric banding surgery. However, the individual values of both FABP expressions significantly increased after weight loss.

On the other hand, short-term overfeeding in healthy women [39] was associated with increased expression and activation of PPRAγ, as shown by increased expression of A-FABP and its correlation with PPARγ expression. The fat mass gain was positively related to PPARγ and A-FABP mRNA changes.

There are regional differences in adipose tissue FABP expression that might be influenced by obesity [40,41]: in obese individuals A-FABP mRNA and protein were higher in SAT compared to visceral adipose tissue (VAT), which was in accordance with higher rates of basal lipolysis detected in SAT adipocytes [41]. E-FABP expression was comparable between the depots in obese individuals. Lean subjects displayed higher E-FABP expression in VAT, A-FABP protein and mRNA were similar in both depots. The authors concluded that the A-FABP/E-FABP ratio might be an important measure being higher in SAT in both lean and obese subjects. In this study, expressions of both FABPs were suggested to be higher in lean subjects; however, the groups were not directly compared. In another group of lean and overweight subjects, higher A-FABP expression in SAT compared to VAT was also demonstrated [42].

Gender difference in FABP expression was suggested by Fisher et al. [37], who described higher A-FABP/E-FABP ratio in men compared to women in both lean and obese subgroups. Taking into account the separate FABP expression the effect of gender was not significant.

Other regulatory mechanisms (insulin, FFA, thiazolidinediones) have not been tested in vivo with concern to human adipose tissue.

3.2. *Macrophages*

Both A-FABP and E-FABP are also present and expressed in macrophages [24,43] that show striking similarities to adipocytes in their biology and functions—both are able to accumulate lipids, secrete cytokines and express number of the same genes involved in lipid metabolism and inflammation [6].

First evidence on FABP functions and regulations in macrophages is derived from cell cultures—primary isolated human monocytes or monocyte THP-1 cell lines. A-FABP expression was not detected in the resting cells but became evident following differentiation stimulation with phorbol myristate acetate [43], oxidized LDL [44], PPARγ activators [45] or lipopolysacharide/Toll like receptor activation [46]. On the contrary, the oxidized LDL-induced A-FABP expression was attenuated following treatment with atorvastatin [47].

E-FABP is regulated in an identical manner as A-FABP in cell cultures [24]. However, unlike adipocytes, macrophages do not exhibit the compensatory increase in E-FABP expression in A-FABP deficient mice [43]. Although being a minor isoform in adipocytes, E-FABP protein levels are comparable to A-FABP in macrophages.

Studies dealing with molecular mechanisms of FABP action in macrophages have shown that A-FABP deficiency reduced

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cholesterol-ester accumulation as well as production of inflammatory cytokines both in the resting state and during differentiation, but it increased the cellular FFA [43]. In the absence of A-FABP, the macrophage PPARγ activity is enhanced, leading to elevated CD36 expression and stimulation of cellular lipoprotein uptake [48]. The proteins of cholesterol efflux pathway are also up-regulated in A-FABP deficiency and thus cholesterol release is preferred to accumulation. In parallel, A-FABP deficient macrophages display reduced IκB kinase and NF-κB activity resulting in suppression of inflammatory functions [48]. In accordance with these findings, an overexpression of A-FABP in human macrophages drives cholesterol ester accumulation and triggers foam cell formation [7,37].

In a rodent model of accelerated atherosclerosis – apolipoprotein E-deficient (apoE$^{-/-}$) mice, the ablation of aP2 gene has led to substantial reduction of vascular atherosclerotic lesions in the absence of differences in serum lipids or insulin sensitivity [43]. This effect can be attributed to macrophage-specific action of aP2 in formation of atherosclerotic lesions, as it was confirmed by bone marrow transplantation studies [24,43].

Combined aP2 and mal1 deficiency in apoE$^{-/-}$ mice has led to further dramatic reduction of atherosclerosis development compared to aP2$^{-/-}$ mice and wild type mice with the same background [49]. Moreover, the aP2 and mal1 null mice also have significantly improved survival (by 67%) in apoE$^{-/-}$ background even when challenged with the Western hypercholesterolaemic diet for 1 year, probably due to increased stability of atherosclerotic plaques [49].

3.3. *Skeletal muscle*

Recently it has been shown that A-FABP is also present and expressed within skeletal muscle fibers [50], although in markedly lesser amount than H-FABP, which is generally accepted as dominating FABP in skeletal muscle (the mRNA H-FABP: A-FABP ratio was 10:1). Interestingly A-FABP mRNA and protein were more abundant in the endurance trained subjects, suggesting a physiological role of A-FABP in metabolic adaptation.

3.4. *Plasma*

Although A-FABP has been traditionally thought to be a cytoplasmic protein, Xu and coworkers have demonstrated using tandem mass spectrometry-based proteomic analysis that A-FABP is released from 3T3-L1 adipocytes into extracellular medium in the absence of any obvious cell damage [51]. The presence of A-FABP in human plasma was confirmed, showing comparable or higher concentrations than those of several major adipokines (~10–60 ng/ml) [51]. Adipose tissue is considered as a source of circulating A-FABP; however, the relative contribution of adipocytes versus macrophages (resident in adipose tissue or circulating) to plasma concentrations of A-FABP has not been elucidated so far. The only study in vivo, showing adipose tissue expressions and plasma concentration of A-FABP in parallel in animal model, revealed a positive correlation between serum A-FABP and its expression levels in visceral, subcutaneous and epididymal fat depots [52]. The human data on relation between A-FABP expression in adipose tissue and its serum concentration are lacking.

Mechanism of A-FABP secretion from cells remains also unclear, since the protein has no ordinary secretory signal peptide and its molecular weight is relatively low [53].

Similarly, the physiological functions of circulating A-FABP remain to be determined. There is increasing evidence on the predictive role of increased serum A-FABP in metabolic syndrome and cardiometabolic risk, which is based on population studies discussed bellow. However, at the molecular level no data explaining a causal relationship are available at present. It is speculated that circulating A-FABP might function as a lipid hormone transporter or in hormone-like fashion to modulate systemic insulin sensitivity and energy metabolism [51,52]. Further intriguing questions have been raised: what are factors influencing the balance between serum and tissue A-FABP? How can an increase in circulating A-FABP lead to progression of cardiometabolic risk? [52].

3.4.1. *Regulations of circulating A-FABP*

Serum A-FABP concentrations were reported to be positively and independently associated with parameters of adiposity, hyperglycaemia, insulin resistance (assessed as HOMA index) and metabolic syndrome abnormalities in an Asian population in cross-sectional [51] and longitudinal [52,54] studies, as well as in a cohort of Caucasian population [53]. Plasma A-FABP increased significantly with the increasing number of prevalent components of metabolic syndrome [30,51,55]. Higher circulating A-FABP levels in comparison with lean subjects were detected in overweight/obese patients [51,56] and children [57], in patients with metabolic syndrome [53] and/or type 2 diabetes [30,58]. In subjects with impaired glucose homeostasis both increase [52] and no difference [58] in plasma A-FABP have been reported.

Another specific example of metabolic syndrome and/or lipodystrophy is represented by HIV-infected patients. In this cohort, plasma A-FABP is also strongly correlated with metabolic syndrome abnormalities, including insulin levels [55]. Patients in the highest quartile of A-FABP presented 6-fold increased odds ratio for metabolic syndrome and 3-fold increased odd for lipodystrophy.

The longitudinal studies even showed predictive values of high A-FABP levels for development of metabolic syndrome over the course of 5 years [54] and for prevalence of type 2 diabetes over the course of 10 years [52], which were independent on obesity, insulin resistance or glycaemic indexes. However, the latter study had a relatively high rate of loss to follow-up (136 of 544 subjects were lost at 10 years). Those patients did not differ in baseline characteristics from the subjects who returned to follow-up except for older age, but they were assumed to be free from type 2 diabetes for the analysis at 10 years. This might have introduced a substantial bias in the analysis.

The inverse association between plasma A-FABP and adiponectin [51,52,54] and the positive correlation with high sensitivity CRP [30,52,54] (adjustment for adiposity taken into account) suggested a contribution of A-FABP to systemic inflammation. On the contrary, another study shows a positive correlation between A-FABP and adiponectin con-

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centrations [30]. Leptin was found to be positively correlated to A-FABP levels in a cohort of obese children [57].

Sexual dimorphism of plasma A-FABP is consistently reported [30,51,53,54,58,59] with higher levels in women compared to men, except for one study in obese children [57]. Possible explanations for the gender difference include higher body fat percentage in women or sex differences in regional fat distribution, as women generally have more SAT than men, whereas men have more VAT. Several lines of evidence have shown the increased A-FABP expression in SAT compared to VAT [40–42]. Another potential underlying mechanism might represent the A-FABP regulation by sex hormones [58]. The persistence of the gender difference in postmenopausal women suggested that estrogen might not be an important regulator. The potential suppressive role of testerone (as it is in the case of adiponectin secretion) is being investigated [58].

Weight loss induced by bariatric surgery in obese adults [56] or lifestyle intervention in obese children [57] led to significant reduction of plasma A-FABP. A-FABP plasma concentrations and their changes were only related to parameters of adiposity but not to insulin resistance in both studies.

Pharmacological interventions have been also shown to influence circulating A-FABP in humans in vivo. Three months treatment with atorvastatin in hyperlidaemic non-diabetic subjects substantially decreased plasma A-FABP independently on changes in total cholesterol levels [59]. Another cross-sectional study in subjects with type 2 diabetes [30] found no difference in A-FABP concentrations in statin-treated subjects but reported increased A-FABP levels in TZD-treated subgroup. In the prospective study with 12 weeks pioglitazone treatment in type 2 diabetes [30], the authors also reported significant increase in plasma A-FABP that was paralleled by increased PPARγ activity in peripheral blood mononuclear cells. This finding is in accordance with in vitro data [29,45] and could be explained by the fact, that TZD through PPARγ activation induce adipocyte differentiation and intra-cellular fat accumulation [30].

3.4.2. *E-FABP in plasma*

Much fewer evidence is available for circulating E-FABP. Increased plasma concentrations are reported in morbidly obese subjects [56]. After gastric banding-induced weight loss plasma E-FABP decreased bellow the concentration found in lean subjects.

4. Human genetic studies

In humans a functionally significant genetic variant of A-FABP locus has been identified [60]. The T-87C allele in the promoter region of A-FABP gene disturbs the CAAT box/enhancer-binding protein binding and thus reduces the transcriptional activity of the A-FABP promoter, resulting in decrease in A-FABP expression in adipose tissue. This "loss-of-function" allele was confirmed in vivo—its presence results in significantly reduced A-FABP expression in SAT of carrier subjects. The prevalence of this allele is rather low in general population (~4%). In population genetic studies with 7899 participants, the carriers of this T-87C polymorphism had lower triglyceride levels, reduced risk for coronary heart disease and in obese subpopulation the risk for type 2 diabetes was also reduced. No other association between the studied single nucleotide polymorphism and inflammatory or metabolic syndrome markers has been found.

5. A-FABP as a target for treatment of insulin resistance and atherosclerosis

Recently, a potent selective inhibitor of A-FABP – the compound BMS309403 – has been developed that competitively inhibits binding of endogenous FA within the internal cavity of FABP [61]. In vitro the A-FABP inhibitor reduced macrophage transformation into foam cells, as shown by 40% lower cholesterol ester accumulation, which was attributed to substantial increase in cholesterol efflux. Similarly to knock-out models, treatment with BMS309403 led to reduced expression of inflammatory cytokines in macrophages. In vitro treatment of adipocytes resulted in decrease in FA uptake. Mice models of genetic and dietary obesity treated with A-FABP inhibitor presented lower glucose, insulin and triglycerides levels, increased adiponectin, but also increased FFA concentrations. The improvement in insulin sensitivity and glucose tolerance indicated by these results was proven also in hyperinsulinaemic–euglycaemic clamp studies (decrease in hepatic glucose production and increased glucose uptake in skeletal muscle and adipose tissue). Treated mice exhibited attenuated macrophage infiltration as well as cytokine expression in adipose tissue, diminished triglyceride accumulation in liver and reduced activity of cellular inflammatory pathways in both tissues. In apoE knock-out mice, A-FABP inhibition substantially reduced atherosclerotic lesions, regardless of no changes in body weight, glucose and lipid metabolism.

6. Role of adipocyte/macrophage FABP in metabolic syndrome in humans

Although experimental data on A-FABP in knock-out mice provide an exciting insight into its central regulatory role in metabolic syndrome, even with the option of treatment by selective inhibition of A-FABP, several discrepancies and questions based on available human studies arise.

6.1. *What is the relation between plasma and tissue levels and expressions of A-FABP? What are the sources of circulating A-FABP and how it is released from cells?*

In humans, plasma concentrations and adipose tissue expression of A-FABP have never been shown in parallel. The evidence on plasma A-FABP is in accordance with experimental data, showing the association of high A-FABP levels with metabolic syndrome abnormalities, body mass and insulin resistance [51,53,54] or a decrease in A-FABP after weight loss [56,57]; on the other hand, the studies focused on adipose tissue expression are contradictory. They report no difference in A-FABP expression or even a decrease in E-FABP expression in obese subjects [36,37], as well as increased

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expression of both FABPs after weight loss [37]. Furthermore, no consistent association between A-FABP expression and measures of adiposity or insulin resistance was found [36,38]. These findings implicate involvement of other source of circulating A-FABP – macrophages. Their contribution to whole-body A-FABP balance has not been elucidated so far.

Another related question would be the mechanism of cellular secretion of A-FABP, since it might be involved in maintaining of balance between serum and tissue levels. Therefore, the clarification of regulations of FABP expression in human adipose tissue and macrophages together with its plasma concentrations awaits further studies.

6.2. *What are the physiological functions of circulating A-FABP and how do they differ in metabolic diseases and atherosclerosis?*

A-FABP might participate in transport of FFA and lipid hormones and thus modulate systemic insulin sensitivity and energy metabolism [54]. Whether the causative mechanisms of these functions at the molecular level are similar to those proposed in mice, it remains to be elucidated.

6.3. *What are the possible consequences of long-term A-FABP inhibition that is suggested as a potent treatment of metabolic syndrome and atherosclerosis in mice?*

The high evolutionary conservation of FABPs indicates that they belong to thrifty genes that were important for survival. Thus A-FABP possibly ensures sufficient lipid storage in adipocytes and potent inflammatory response in macrophages [62], which may have impact on the long-term body weight regulation or in conditions requiring strong inflammatory response.

6.4. *What is the physiological role of E-FABP – the minor isoform derived from adipose tissue and macrophages?*

Few data on E-FABP in humans are available. The interesting finding showing that E-FABP expression might account for the differences between fat depots and changes with adiposity [37,40] needs to be confirmed by further studies.

6.5. *What is the relation of A-FABP to the individual components of metabolic syndrome?*

In different human studies, this relation was not expressed to the same extent. This might partially lie in difference in ethnicity or in the size of population studied. The association with measures of obesity and dyslipidaemia is consistent for all studies mentioned above. However, no association with parameters of insulin resistance was observed in Caucasian cohort of type 2 diabetes [30] or in a specific cohort of insulin resistance—in polycystic ovary syndrome [63]. Considering that insulin resistance was mostly assessed as HOMA index, for more precise discrimination the insulin clamp technique should be used. Similarly, the association between A-FABP and impairment of glucose homeostasis is not definite with studies reporting both A-FABP increase [52] and no difference [58].

6.6. *Finally, is the regulation of A-FABP by currently available therapies relevant for treatment of metabolic syndrome?*

A study with TZD treatment has brought a confusing element about our knowledge of A-FABP. The TZD-treated patients showed higher A-FABP plasma concentrations, probably due to PPARγ stimulated adipocyte differentiation and intracellular fat accumulation [30]. While this mechanism appears to be logical, supported also by experimental data [29,45], the clinical relevance of such ‘‘negative’’ effect of a potent insulin-sensitizing drug awaits further clarification.

7. Role of adipocyte/macrophage FABP in atherosclerosis in humans

The proof of A-FABP expression in macrophages together with clarification of A-FABP's role in macrophage metabolism [43,64] provided a very clear concept of the role of FABP in inflammation and development of atherosclerosis, which is supported by the data in apoE deficient mice [24,43,49].

The current evidence in vivo in humans is not extensive. In a cohort of Chinese women but not in men, serum A-FABP was shown to be an independent determinant of carotid intima-media thickness as a measure of atherosclerosis [58]. In women but not in men, higher A-FABP levels were associated with the presence of plaques. On the contrary, another study in Caucasian population with type 2 diabetes showed that circulating A-FABP was not associated with clinical or subclinical atherosclerosis in both genders [30].

Moreover, atorvastatin treatment has been show to reduce A-FABP plasma concentrations [59] suggesting that this might be one of the pleiotropic effects of statin drugs in atherosclerosis.

The involvement of A-FABP in atherosclerosis is also supported by the genotype–phenotype study [60], demonstrating that the carriers of T-87C polymorphism have reduced risk for coronary heart disease.

Further studies in large cohorts, in different ethnicities, different levels of glucose intolerance, clinical and subclinical atherosclerosis as well as longitudinal studies are needed to confirm the role of A-FABP in atherosclerosis in vivo in humans.

8. Conclusions

Adipocyte/macrophage FABP clearly links several mechanisms and pathways that are involved in the development of obesity, metabolic syndrome and atherosclerosis. To translate these important data from mice models to humans will require further comprehensive investigations. Whether circulating adipocyte/macrophage FABP represents a biomarker of obesity, metabolic syndrome and atherosclerosis or whether it is a causative factor of metabolic and inflammatory dysregulation, which can be effectively and safely inhibited, remains to be elucidated.

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Conflict of interest

There are no conflicts of interest.

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