Adipose tissue secretion and inflammation

Characterization of novel adipokines and the impact of hypoxia on adipocyte function

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« Unsere größte Schwäche liegt im Aufgeben. Der sicherste Weg zum Erfolg ist immer, es doch noch einmal zu versuchen. »

Thomas A. Edison

Zusammenfassung

Adipositas ist ein globales Gesundheitsproblem, das mit einer Vielzahl pathologischer Krankheitsbilder, wie Typ 2 Diabetes, dem metabolischen Syndrom und Herz-Kreislauf-Erkrankungen einhergeht. In diesem Zusammenhang ist die Zunahme an Fettgewebsmasse eng mit der Entwicklung dieser Erkrankungen verbunden. Es ist heutzutage weitgehend akzeptiert, dass das Fettgewebe nicht nur als Energiespeicher dient, sondern vielmehr ein endokrines Organ repräsentiert, welches eine Vielzahl biologisch aktiver Proteine, die sogenannten Adipokine, freisetzt. Diese Adipokine spielen eine wichtige Rolle bei der Kommunikation zwischen Fettgewebe und peripheren Geweben, wie der Leber oder dem Skelettmuskel sowie bei der Entwicklung pathologischer Prozesse, wie zum Beispiel einer Insulinresistenz und Inflammation. Die Zahl neu-identifizierter Adipokine steigt stetig auf Grund zahlreicher Proteom-basierter Analysen, die Aufschluss über das vollständige Adipozyten-Sekretom geben sollen. Eine zwangsläufige Herausforderung solcher Sekretomanalysen ist eine anschließende notwendige Validierung der identifizierten Faktoren als tatsächliche Adipokine sowie deren funktionelle Charakterisierung im Zusammenhang der Fettgewebsfunktion und Adipositas-relevanter Erkrankungen. Aus diesem Grund war das erste Ziel dieser Arbeit, fünf potentielle neue Adipokine zu validieren und zu charakterisieren, welche in einer vorhergehenden Sekretomanalyse identifiziert wurden. Die fünf Kandidaten waren Hämoxygenase-1 (HO-1), αB-Crystallin (CRYAB), Komplement-Faktor H (CFH), *cartilage intermediate layer protein* (CILP), sowie Dipeptidylpeptidase 4 (DPP4). Für alle fünf Kandidaten konnte eine differenzierungsabhängige Expression sowie ihre Freisetzung aus humanen Adipozyten gezeigt werden. Ein Vergleich der Expression mit Fettgewebs-spezifischen Makrophagen identifizierte Adipozyten als potentielle Hauptquelle für die Expression dieser Adipokine im Fettgewebe. Desweiteren konnten bekannte Regulatoren der Fettgewebsfunktion, wie z.B. Troglitazon, Insulin, TNF-α und Hypoxie, als Faktoren für die Regulierung der Expression und Sekretion dieser Adipokine identifiziert werden. Im Serum adipöser Probanden konnten im Vergleich zu schlanken Probanden erhöhte Level an HO-1, CFH und DPP4 gemessen werden, während entsprechende CILP Level vermindert waren.

Desweitern identifizierte die Sekretomanalyse *pigment epithelium-derived factor* (PEDF) als hoch-abundantes Protein in konditioniertem Medium (CM) von humanen Adipozyten. Es zeigte sich, dass PEDF hauptsächlich von Adipozyten sekretiert wurde, während andere Zelltypen wie Makrophagen, Skelettmuskelzellen und glatte Muskelzellen nur geringe Mengen an PEDF freisetzten. Troglitazon, Insulin, TNF-α und Hypoxie konnten als Regulatoren der PEDF Expression und Sekretion identifiziert werden. Es zeigte sich zudem, dass PEDF Insulinresistenz in humanen Adipozyten, Skelettmuskelzellen und glatten Muskelzellen induzierte.

Als zweites Ziel dieser Arbeit sollte die Rolle eines verminderten Sauerstoffangebotes auf das Sekretionsverhalten, die Inflammation und die Adipogenese von humanen Adipozyten untersucht werden. Bei Adipositas wurde bereits das Auftreten einer Hypoxie im humanen sowie murinen Fettgewebe beschrieben, welche positiv mit einer chronischen Inflammation des Fettgewebes korrelierte. Zur Untersuchung der Auswirkung einer starken Hypoxie von 1% O_2 in humanen Adipozyten wurde ein entsprechendes Protokoll etabliert. Hierbei konnten bereits bekannte Effekte auf die Expression und Sekretion bestimmter Proteine in Adipozyten reproduziert werden, wie z.B. eine erhöhte Sekretion von IL-6, Leptin und VEGF sowie eine verminderte Freisetzung von Adiponektin. Desweiteren zeigte sich eine potentielle anti-inflammatorische Wirkung der Hypoxie anhand einer verminderten TNF-α-induzierten NF-κB Signalwirkung. Diese führte zu einer verminderten Freisetzung der pro-inflammatorischen Chemokine MCP-1 und IL-8.

Für das humane Fettgewebe konnten physiologische Sauerstoffspannungen zwischen 3-11% O_2 nachgewiesen werden. Standard Protokolle sehen eine Differenzierung von Präadipozyten zu Adipozyten unter Raumluft vor, welche durch den deutlich höheren O_2-Anteil der Luft (ca. 21% O_2) nicht den jeweiligen Sauerstoffbedingungen im humanen Fettgewebe entspricht. Um physiologische Bedingungen nachzuahmen, wurden humane Adipozyten bei 10% und 5% O_2 im Vergleich zu 21% Raumluft differenziert. Anhand der Expression diverser Differenzierungsmarker konnte gezeigt werden, dass unter allen drei Bedingungen die Differenzierung der Adipozyten vergleichbar war. Dahingegen zeigten sich entscheidende Unterschiede im Sekretionsverhalten der unter geringeren Sauerstoffspannungen

differenzierten Adipozyten, da beispielsweise die Sekretion von Adiponektin deutlich erhöht war. Weiterhin zeigte sich sowohl eine basale, als auch Isoproterenol-induzierte Steigerung der Lipolyse in Adipozyten, die unter 10% und 5% O_2 differenziert wurden im Vergleich zu unter Raumluft kultivierter Adipozyten.

Durch die Validierung sowie Charakterisierung neuer und abundanter Adipokine konnte im Rahmen dieser Dissertation die wichtige Rolle des Fettgewebes als zentrales endokrines Organ, insbesondere im Kontext der Adipositas, unterstrichen werden. Desweiteren wurde gezeigt, dass verminderte Sauerstoffspannungen zu einer geringeren TNF-α-induzierten Inflammation in Adipozyten führten. Auch während der Adipogenese spielte die Sauerstoffkonzentration eine wichtige Rolle, da sowohl das Sekretionsverhalten als auch generelle Funktionen, wie die Lipolyse, beeinflusst wurden.

Summary

Obesity is a major global health burden that is associated with a number of pathological disorders, including type 2 diabetes, the metabolic syndrome and cardiovascular diseases. In this context, enlargement of adipose tissue (AT) is closely related to the development of these pathologies. Nowadays, it is well established that AT functions not only as a fuel storage site, but also represents a major endocrine organ, which is capable to release a variety of biologic active proteins, collectively named adipokines. These adipokines are known as important mediators for the inter-organ crosstalk between AT and peripheral tissues such as the liver or the skeletal muscle. Pathological processes are influenced by adipokines, including insulin resistance and inflammation. The number of newly identified adipokines rises continuously, as various proteomic approaches have been conducted to obtain a more detailed insight into the complex nature of the adipocyte secretome. The challenge in this context is the validation of these factors as confirmed adipokines, and their further characterization for a potential role in AT function and obesity-related disorders. Therefore, the first aim of this thesis was to validate and characterize five potential novel adipokines, previously identified by a proteomic approach investigating the secretome of human primary adipocytes. These factors were heme-oxygenase-1 (HO-1), αB-crystallin (CRYAB), complement factor H (CFH), cartilage-immediate layer protein (CILP), and dipeptidyl peptidase 4 (DPP4). All five candidates were expressed in a differentiation-dependent manner and released from human adipocytes. Comparison of the specific expression to AT-derived macrophages demonstrated adipocytes as the most likely source of these adipokines from AT. Moreover, important regulators of AT, including troglitazone, insulin, TNF-α, and hypoxia, were capable to affect the expression and secretion level of a subset of these novel adipokines. Serum analyses from lean and obese individuals revealed elevated circulating levels of HO-1, CFH and DPP4, while CILP levels were decreased in obesity. Furthermore, DPP4 could be identified as a potent mediator of adipocyte-specific insulin resistance.

In addition, proteomic profiling identified pigment epithelium-derived factor (PEDF) as a high abundant adipokine, which was predominantly

expressed and released by human adipocytes and only by small amounts from other cell types including AT-derived macrophages, skeletal muscle cells, and smooth muscle cells. PEDF expression and release was regulated by factors such as troglitazone, insulin, TNF-α, and hypoxia. Moreover, PEDF was capable to induce insulin resistance in human adipocytes, skeletal muscle cells and smooth muscle cells.

A second aim of this thesis was to address the role of reduced oxygen levels on adipocyte secretion, inflammation and development. Hypoxia has been shown to occur in local areas of human and murine obese AT, which correlated to the chronic low-grade inflammation of obese AT. A protocol for the in vitro investigation of acute and severe hypoxia using 1% O_2 was established for human adipocytes. Here, known effects on adipocyte protein expression and release could be reproduced, including elevated IL-6, leptin, and VEGF release, as well as decreased adiponectin secretion. Moreover, a potential anti-inflammatory effect was observed, since hypoxia in human adipocytes attenuated TNF-α-induced NF-κB signalling. This attenuation led to a decreased release of the pro-inflammatory chemokines MCP-1 and IL-8.

Moreover, human AT oxygenation has been shown to range between 3-11% O_2. However, the standard *in vitro* procedure for adipocyte differentiation is performed at ambient air with higher oxygen tensions than observed in human AT. In order to mimic physiological oxygen conditions, adipocyte differentiation was performed at 10% and 5% O_2 in comparison to 21% O_2. Adipocyte differentiation was similar at all three conditions, as demonstrated by comparable expression of distinct differentiation markers. However, adipokine release by adipocytes differentiated at 10% and 5% O_2 demonstrated significant variations compared to 21% O_2. In particular, adiponectin secretion was markedly up-regulated at reduced oxygen levels. Furthermore, the basal and isoproterenol-stimulated lipolysis was significantly increased at physiological oxygen tensions.

Conclusively, this thesis presented the validation and characterization of novel adipokines in human adipocytes, underlining the importance of AT in releasing obesity-associated factors. Furthermore, reduced O_2 levels during adipocyte culture could be identified as a positive regulator of inflammation during acute hypoxia, as well as a molecular regulator of adipogenesis and subsequent adipocyte secretion and function.

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ACE	angiotensin converting enzyme
AT	adipose tissue
ATGL	adipose triglyceride lipase
ATM	adipose tissue macrophages
BMI	body mass index
BMSC	bone marrow stromal cells
CGI-58	Comparative Gene Identification-58
cIAP	cellular inhibitor of apoptosis protein
CLS	crown-like structures
COX	cytochrome c oxidase
CRP	c-reactive protein
DAG	diacylglycerol
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
ETC	electron transport chain
FFA	Free fatty acid
FIH-1	factor inhibiting HIF-1
GLUT	glucose transporter
HGF	hepatocyte growth factor
HIF-1	hypoxia-inducible factor-1
HRE	hypoxia-response elements
HSL	hormone-sensitive lipase
IDF	International Diabetes Federation
IFG	impaired fasting glucose
IFN-γ	interferon gamma
IGF	insulin-like growth factor
IGT	impaired glucose tolerance
IKK	IκB kinase
IL	interleukin
IR	insulin receptor
IRS	insulin receptor substrate
IκBα	inhibitor of kappa B alpha
MAG	monoacylglycerol
MCP-1	monocyte chemotactic protein-1
MCT	monocarboxylate transporters

MGL	monoglyceride lipase
MIF	macrophage migration inhibitory factor
MMP	matrix metalloproteinases
MSC	mesenchymal stem cells
NEFA	non-esterified fatty acids
NEMO	NF-κB essential modulator
NF-κB	nuclear factor-kappaB
PAI-1	plasminogen activator inhibitor-1
PAMP	pathogen-associated molecular pattern
PDE3B	phosphodiesterase 3B
PDK-1	phosphoinositide dependent kinase-1
PEDF	pigment epithelial growth factor
PHD	prolyl hydroxylase domain proteins
PI3K	phosphatidylinositol-3-kinase
RBP4	retinol binding protein 4
RIP1	receptor-interacting protein 1
SH2	Src-homology-2
T1D	type 1 diabetes
T2D	type 2 diabetes
TAG	triacylglycerol
TCA	tricarboxylic acid cycle
TGF-β	transforming growth factor-beta
TIMP	tissue inhibitor of metalloproteinases
TLR	toll-like receptor
TNF-R	tumour necrosis factor-receptor
TNF-α	tumour necrosis factor-alpha
TRADD	TNF receptor-associated protein with a death domain
TRAF	TNF receptor-associated factor
VEGF	vascular endothelial growth factor
WHO	World Health Organisation
ZAG	zinc-alpha2-glycoprotein

Chapter 1

General Introduction

1.1. Type 2 Diabetes Mellitus

1.1.1. Pathophysiology of type 2 diabetes

Diabetes mellitus is a heterogeneous disorder that is characterized by common elements of hyperglycaemia and glucose intolerance resulting from defects in insulin action and/or insulin secretion. Long-term consequences of this disease are damage, dysfunction and failure of various organs of the human body. It could be classified mainly into type 1 and type 2 diabetes. Type 1 diabetes (T1D) is a chronic disease that is caused by an organ-specific immune destruction of the insulin-producing β-cells in the pancreas, resulting in a long-life requirement of exogenous insulin.[1] It usually develops in the youth or adolescence under the age of 18 and is discussed to originate from a combination of genetic predisposition and not yet fully determined environmental factors.[2] T1D accounts for 5–10% of all diabetes cases, but its incident rates are increasing especially in very young children, supporting the hypothesis that environmental factors may play a major role in the development of the disease.[3]

Type 2 diabetes (T2D) represents the predominant diabetes form as it accounts for at least 90 % of all cases and is associated with obesity, overnutrition and physical inactivity. The disease is characterized by a metabolic disorder of energy homeostasis. Besides hyperglycaemia also dyslipidaemia, increased endogenous glucose production, insulin resistance and β-cell dysfunction could occur.[4] In the insulin resistant

state and because of fuel surfeit pancreatic islets respond with increased insulin production and secretion, that could lead to the exhaustion of β-cells and finally to the loss of β-cell mass.[5] Prior to its manifestation, a condition involving impaired fasting glucose (IFG) and impaired glucose tolerance (IGT), termed as pre-diabetes, could be present for years or even decades without clinical symptoms and is associated with the incidence of developing T2D and cardiovascular diseases (CVD).[6,7] Structured lifestyle interventions, including loss of body weight and increased physical activity, have been demonstrated to prevent or delay the onset of T2D in patients with IGT and IFG.[8,9] The importance of diabetes prevention becomes clear, when considering the possible long-term complications such as retinopathy with potential loss of vision, nephropathy leading to renal failure, peripheral neuropathy with risk of foot ulcers and amputations, and atherosclerotic CVD.[7] Glycemic control is a major target in the treatment of T2D. This could be achieved in some individuals by weight reduction, exercise and/or oral glucose-lowering agents, while individuals exhibiting β-cell destruction require exogenous insulin.[7]

Diabetes mellitus is a global health burden and one of the most non-communicable diseases worldwide. According to the International Diabetes Federation (IDF), in 2011 a number of 366 million adults worldwide between the age of 20 and 79 years are estimated to be diabetic, while an additonal 280 million people exhibit impaired glucose tolerance (IGT). In 2030, the number of diabetics is projected to increase by 50.7% to 552 million people, reflecting 9.9% of all adults worldwide. This increase equates to approximately three new diabetic patients every 10 seconds.[10] According to the current IDF diabetes atlas, the diabetes prevalence in Germany is estimated to about 5 million people, representing 8% of the adult population. This number might further increase to 5.6 million (9.5) adults until 2030. This rate is relatively low compared to low and middle income countries such as Saudi Arabia, where the diabetes prevalence accounts for a total of 16.2% in 2011 and is estimated to increase up to 20.8% in 2030.[11] It is likely that the actual number of people with diabetes is much higher, as studies such as the KORA survey, which was conducted in southern Germany, demonstrated that half of all diabetes cases were undetected prior to the study.[12]

1.1.2. Obesity and its role in diabetes

In 2008, the World Health Organization (WHO) estimated 1.5 billion adults worldwide as being overweight with more than 200 million men and nearly 300 million women being obese.[13] Overweight and obesity are characterized by an abnormal or excessive fat accumulation and is commonly classified by the body mass index (BMI), the quotient of a person's weight in kilogram and the square of his height in meters. According to the WHO, a person is considered as overweight with a BMI greater or equal to 25 and obese with a BMI greater or equal to 30.[13] In general, obesity is suggested to be a result of a complex interaction of environmental factors and genes.[14] Besides the modern lifestyle characterized by reduced physical activity and increased energy-intake, a number of genes might be involved, favouring the storage of fat.[14] Obesity is associated with various diseases, including diabetes, hypertension, CVD, in particular heart disease and stroke, as well as with certain types of cancer such as breast or colon cancer.[15,16]

44% of the diabetes burden is attributed to obesity and the risk for developing T2D is nine-fold higher in obese compared to lean men.[13,17] As diabetes is considered to be an obesity-induced disease, Astrup and Finer defined the term 'diabesity'.[18] Lifestyle intervention represents an important therapeutic option as a number of intervention studies revealed that increased physical activity and even a moderate reduction in body weight of 5% could prevent T2D in obese individuals.[19] The importance of weight reduction and prevention of obesity is even more important when considering the raising prevalence of obesity-induced T2D in children and adolescence.[20,21] It is reported that the progression from insulin resistance to T2D is faster in obese children than in obese adults, and several metabolic and cardiovascular complications are associated with T2D even in young age groups.[22]

The pathological effect of obesity seems to be determined not only by the quantity of fat accumulation but also on its distribution, as the risk for developing T2D and CVD is correlated with a higher amount of abdominal fat.[23-25] Abdominal obesity is characterized by the quantity of visceral fat surrounding the inner organs. Its mass is more related to genetic determinants than subcutaneous fat, as demonstrated by family and twin studies.[26,27] Visceral fat accumulation is associated with a number of metabolic abnormalities such as glucose intolerance, lower high density

lipoprotein cholesterol (HDL), elevated triglycerides and hypertension. This was initially described by Reaven et al. as syndrome X.[28,29] Today this syndrome is known as the metabolic syndrome and is defined by the IDF as central obesity, measured by waist circumference depending on the ethnicity of the patient (for Europeans: ≥ 80 cm in females, ≥ 94 cm in males) together with two of four additional criteria: 1. raised triglyceride levels (≥ 150 mg/dl), 2. reduced HDL levels (≤ 50 mg/dl in females, ≤ 40 mg/dl in males), 3. raised blood pressure (≥ 130/85 mm Hg), and 4. increased fasting plasma glucose (≥ 100 mg/dl) or previously diagnosed T2D.[30] Non-diabetics with the metabolic syndrome are at a fivefold higher risk to develop T2D, as commonly in this state IFG or IGT is already present.[31]

Obesity is associated with a state of chronic, low-grade inflammation. It is suggested that inflammation is the link between obesity-induced insulin resistance and related disorders.[32,33] A variety of bioactive factors, collectively named adipokines, are released by adipose tissue (AT) and these adipokines exert diverse functional roles including pro- and anti-inflammatory properties.[34] Visceral fat is regarded as the more active fat depot, as the release of diverse adipokines, playing important roles in obesity-related disorders, is higher from visceral than subcutaneous fat.[35] Moreover, lipolysis is increased in this tissue, contributing to high circulating levels of free fatty acids (FFA). Moreover, the anti-lipolytic activity of insulin is impaired in visceral AT, indicating that this tissue is more insulin resistant than subcutaneous fat.[36-40] Weight loss is associated with a rapid reduction of visceral fat mass in obese subjects,[41] but there is no direct evidence providing a causal link between visceral obesity and insulin resistance. Data from animal models reported improvements in insulin sensitivity after omental fat resection.[42,43] However, a recent human study showed that surgical removal of omental fat did not reduce insulin resistance, suggesting that increased visceral fat mass alone is not the cause of metabolic dysfunction.[44] Nevertheless, an intriguing observation is that not all obese subjects are affected by obesity-induced metabolic diseases, referred to as metabolically healthy obese.[45] This obese subgroup is characterized by less visceral fat, better insulin sensitivity, higher levels of the anti-inflammatory adipokine adiponectin, and also by a better lipid, inflammation, hormonal and immune profile.[45] It is suggested that mechanisms affecting inflammation and adipokine

release beyond a positive caloric balance determine the pathological metabolic consequences in patients with obesity.[46]

1.2. Adipose tissue dysfunction

1.2.1. Adipose tissue composition

AT consist of two distinct tissues, brown AT and white AT. Brown AT is a highly active tissue that produces heat due to cold-induced thermogenesis.[47] Until recently, brown AT was assumed to be present in significant amounts mainly in neonatals, as during the first years of life rapid involution of this tissue occurs, leaving only minimal amounts of brown AT in healthy human adults.[48] However, current studies demonstrated active brown AT in 7.3% of adult women and 3.1% of adult men that was limited to the trunk.[49-51] Brown AT activity seems to be not only gender but also BMI dependent as van Marken Lichtenbelt showed reduced activity of this tissue in overweight and obese men.[51] In contrast to brown AT, white AT is a highly efficient storage site for fuels in form of triglycerides and can account for more than 45% of body weight in obese individuals.[52] Nowadays, AT is recognized as a major endocrine organ since in 1987 adipsin and later in 1994 leptin were identified as factors expressed and secreted by AT.[53,54] Until today numerous adipokines are known to be released by AT and it is suggested that only factors secreted by adipocytes and not from other AT-located cells should be classified as adipokines.[34] The number of adipokines is increasing continuously, as several studies using proteomic approaches are investigating AT protein expression and secretion in rodent and human cell models to identify novel adipokines.[55-58]

The AT is composed of a number of heterogeneous cells including mature adipocytes, preadipocytes, fibroblasts, endothelial cells, adipose-derived stromal/stem cells as well as diverse immune cells, such as macrophages, natural killer cells or B- and T-lymphocytes.[59-62] To keep energy homeostasis, AT can undergo relatively rapid and dynamic tissue remodelling in response to alterations in energy demand and supply through adipocyte hypertrophy and hyperplasia.[63] Adipocyte hypertrophy

is characterized by an increase in adipocyte size due to elevated triglyceride accumulation. In situations such as genetic obesity or a high-fat diet, adipocytes have the capacity to increase their volume 6-7 times.[64] In contrast, adipocyte hyperplasia is reflected by a higher number of smaller adipocytes. It could be shown that adipocyte size correlates positively with insulin resistance as well as with an increased risk for the development of T2D.[65-67]

AT is infiltrated with immune cells such as macrophages. The amount of these AT-located macrophages (ATM) was shown to increase with obesity, demonstrating higher infiltration rates in visceral than subcutaneous fat depots.[68-70] AT infiltration of ATM is suggested to be mediated by diverse mechanisms, including overproduction of chemokines such as monocyte chemotactic protein (MCP)-1, that promote macrophage mobilization, as well as by activation of inflammatory pathways in response to FFAs.[71,72] Adipocyte cell death was also demonstrated to induce macrophage recruitment, as macrophages were shown to surround dead adipocyte in so-called 'crown-like structures' (CLS). These CLS were observed in AT of lean and obese mice and humans, but the number of CLS were 30 times higher in obese compared to lean AT.[64,73,74] Obesity also induces a phenotypic switch in macrophage polarization. It is reported that ATM are characterized by the alternatively activated M2 phenotype, which produces anti-inflammatory cytokines such as IL-10 and IL-1Ra. In contrast, recruited macrophages are represented by the classically activated M1 phenotype, which is associated with the production of pro-inflammatory cytokines, including tumour necrosis factor (TNF)-α and interleukin (IL)-6.[36,75] Increased ATM accumulation and adipocyte hypertrophy are associated with a dysregulation of AT secretion, promoting a more prominent inflammatory environment. It is suggested that a cross-talk between adipocytes and macrophages plays an important role in the development of insulin resistance.[36,76]

1.2.2. Adipose tissue secretion and its dysregulation

Adipocytes release substantial amounts of adipokines, which could act either locally in an autocrine or paracrine manner on adipocytes themselves or other cell types localized in AT, or in an endocrine way by entering the circulation and affecting peripheral tissues.[76] Different adipokines exert diverse physiological functions, as they are involved in the regulation of the immune system, blood pressure, vascular haemostasis, lipid metabolism, glucose homeostasis, and angiogenesis (see Fig. 1.1).[77] Dysregulation of the vast majority of adipokines is observed in obesity, T2D, and the metabolic syndrome, as inflammation- and insulin resistance-related adipokines are up-regulated, while anti-inflammatory and insulin-sensitizing adipokines are down-regulated.[33]

Leptin was first identified in 1994 as a factor released by AT of obese mice, which signals to the brain, promoting the inhibition of food intake and therefore decreasing weight.[54] Leptin deficiency in mice (*ob/ob* mice) resulted in severe obesity, diabetes and diverse neuroendocrine anomalies such as infertility, which could be restored by exogenous leptin replacement.[78-80] The effect of leptin is mediated by its specific receptor in the hypothalamus, leading to the suppression of orexigenic peptides (e.g. neuropeptide Y) and the increase in anorexigenic peptides (e.g. corticotrophin-releasing hormone).[81] The role of leptin as an anti-obesity hormone is questionable as leptin serum levels are elevated in obese subjects and resistance to leptin in the hypothalamus is described.[81,82] It is reported that leptin transport across the blood-brain barrier is diminished in obesity, possibly representing a mechanism for leptin resistance.[83] Furthermore, increased endoplasmic reticulum (ER) stress in the hypothalamus of obese mice was demonstrated to inhibit leptin signalling, which could be restored by the administration of chemical chaperones decreasing ER stress.[84] Moreover, leptin displays metabolic effects such as stimulation of lipolysis, fatty acid oxidation, and glucose uptake, although these effects are partially mediated by central activation of the hypothalamic-sympathetic nervous system axis.[85-87]

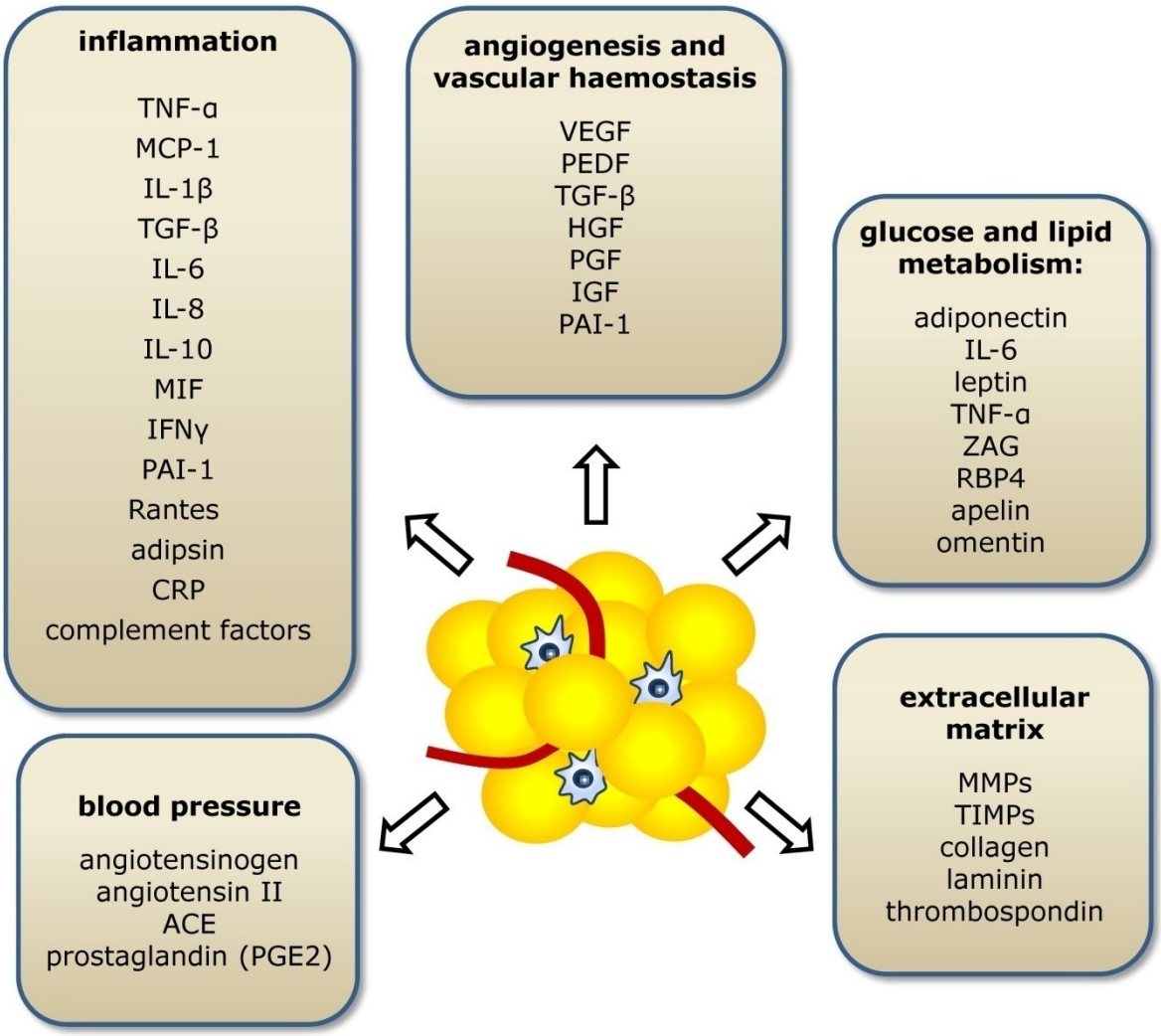

Figure 1.1 Overview of adipokines released by adipose tissue and their role in adipose tissue biology

TNF, tumour necrocis factor; MCP, monocyte chemotactic protein; IL, interleukin; TGF, transforming growth factor; MIF, macrophage migration inhibitory factor; IFN, interferon; PAI, plasminogen activator inhibitor; Rantes, regulated upon activation, normal T-cell expressed, and secreted; CRP, c-reactive protein; ACE, angiotensin converting enzyme; VEGF, vascular endothelial growth factor; PEDF, pigment epithelial growth factor; HGF, hepatocyte growth factor; IGF insulin-like growth factor; ZAG, zinc-alpha2-glycoprotein; RBP, retinol binding protein; MMP, matrix metalloprotease; TIMP, tissue inhibitor of metalloproteinases

Adiponectin is a classical adipokine mainly released by adipocytes, which is known to exert anti-inflammatory, anti-diabetic, and anti-atherogenic properties.[88] It is highly abundant in the human circulation with plasma concentrations ranging between 3-30µg/ml, accounting in average for about 0.01% of all plasma proteins.[89] Adiponectin plasma levels are higher in women than in men and are decreased in pathologies such as the metabolic syndrome, insulin resistance, and obesity.[90,91] In this context, a strong inverse correlation between plasma adiponectin levels and visceral fat accumulation was reported.[92] Adiponectin expression and secretion is highly regulated and the underlying mechanisms are intensively investigated but yet not fully unraveled.[93] There is evidence indicating that it is down-regulated by cytokines, such as TNF-α and IL-6, whereas peroxisome proliferator-activator receptor gamma (PPARγ) agonists such as thiazolidinediones increase it.[94-96] Adiponectin exerts pleiotropic effects via its receptors AdipoR1, AdipoR2, and T-cadherin, which are expressed in diverse peripheral tissues and organs in addition to AT.[88] The insulin sensitizing effect of adiponectin is suggested to be mediated by sequential activation of AMP-activated protein kinase AMPK, p38 mitogen-activated protein kinase (MAPK), and PPARα.[97,98] Adiponectin-mediated activation of these factors in rodents has been demonstrated to regulate glucose metabolism and insulin sensitivity, leading to higher glucose utilization and increased fatty acid oxidation, involving glucose transporter (GLUT) 4 translocation.[97,99,100] Interestingly, adiponectin gene polymorphisms seem to be associated with a predisposition for T2D, as the most common single nucleotide polymorphisms (SNPs) in the adiponectin gene are associated with insulin sensitivity and glucose tolerance as well as with reduced adiponectin plasma levels.[101,102] According to preclinical studies, adiponectin might be a reliable biomarker for the metabolic syndrome and its related diseases.

TNF-α is a pro-inflammatory cytokine, which is predominantly released by ATM.[68] Serum levels of TNF-α have been shown to be elevated in obesity and insulin resistant T2D.[103,104] It is well established that TNF-α induces insulin resistance in obese rodents by the activation of inflammatory pathways.[105,106] In humans, this role is not as clear, as studies using antibodies to neutralize TNF-α *in vivo* failed to restore insulin sensitivity in obese individuals, even though an improvement in circulating inflammatory markers such as C-reactive protein (CRP) was observed.[107-

[109] It is suggested that this lack of benefit might be due to the predominant role of TNF-α as a local factor, which exhibits autocrine and paracrine actions on its producing tissues.[33] Overproduction of TNF-α might be interpreted as an attempt to limit AT expansion, as TNF-α was shown to induce lipolysis and insulin resistance to impair preadipocyte differentiation and to increase adipocyte apoptosis.[110] Besides induction of its own synthesis, TNF-α is a powerful stimulator of other pro-inflammatory cytokines and chemokines such as plasminogen activator inhibitor-1 (PAI-1), IL-6, or MCP-1.

MCP-1 is an important chemokine and its serum levels are related to obesity, insulin resistance, and diabetes.[111-113] Moreover, MCP-1 is suggested to be a strong mediator for macrophage infiltration into AT. It is associated with the pathogenesis of insulin resistance as it impairs insulin signalling in skeletal muscle cells and 3T3-L1 adipocytes.[113-115]

AT is very well vascularised and various adipokines involved in angiogenesis are released by adipocytes, including vascular endothelial growth factor (VEGF), plasminogen activator inhibitor (PAI)-1, matrix metalloproteinases (MMP), angiopoetins, transforming growth factor (TGF)-β and placental growth factor (PIGF).[116] VEGF plays a pivotal role as growth factor for endothelial cells and is essential for the formation of immature vessels.[117] Knock-out of VEGF or its receptors VEGFR1 and VEGFR2 results in embryonic lethality. These mice are virtually devoid of vascular structures or show impaired vessel assembly.[117] Angiogenesis and adipogenesis seem to be correlated to each other, as recent studies demonstrated that inhibition of angiogenic factors also inhibited adipogenesis in mice.[118,119] PPARγ, generally known as an important factor in cell differentiation and particularly in adipogenesis, has been shown to be also involved in angiogenesis.[120,121] In obesity, VEGF serum levels are increased,[122] and also other factors involved in angiogenesis and tissue remodelling are up-regulated in the obese state[123]. Moreover, VEGF seems to play a role in the attraction and activation of monocytes, as VEGF-induced monocyte chemotaxis was reported.[124,125] Monocytes are known as important cells involved in angiogenesis and tissue remodelling.[126] Interestingly, in diabetic patients the effect of VEGF on monocytes is impaired due to a defect in the VEGFR1-mediated signalling cascade, which is suggested to reflect certain type of VEGF resistance.[127,128]

1.2.3. Insulin and inflammatory signalling in adipocytes

Blood glucose concentration is controlled in a narrow range of 4 to 7nM in healthy individuals.[129] Therefore, blood glucose levels have to be maintained by the precise orchestration of hepatic glycogenolysis and gluconeogenesis, as well as by glucose disposal via skeletal muscle and AT. Insulin plays a central role in blood glucose homeostasis, as insulin inhibits hepatic glucose production and induces glucose uptake by the skeletal muscle and AT after a meal. One of the most important steps of glucose metabolism is the regulation of insulin-sensitive GLUT4 trafficking via the PI3K/Akt pathway. GLUT4 is stored in intracellular storage pools but is translocated to the plasma membrane upon insulin stimulation, where subsequently glucose is taken up.[130] In this context, AT represents a pivotal organ for whole body glucose metabolism, since it has been shown that adipose-specific GLUT4 knockout mice developed insulin resistance not only in AT but secondarily also in skeletal muscle and liver, even though GLUT4 expression was not affected in these tissues.[131] A second mouse model demonstrated that adipose-specific GLUT4 over-expression increased whole body insulin sensitivity, which could even reverse insulin resistance induced by GLUT4 knockout in skeletal muscle.[132] Moreover, it has been reported that insulin resistance in obese and diabetic humans correlates with reduced GLUT4 expression levels in AT.[133,134]

Insulin signalling is initiated by binding of insulin to the α-subunit of its specific insulin receptor (IR) on the cell surface. This event results in autophosphorylation of the intracellular β-subunit of the IR and subsequent activation of the receptor intrinsic tyrosine kinase. The activated tyrosine kinase phosphorylates specific intracellular substrates, including insulin receptor substrate (IRS) proteins 1-4.[130] In humans, IRS1 and IRS2 are the most widely expressed and studied isoforms, while IRS4 is mainly expressed in the pituitary and thyroid gland, Although IRS3 is present in rodent tissues, humans seem to not possess a functional IRS3 gene.[135,136] Phosphorylated tyrosine residues of IRS1/2 act as docking sites for Src-homology-2 (SH2) domain containing proteins, such as phosphatidylinositol-3-kinase (PI3K). PI3K is bound by IRS1/2 at the p85 regulatory subunit, which leads to the activation of the catalytic p110 subunit and subsequent activation of the kinase. Activated PI3K catalyzes the conversion of phophatidylinositol-(4,5)-biphosphate (PIP2) to

phosphatidylinositol-(3,4,5)-triphosphate (PIP3). PIP3 is a second messenger which activates diverse targets, including serine/threonine protein kinase B, which is also known as Akt.[137] As PIP proteins are membrane bound phospholipids, Akt is targeted to the cell membrane, where it is activated by upstream kinases at two specific phosphorylation sites. First, phosphoinositide dependent kinase-1 (PDK-1) phosphorylates Akt at the catalytic domain at residue Thr308, which leads to a basal Akt activation of approximately 10%. For full activation, Akt needs to be phosphorylated at its regulatory domain at residue Ser473 by the mammalian target of rapamycin complex 2 (mTORC2).[138] Upon activation, Akt is released from the membrane and phosphorylates various substrates in the cell, regulating diverse processes such as cell survival, proliferation, and energy metabolism.[138] For the latter, Akt plays a key role in insulin-stimulated glucose uptake. To this end, a key substrate of Akt, AS160, induces the translocation of GLUT4 vesicles to the plasma membrane and subsequent glucose uptake upon its phosphorylation.[139]

The insulin signalling cascade can be disturbed in a variety of ways, but main contributors are inflammatory mediators released by immune cells and AT such as MCP-1, IL-1, IL-6, and TNF-α.[140] TNF-α has already been identified in the early nineties as a mediator of insulin resistance in 3T3-L1 adipocytes and in an obese mouse model.[105,141] This inflammatory cytokine exerts its insulin-inhibitory effect in various ways. In adipocytes, TNF-α down-regulates the expression of GLUT4 and IRS-1, while in skeletal muscle not GLUT4 expression but its translocation to the plasma membrane is decreased by TNF-α.[142,143] Moreover, TNF-α affects IRS proteins by the induction of proteasome-mediated IRS degradation, phosphatase-mediated dephosphorylation and phosphorylation of specific inhibitory serine sites, subsequently leading to the inhibition of IRS interaction with the insulin receptor.[144] The latter mechanism is mediated by diverse inflammatory and stress signalling pathways such as JNK, p38 MAPK, extracellular signal-regulated kinase (ERK) 1/2, and IKKβ/NF-κB.

NF-κB is a family of dimeric transcription factors, which are composed of five different subunits, namely RelA (p65), RelB, cRel, p50, and p52. NF-κB plays a major role in the immune response, but it also regulates other important cellular processes such as cell survival, differentiation, and proliferation.[145] In the unstimulated state, NF-κB proteins are sequestered in the cytoplasm either by the family of inhibitor of kappa B

(IκB) proteins or by other related ankyrin repeat-containing proteins, depending on the activation pathway. The classical or canonical pathway is predominantly reflected by the activity of the RelA/p50 dimer, which in the unstimulated state is bound to inhibitory IκB proteins, in particular IκBα. Signalling molecules of the TNF family, such as TNF-α, IL-1, or LPS, bind to cell membrane receptors, including TNF-receptors (TNF-Rs) or toll-like receptors (TLRs). This event induces a signalling cascade, which subsequently activates the IκB kinase (IKK) complex. TNF-α classically binds to TNF-R1, leading to the formation of a signalling complex which includes proteins such as TNF receptor-associated factor (TRAF), TNF receptor-associated protein with a death domain (TRADD), serine-threonine kinase receptor-interacting protein 1 (RIP1), and cellular inhibitor of apoptosis protein (cIAP).[146] This core-signalling complex activates the IKK complex which induces degradation of IκBα, leading to the release and nuclear translocation of active NF-κB.[146,147] The IKK complex contains the kinases IKKα, IKKβ, and the regulatory component IKKγ, also known as NF-κB essential modulator (NEMO).[148] IκBα is phosphorylated by the IKKβ subunit which triggers IκBα ubiquitation and subsequent degradation by the 26S proteasome, which is essential for the dissociation of the activated NF-κB dimer.[149,150] An alternative or non-canonical NF-κB pathway depends on the activity of IKKα. Here, IKKα phosphorylates the inactive NF-κB precursor proteins p100 and p105, triggering their proteasomal processing to the active subunits p52 and p50, respectively. Additionally, p100 functions as an IκB-like molecule which inhibits translocation of RelB into the nucleus.[151] As different NF-κB dimers have different target genes, the two signalling pathways have non-redundant functions. While the canonical pathway is mainly involved in the innate immunity, the non-canonical regulates lymphoid organogenesis, dendritic cell activation, B-cell survival and maturation, as well as bone metabolism.[146,147]

In the immune response, NF-κB is activated upon stimulation by cytokines and pathogen-associated molecular patterns (PAMPs) of cell surface receptors, including TLRs and TNF-Rs. Its specific target genes promote immune cell proliferation, differentiation, and the release of antimicrobial molecules and cytokines necessary for the immune response.[145] In particular, the differentiation process from recruited monocytes towards activated macrophages at sites of infection and

inflammation is induced by exposure to the local cytokine milieu in an NF-κB dependent manner.[152,153] These activated cells further produce pro-inflammatory cytokines such as IL-1, IL-6, interferon (IFN)-γ, and TNF-α, attracting more immune cells and stimulating the differentiation of these. Cell-intrinsic and -extrinsic mechanisms, such as increased repression of NF-κB by IκBs as well as the short half-life of pro-inflammatory and anti-inflammatory cytokines, e.g. IL-10, attenuate the inflammatory response. These mechanisms are dysregulated in conditions of chronic inflammation as observed in AT under obesity. Here, cytokines are released from local cells, such as adipocytes and macrophages, can lead to the systemic recruitment and activation of immune cells promoting a state of chronic unresolved inflammation.[152]

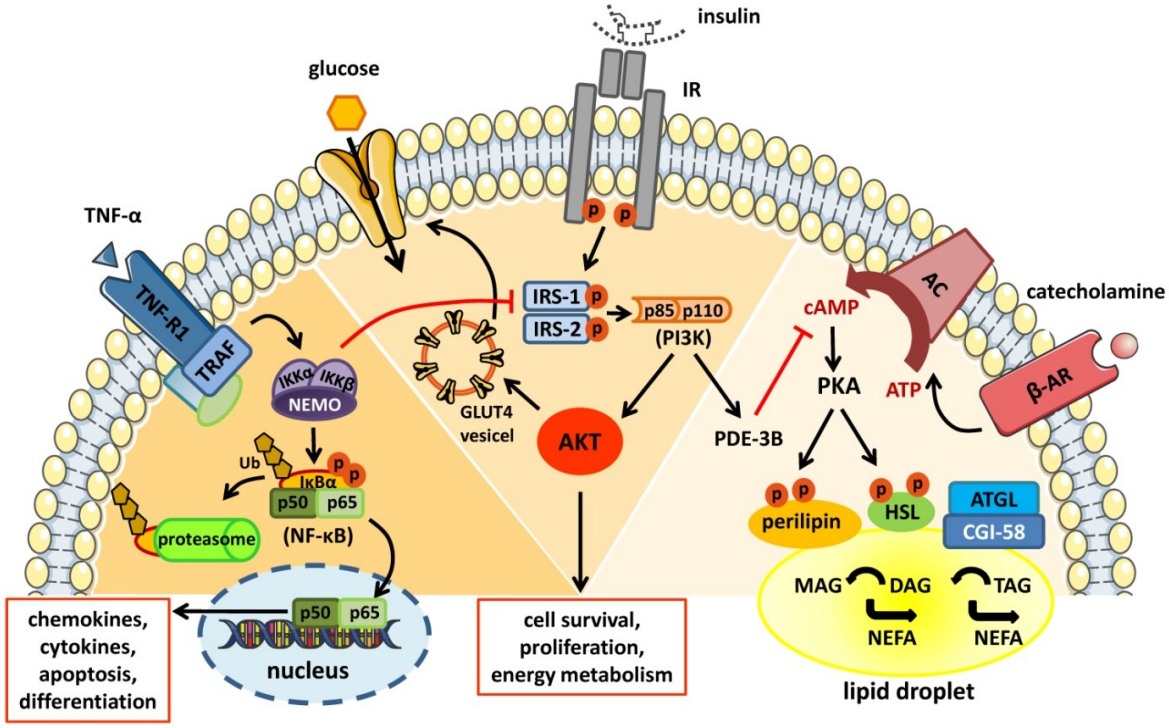

Figure 1.2 Schematic overview on signalling pathways in the adipocyte

Nuclear factor-kappa B (NF-κB) signalling via tumour necrosis factor-α (TNF-α)-induced TNF-receptor 1 (TNF-R1) activation and subsequent NF-κB-dimer translocation into the nucleus and transcriptional activity; insulin signalling via insulin-induced insulin receptor (IR) autophosphorylation and subsequent Akt activation with following glucose transporter 4 (GLUT4) translocation form intracellular vesicels to the plasma membrane and glucose uptake; lipolytic pathway via catecholamine-induced activation of β-adrenergic receptors (β-AR) and ensuing hydrolysis of triacylglycerol (TAG) into glycerol and non-esterified fatty acids (NEFA)

1.2.4. Adipose tissue lipolysis

One important function of AT is the regulation of energy homeostasis by controlling the storage of triacylglyceroles (TAG) in lipid droplets of adipocytes on the one hand and their breakdown into non-esterified fatty acids (NEFA) and glycerol upon energy demand on the other hand.[154] Lipolysis describes the mechanism of fatty acid hydrolysis from the glycerol backbone and depends on designated lipases.[155] In adipocytes, full hydrolysis of TAG is mediated by three distinct lipases: adipose triglyceride lipase (ATGL), which catalyzes the first and rate-limiting step by hydrolyzing TAG into diacylglycerol (DAG) and NEFA[156], hormone-sensitive lipase (HSL), which is rate-limiting in DAG metabolism[157], and finally monoglyceride lipase (MGL), which cleaves the remaining fatty acid from the monoacylglycerol (MAG) molecule into glycerol and NEFA[158]. For many years, HSL was believed to act as the most important lipase, as it is regulated by a variety of catabolic hormones, such as catecholamines (adrenaline, noradrenaline), glucagon, and insulin.[159] Moreover, HSL is specific to a number of substrates including TAG, DAG, MAG, cholesterylester, or short chain carbonic esters.[155,160] It was assumed that HSL deficiency would result in TAG accumulation and enhanced obesity. In contrast, a knock-out mouse model showed normal body weight as well as a retained lipolytic activity in TAG hydrolysis of 40% and increased DAG accumulation in several tissues.[161] In 2004, ATGL has been identified independently by three groups as the missing enzyme which selectively hydrolyzes TAG.[156,162,163] ATGL is regulated by β-adrenergic stimulation as well, however for full activation the enzyme requires a coactivator called Comparative Gene Identification-58 (CGI-58).[164] The importance of this coactivator is illustrated by the neutral lipid storage disease Chanarin-Dorfman syndrome, which is caused by mutations in the gene of CGI-58 resulting in its dysfunction and an abnormal accumulation of TAG in diverse tissues.[165] Furthermore, mutations in the ATGL gene are associated with systemic TAG accumulation and cardiac myopathy.[166]

Stimulation of lipolysis is mediated via three β-adrenergic receptor subtypes (β1-3), which are activated by binding of catecholamines. In human AT, the major lipolytic activity is mediated through β1- and β2-adrenergic receptors.[167] α2-adrenergic receptors are involved in anti-lipolytic regulation of adipocyte lipolysis and also exhibit a high binding affinity to catecholamines, particularly to adrenaline.[168] In subcutaneous

AT, α2-adrenergic receptors show a significantly higher expression than β-adrenergic receptors, while in visceral AT expression of both receptors is balanced.[169] This implies that adipocyte lipolysis depends on a functional balance between β- and α2-adrenergic stimulation by catecholamines.[168] Insulin exerts a strong inhibitory effect on adipocyte lipolysis by reducing HSL activity as well as down-regulating ATGL expression.[170] In addition to catecholamines and insulin also other endocrine factors are regulating AT lipolysis, including natriuretic peptides and glucocorticoids. However, also autocrine and paracrine factors released by AT, such as TNF-α, adenosine, or prostaglandins, are capable to regulate lipolysis locally.[170]

In the fasted state, lipolysis is stimulated upon binding of catecholamines to the β-adrenergic receptors, inducing a signalling cascade. These receptors are coupled to stimulatory G proteins which interact with adenylyl cyclase (AC) to generate the second messenger cyclic AMP (cAMP).[171] Binding of cAMP to the protein kinase A (PKA) increases its activity and subsequently leads to the phosphorylation of HSL and the lipid coating protein perilipin A.[170] Phosphorylated HSL translocates from the cytosol to the lipid droplet, where its full activation is mediated by interaction with PKA-activated perilipin A.[172] Perilipin A restricts the access of lipases to the lipid droplet, but upon phosphorylation it provides increased access to the lipid droplet surface.[173] In the unstimulated state CGI-58, the coactivator of ATGL, is bound to perilipin A. Upon PKA-dependent perilipin A stimulation, CGI-58 is released, subsequently allowing the formation of the ATGL-CGI-58 complex followed by ATGL activation.[164] In the fed state, insulin suppresses lipolysis in a cAMP-dependent and independent mechanism.[174] To this end, insulin activates the enzyme phosphodiesterase 3B (PDE3B) via PI3K-signalling, leading to degradation of cAMP and decreased PKA-activity[175], resulting in reduced HSL and perilipin A activity. cAMP-independent suppression of lipolysis involves the insulin-stimulated phosphorylation and activation of the enzyme protein phosphatase-1 which dephosphorylates and deactivates HSL.[176]

In obesity basal lipolysis is increased, while catecholamine-induced lipolysis is reduced.[177,178] This effect might be mediated by many pathophysiological conditions. It has been shown that HSL as well as ATGL and perilipin A expression is reduced in AT of obese individuals.[179-181] Moreover, insulin resistance is assumed to be an important cause for

reduced suppression of lipolysis, an effect observed in studies from obese rats as well as in women with visceral obesity.[182-184] However, some studies demonstrated sustained anti-lipolytic activity of insulin even though glucose metabolism was impaired in these subjects.[185,186] Dysregulation of adipokines such as leptin and TNF-α might be involved in increased lipolysis, as leptin interferes with the metabolic effects of insulin[187], while TNF-α is reported to reduce perilipin A protein levels in adipocytes[188].

1.3. Adipose tissue hypoxia

1.3.1. Pathology of hypoxic tissues

Oxygen tensions in the body are different from ambient air, which is about 21% O_2, and vary between different tissues. Between the lung parenchyma and the circulation, as well as irrigated parenchymal tissues like the liver, kidney or the heart, O_2 concentrations vary between 14% and 4%.[189-192] Tissues which are relatively less irrigated show even less oxygen levels, such as the brain, which varies between 0.4% and 7% O_2, the eye ranging from 1% to 5% O_2 and the bone marrow niches, which range from below 1% to 6% O_2.[193-195] From a molecular standpoint, these tissue oxygen tensions can be regarded as a hypoxic environment compared to atmospheric oxygen concentration. However, this 'physiological hypoxia' is suggested to be defined as 'in situ normoxia', while the standard cell culture model used in current investigations are performed at atmospheric O_2 and would rather reflect a condition of 'hyperoxia'.[196,197] However, the term used in this work will be adapted to the molecular definition of hypoxia.

Hypoxia may arise in normal tissue development and in pathological conditions where oxygen supply is diminished due to defective vasculature, such as ischemic disorders (cerebral or cardiovascular), atherosclerosis, diabetes, inflammatory disease, psoriasis, pre-eclampsia, chronic obstructive pulmonary disease, and cancer.[198] Adaptation processes are initiated to maintain cell viability and vital metabolism at reduced oxygen availability, and these mechanisms are evolutionally

highly conserved.[199] The transcription factor hypoxia-inducible factor (HIF)-1 is activated under hypoxia and initiates transcription of its target genes, which are involved in angiogenesis and vascular tone, glucose and energy metabolism, cell proliferation, cell survival, and cell death.[200,201] HIF-1 is a heterodimer composed of a constitutively expressed HIF-1β subunit and an oxygen-regulated HIF-1α subunit.[202] HIF-1α is continuously expressed but quickly targeted to proteasomal degradation under aerobic conditions. *In vitro* experiments demonstrated that accumulated HIF-1α protein levels in adipocytes are returned to basal level within 10 minutes after cell exposure to atmospheric oxygen conditions.[203] In this context, HIF-1α is hydroxylated via prolyl hydroxylase domain proteins (PHDs), which are oxygen dependent dioxygenases.[204] This hydroxylation provides binding sites to von Hippel-Lindau proteins which further promote polyubiquitylation of HIF-1α, tagging it for destruction via the 26S proteasomal system.[205] The transcriptional activity of HIF-1α is further inhibited by its hydroxylation via factor inhibiting HIF-1 (FIH-1) at specific arginine residues, which prevents binding of the coactivator protein p300.[204,206] Low oxygen tensions result in inhibition of the involved hydroxylases, leading to the stabilization of HIF-1α, its binding to the coactivator p300, and subsequent translocation to the nucleus. Finally, the heterodimer of HIF-1α/HIF-1β binds to specific hypoxia-response elements (HRE) within target genes, leading to their transcription.[207] Besides HIF-1α, two other human isoforms, HIF-2α and HIF-3α, are known. HIF-2α mediates vascular and erythropoietic responses, while HIF-1α is involved in vascular and metabolic responses.[208] Not much is known about the function of HIF-3α, which is currently under investigation. There is evidence that HIF-3α is involved in the inhibition of HIF-1α and HIF-2α as well as in the regulation of specific hypoxia responsible genes.[209]

Angiogenesis is initiated under hypoxia as an adaptive mechanism to establish re-oxygenation of hypoxic tissues. This effect is mediated by HIF-induced expression of important angiogenic factors such as VEGF and Angiopoetin-2.[210] Energy metabolism is also modified as HIF-1 promotes a switch from oxidative to glycolytic metabolism via two ways: 1. promoting anaerobic glucose metabolism and 2. repressing and rendering tricarboxylic acid cycle (TCA) to result in a more efficient mitochondrial respiration.[198,208] Up-regulation of distinct glucose transporters, glycolytic

enzymes, and lactate dehydrogenase A (LDH-A) results in increased glucose utilization and lactate production as a metabolic end-product. HIF-1-mediated overexpression of pyruvate dehydrogenase kinase 1 (PDK1) results in the suppression of pyruvate conversion into acetyl-CoA, which subsequently reduces carbon flux through the TCA.[211] Moreover, HIF-1 mediates optimization of the electron transport chain (ETC) in order to utilize the limited oxygen supply appropriately and to prevent the generation of ROS such as superoxide radicals. In this context, the subunit cytochrome c oxidase (COX)4-1 is down-regulated and COX4-2 is up-regulated, as this subunit is more efficient in the electron transfer to oxygen, leading to a reduced production of ROS.[212,213]

Adequate regulation of the HIF-1 pathway is important for the development and progression of pathological conditions. HIF-1 plays an important role in wound healing as acute hypoxia initiates repair processes such as angiogenesis, fibroblast proliferation, and cytokine production, while chronic hypoxia is associated with impaired wound healing.[214] In general tumours are severely hypoxic, as they are characterized by rapid proliferation of tumour mass and a distorted and irregular vascular system which is insufficient in oxygen supply.[198] Intratumoural activation of HIF-1 does not only result in the preference for anaerobic glucose metabolism, known as 'Warburg effect', but it is also associated with the transcription of genes involved in tumour invasion, metastasis, or radiation resistance.[215-218] Therefore, over-expression of HIF-1α is associated with increased mortality in common cancers such as breast cancer.[219] Additionally, diabetes is associated with a dysregulation of the HIF-1α pathway. Hypoxia is a hallmark of diabetic complications, including poor wound healing, diabetic retinopathy, neuropathies, cardiovascular and renal diseases.[220] Hyperglyceamia could be demonstrated to compromise the stability and function of HIF-1α, resulting in an impaired response to hypoxia with detrimental consequences for cell and tissue adaptation, which might lead to a worsening of diabetic complications.[220-222]

1.3.2. The hypothesis of adipose tissue hypoxia

AT oxygenation in obesity seems to be impaired, as it is reported that poor vascularization, reduced blood flow, particularly in the postprandial state, and reduced subcutaneous tissue oxygenation occur in obese individuals.[223-227] In 2002, it was reported that low oxygen tension could induce the leptin gene in skin fibroblasts and a human adipocyte cell line.[228-230] In 2003, hypoxia-mediated induction of angiogenic factors such as VEGF in mouse adipocytes was reported.[231] In 2004, Trayhurn et al. proposed the hypothesis that hypoxia in AT might occur and be one trigger for adipokine dysregulation and AT inflammation.[34] There are diverse causes proposed to contribute to AT hypoxia. First, oxygen diffusion through AT might be impaired because of increased adipocyte size. In obesity, hypertrophic adipocytes could increase up to 140-180 µm in diameter, hence exceeding the oxygen diffusion range of 100 µm in tissues.[232,233] It is also suggested that oxygen supply is impaired in AT as angiogenesis may not be sufficient to accommodate the progressive expansion of AT in obesity.[34] Reduced AT blood flow might also contribute to AT hypoxia, as it is well established that blood flow in obese AT is reduced per unit tissue mass and in the postprandial state.[223,234,235] ATM may also be involved in local reduction of AT oxygen tension as macrophages are known to be metabolically active cells with high consumption rates of oxygen, which might contribute to local AT hypoxia.

Evidence for AT hypoxia has been obtained from various mouse models as well as from human studies. Hypoxia in AT of obese mice was demonstrated either by a chemical marker of low oxygen, namely pimonidazole hydrochloride, or by measurement with an O_2 microelectrode.[236-239] These studies revealed that oxygen levels could decrease to 2% O_2 in AT of obese or high fat (HF) fed mice, while in the respective lean phenotype oxygen tensions of about 6% O_2 were detected. Moreover, AT hypoxia in these mice correlated positively with AT expression levels of inflammatory markers such as TNF-α, IL-1, PAI-1, or IL-6 as well as reduced adiponectin levels. Currently, two human studies described differences between lean and obese AT oxygenation[240,241]. Pasarica et al. demonstrated an average oxygen tension of 7.3% O_2 in subcutaneous AT of lean individuals whereas AT of obese individuals displayed a moderately reduced oxygenation of 6.2% O_2.[242] The second

human study of Goossens et al. provided a contrary picture as they reported elevated oxygen levels in WAT of obese (8.9% O_2) compared to lean individuals (5.9% O_2).[243] The latter study argued that O_2 consumption of WAT might be reduced because of diminished mitochondrial biogenesis and mass in the obese state, as it was previously shown in diabetic Zucker rats and diverse obese mouse models.[244-246] Moreover, it has to be noted that different methods of oxygen measurement were used in these two studies and both could neither verify nor exclude oxygen variations or local alterations in oxygen supply. However, these studies demonstrated oxygen levels ranging between 3% and 11% O_2 in human subcutaneous WAT.

1.3.3. Hypoxia-induced alterations in adipocyte function

In vitro studies revealed that adipocyte function is affected by hypoxia, in particular adipokine expression and secretion is modified towards increased amounts of inflammation-related factors. Moreover, glucose uptake, lipid turnover, and insulin sensitivity is altered under hypoxia treatment.

Human and murine adipocytes exhibit increased expression and secretion of adipokines such as IL-6, leptin, and VEGF, while adiponectin levels are decreased under hypoxia (Fig. 1.2).[203,236] This profile is similar to the dysregulation of adipokines observed in the obese state, supporting the hypothesis that hypoxia may be one trigger of AT inflammation. Additionally, other adipokines involved in angiogenesis (PAI-1), extracellular matrix (ECM) remodelling (MMP2, MMP9) and fibrosis (collagen-1,-3,-4,-6) are regulated by hypoxia.[247-250]

Hypoxia also affects macrophages, as the release of pro-inflammatory cytokines such as TNF-α, VEGF, IL-6, or IL-1 is elevated under low oxygen tensions, which might further promote AT inflammation.[251] A number of inflammatory genes are targets of NF-κB and it is well established that hypoxia initiates NF-κB activity in various cells types, including cancer cells, immune cells, or cardiac myocytes.[252-255] Increased NF-κB activity has also been shown in murine 3T3-L1 adipocytes[236], but until today there is no direct evidence for this mechanism in human primary adipocytes.

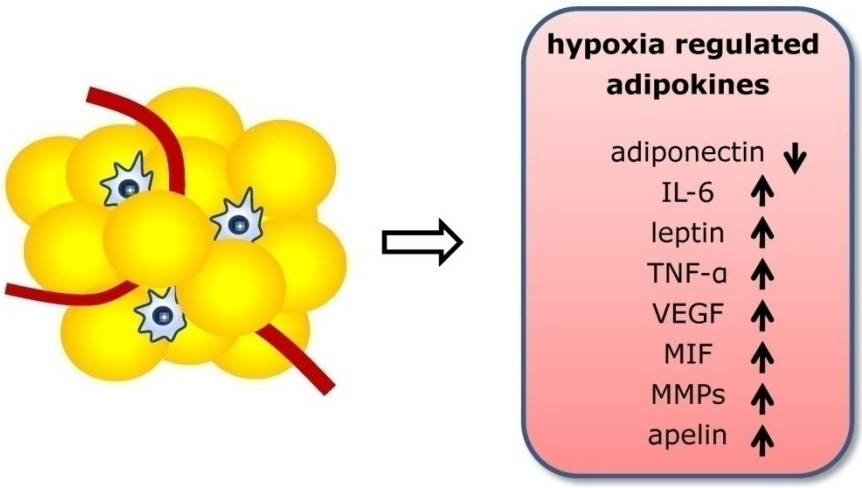

Figure 1.3 Overview on hypoxia-regulated adipokines in adipose tissue

IL, interleukin; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor; MIF, macrophage migration inhibitory factor; MMP, matrix metalloproteinase

Glucose metabolism is altered under hypoxia, as basal glucose uptake is increased and a switch towards anaerobic glycolysis occurs in hypoxic adipocytes. This effect is meditated by an increased expression of the GLUT1 and specific glycolytic enzymes.[256,257] As a result of anaerobic glycolysis, the metabolic endproduct is lactic acid which has to be removed from the intracellular compartment via specific monocarboxylate transporters (MCTs) to prevent cell damage because of a lactate-induced reduction of the intracellular pH. It has been demonstrated in human adipocytes, that in addition to lactate accumulation in the cell culture supernatant, the expression level of distinct MCTs is increased under hypoxia.[258] Moreover, hypoxia is suggested to contribute to adipocyte insulin resistance, as studies in human adipocytes and mouse 3T3-L1 adipocytes revealed impaired insulin signalling and glucose uptake under severe hypoxia of 1% O_2.[239,259] Additionally, lipid metabolism is affected by hypoxia. It has been reported that reduction of oxygen pressure in the air, such as at high altitudes, as well as reduced AT oxygenation increase adipocyte lipolysis *in vivo* and *in vitro*.[239,259-261] Therefore, it is likely that AT hypoxia may contribute to obesity-related dyslipidaemia by at least two mechanisms, the induction of basal lipolysis and possibly by affecting the anti-lipolytic action of insulin via hypoxia-induced adipocyte insulin resistance.

1.4. Objectives

As outlined in the sections above, obesity is a major public health problem worldwide, which is associated with a variety of diseases including T2D, atherosclerosis, and the metabolic syndrome. Since AT is accepted to act as an endocrine organ, the role of its released factors is central to numerous studies investigating the impact of these adipokines on peripheral organs, and their role in the development of various diseases. The number of adipokines is increasing continuously due to various proteomic approaches identifying potential novel adipokines. One challenge of the outcome of such proteomic approaches is the validation of these factors as confirmed adipokines released by adipocytes, and their characterization as potent factors involved in the development of obesity-related diseases. Furthermore, in the last 10 years the hypothesis of AT hypoxia in the context of obesity was proposed and suggested to be involved in the dysregulation of AT secretion and functionality. As outlined in chapter 1.3, hypoxia is well known to be a hallmark of various diseases, and its role in tumor progression and wound healing is well established. However, its role and the related mechanisms in AT inflammation are until today not completely understood.

The starting point of this thesis was the validation and characterization of potential novel adipokines, identified in a previous proteomic approach, which analysed the secretome of human adipocytes. As a second aspect, the role of reduced oxygen tension on adipocyte function and adipocyte development should be investigated.

- AT releases a variety of different adipokines and the number of reported adipokines is increasing. A comprehensive proteomic approach by means of 1D-gelelectrophoresis with an ongoing LC-MS/MS analysis and a 2D-gelelectrophoresis with subsequent MALDI-MS/MS investigated the secretome of human primary adipocytes using adipocyte conditioned medium (CM). This secretome analysis identified more than 300 different proteins potentially released from adipocytes. Intensive literature search revealed 44 putative novel adipokines and five of these were selected for further validation and characterization as adipokines.

- DDP4 is known to be a potent drug target in T2D treatment. Identification of DPP4 as a novel adipokine underlined the importance of AT in contributing to the release of pivotal factors affecting diseases such as T2D. An intensive characterization of the expression and secretion pattern of DPP4 during adipogenesis, as well as its autocrine and paracrine effects on various cell types was a second objective of this thesis.

- AT releases not only a vast number of adipokines, but some of these are released at especially high amounts. One high abundant adipokine is PEDF, whose role as a positive and negative regulator of diverse diseases has been demonstrated in recent studies reported in the literature. The role of PEDF as an autocrine and paracrine regulator of adipocyte and skeletal muscle cell function was the third objective of this thesis.

- AT hypoxia has obtained much attention as a possible trigger for the well-known chronic low grade inflammation observed in obesity. Studies in murine adipocytes have shown an altered secretory profile of these cells towards an inflammatory response under hypoxic conditions. Moreover, it is known that hypoxia can induce inflammatory pathways, such as the NF-κB pathway. In the second part of this thesis one objective was to elucidate the role of acute severe hypoxia on adipokine secretion and adipocyte inflammation in human primary adipocytes.

- Current investigations on adipocyte secretion and function are using experimental settings, which include the differentiation and cultivation of adipocytes at ambient air and a following incubation under acute and severe hypoxia of 1% O_2. As AT oxygen tensions have been shown to range between 3-11% O_2 in lean and obese AT, it is apparent that conventional culture and hypoxic treatment do not reflect the physiological situation. The last objective was to establish adipocyte differentiation at more physiological oxygen conditions and to investigate differences in the secretory capacity and functionality between adipocytes cultivated in a more physiological condition compared to the conventional culture procedure.

Chapter 2

Study 1

Identification and validation of novel adipokines released from primary human adipocytes ‡

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Abbreviations: 1/2D-GE, one/two dimensional gel electrophoresis; CFH, complement factor H; CILP, cartilage intermediate layer protein; CM, adipocyte conditioned media; CRYAB, αB-crystallin; ESI, electrospray ionization; HO, heme oxygenase; LC, liquid chromatography; MALDI, matrix assisted laser desorption/ionization; MS, mass spectrometry; PEDF, pigment epithelium-derived factor; TOF, time of flight; TGF, transforming growth factor; TNF, tumor necrosis factor

Abstract

Adipose tissue is a major endocrine organ, releasing signaling and mediator proteins, termed adipokines, via which adipose tissue communicates with other organs. Expansion of adipose tissue in obesity alters adipokine secretion which may contribute to the development of metabolic diseases. Although recent profiling studies have identified numerous adipokines, the amount of overlap from these studies indicates that the adipokinome is still incompletely characterized. Therefore, we conducted a complementary protein profiling on concentrated conditioned medium derived from primary human adipocytes. SDS-PAGE/LC-ESI-MS/MS and 2-dimensional SDS-PAGE/MALDI-MS identified 347 proteins, of which 263 were predicted to be secreted. Forty-four proteins were identified as novel adipokines. Furthermore, we validated the regulation and release of selected adipokines in primary human adipocytes and in serum and adipose tissue biopsies from morbidly obese patients and normal weight controls. Validation experiments conducted for complement factor H, αB-crystallin, cartilage intermediate-layer protein and heme oxygenase-1 show that the release and expression of these factors in adipocytes is regulated by differentiation and stimuli which affect insulin sensitivity, as well as by obesity. Especially heme oxygenase-1 reveals to be a novel adipokine of interest. *In vivo*, circulating levels and adipose tissue expression of HO-1 are significantly increased in obese subjects as compared to lean controls. Collectively, our profiling study of the human adipokinome expands the list of adipokines, and further highlights the pivotal role of adipokines in the regulation of multiple biological processes within adipose tissue and their potential dysregulation in obesity.

Introduction

Obesity has become a critical global health problem which frequently associates with the development of chronic diseases including type 2 diabetes and cardiovascular dysfunction.[1] It is now considered that adipose tissue is one of the major endocrine organs[2,3] besides acting as a lipid depot providing an important function in storage and release of energy rich substrates. By secreting a huge diversity of signaling and mediator molecules, termed adipokines, adipose tissue communicates with other tissues, such as liver, skeletal muscle, heart, brain, and vasculature.[3-6] Recent data indicate that these adipokines create a complex interconnected network mediating the crosstalk between these tissues.[7,8] In obesity, enlargement of adipose tissue has been linked to a dysregulation of adipokine secretion and adipose tissue inflammation.[9] This switch to a chronic state of low-grade-inflammation represents a critical pathogenic link between obesity and the development of multifactorial diseases, like type 2 diabetes and the metabolic syndrome.

Because of the relevance of adipose tissue in the progression of these common diseases, multiple unbiased, proteomic approaches have characterized the secretome from both rodent and human adipocytes and adipose tissue.[10-17] These studies have emphasized the complex nature of the adipokinome and identified hundreds of adipokines. However, the amount of overlap between the identified adipokines thus-far indicates that the adipokinome is still incompletely characterized.

Furthermore, it is largely unclear whether novel adipokines found in these approaches are dysregulated in obesity and type 2 diabetes. Because differentiated primary human adipocytes secrete factors which induce insulin resistance in skeletal muscle cells and aberrant proliferation of smooth muscle cells,[18,19] we have conducted an extensive proteomic profiling of conditioned media (CM) derived from differentiated, primary human adipocytes.

This resulted in the identification of 347 proteins, of which to the best of our knowledge 44 have not been described as adipokines before. We subsequently assessed the regulation of i) complement factor H (CFH), ii) αB-crystallin (CRYAB), iii) cartilage intermediate-layer protein (CILP) and iv) heme oxygenase-1 (HO-1) in both primary human adipocytes and serum and adipose tissue samples from patients with obesity versus

control with normal body weight. The identified proteins have been implicated in multiple biological processes, thereby further highlighting the regulatory role of adipokines in intra-organ cross-talk.

Experimental Procedures

Adipocyte isolation and culture

Subcutaneous adipose tissue was obtained from lean or moderately overweight women (for proteomic analysis n=5, body mass index 28.5 ± 5.7 including one obese woman, and aged 24.9 ± 1.6 years, for biochemical analysis n=13, body mass index 26.2 ± 0.6, and aged 44.6 ± 3.3 years) undergoing plastic surgery. The procedure was approved by the ethical committee of the Heinrich-Heine-University (Duesseldorf, Germany) and written consent was obtained from each donor. All subjects were healthy, free of medication and had no evidence of diabetes according to information of the respective family doctor and were known not to be affected by HIV, hepatitis C and tuberculosis. Pre-adipocytes were isolated by collagenase digestion of adipose tissue as previously described by us.[18] Isolated cell pellets were re-suspended in Dulbecco's modified Eagles/Hams F12 (DMEM/F12) medium supplemented with 10 % FCS, seeded in 75 cm² culture flasks or six-well culture dishes and maintained at 37° C with 5 % CO_2. After overnight incubation, cultures were washed and further incubated in an adipocyte differentiation medium (DMEM/F12, 33 µmol/l biotin, 17 µmol/l d-panthothenic-acid, 66 nM insulin, 1 nM triiodo-L-thyronine, 100 nM cortisol, 10 µg/ml apo-transferrin, 50 µg/µl gentamycin, 15 mmol/l HEPES, 14 nmol/l NaHCO3, pH 7.4) for 15 days with medium change every 2-3 days and addition of 5 µM troglitazone for the first 3 days. The degree of differentiation was determined by oil red staining and induction of adiponectin expression. Differentiated adipocytes were used for the generation of adipocyte-conditioned media (CM), as described previously.[20] Macrophages were isolated from human adipose tissue, using a method described by Curat et al.[21] For hypoxia treatment, differentiated adipocytes were incubated with a gas mixture containing 1 % O_2, 5 % CO_2 and 94 % N_2 in MIC-101

modular incubator chambers (Billups-Rothenburg, Del Mar, USA) at 37 °C for indicated times.

Sample preparation for secretome analysis

For sample preparation CM from 5 different donors (200 ml derived from $7x10^7$ adipocytes) were collected. CM was first analyzed for their capacity to induce insulin resistance of the level of insulin-stimulated Akt phosphorylation in skeletal muscle cells as described previously[18,19] and only active CM inducing insulin resistance were further used. Prior to protein profiling studies these CM were pooled and centrifuged for 20 min at 40,000 × *g* at 4 °C. Subsequently, supernatants were concentrated to a final volume of 200 µl using Amicon™ Ultra 15 centrifugal filter devices (Millipore, Billerica, USA) with a cut-off mass of 3,000 Dalton. Protein concentrations were measured using Advanced Protein Assay (Cytoskeleton, Denver, USA). Concentrated CM (4.9 mg/ml) was diluted 1:3 in a buffer containing 25 mM Tris, 4 % CHAPS (w / v), 7 M urea and 2 M thio-urea and stored as aliquots at –80°C until use.

SDS-PAGE and protein identification by LC-MS

For LC-MS analysis aliquots of concentrated CM was initially separated by one-dimensional SDS-PAGE. 20 µg of protein was mixed with 10 µl of 2 x SDS PAGE sample buffer and 3 µl of 60 mM DTT. The samples were boiled for 10 min at 96 °C and cooled down to room temperature. After addition of 3 µl of 150 mM iodine acetamide, the samples were incubated for 30 min in the dark before they were subjected to SDS PAGE on a 5 % to 15 % gradient gel. Proteins were visualized with Imperial Protein Stain (Thermo-Fisher, Bonn, Germany). The stacking gels were removed and the entire lanes (60 x 5 x 0.75 mm) were divided into 24 equally sized portions based on different molecular weights. The bands were chopped into small cubes and washed three times with acetonitrile-water (1:1). Acetonitrile was added and removed to dehydrate the gel pieces before they were dried for 5 min in a speed vac. The dry gel pieces were rehydrated in an ice cold solution of 12.5ng/µl Trypsin (sequencing grade, Promega) in 10 mM NH_4HCO_3. After 45 min on ice, excessive trypsin solution was replaced by 20 µl of buffer without enzyme and

proteins were digested at 37 °C for 4 h. The digest was stopped by the addition of 20 µl 10 % formic acid (FA). Peptides were extracted for 30 min and the volume was reduced to 20 µl before the extracts were stored at -80 °C.

Liquid chromatography (LC)-MS data were acquired on a HCT ETD II ion trap mass spectrometer (Bruker Daltoniks, Bremen, Germany) equipped with a nano ESI source (Bruker Daltonics, Bremen, Germany). Samples were introduced by an easy nano LC system (Proxeon, Odense, Denmark) using a 0.1-by-200-mm analytical column, self-packed with ReproSil-Pur C18-AQ, 5 µm (Dr. Maisch, Ammerbuch, Germany). 18 µl sample were aspirated into the sample loop and a total of 25 µl was loaded onto the column using a flow rate of 2 µl/min. Loading pump buffer was 0.1 % FA. Peptides were eluted with a gradient of 0 % to 35% acetonitrile (ACN) in 0.1% FA over 170 min at a column flow rate of 500 nl/min. Subsequently the ACN content was raised to 100 % over 2 min and the column was regenerated with 100 % ACN for additional 8 min.

Data-dependent acquisition of MS and tandem MS (MS/MS) spectra was controlled by the Compass 1.3 software. MS1 scans were acquired in standard enhanced mode. Five single scans in the mass range from *m/z* 400 to m/z 1400 were combined for one survey scan. Up to three doubly and triply charged ions rising above a given threshold were selected for MS/MS experiments. Ultra scan mode was used for the acquisition of MS2 scans in the mass range from m/z 100 m/z 1600 and three single scans were added up. The ion charge control value was set to 250,000 for all scan types. Raw data were processed with Data Analysis 4.0 (Bruker Daltoniks, Bremen, Germany) and xml formated peak lists were transferred to Proteinscape 2.1 (Bruker Daltoniks, Bremen, Germany).

MASCOT 2.2 (Matrix Science Ltd, London, UK) was used to search a composite decoy database which was built from SwissProt_57.4 (468851 sequences; 166,149,756 residues). The composite database was generated with the Perl script *makeDecoyDB* (Bruker Daltoniks, Bremen, Germany) which added a randomised sequence and a tagged accession number for each entry. The tagged accessions were used for the calculation of false positive rate in Proteinscape 2.1. Searches were submitted via Proteinscape (Bruker Daltoniks, Bremen, Germany) and the following parameter settings: enzyme “trypsin”, species “human”, fixed modifications “carbamidomethyl”, optional modifications “Methionine

oxidation" and missed cleavages "2". The mass tolerance was set to 0.4 Da for peptide and fragment spectra. Protein lists were compiled in Proteinscape. Peptide hits were accepted when the ion score exceeded a value of 20. Protein hits required at least one peptide hit exceeding a peptide score of 40. In addition, the hits to decoy entries were used to calculate a minimal protein score which is required to keep the false positive rate below 2 % on the protein level[22].

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and protein identification by MALDI-MS

For 2D-PAGE analysis aliquots (150µg) of concentrated CM were separated in the first dimension by isoelectric focusing (IEF) using pH 4 – 7 and pH 6 - 9 linear IPG strips performed on a MultiPhor II electrophoresis unit (GE-Healthcare, Freiburg, Germany) and in the second dimension by large format SDS-PAGE (12%) as previously described[23]. Subsequent to electrophoretic separation, gels were stained with a ruthenium fluorescent stain[24] and protein pattern were visualized by laser scanning using blue laser source (457 nm) on a Typhoon 9400 (GE-Healthcare, Freiburg, Germany) and a resolution of 100 µm. Detection of protein spots and calculation of relative spot abundances was carried out automatically using Proteomweaver 4.0 image analysis software (BioRad, Munich, Germany).

Protein spots exceeding intensity levels of 0.2 and matching gel sets of six individual 2D-gels were selected for protein identification by MALDI-MS. Proteins matching these criteria subsequently were excised from four replicate gels (4-7 and 6-9 each) using a Gelpix spot picker (Genetix, Dornach, Germany). For in-gel digestion gel pieces were washed for 10 min in digestion buffer (10 mM NH_4HCO_3) and digestion buffer containing 50% acetonitrile (1:1, v/v). Acetonitrile was added and removed to dehydrate the gel pieces. The dry gel pieces were rehydrated in an ice cold solution of 3.5 ng/µl Trypsin (sequencing grade, Promega) in 10 mM NH_4HCO_3. Proteins were digested at 37 °C for 4 h. Peptides were extracted for 30 min with 10 µL of 0.1% TFA anddirectly applied to a MALDI Pre-spotted AnchorChip target (Bruker Daltoniks, Bremen, Germany) according to the manufacturer's instructions.

Subsequently, samples were analyzed in a time-of-flight Ultraflex-Tof/Tof mass spectrometer (Bruker Daltoniks, Bremen, Germany). Acquired mass spectra were automatically calibrated and annotated using Compass 1.3 software (Bruker Daltoniks, Bremen, Germany) and xml formated peak lists were transferred to Proteinscape 2.1 (Bruker Daltoniks, Bremen, Germany). Since all experiments were performed on primary human adipocytes, MS spectra from each individual spot were used to search a human sub-set of Swiss-Prot (Sprot_57.4, 20401 protein entries) non-redundant database using Mascot search engine (Version 2.2, Matrix Science Ltd, London, UK) in consideration of the following settings: enzyme "trypsin", species "human", fixed modifications "carbamidomethyl", optional modifications "Methionine oxidation" and missed cleavages "1". Mass tolerance was set to 50 ppm. Using these settings, a mascot score of greater than 70 was taken as significant ($p < 0.01$). Calculated p*I* and molecular mass data were obtained by Mascot. For peptides matching to different isoforms or multiple members of a protein family, we used the following reporting criteria: The experimental p*I* and molecular mass taken from the 2D-gels were compared with the theoretical data of the different isoforms / protein members. If no conflicts in molecular mass or p*I* were found, the isoform / protein member with the highest mascot score was reported. For verifying the results each protein spot was picked and identified from at least three physically different 2D-gels.

Prediction and annotation of secretory proteins

Secretory protein prediction and functional annotation was done by using different, independent ways. First protein information of all identified proteins was extracted from the Swiss-Prot/TrEMBL database (http://www.expasy.ch/sprot/). Gene name were usedto screen the Bio-GPS gene annotation portal (http://biogps.gnf.org) for expression data in adipose tissue (Suppl. Table 1). To assess secretory properties, subsequently protein sequences were analyzed by Ingenuity IPA8.5, SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and SecretomeP 2.0. (http://www.cbs.dtu.dk/services/SecretomeP/). Protein location annotations received from Ingenuity are displayed in Suppl. Table 1. To assign proteins as putative secretory passing prediction thresholds for

SignalP 3.0 (*D* score cut-off: 0.43) predicting a signal peptide or SecretomeP 2.0 (NN score cut-off: 0.5) predicting non-classical secretory proteins without signal peptide were set as mandatory (Suppl. Table 1). In addition to that protein ID's were applied to NCBI/Pubmed (http://www.ncbi.nlm.nih.gov/pubmed) literature screening in order to classify and compare identified proteins with published adipokinome studies (Suppl. Table 1).

Clinical study for the analysis of adipokine concentration in serum and adipokine expression in subcutaneous and visceral adipose tissue.

23 male obese patients scheduled for bariatric surgery and 17 lean age-matched controls undergoing an elective abdominal surgery were recruited at Ghent University Hospital. For all patients, anthropometric and routine blood parameters were assessed (Suppl. Table 2). Fasting blood samples were collected and frozen at -80°C, adipose tissue biopsies fixed for microscopic evaluation of adipocyte surface area analysis or frozen at -80°C for protein expression analysis. The study protocol was approved by the local Ethics Committees and all participants gave written, informed consent (Registration no B67020084018).

Determination of adipose tissue cell size

Visceral and subcutaneous adipose tissue specimens were obtained from the patients at the end of the surgical intervention. Adipose tissue was fixed in buffered 4% formol solution (Klinipath, Olen, Belgium) at room temperature. Further fixation, dehydration, cleaning and paraffin impregnation of tissues was performed (Tissue TekVip, Sakura, Heppenheim, Germany) and tissues were embedded with TBS 88 Paraffin Embedding System (Medite, Burgdorf, Germany). Haematoxylin-eosin staining and film cover slipping of 3µm slides was completed by a Tissue TekPrisma (Sakura, Heppenheim, Germany). Adipocyte size was assessed by using the approach of Tchoukalova et al.[25] Digital photographs of the paraffin slides were taken with a Mirax Midi camera (Zeiss, Jena, Germany) and the average surface area of 10 adipocytes of each slide was calculated using the Mirax Viewer software (Zeiss, Jena Germany).

Immunoblotting

Adipocytes and macrophages were treated as indicated and lysed in a buffer containing 50 mM HEPES, pH 7.4, 1 % TritonX100, Complete protease inhibitor and PhosStop phosphatase inhibitor cocktail (both Roche, Mannheim, Germany). After incubation for 2 h at 4 °C, the suspension was centrifuged at 10,000 x *g* for 15 min. Thereafter, 5-10 µg of adipocyte lysates were separated by SDS-PAGE using 10 % horizontal gels and transferred to polyvinylidenefluorid membranes in a semidry blotting apparatus. Membranes were blocked with Tris-buffered saline containing 0.1 % Tween and 5 % nonfat dry milk and subsequently incubated overnight with a 1:1000 dilution of the appropriate antibodies. After washing, filters were incubated with secondary HRP-coupled antibody and processed for enhanced chemiluminescence detection using Immobilon HRP substrate (Millipore, Billerica, MA, USA). Signals were visualized and evaluated on a LUMI Imager (Boehringer, Mannheim, Germany) or VersaDoc 4000 MP (BioRad, Munich, Germany) work station. Detection of actin was used to normalize for protein loading. Analysis of serum probes by Western Blot was done by application of defined serum volumes not requiring normalization for protein loading. Antibodies were purchased from Abcam (CFH, CRYAB, HO-1, actin) and R&D Systems (CILP).

ELISA

HO-1 release by cells and serum concentration was determined by ELISA (R&D Systems, Stressgene). The assay was performed in duplicate according to the manufacturer's instructions.

Gene expression

RNA was isolated from 100-200mg adipose tissue biopsy with an RNeasy mini kit for lipid rich tissue using the Qiacube robot (Qiagen, Hilden, Germany). DNA remove was done by a DNAse1 digestion followed by a clean-up with RNeasy mini column using the Qiacube robot. For cDNA synthesis 500 ng total RNA was applied to Superscript first-strand cDNA Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's

instructions. Measurements of relative CILP1 expression were performed on a StepOne Plus Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) using Sybr Green and Qiagen Quantitect primer assay for human CILP1. The cycling conditions comprised a polymerase activation at 95°C for 10 min followed by 40 cycles at 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. mRNA expression was normalized to RPS18 mRNA content and expressed as arbitrary units.

Presentation of data and statistics

Data are expressed as mean ± SEM. The Shapiro-Wilcoxon test was used to test the Gaussian distribution of biological parameters. Student's t test was used for all experiments where only two experimental groups were compared, analysis of variance (ANOVA) followed by p for linear trend post-test were used for experiments where more than two experimental groups were compared. Correlations were performed by Pearson. All statistical analyses were done using JMP statistics software (SAS Institute Inc., Cary, NC, USA) or Prism (GraphPad, La Jolla, CA, USA) considering a P value of less than 0.05 as statistically significant. Corresponding significance levels are indicated in the figures.

Results

Proteomic profiling of the adipocyte secretome

As summarized in Figure 1, two complementary, orthogonal approaches, i.e. 1-dimensional SDS-PAGE/LC-ESI-MS/MS and 2-dimensional SDS-PAGE/MALDI-MS were used to profile the conditioned medium (CM) derived from primary human adipocytes. To account for biological variability CMwas collected from five different donors.Prior to protein profiling,CMs derived from the different donors were pooled to make profiling more feasible. Subsequently, CM was concentrated by a factor of 1,000 in order to achieve a protein concentration in the mg/ml range. Using LC-ESI-MS/MS analysis, automated data processing by Proteinscape™ 2.1 led to the consistent identification of 341 unique

protein species in protein slices (24 slices per lane) derived from two replicate 5-15% gradient SDS-PAGE gels with a false positive rate below 2 % (Table S1 in Supporting Information). Complementary to this approach, the CM was analyzed using 2D-SDS-PAGE/MS. Image analysis of the merged large format 2D-gels covering a pH range of 4-9 (overlapping gel couple pH 4-7 and pH 6-9, exemplarily shown in Suppl.Fig.2) reproducibly detected more than 1,200, non-redundant protein spots within the CM. Consistently detected protein spots were excised from four replicate gel-couples, subjected to in-gel digestion and MALDI-MS. Proteinscape^{TM} 2.1 data processing identified a total of 351 protein spots which were consistently found in at least three technical replicate gels. Subsequent analysis assigned these 351 spots to 89 different protein species (TableS1 in Supporting Information).

Overall the combined MS data resulted in the identification of 347 distinct protein species. Of these, 83 proteins were detected via both approaches (TableS1 in Supporting Information).

Secretory protein selection and comparison with reported secretomes

We used different web-based bioinformatic tools (Swiss-Prot/TrEMBL, BioGPS, Ingenuity IPA8.5, SignalP3.0, SecretomeP2.0, NCBI PubMed) to assess whether the 347 proteins could be secretory proteins. Applying these consecutive filter methods, 263 proteins (263/347, 76%) were predicted to be or have been annotated as secretory proteins (TableS1 in Supporting Information). Of these, 219 proteins have been described in previous profiling studies on adipocytes or adipose tissue (Table 1, supplementary Table 1). However, to the best of our knowledge, the 44 proteins listed in Table 2 have not been reported in previous approaches, and can therefore be considered as potential novel adipokines (Table 2).

Validation of novel adipokines using human adipocytes and adipocyte-conditioned medium

The available web-based bio-informatical and Gene Onthology (GO) Biological Process and Molecular Function annotation tools, ascribe a wide array of biological functions to the adipokines identified in this study. Depending on the immediate availability of suitable biochemical tools, we

therefore selected 4 adipokines eliciting completely distinct biological functions for further validation. These are (i) complement factor H (CFH), which has been implicated in inflammation, (ii) αB-crystallin (CRYAB), which has been implicated in apoptosis, (iii) cartilage intermediate layer protein (CILP), which participates in extracellular matrix structure and remodeling and (iv) heme oxygenase-1 (HO-1), a component of the oxidative stress response.

Although CFH and CRYAB were recently described as novel adipokines[15-17] these proteins have not been validated so far. Western blot analysis of concentrated CM confirmed the presence of CRYAB, CFH, HO-1 and CILP, thereby validating the secretion of these factors from primary human adipocytes (Fig 2A). Like adiponectin which was used as a control marker for adipocyte differentiation, protein expression of CRYAB, and CILP gradually increased during the transition of pre-adipocytes in mature primary human adipocytes (Fig. 2B). Protein expression of HO-1 peaked at day 7 of differentiation and was slightly decreased in mature cells (day 14; Fig. 2B). The abundance of CFH gradually decreased during adipocyte differentiation (Fig. 2B, Fig. 3A). Finally, all of these factors are more highly expressed in adipocytes as compared to macrophages isolated from human adipose tissue (Fig. 2C).

Although CFH protein expression is higher in preadipocytes as compared to mature adipocytes (Fig. 3A) CFH expression can be upregulated in adipocytes by troglitazone, TNFα, insulin and hypoxia (Fig. 3B). Furthermore, CFH levels in serum from obese subjects, as determined by Western blotting, were elevated versus subjects with normal body weight (Fig. 3C).

CRYAB expression is strongly induced during adipogenesis (Fig. 3D) reaching levels which are 10-times higher in mature adipocytes than in preadipocytes. CRYAB expression is not regulated by troglitazone, TNFα, insulin, adiponectin or hypoxia (data not shown) and the amount of CRYAB detected in serum by Western Blotting is not significantly different between lean and obese subjects (Fig. 3E).

CILP is a completely novel adipokine first identified by us. The antibody used to validate CILP recognizes both the 140-kDa precursor form as well as the 90-kDa secreted N-terminal form. The abundance of the CILP precursor significantly increased during differentiation reaching about 10-times higher concentrations in mature adipocytes as compared

to preadipocytes (Fig. 4A). Although the same trend could be observed for the abundance of the 90 kDa secreted product, this increase failed to reach significance. It seems plausible to ascribe this to release of the 90-kDa form during differentiation based on prevalent abundance of this form in CM (Fig. 2B). Western blot analysis revealed that the 140 kDa precursor form is significantly decreased by troglitazone while the 90 kDa secreted product is significantly inhibited by TNFα in cultured adipocytes (Fig. 4B). Immunoblotting failed to produce reproducible results in adipose tissue biopsies (data not shown). However, using real-time PCR, we observed that expression of CILP1 mRNA was up to 30-fold increased in subcutaneous versus visceral adipose tissue (Fig. 4C). Finally, plasma levels of CILP were significantly decreased in serum from obese males versus normal weight controls (Fig. 4D). Notably, only the secreted 90 kDa form was observed in serum.

Protein expression of HO-1 significantly increases with adipocyte differentiation (Fig.5A and 5B). While HO-1 expression is not affected by TNFα (data not shown), its release is significantly decreased by this pro-inflammatory factor (Fig.5C). ELISA analysis showed that circulating levels of HO-1 were significantly increased in obese subjects as compared to lean controls (Fig. 5D). Interestingly, HO-1 serum concentrations correlate with subcutaneous adipocyte size (Fig. 5E).

Measurement of CRYAB and HO-1expression in adipose tissue biopsies from subcutaneous and visceral adipose tissue of lean and obese men revealed that both proteins are significantly higher expressed in adipose tissue of obese subjects (Fig.6A and 6B). While the increase in HO-1 expression in visceral fat is similar in lean and obese subjects (Fig. 6B) CRYAB expression in visceral adipose tissue of obese subjects is significantly higher as compared to lean controls (Fig. 6A).

Discussion

Proteomic Profiling of the Adipocyte Secretome

Detailed characterization of the human adipokinome is necessary to increase our understanding of the role of adipocytes in disease pathophysiology. Therefore, we profiled the adipokinome from primary human adipocytes. To enable a more general analysis and considering the substantial biological variability in humans, we collected and pooled CM derived from five different donors. For proteomic profiling, concentrated protein samples equivalent to 200 ml CM were analyzed by two orthogonal techniques, i.e. 1DE-LC-ESI-MS/MS and 2DE-MALDI-MS, which led to the identification of 347 different proteins. Combining these two complementary approaches resolves limitations given by each single method and together with the huge sample amount may provide a more comprehensive strategy to catalogue and compare the complex nature of the adipose tissue secretome. Although, almost all proteins detected via 2DE-MALDI-MS (83 of 89) were also observed by LC-MS analysis (341 proteins identified), gel based examination provides the opportunity to display unrivaled additional information concerning protein isoforms or putative protein modifications and 2DE maps also can serve as a reference for further quantitative profiling studies (e.g. DIGE). This is illustrated by our profiling results, where each identified protein species is represented in average by more than three distinct spots (351 protein spots, 89 distinct protein species) varying in molecular mass or pI. Based on this information we already described pigment epithelium-derived factor (PEDF) as a major biological active secretion product of the human adipocyte (present in 15 spots and accounting for 6% of total spot intensities)[26]. Furthermore, we also discovered dipeptidyl peptidase 4 (DPP4) as novel adipokine[27], thereby further confirming the usefulness of our approach.

A major challenge in secretome analysis is to discriminate between actually secreted proteins and those, which may be contaminants introduced due to the operational procedure. To address this issue, we filtered the identified proteins for properties disclosing them as secretory proteins. Testing all proteins by hierarchical analysis, including screening of expression data and literature, as well as using signal sequence

prediction programs led to the identification of 263 putative secretory proteins. Applying this strategy utilizing mainly theoretical analysis tools assigned 84 proteins as 'non-secretory'. A major limitation of this analysis strategy is the risk to lose interesting target proteins without further validation. In order to uncover those proteins and achieve additional information usage of BrefeldinA treatment at 20°C has been described to monitor active secretion.[28,29] BrefeldinA blocks major ER/Golgi dependent as well as independent secretion pathways and therefore facilitates identification and discrimination of genuine secreted proteins. On the other hand proteins which are released by cleavage processes like DPP4,[27] or proteins secreted by mechanisms not affected by Brefeldin A will not be covered. Nevertheless, the high number of 263 identified proteins in our study demonstrates the efficiency of our integrated profiling approach, which is in line with very recently published studies of the human adipocyte secretome.[15,17] Comparison analysis reveals, that 219 (83%) of our putative secretory proteins were already reported in previous studies (Table 1), based on secretome analysis from other origins, such as 3T3-L1 adipocytes, tissue explants or isolated adipocytes. A very recent study conducted by Rosenow et al.,[16] applying an analogous approach to the human SGBS adipocyte cell line identified only 80 secreted proteins, potentially indicating limitations of the used cell model.

Although about 80% of the proteins are already published, our approach has identified 44 additional proteins (Table 2), which we consider as novel adipokines secreted from the human adipocyte. Interestingly to date more than 700 different proteins are described to be potentially secreted from the adipose tissue irrespective of species differences. Though detection and identification of putative novel adipokines is the irreplaceable basis, this alone will not be sufficient to enhance our current knowledge of the endocrine function of adipose tissue. Therefore, the candidate proteins have to be validated further regarding their expression, secretion and function.

Validation of novel Adipokines

To address their putative biological relevance, we have chosen 4 candidate proteins for further validation experiments. CFH, CRYAB, CILP and HO-1 are representatives of different critical functional pathways

(inflammation, apoptosis, extracellular matrix structure and oxidative stress) that have been demonstrated to be dysregulated in obesity.[15,30]

Complement factor H (CFH) is an example of a large group of adipocyte-secreted proteins being involved in inflammation. Showing a significant reduction of CFH protein expression during adipogenesis, we could corroborate findings from two other studies demonstrating at the mRNA level that this factor is predominantly expressed in preadipocytes although also present in adipocytes.[31,32] In addition, CFH in serum is elevated in insulin-resistant subjects, and CFH expression was found to be elevated in subcutaneous adipose tissue.[31] Consistently, we found higher CFH content in blood of obese men and an up-regulation of CFH in adipocytes treated with TNFα, insulin and hypoxia. Collectively, these data point to a possible higher contribution of adipose tissue to circulating CFH levels in obesity due to adipose tissue inflammation. If adipocytes or preadipocytes contribute to increased adipose CFH levels in obesity remains an open question that requires further analysis.

The protection against apoptosis and oxidative stress is a function of αB-crystallin (CRYAB). It is known that CRYAB is expressed in adipose tissue.[33] Here, we confirm CRYAB as an adipokine, in agreement with a recent report by Kim et al.[15] In addition the present study also demonstrates that CRYAB is increased during adipogenesis. Although CRYAB was not affected by stimuli that affect insulin sensitivity in adipocytes, CRYAB expression was found to be significantly increased in subcutaneous and visceral adipose tissue of obese patients as compared to lean controls. Therefore, further characterization of CRYAB regulation in adipose tissue of obese patients and patients with the metabolic syndrome could be of potential interest. Especially, the increased relative abundance of CRYAB in visceral adipose tissue of obese patients could be important, since visceral adipose tissue is a critical player in the development of obesity-related complications whereas subcutaneous adipose tissue may act merely as an innocent "bystander".[34] It should further be noted that CRYAB concentrations measured by Western Blot in serum are not different between lean and obese patients, suggesting that also other tissues such as heart and skeletal muscle regulate circulating levels of CRYAB.[35,36]

Cartilage intermediate-layer protein (CILP) is a secreted glycoprotein that resides in the extracellular matrix[37,38] maintaining cartilage

homeostasis.[39] Although CILP expression was originally suggested to be restricted to cartilage,[37,40-43] we here confirm Bio-GPS data showing that CILP is highly expressed in adipose tissue. Furthermore, we demonstrate for the first time that CILP is also secreted by primary human adipocytes. The gene product of CILP is a 140 kDa precursor protein for two secreted, proteolytically generated products, a 90 kDa N-terminal CILP and a 62 kDa C-terminal domain. The N-terminal CILP domain polypeptide was shown to function as an IGF-1 antagonist and binding partner for TGF-β1.[44,45] Several studies could show that CILP dysfunction contributes to various diseases affecting the cartilage. In this regard, the expression of CILP rises substantially in association with aging and also in the early stages of osteoarthritis and rheumatoid arthritis.[37] CILP acts as negative regulator of TGF-β1 by binding it directly *in vitro*, thereby disrupting normal TGF-β activity.[45] Furthermore, TGF-β strongly inhibits adipogenesis and the amount of fat in adipocytes.[46] Because CILP plasma levels are decreased in obese men, one could speculate that this decrease could result in a higher TGF-β1 activity, and thus could result in an abrogation of adipogenesis. Accordingly, Fain et al. could demonstrate that TGF-β1 release by human adipose tissue is enhanced in obesity.[47] Yet, whether CILP levels are associated with TGF-β1 remains to be characterized.

Heme oxygenase-1 (HO-1) is an ubiquitously expressed enzyme that is involved in the reduction of oxidative stress and inflammation.[48] It can be found in the circulation. Recent studies showed elevated plasma levels in several chronic disorders like Parkinson's disease and hemophagocytic syndrome and in newly diagnosed type 2 diabetics.[49-51] Until today the sources of circulating HO-1 are unknown and it is postulated that plasma HO-1 is due to 'leakage' of the enzyme from tissues to the plasma compartment.[49,52] Here, we demonstrate that HO-1 is an adipokine expressed and released by human primary adipocytes in a differentiation dependent manner and that circulating levels of HO-1 are increased in obese men correlating with the size of subcutaneous adipocytes. Our data further suggest that adipocytes actively secrete HO-1 and possibly release it into the circulation and that this secretory activity is dependent on adipocyte size.

We also demonstrate that HO-1 secretion is down-regulated by TNFα with no effect on its expression level which could be a time-course dependent effect. As other studies done in human peripheral monocytes

and human chondrocytes could demonstrate a TNFα-induced reduction of HO-1 expression[53,54] as well as HO-1 induction in an animal model leads to a reduction in the circulating TNFα level [55], it is assumable that there is a direct link between HO-1 and TNFα, which has to be further investigated.

It is described that HO-1 reduces adipogenesis, and its induction in animal models is associated with decreased body weight, improved adipokine profile and insulin sensitivity.[56]These observations imply that HO-1 seems not to be only a marker of cellular stress[57] but also acts as an important factor that helps to maintain cellular and tissue homeostasis. As our results show that HO-1 increases during adipocyte differentiation and is elevated in subcutaneous and visceral fat of obese patients, we suggest that HO-1 might play an important role in adipose tissue inflammation. Moreover, the elevated plasma levels in obese patients described in this study and elevated levels of circulating HO-1 in newly diagnosed type 2 diabetics[49] suggests that HO-1 may be a potent biomarker for obesity and obesity-associated disorders like the metabolic syndrome and type 2 diabetes.

Conclusion

Taken together the presented study contributes to our understanding of the complex endocrine function of human adipocytes. Extensive profiling led to the identification of 263 proteins to be released from adipocytes, including 44 putative novel adipokines. Functional studies of four selected adipokines, i.e. CFH, CRYAB, HO-1 and CILP provide valuable clues regarding their regulation during adipogenesis and the relationship of their circulation levels to obesity. Especially HO-1 exhibiting significantly elevated plasma levels in obesity and increased expression in visceral fat of obese patients may be an attractive candidate adipokine for further investigations in the field of obesity and obesity-associated disorders like the metabolic syndrome and type 2 diabetes. For the novel adipokines presented in this study, additionalbasic research and clinical studies could provide insight into their involvement in obesity-related diseases and their potential use as biomarkers.

Acknowledgements

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Study	Source	Identified proteins	Common with our study
Wang *et al*. Cell Mol. Life Sci. 61(18):2405-2417; (**2004**)	3T3 L1 cells mouse	**41**	**35**
Chen *et al*. J Proteome Res, 4(2):570-7; (**2005**)	fat pads rat	**183**	**63**
Alvarez-Llamas *et al*. Mol Cell Proteomics, 6(4):589-600; (**2007**)	visceral fat tissue explants human	**259**	**149**
Zvonic *et al*. Mol Cell Proteomics, 6(1):18-28; (**2007**)	huASC human	**101**	**54**
Molina *et al*. J Proteome Res. 8(1): 48-58; (**2009**)	3T3 L1 cells mouse	**147**	**70**
Kim *et al*. Proteomics, 10(3): 394-405; (**2010**)	hSVF cells human	**474**	**173**
Rosenow *et al*. J Proteome Res. Oct 1;9(10):5389-401; (**2010**)	SGBS cells human	**241**	**125**
Zhong *et al*. J Proteome Res. Oct 1;9(10):5228-38. (**2010**)	adipocytes human	**420**	**195**

Table 2.1 Literature comparison

Swissprot accession	Name	
O75339	Cartilage intermediate layer protein 1	
P09601	Heme oxygenase 1	
P08603	Complement factor H	recently described by Kim *et al.* Proteomics 2010
P02511	Alpha-crystallin B chain	recently described by Zhong *et al.* JProteomeRes 2010

Swissprot accession	Name	Swissprot accession	Name
O95861	3'(2'),5'-bisphosphate nucleotidase 1	P34059	N-acetylgalactosamine-6-sulfatase
Q13510	Acid ceramidase	O43505	N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase
P23526	Adenosylhomocysteinase	O00533	Neural cell adhesion molecule L1-like protein
P01009	Alpha-1-antitrypsin	P19021	Peptidyl-glycine alpha-amidating monooxygenase
Q8NCW5	Apolipoprotein A-I-binding protein	Q9BTY2	Plasma alpha-L-fucosidase
Q07812	Apoptosis regulator BAX	P13796	Plastin-2
P43251	Biotinidase	Q96SM3	Probable carboxypeptidase X1
Q96CX2	BTB/POZ domain-containing protein KCTD12	Q92520	Protein FAM3C
Q13231	Chitotriosidase-1	Q92954	Proteoglycan 4
Q6UVK1	Chondroitin sulfate proteoglycan 4	P34096	Ribonuclease 4
P27487	Dipeptidyl peptidase 4	P07998	Ribonuclease pancreatic
Q9NZ08	Endoplasmic reticulum aminopeptidase 1	Q6FHJ7	Secreted frizzled-related protein 4
P30043	Flavin reductase	Q9NS98	Semaphorin-3G
O75223	Gamma-glutamylcyclotransferase	Q9HAT2	Sialate O-acetylesterase
P00367	Glutamate dehydrogenase 1, mitochondrial	O75094	Slit homolog 3 protein
O76003	Glutaredoxin-3	Q6EEV6	Small ubiquitin-related modifier 4
Q9UIJ7	GTP:AMP phosphotransferase mitochondrial	Q9H3U7	SPARC-related modular calcium-binding protein 2
P01857	Ig gamma-1 chain C region	Q03167	TGF-beta receptor type III
P01859	Ig gamma-2 chain C region	P04066	Tissue alpha-L-fucosidase
P01591	Immunoglobulin J chain	Q15661	Tryptase beta-1
O95865	N(G),N(G)-dimethylarginine dimethylaminohydrolase 2	O76076	WNT1-inducible-signaling pathway protein 2

Table 2.2 Novel Adipokines.

All of the proteins were identified from the adipokinome and predicted as potentially secretory by signalP or secretomeP

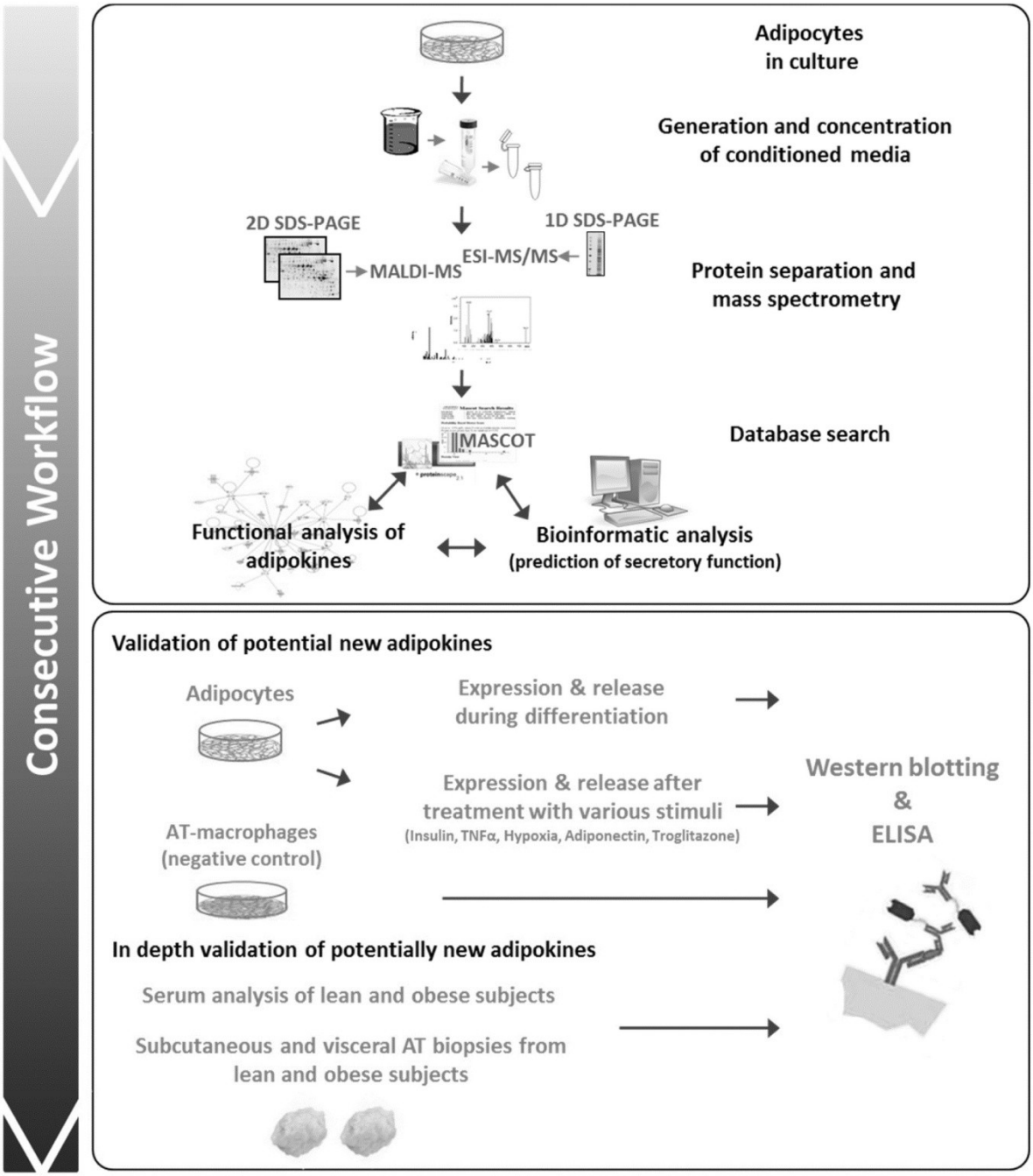

Figure 2.1 Flow chart – work scheme

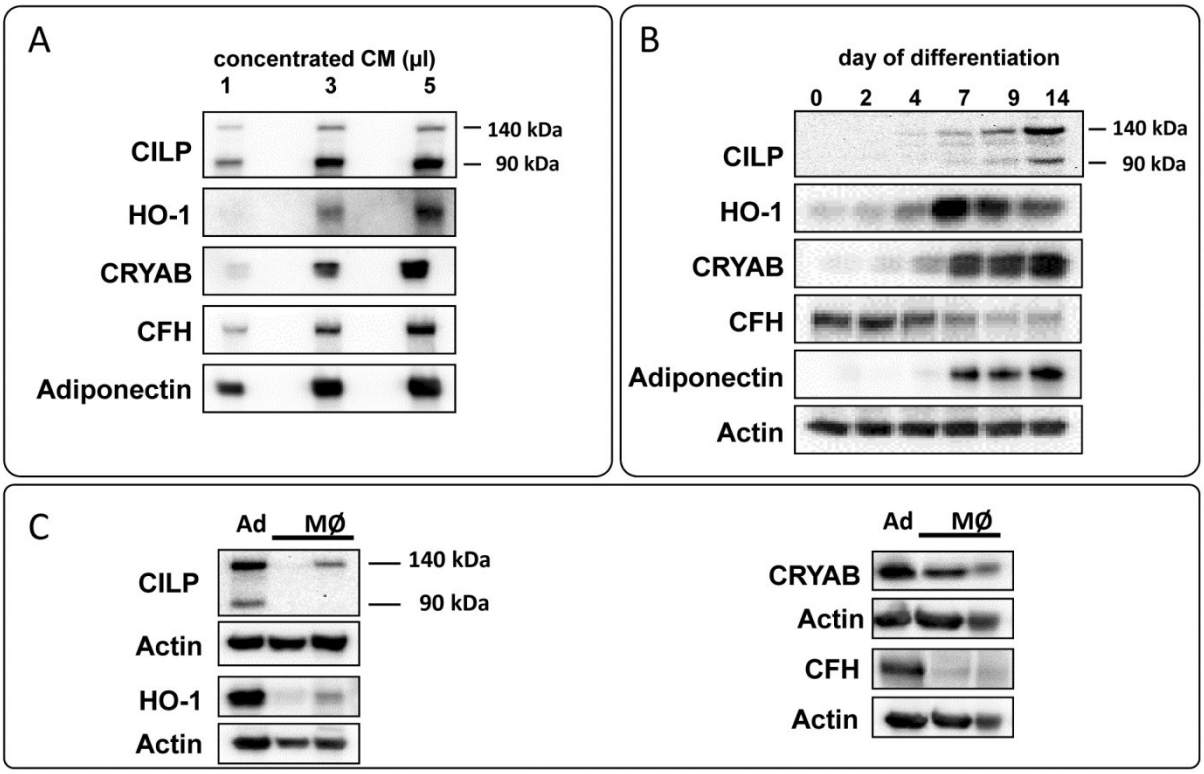

Figure 2.2 Representative Western Blots of CILP, HO-1, CRYAB and CFH protein level and release by human primary adipocytes.

Adipocytes were differentiated and concentrated CM was generated as described in materials and methods. (A) Release of novel adipokines at day 14 of differentiation. 1-5 µl of concentrated CM were analyzed by SDS-PAGE and Western blotting. (B) Protein level of novel adipokines during adipocyte differentiation. 10 µg of total lysates were analyzed by SDS-PAGE and Western blotting. Signals were detected by ECL. (C) 10 µg of total lysates derived from adipocytes (Ad) and macrophages (MØ) were analyzed by SDS-PAGE and Western blotting with subsequent signal detection by ECL.

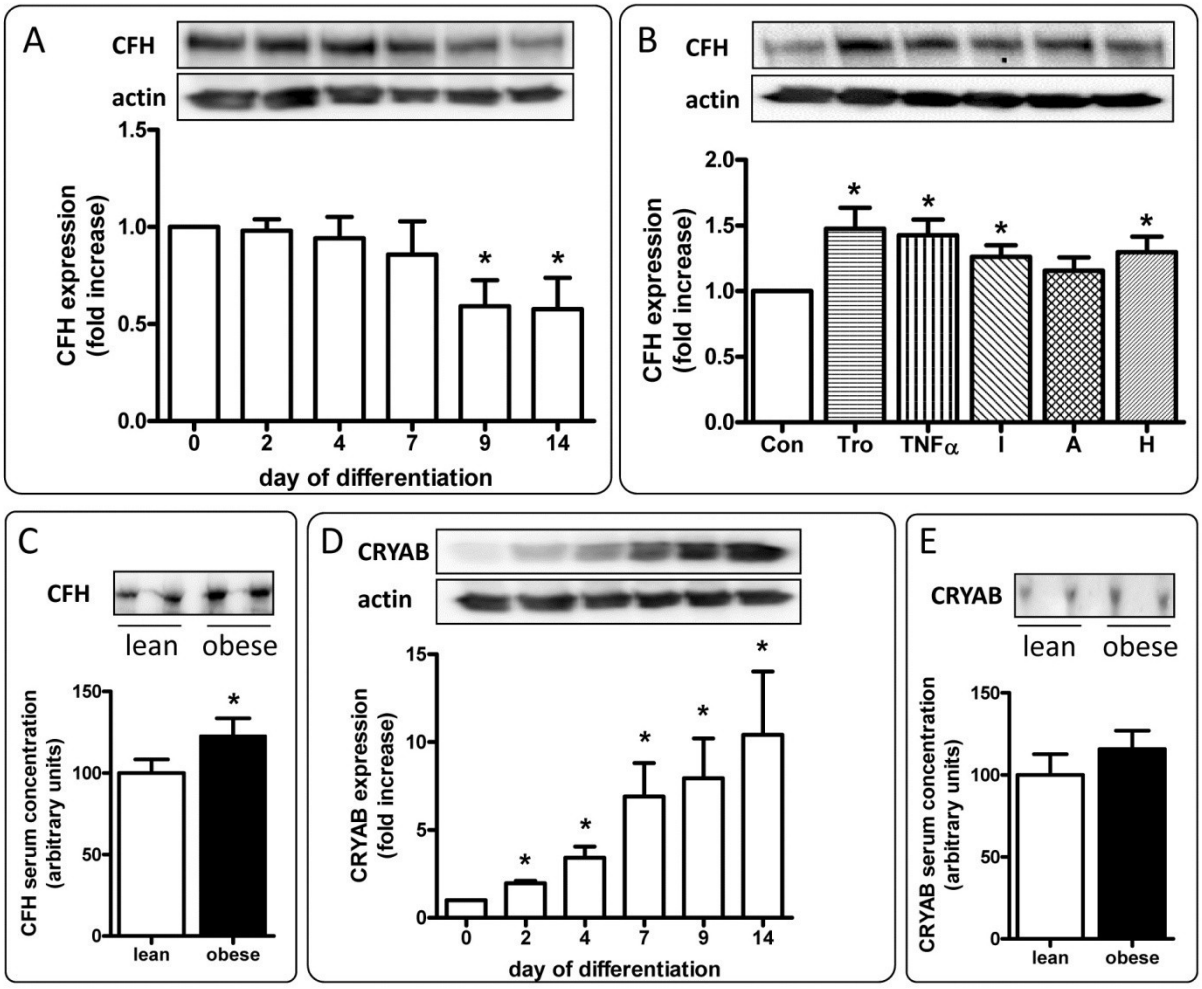

Figure 2.3 Protein level of CFH and CRYAB in adipocytes and relative serum concentrations in lean and obese subjects.

Human primary adipocytes were differentiated as described in materials and methods. CFH (A) and CRYAB (D) protein level during differentiation were analyzed by SDS-PAGE and Western blotting. Data were normalized to the protein level of actin and are expressed relative to day 0. Data are mean values ± SEM, n≥5, *p<0.05 vs. preadipocytes. (B) Regulation of CFH protein expression. Differentiated adipocytes were treated with 5 µmol/L troglitazone (Tro), 10 ng TNFα, 50 mmol/L insulin (I), 5nMadiponectin (A) or incubated under hypoxic conditions (H) for 24 h. CFH protein level was analyzed by SDS-PAGE and Western blotting. Data were normalized to the protein level of actin and are expressed relative to unstimulated control (Con). Data are mean values ± SEM, n=6, *p<0.05 vs. control. (C, E) Relative serum concentration of CFH and CRYAB were determined in samples obtained from lean (n=12) and obese (n=9) subjects participating in study 1. Sera samples were diluted and analyzed by SDS-PAGE and Western blotting. Data are mean values ± SEM, *p<0.05 vs. lean controls.

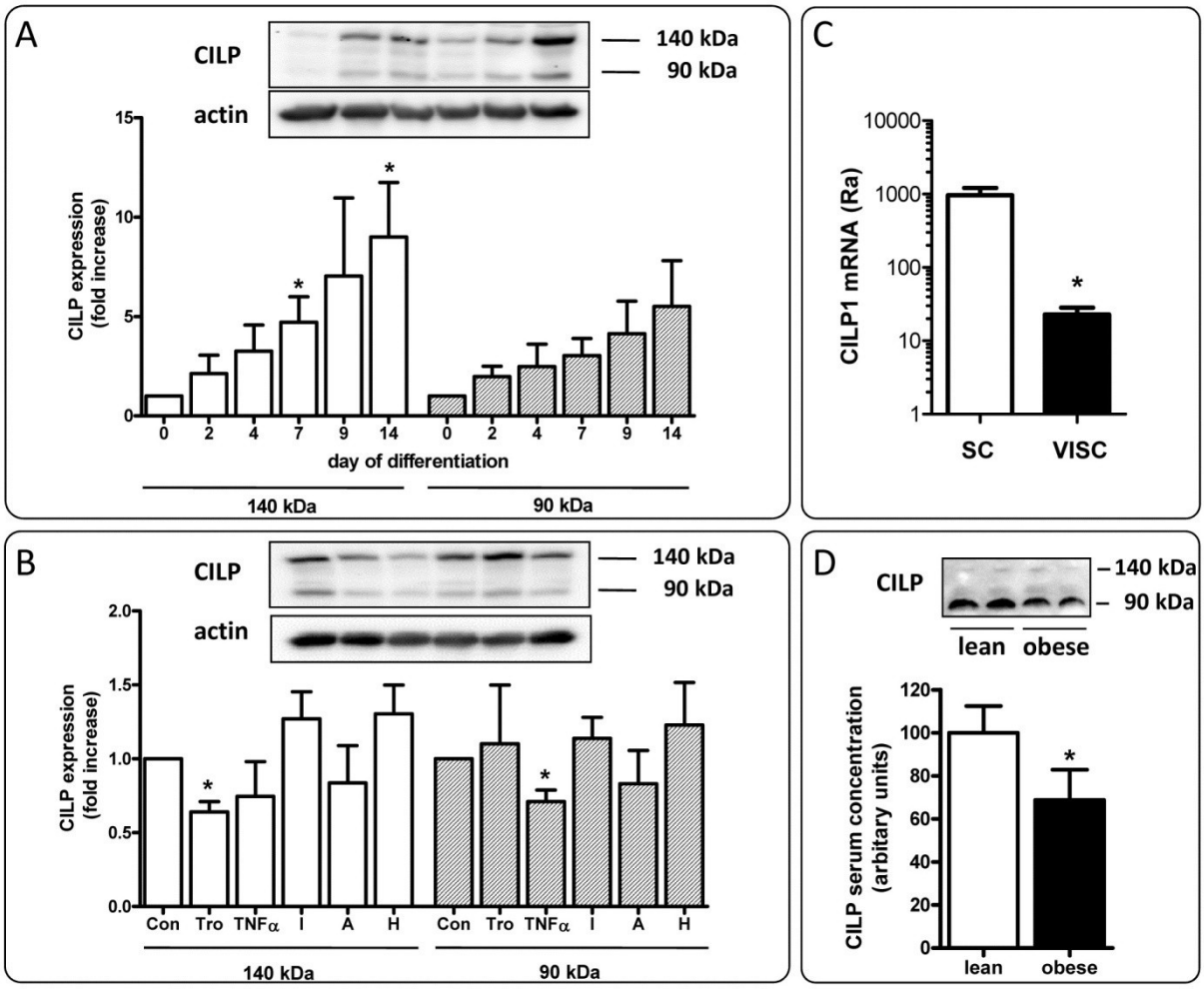

Figure 2.4 Protein level of CILP in adipocytes and relative CILP serum concentration in lean and obese subjects.

(A) Human primary adipocytes were differentiated as described in materials and methods, and CILP protein level during differentiation was analyzed by SDS-PAGE and Western Blot. Data were normalized to the protein level of actin and are expressed relative to day 0. Data are mean values ± SEM, n≥3, *p<0.05 vs. pre-adipocytes. (B) Differentiated adipocytes were treated as described in legend to fig.3. CILP protein level was analyzed by SDS-PAGE and Western blotting. Data were normalized to the protein level of actin and are expressed relative to unstimulated control (Con). Data are mean values ± SEM, n=5, *p<0.05 vs. control. (C). CILP-1 mRNA expression (Ra) in human adipose tissue. Expression of CILP-1 was determined by real-time PCR in subcutaneous (sc) and visceral (visc) adipose tissue biopsies collected during abdominal surgery of normal weight males (n=12). Data were normalized for RPS18 expression and expressed as mean ± SEM. *p<0.05 vs. subcutaneous adipose tissue. (D) Relative serum concentration of CILP was determined in samples obtained from lean (n=12) and obese (n=9) subjects participating in study 1. Sera samples were diluted and

analyzed by SDS-PAGE and Western blotting. Data are mean values ± SEM, *p<0.05 vs. lean controls.

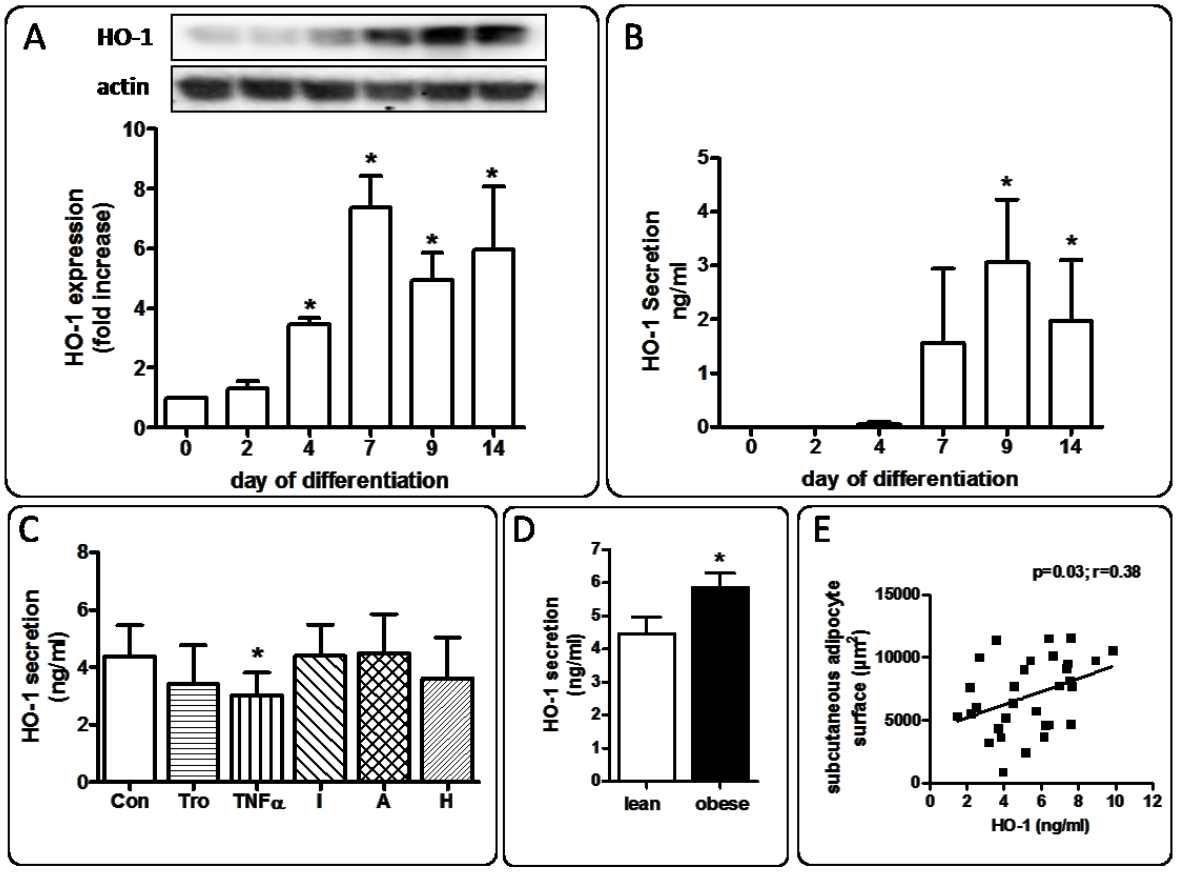

Figure 2.5 HO-1 protein level in adipocytes and relative HO-1 serum concentration in lean and obese subjects.

(A) HO-1 protein level during adipocyte differentiation was analyzed by SDS-PAGE and Western blotting. Data were normalized to the protein level of actin and are expressed relative to day 0. Data are mean values ± SEM. n≥5, *p<0.05 vs. pre-adipocytes. (B) Secretion of HO-1 during differentiation of adipocytes was analyzed by ELISA. Data are mean values ± SEM, n=5. (C) Differentiated adipocytes were treated for 24 h as described in legend to fig. 3B. HO-1 secretion was measured by ELISA. Data are mean values ± SEM, n=6. (D) Sera from lean (n=20) and morbidly obese men (n=20) participating in study 1 were analyzed for their HO-1 concentration by ELISA. Data are mean values ± SEM, *p<0.05 vs. lean group. (E) Linear regression analysis of HO-1 serum concentration and size of subcutaneous adipocytes (p=0.031; r=0.40).

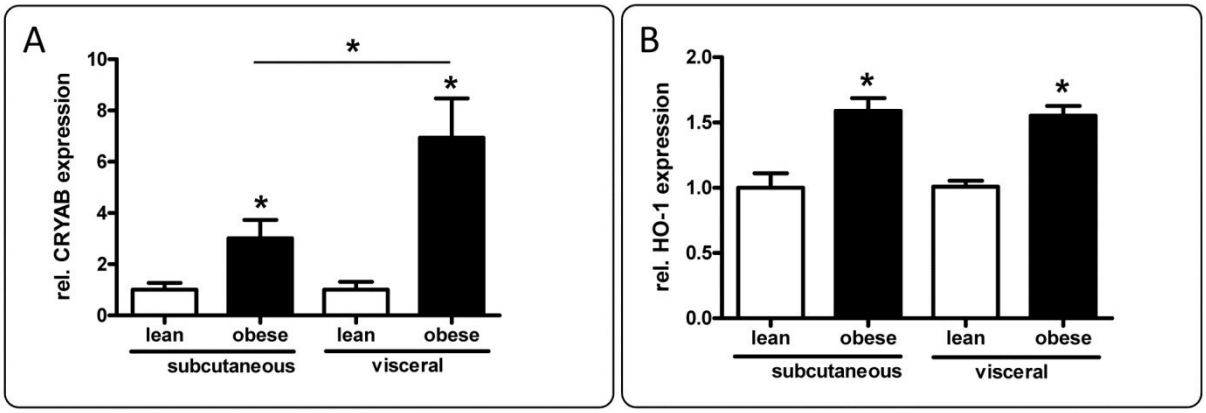

Figure 2.6 CRYAB and HO-1 protein expression in subcutaneous and visceral adipose tissue from lean and obese patients.

CRYAB (A) and HO-1 (B) level were determined in adipose tissue lysates from paired subcutaneous and visceral adipose tissue of lean (n=9) and obese (n=15) patients. 5 µg of tissue lysates were analyzed by SDS-PAGE and Western blotting. Data were normalized to the protein level of GAPDH and are expressed relative to subcutaneous adipose tissue of lean subjects. Data are mean values ± SEM, *p<0.05 vs. lean.

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Chapter 3

Study 2

Dipeptidyl peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome ‡

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Abbreviations: BMI, body mass index; BSA, bovine serum albumin; DPP4, dipeptidyl peptidase 4; FCS, fetal calf serum; GLP, glucagon-like peptide; MCP, monocyte chemotactic protein; NPY, neuropeptide Y; PAI, plasminogen activator inhibitor; TNF, tumor necrosis factor

Abstract

Objective: Comprehensive proteomic profiling of the human adipocyte secretome identified DPP4 as a novel adipokine. This study assessed the functional implications of the adipokine DPP4 and its association to the metabolic syndrome. *Research Design and Methods:* Human adipocytes, skeletal and smooth muscle cells were used to monitor DPP4 release and assess the effects of soluble DPP4 on insulin signalling. In lean and obese subjects, depot-specific expression of DPP4 and its release from adipose tissue explants was determined and correlated to parameters of the metabolic syndrome. *Results:* Fully differentiated adipocytes exhibit a substantially higher release of DPP4, when compared to preadipocytes or macrophages. Direct addition of DPP4 to fat, skeletal and smooth muscle cells impairs insulin signalling. A 5-fold higher level of DPP4 protein expression was seen in visceral as compared to subcutaneous fat of obese patients, with no regional difference in lean subjects. DPP4 serum concentrations significantly correlated with adipocyte size. Using adipose tissue explants from lean and obese subjects, we observed a 2-fold increase in DPP4 release that strongly correlated with adipocyte volume and parameters of the metabolic syndrome, and was decreased to the lean level after weight reduction. DPP4 released from adipose tissue strongly correlated positively with an increasing risk score for the metabolic syndrome. *Conclusion:* DPP4 is a novel adipokine which may impair insulin sensitivity in an auto- and paracrine fashion. Further, DPP4 release strongly correlates with adipocyte size, potentially representing an important source of DPP4 in obesity. We therefore suggest that DPP4 may be involved in linking adipose tissue and the metabolic syndrome.

Keywords

Adipocyte secretome; proteomics; adipokines; DPP4; obesity; metabolic syndrome

Introduction

Obesity is the hallmark of the metabolic syndrome and represents a major global health problem that frequently associates with the development of chronic diseases, including type 2 diabetes and cardiovascular disease.[1] A complex inter-organ crosstalk scenario between adipose tissue and other central and peripheral organs underlies the progression of these diseases, with adipose tissue being on top of the crosstalk hierarchy.[2] This is due to the huge diversity of signalling and mediator molecules released from adipose tissue, which is now considered as one of the major endocrine organs.[3,4] Recent data show that adipokines, which are proteins and peptides released by various adipose tissue cells, create a complex interconnected network of feedback loops.[5] Enlargement of adipose tissue leads to dysregulation of adipokine secretion, representing a potential critical pathogenic link between obesity, insulin resistance and type 2 diabetes.[1] Therefore, we conducted a comprehensive proteomic profiling of conditioned media derived from differentiated, primary human adipocytes. This resulted in the identification of novel adipokines, including the exoprotease dipeptidyl peptidase (DPP) 4.

DPP4 is an ubiquitously expressed transmembrane glycoprotein, which cleaves N-terminal dipeptides from a variety of substrates including growth factors and hormones, neuropeptides and chemokines.[6] Two substrates of DPP4, glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) are released from the intestinal mucosa and are responsible for about 60% of post-prandial insulin secretion, the so-called incretin effect.[7] Since GLP-1 remains active under hyperglycemic conditions in type 2 diabetes, DPP4 has gained considerable interest as a therapeutic target, and a variety of DPP4-inhibitors that prolong the insulinotrophic effect of GLP1 are now in clinical use as anti-diabetic drugs.[8] Substantial DPP4 activity is also found in plasma and other body fluids due to a soluble form of DPP4 lacking the cytoplasmic tail and the transmembrane region of this protein.[9] Both, the membrane abundance and the circulating activity of DPP4 have been found to be altered in a variety of neurological and inflammatory diseases.[6] However, although a fraction of soluble DPP4 most likely originates from cells of the immune

system,[10] the major source of circulating DPP4 and its regulation remain unknown.

Furthermore, essentially no data are currently available regarding the potential effects of soluble DPP4 on insulin target tissues including muscle and fat. In the present investigation we combined *in vitro* experiments with two independent clinical studies, aiming to validate DPP4 as a novel adipokine and to characterize the association of DPP4 to different parameters of the metabolic syndrome. Here we show that i) DPP4 is a novel adipokine released from differentiated human adipocytes and that it may exert auto- and paracrine effects leading to insulin resistance, ii) DPP4 expression is substantially elevated in visceral fat of obese subjects and that serum DPP4 correlates with adipocyte size and all parameters of the metabolic syndrome, and that iii) adipose tissue explants from obese subjects release substantially more DPP4 with a prominent decrease after weight reduction. In light of the well-accepted interference of DPP4 with the incretin system, we now suggest that DPP4 may play a role in linking obesity to insulin resistance and the metabolic syndrome.

Materials and Methods

Materials

Reagents for SDS-PAGE were supplied by GE Healthcare (Freiburg, Germany) and by Sigma (München, Germany). Polyclonal antibodies for adiponectin and actin were supplied by Abcam (Cambridge, UK). HRP-conjugated goat anti-rabbit and goat anti-mouse IgG antibodies came from Promega (Mannheim, Germany). Collagenase NB4 was obtained from Serva (Heidelberg, Germany). Troglitazone, TNF-α, BSA (fraction V, fatty acid free, low endotoxin) were obtained from Sigma (München, Germany). Adiponectin was purchased from Biovendor (Heidelberg, Germany). Complete protease inhibitor cocktail and PhosStop phosphatase inhibitor cocktail were from Roche (Mannheim, Germany). FCS was supplied by Gibco (Invitrogen, Carlsbad, CA, USA). All other chemicals were of the highest analytical grade commercially available and were purchased from

Sigma. Human recombinant DPP4 was purchased from R&D Systems and a polyclonal antibody from Abnova (Heidelberg, Germany). The specific DPP4 inhibitor K579 was purchased from Biozol (Eching, Germany).

Clinical studies of DPP-4 concentration in serum and DPP-4 release from adipose tissue.

For all studies, protocols were approved by local Ethics Committees and all participants gave written, informed consent.

Study 1: 20 male obese patients and 20 lean controls were recruited at Gent University (clinical trial number B67020084018). For all patients, anthropometric and routine blood parameters were assessed. Fasting blood samples were collected and adipose tissue biopsies fixed for microscopic evaluation of adipocyte surface area analysis.

Study 2: Nineteen obese (BMI, ≥30 kg/m^2) otherwise healthy and ten lean (BMI < 25 kg/m^2) healthy women were recruited at Karolinska Institute and investigated in the morning after an overnight fast. Sixteen obese women were re-investigated 18-24 month after gastric bypass in a weight stable period for at least three months according to self report (reduction of BMI from 43.0 to 27.9 kg/m^2). A venous blood sample was obtained for the analysis of glucose and insulin to be used as estimation of insulin sensitivity *in vivo*, HOMA index as described.[11] Thereafter, abdominal subcutaneous adipose tissue biopsies were obtained by needle aspiration as described.[12] One part of the tissue was used for measurements of DPP4 release as described.[13] Methodological experiments revealed that DPP4 release was linear with time for at least 3 h suggesting no important cell damage (data not shown). Another part of the tissue was subjected to collagenase treatment and mean adipocyte volume and weight were determined as described.[14]

For calculation of the risk score for the Metabolic Syndrome, we used ATPIII definitions which are the following: 1. fasting glucose > 110 mg/dl or diagnosis of type 2 diabetes, 2. blood pressure > 135/85 mmHg, 3. serum triglycerides > 150 mg/dl, 4. HDL-cholesterol < 40 mg/dl for men and < 50 mg/dl for women, and 5. abdominal obesity characterized by a waist >102 cm for men and > 88 cm for women. The risk score is equal to the number of criteria fulfilled.

Homeostasis model assessment (HOMA) for insulin resistance (IR) was determined in all patients except those treated with insulin by a mathematical transformation of fasting blood glucose and insulin measurements (HOMA = insulin (µU/ml) x glucose (mmol/l) / 22.5).

Adipocyte isolation and culture

Subcutaneous adipose tissue was obtained from lean or moderately overweight women undergoing plastic surgery for mammary reduction or breast reconstruction with subcutaneous abdominal adipose tissue. The procedure was approved by the ethical committee of the Heinrich-Heine-University (Düsseldorf, Germany). All subjects were healthy, free of medication and had no evidence of diabetes according to routine laboratory tests. Preadipocytes were isolated by collagenase digestion of adipose tissue as previously described by us.[15] Isolated cell pellets were resuspended in Dulbecco's modified Eagles/Hams F12 (DMEM/F12) medium supplemented with 10 % FCS. After overnight incubation, cultures were washed and further incubated in an adipocyte differentiation medium (DMEM/F12, 33 µmol/l biotin, 17 µmol/l d-panthothenic-acid, 66 nM insulin, 1 nM triiodo-L-thyronine, 100 nM cortisol, 10 µg/ml apo-transferrin, 50 µg/µl gentamycin, 15 mmol/l HEPES, 14 nmol/l NaHCO3, pH 7.4) for 15 days with medium change every 2-3 days and addition of 5 µM troglitazone for the first 3 days. The degree of differentiation was determined by oil red staining and induction of adiponectin expression. Differentiated adipocytes were used for the generation of adipocyte-conditioned media (CM), as recently described by us.[15] Briefly, after *in vitro* differentiation, adipocytes were washed and incubated for 48 h in α-modified Eagles medium followed by collection of the medium. Macrophages were isolated from human adipose tissue and cultured, using a method described by Curat et al.[16] For hypoxia treatment, differentiated adipocytes were incubated with a gas mixture containing 1 % O_2, 5 % CO_2 and 94 % N_2 in MIC-101 modular incubator chambers (Billups-Rothenburg, Del Mar, USA) at 37 °C for indicated times.

Skeletal muscle cell culture

Primary human skeletal muscle cells of healthy Caucasian donors were supplied as proliferating myoblasts (5 x 10^5 cells) and cultured as described previously.[15] For an individual experiment, myoblasts were seeded in six-well culture dishes (9.6 cm2/well) at a density of 10^5 cells per well and were cultured in α-modified Eagles/Hams F12 medium containing Skeletal Muscle Cell Growth Medium Supplement Pack up to near confluence. The cells were then differentiated and fused by culture in α-modified Eagles medium for 4 days and used for experiments.

Smooth muscle cell culture and proliferation

Primary human coronary artery smooth muscle cells were obtained from PromoCell (Heidelberg, Germany). Cells from four different donors were supplied as proliferating cells and kept in culture according to the manufacturer's protocol. For all experiments, subconfluent cells of passage 3 were used. Cells were characterized as smooth muscle cells by morphologic criteria and by immunostaining with smooth muscle α-actin.

Immunoblotting

Adipocytes and macrophages were treated as indicated and lysed in a buffer containing 50 mM HEPES, pH 7.4, 1 % TritonX100, Complete protease inhibitor and PhosStop phosphatase inhibitor cocktail. After incubation for 2 h at 4 °C, the suspension was centrifuged at 10.000 x *g* for 15 min. Thereafter, 5-10 µg of adipocyte lysates were separated by SDS-PAGE using 10 % horizontal gels and transferred to polyvenylidene fluorid filters in a semidry blotting apparatus. Filters were blocked with Tris-buffered saline containing 0.1 % Tween and 5 % nonfat dry milk and subsequently incubated overnight with a 1:1000 dilution of the appropriate antibodies. After washing, filters were incubated with secondary HRP-coupled antibody and processed for enhanced chemiluminescence detection using Immobilon HRP substrate (Millipore, Billerica, MA, USA). Signals were visualized and evaluated on a LUMI Imager (Boehringer, Mannheim, Germany) or VersaDoc 4000 MP (BioRad, Munich, Germany) work station.

ELISA

DPP-4 secretion by human primary adipocytes and macrophages was determined using ELISA kits purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany) and Stressgene (Lörrach, Germany). The assays were performed in duplicates according to the manufacturer's instructions.

Presentation of data and statistics

Data are expressed as mean ± SEM. The Shapiro-Wilcoxon test was used to test the Gaussian distribution of biological parameters. Student's t test, analysis of variance (ANOVA) followed by p for linear trend post-test when appropriate were used for comparison between groups. Correlations were performed by Pearson. For adjustment (BMI, age), we applied a multiple linear regression modelling using least squares means. All statistical analyses were done using JMP statistics software (SAS Institute Inc., Cary, NC, USA) or Prism (GraphPad, La Jolla, CA, USA) considering a P value of less than 0.05 as statistically significant. Corresponding significance levels are indicated in the figures.

Results

DPP4 is a novel adipokine exhibiting regulated release from human adipocytes

Comprehensive proteomic profiling of the adipocyte secretome led to the identification of 347 proteins, with 263 proteins being predicted or annotated as secretory proteins (data to be presented elsewhere). Although about 80% of these proteins have been reported in earlier studies,[17-19] our approach has identified more than 40 novel adipokines including DPP4.

To validate this novel adipokine, we used in vitro differentiated human adipocytes and macrophages isolated from adipose tissue. DPP4 expression in human adipocytes is significantly increased during differentiation with a maximum reached at day 7 (4-fold over

undifferentiated control) (Fig. 1A). DPP4 expression is paralleled by a marked release of this adipokine (Fig. 1B), which was significantly elevated as compared to the undifferentiated control starting at day 4 and increasing up to day 9 (1.1 ng/ml released over 24h by 3.5 x 10^5 cells). DPP4 in the supernatant of adipocytes was quantified by ELISA and additionally confirmed by Western blotting (Fig. 1B). We further analyzed the release of DPP4 with prominent regulators of adipocyte secretory activity such as troglitazone, TNF-α, insulin and adiponectin.[15,20,21] As shown in Fig. 1C, DPP4 release is significantly up-regulated by TNF-α and insulin. In addition to adipocytes, adipose tissue-derived macrophages release measurable amounts of DPP4 (Fig. 1D). This, however, is only 1/3 compared to adipocytes, pointing to a major contribution of adipocytes to DPP4 output from adipose tissue.

Soluble DPP4 exerts direct effects on fat and muscle cells

The soluble form of DPP4 may bind to the extracellular matrix[22] and affect a variety of cells, yet this has not been investigated so far. To assess potential direct effects of soluble DPP4 on peripheral cells, we studied insulin signalling in adipocytes and skeletal muscle cells. DPP4 treatment of human adipocytes results in a dose-dependent decrease in insulin-stimulated Akt phosphorylation which reached significance using a dose of 200 ng/ml (Fig. 2A). This demonstrates an autocrine effect of DPP4 on adipocytes. It should be noted that circulating DPP4 concentrations were found in the range of 200 to 600 ng/ml in healthy patients. The effect of DPP4 on insulin-stimulated Akt phosphorylation can be completely blocked by a specific DPP4 inhibitor (Fig. 2B). Validation experiments using this compound prove inhibition of DPP4 *in vitro* which remained unaltered for a period of at least 8 h (data not shown). Similar to adipocytes, DPP4 also induces insulin resistance in skeletal muscle cells at the level of Akt phosphorylation in a dose-dependent way but less prominent compared to adipocytes (Fig. 2C-D). To prove if DPP4 has a functional impact not only on insulin signalling, we determined DPP4-stimulated proliferation and insulin signalling in primary human smooth muscle cells. In addition to the induction of insulin resistance at the level of Akt in this cell type, DPP4 induced a 1.6 fold increase in cell

proliferation which can be completely blocked by the DPP4 inhibitor (Fig. 3A-C).

DPP4 is elevated in serum of obese patients and correlates with various anthropometric and clinical parameters (Clinical study 1)

Measuring DPP4 in serum from age-matched lean and morbidly obese man (patient characteristics in supplementary table 1) revealed that obese subjects are characterized by significantly increased DPP4 concentrations (Fig. 4A). DPP4 expression in adipose tissue biopsies from the same patients revealed that DPP4 protein expression is regulated by both the fatness of the individual and the adipose tissue depot (Fig. 4B). While there is only a trend for higher DPP4 expression in visceral fat of lean subjects, obese patients are characterized by significantly higher DPP4 in visceral adipose tissue as compared to subcutaneous adipose tissue. Furthermore, expression of DPP4 in both depots is significantly higher in obese subjects as compared to lean ones. DPP4 levels positively correlate with BMI, the size of subcutaneous and visceral adipocytes, insulin and leptin, while a negative correlation with age and adiponectin could be found (Fig. 5A-G). Adjusting DPP4 for age has no impact on these correlations. However, when adjusted for BMI, DPP4 serum concentrations only significantly correlate with the size of subcutaneous adipocytes ($p=0.04$, $r=0.32$) pointing to a very close relation between the size of adipocytes and the release of this adipokine.

DPP4 is released from subcutaneous adipose tissue in vitro (Clinical study 2)

We investigated lean and obese after weight reduction and we analyzed the release of DPP4 from whole adipose tissue. Adipocytes from lean subjects are significantly smaller than those from obese patients (Fig. 6A). Surgery induced weight loss reduced the average size of adipocytes below the size from lean subjects. DPP4 release is significantly increased from adipose tissue of obese subjects as compared to lean ones (Fig. 6B) while weight reduction by bariatric surgery normalized the DPP4 release to the lean level. This was paralleled by a significant reduction in the circulating DPP4 level, supporting the notion that adipose tissue is an

important source of serum DPP4 (Fig. 6C). In the group of lean and obese subjects, DPP4 release from adipose tissue significantly correlates with BMI, waist circumference, percent body fat, triglycerides, HOMA, adipocyte volume and leptin while the correlation is negative with HDL-cholesterol (Fig. 6D-K). All these factors are denominators of the metabolic syndrome. Notably, leptin shows similar correlations with the above mentioned parameters except for triglycerides where no correlation could be found. Importantly, the release of DPP4 from adipose tissue correlates with many parameters that correlate with circulating DPP4 concentrations. There is also a strong correlation between adipose secretion of leptin and DPP4 (Fig. 6K).

DPP4 serum concentrations and release from adipose tissue is significantly related to the risk for having the metabolic syndrome

In both obese patient groups providing data for circulating DPP4 levels and for DPP4 release from adipose tissue explants, the respective concentrations of DPP4 are significantly linked to a risk score for the metabolic syndrome of equal to or greater than 3 (at risk), as calculated according to the ATP-III guidelines (Fig. 7A-B). Performing the same analysis for the circulating levels of leptin, MCP-1, RANTES, PAI-1, chemerin and hs-CRP, we could not find any such relationship with the metabolic syndrome (data not shown). Conversely, adiponectin serum levels are significantly decreased in patients at risk (data not shown). Including the lean subjects in this type of analysis does not change the outcome of this analysis and it should be noted that the relationship of DPP4 with the risk score for the metabolic syndrome in the obese subjects is independent from BMI.

Discussion

Our proteomics approach identified DPP4 as a novel adipokine released by fully differentiated human adipocytes. This was confirmed by Western blot, ELISA and determination of enzymatic activity. DPP4 release increased substantially upon fat cell differentiation, and comparison to preadipocytes and adipose tissue macrophages showed, that adipocytes most likely represent the major source of DPP4 released from the intact organ to the circulation. DPP4 is a multifunctional, type II integral membrane glycoprotein exhibiting ubiquitous expression including adipose tissue,[23] being highly abundant in the kidney, on T lymphocytes and endothelial cells.[22] DPP4 is certainly different from many other adipokines, in that i) the protein is not secreted but released from the plasma membrane as soluble DPP4 subsequent to proteolytic cleavage,[24] ii) DPP4 exerts dual functions both as a regulatory protease and a binding protein, and iii) this protein is already an established target for treatment of type 2 diabetes,[8] supporting our notion that DPP4 may potentially link adipose tissue to type 2 diabetes and the metabolic syndrome. Regulators of DPP4 release are presently unknown, but we show here that both insulin and TNF-α augment the shedding of soluble DPP4 by about 50% despite an unaltered expression. Thus, factors related to insulin resistance and adipose tissue inflammation enhance the release of this novel adipokine from the fat cell. In addition to the endocrine effects of DPP4 released to the circulation, both cell surface resident and soluble DPP4 may have multiple auto-/paracrine functional implications for adipose tissue physiology. First, DPP4 recruits adenosine deaminase to the cell surface,[25] which may modulate the well-established anti-lipolytic effects of adenosine. Second, DPP4 is a very strong inhibitor of the anti-lipolytic activity of neuropeptide Y (NPY),[23] which is one of the best peptide substrates of the enzyme.[26] Therefore, enhanced abundance of both resident and soluble DPP4 within adipose tissue of obese subjects may substantially augment the lipolytic activity of enlarged adipocytes. Finally, DPP4 inactivates or alters the specificity of many chemokines including RANTES, eotaxin, MDC, SDF-I and many others,[22] making it likely that DPP4 plays a yet undefined functional role in the intra-organ crosstalk between macrophages, adipocytes and other components of the stroma-vascular fraction.

So far, the direct effects of soluble DPP4 on isolated cells have not been investigated, although it binds to the extracellular matrix and may exert signalling functions.[22] We demonstrate here for the first time that DPP4 consistently impairs insulin signalling at the level of Akt in three different primary cell types, namely adipocytes, skeletal muscle and smooth muscle cells. Enzymatic activity of DPP4 appears to be involved in this process, but DPP4 inhibitors may also affect the binding properties of sDPP4 to a putative receptor. This issue is currently under investigation in our laboratory. It may be speculated that DPP4 exerts an autocrine action on adipocytes, which may be of particular interest for perivascular fat, where DPP4 may also act in a paracrine/endocrine fashion on the vascular wall. Importantly, DPP4 induces proliferation of human vascular cells in parallel to an impairment of insulin signalling, suggesting a potential role in obesity-associated vascular complications. In this study we used DPP4 concentrations that match circulating levels in both lean and obese humans as measured by us. As obese patients are characterized by significantly increased circulating DPP4 it may be speculated that DPP4 may interfere with insulin sensitivity not only in adipose tissue but also in other insulin-sensitive peripheral organs. This would substantially extend the current view of DPP4 as a target for treatment of type 2 diabetes. Future work will be needed to address the mechanism and the functional role of these effects in the pathogenesis of insulin resistance and obesity-associated complications.

Serum DPP4 is altered in many pathophysiological conditions such as different types of cancer, allergic asthma or hepatitis C (for review[10]). To the best of our knowledge, this is the first study to analyze circulating DPP4 in the context of obesity and adipose tissue. Morbidly obese men are characterized by elevated DPP4 levels as compared to lean controls. DPP4 serum concentrations are significantly correlated with BMI, fat mass, size of adipocytes in subcutaneous and visceral fat as well as with the adipocyte hormones adiponectin (negatively) and leptin, showing that DPP4 is not only related to increased body weight but also to other important parameters of adipose tissue in particular. Interestingly, DPP4 is negatively associated with age but all of the above mentioned parameters are still significantly correlated with DPP4 even after adjustment for age. Differently, BMI-adjustment causes the disappearance of most of these correlations except for the size of subcutaneous adipocytes. In addition to

circulating DPP4, the protein expression of this adipokine is significantly different not only between lean and obese subject but also between their fat depots. Former studies report contradicting data as both decreased and increased mRNA expression of DPP4 in adipose tissue of obese men was described.[23,27] At the protein level, we now clearly demonstrate that obesity leads to a prominent induction of DPP4 abundance in both subcutaneous and visceral adipose tissue, and that in obese subjects the visceral fat exhibits the highest DPP4 level. We therefore conclude that enlargement of visceral adipocytes in obesity may substantially contribute to the augmented level of circulating DPP4 in obese patients. It is noteworthy that we measured DPP4 serum concentration and not its activity. However, in additional experiments, other samples from the same patients were used to determine DPP4 activity that is significantly correlated with circulating DPP4 levels (data not shown). Thus, DPP4 activity is also significantly increased in obese as compared to lean subjects.

DPP4 expression in adipose tissue is increased in obese as compared to lean individuals, a fact that is reflected by an increased release of DPP4 from adipose tissue explants of obese patients as compared to lean controls. Similar to circulating DPP4, its release from adipose tissue correlates with various classical markers for the metabolic syndrome, namely BMI, waist circumference and plasma triglycerides, HOMA as an index of insulin resistance, as well as with fat cell volume and the adipokine leptin. In addition, DPP4 release can be reversed to normal levels by surgery-induced weight loss which is also reflected by DPP4 being significantly reduced in serum of these patients. Except for one study reporting on DPP4 levels in obese children before and after weight loss,[28] this is the first description of significantly decreased DPP4 levels after weight loss in adults induced by obesity surgery. Thus in obesity, both circulating levels of DPP4 and DPP4 release by adipose tissue are increased but can be reduced to control levels by substantial weight loss.

Both circulating DPP4 and DPP4 release by adipose tissue correlate strongly with the number of risk factors for developing the metabolic syndrome. Thus, DPP4 may be of relevance as a novel biomarker of the metabolic syndrome and for detection of obese subjects at high risk for obesity-associated complications. Future studies are needed to address this important issue and to define the molecular pathways that link

adipose DPP4 to the metabolic syndrome and type 2 diabetes. An adipose-specific knockout of DPP4 would be required to prove a causal role of this protein and this mouse model is currently under development in our laboratory. However, several lines of evidence support our notion that the novel adipokine links obesity to the metabolic syndrome. First, DPP4 impairs the function of the incretin system which is of key importance for glucose homeostasis.[29] Additionally, incretin-based therapies are known to preserve beta-cell function and to exert salutary effects on blood pressure and lipid profile.[30] Second, DPP4 inhibitors are well known to improve glucose tolerance in animal models of obesity.[31] More importantly, chronic DPP4 inhibition in ZDF rats was shown to delay the onset of type 2 diabetes.[31] Finally, preclinical data suggest that GLP-1 is cardioprotective[32] and DPP4 inhibition was shown to improve cardiovascular outcomes in rodents[33]. Our data strongly support the current view[20] that adipocytes and specifically adipose tissue play a major, most likely causative role in the pathogenesis of metabolic diseases.

In summary, we show here that DPP4 is a novel adipokine that is substantially over-expressed in visceral fat from obese subjects and exhibits an augmented release in obesity. Soluble DPP4 exerts auto- and paracrine effects and impairs insulin signalling. We further observe a tight correlation of DPP4 release to adipocyte cell size and plasma levels of DPP4 strongly correlate with the risk of having the metabolic syndrome. We therefore suggest that DPP4 is a novel biomarker and a potential link between obesity and the metabolic syndrome.

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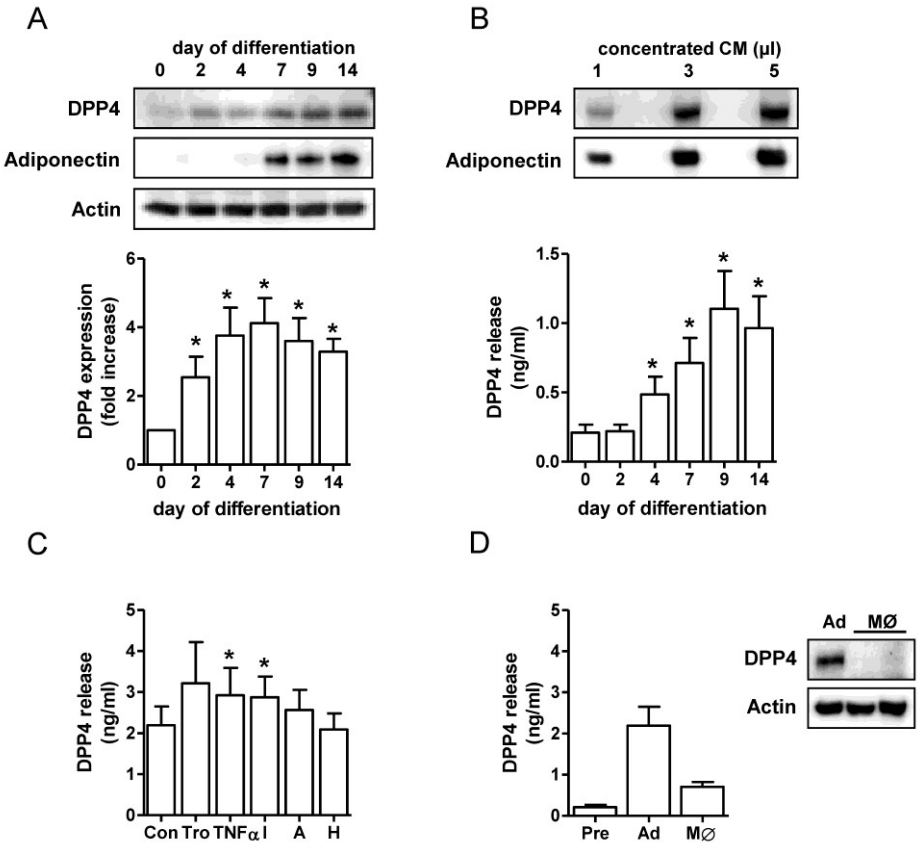

Figure 3.1 DPP4 protein level and release during adipocyte differentiation and after stimulation with different regulatory factors.

(A) Human primary adipocytes were differentiated as described in materials and methods, and DPP4 protein level during differentiation was analyzed by SDS-PAGE and Western Blot. Adiponectin expression served as a control of differentiation. Data were normalized to the protein level of actin and are expressed relative to day 0. Data are mean values ± SEM, n≥5, *p<0.05 vs. preadipocytes. (B) Detection of DPP4 at day 14 of differentiation using 1-5 µl of concentrated CM analyzed by SDS-PAGE and Western Blot. 24-h release of DPP4 by adipocytes determined at different time points of differentiation was analyzed by ELISA. Data are mean values ± SEM, n≥5, *p<0.05 vs. day 0. (C) Differentiated adipocytes were treated with 5 µmol/L troglitazone (Tro), 10 ng TNF-α, 50 mmol/L insulin (I), 5 nM adiponectin (A) or incubated under hypoxic conditions (H) for 24 h. DPP4 release by differentiated adipocytes after indicated 24 h treatments as measured by ELISA. Data are mean values ± SEM, n≥7, *p<0.05 vs. control. (D) DPP4 release by preadipocytes (Pre), differentiated adipocytes (Ad) and adipose tissue-derived and cultured human macrophages (MØ) was analyzed by ELISA. Data are mean values ± SEM, n≥3. 10 µg of total

lysates derived from adipocytes (Ad) and macrophages (MØ) were analyzed by SDS-PAGE and Western Blot, and signals were detected by ECL.

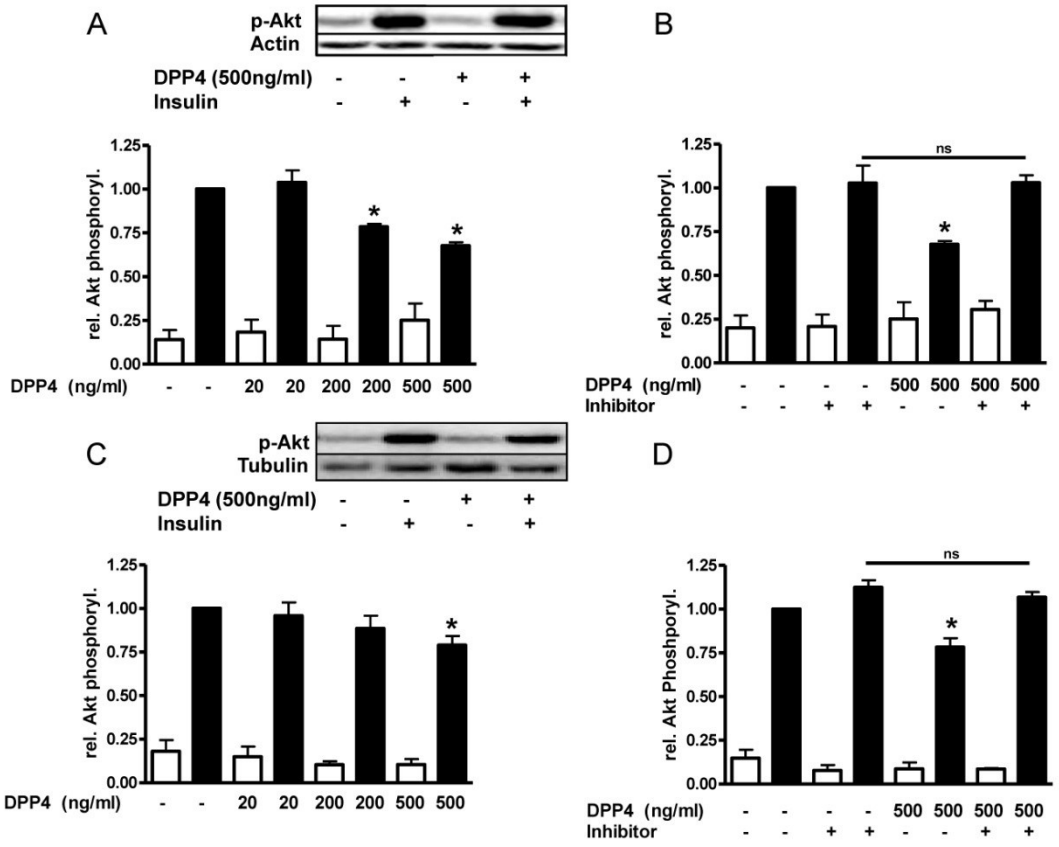

Figure 3.2 Effect of DPP4 on insulin-stimulated Akt phosphorylation in adipocytes and skeletal muscle cells.

Differentiated human adipocytes (A-B) and skeletal muscle cells (C-D) were treated with the indicated amounts of DPP4 without and with concomitant administration of a specific DPP4 inhibitor for 24 h. After stimulation with insulin (100 nM, 10 min) the cells were lysed and 5-10 µg of total lysates were resolved by SDS-PAGE and blotted to PVDF membranes. Membranes were blocked with 5% milk in TBS containing 0.1% Tween-20 and incubated overnight with p-Akt antibody. After incubation with the appropriate HRP-coupled secondary antibody, the signal was detected by ECL. Signals were analyzed on a LUMI Imager Work Station. Data are actin normalized mean values ± SEM (n = 3-8). White bars – basal, black bars – insulin-stimulated. ns – not significant. * significantly different from insulin-stimulated control or indicated situation.

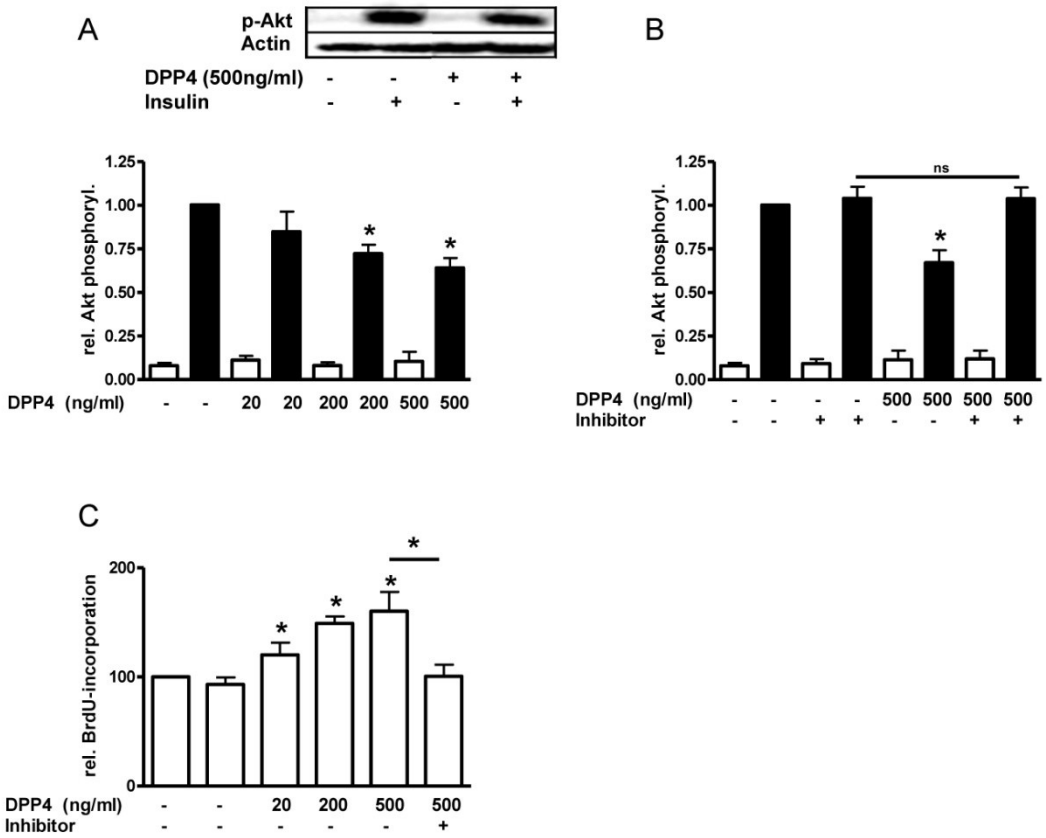

Figure 3.3 Effect of DPP4 on insulin-stimulated Akt phosphorylation and proliferation in smooth muscle cells.

(A-B) Smooth muscle cells were treated with the indicated amounts of DPP4 without and with concomitant administration of a specific DPP4 inhibitor for 24 h. After stimulation with insulin (100 nM, 10 min) the cells were lysed and Western Blots performed as indicated in Fig. 2. Data are actin normalized mean values ± SEM (n = 3-6). White bars – basal, black bars – insulin-stimulated. ns – not significant. * significantly different from insulin-stimulated control or indicated situation. (C) The proliferation of smooth muscle cells was determined by measuring the incorporation of BrdU into DNA. Data are expressed relative to the basal control value, taken as 100 %. Data are mean values ± SEM (n = 3-8). ns – not significant. * significantly different from control or indicated situation.

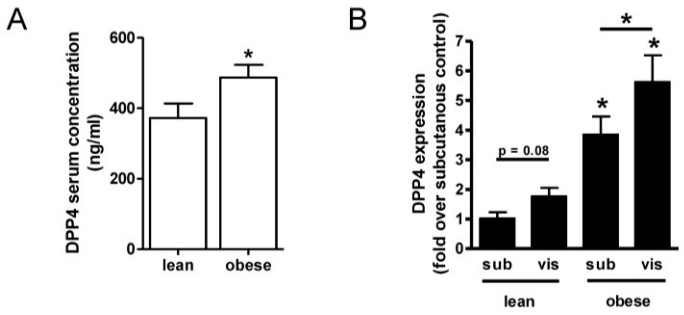

Figure 3.4 DPP4 serum concentration and expression in adipose tissue from lean compared to obese patients (Clinical study 1).

(A) Sera from lean (n=20) and morbidly obese men (n=20) were analyzed for their DPP4 concentration by ELISA. Data are mean values ± SEM, *p<0.05 vs. lean group. (B) DPP4 protein level in adipose tissue biopsies was analyzed by SDS-PAGE and Western Blot. Data were normalized to the protein level of actin and are expressed relative to subcutaneous adipose tissue from lean subjects. Data are mean values ± SEM, n=8 for lean and n=14 for obese patients, *p<0.05 respective subcutaneous or designated group.

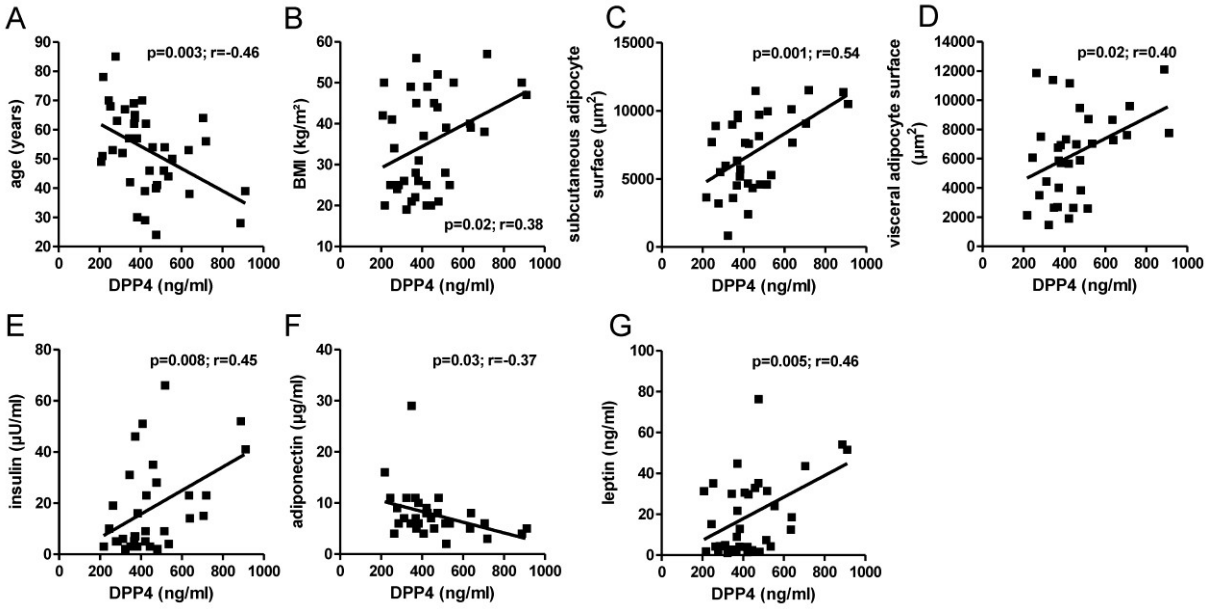

Figure 3.5 DPP4 serum concentrations correlate with various clinical and biochemical parameters (Clinical study 1).

Sera from lean (n=20) and morbidly obese men (n=20) were analyzed for their DPP4 concentration by ELISA. Linear regression analysis of DPP4 serum concentration and patient characteristics like age (A), BMI (B), size of subcutanous (sc) (C) and visceral (vis) adipocytes (D), insulin concentration (E), adiponectin concentration (F) and leptin concentration (G). Statistical evaluation is indicated in each graph.

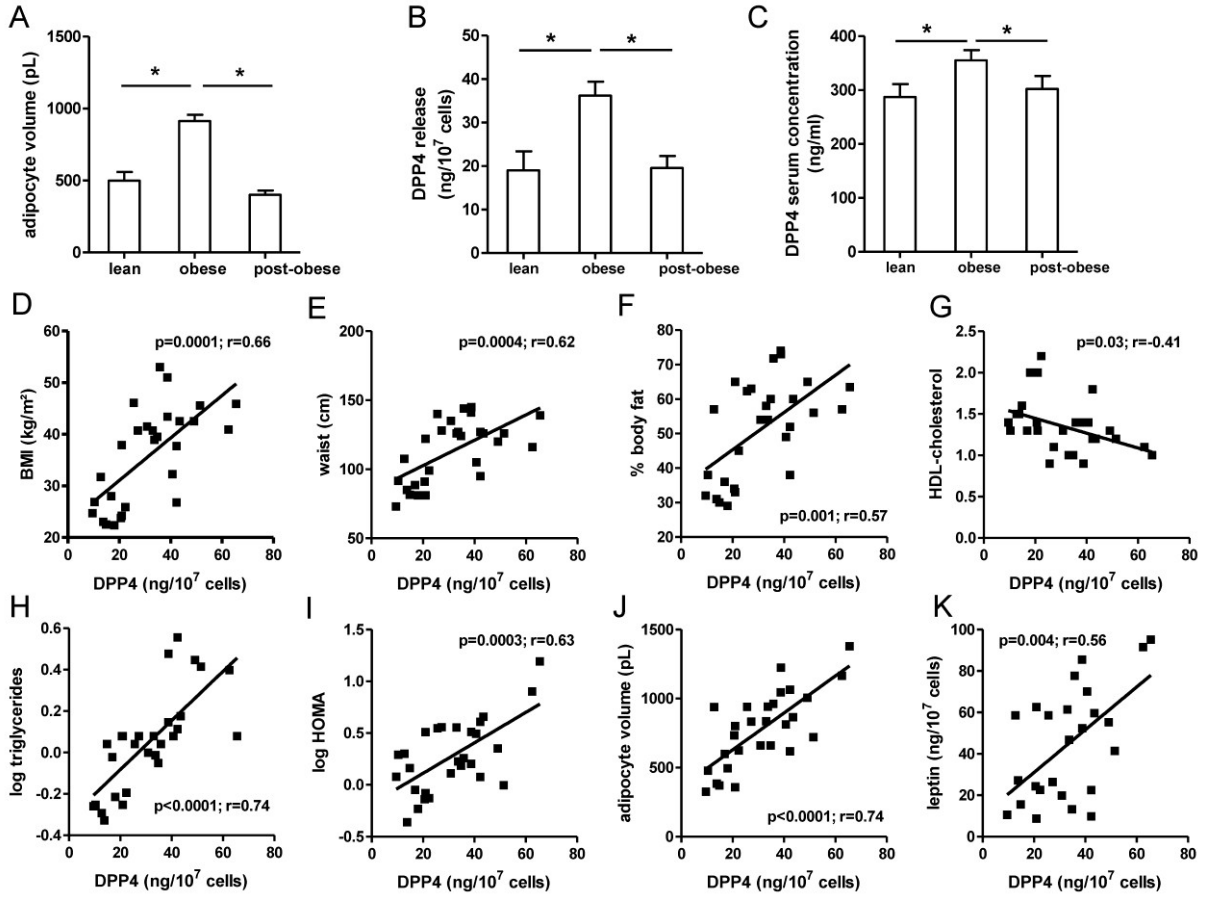

Figure 3.6 DPP4 release of explants obtained from adipose tissue of lean controls and obese patients before and after bariatric surgery as well as linear correlation with various clinical and biochemical parameters (Clinical study 2).

(A-B) Samples of adipose tissue were obtained from lean controls (n=10) and obese patients before (n=19) and after (n=16) bariatric surgery, and used to generate explants as described in material and methods. The size of adipocytes for each subject was measured (A). DPP4 release was analyzed by ELISA and related to the quantity of adipocytes (B). (C) DPP4 serum concentration was measured in lean and in obese patients before and after bariatric surgery. (D-K) Linear regression analysis of DPP4 release per 10^7 cells and patient characteristics like BMI (D), waist circumference (E), percent of body fat (F), HDL-cholesterol concentration (G), triglycerides concentration (H), HOMA (I), adipocyte volume (J) and leptin (K). (A-C) Data are mean values ± SEM . *p<0.05 between respective groups.

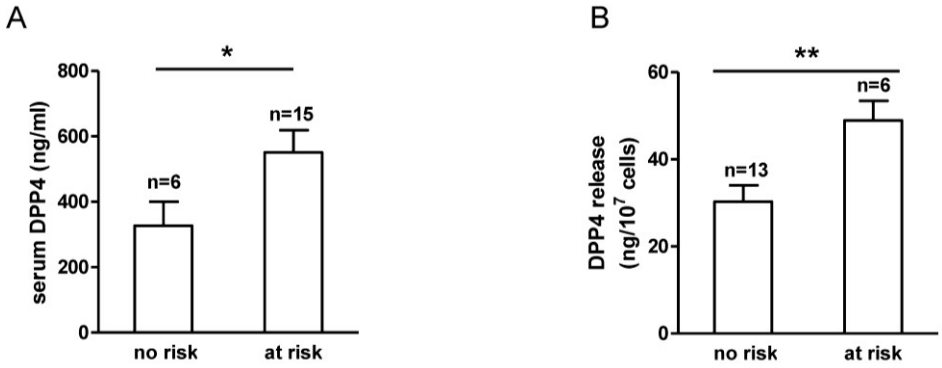

Figure 3.7 DPP4 in serum and release from adipose tissue explants in relation to a risk score for the metabolic syndrome.

A risk score for the metabolic syndrome was calculated for all obese subjects from whom serum and adipose tissue explants were analyzed. Patients with a risk score equal to or higher than 3 were qualified as "at risk". This score was correlated to the respective DPP4 concentrations in serum or culture media. Data was analyzed using ANOVA. Data are mean values ± SEM. *$p<0.05$.

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Chapter 4

Study 3

Pigment epithelium-derived factor is one of the most abundant proteins secreted by human adipocytes and induces insulin resistance and inflammatory signaling in muscle and fat cells [‡]

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Running title: PEDF - a high abundant and inflammatory adipokine

Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; ATGL, adipose triglyceride lipase; BrdU, bromdesoxyuridin; CM, adipocyte-conditioned medium; FCS, fetal calf serum; hSkMC, human skeletal muscle cells; hSMC, human vascular smooth muscle cells; MALDI, matrix-assisted laser desorption/ionization; MAPK, mitogen-activated protein kinase; MS, mass spectrometry; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor- kappa B; PDGF, platelet-derived growth factor; PEDF, pigment epithelium-derived factor; TNF, tumor necrosis factor

Abstract

Objective: PEDF is a multi-functional protein with neurotrophic and anti-angiogenic properties. More recently it became evident that PEDF is up-regulated in patients with type 2 diabetes and also contributes to insulin resistance in mice. During characterization of the secretome of *in vitro* differentiated human adipocytes by 2D-PAGE and MALDI-MS, we found that PEDF is one of the most abundant proteins released by adipocytes. The aim of this study was to investigate the regulation and autocrine function of PEDF in human adipocytes and to determine its paracrine effects on human skeletal muscle cells (hSkMC) and human smooth muscle cells (hSMC). *Methods and Results:* Human primary adipocytes secrete 130 ng/ml PEDF over 24 h from 1 mio cells, which is extremely high as compared to adiponectin, IL-6, or IL-8. This release of PEDF is significantly higher than from other primary cells such as adipose-tissue located macrophages (50-times), hSkMC and hSMC (5-times). PEDF protein expression significantly increases during adipogenesis which is paralleled by increased PEDF secretion. Furthermore, TNF-α and hypoxia significantly downregulate PEDF protein levels. PEDF secretion was significantly reduced by troglitazone and hypoxia and significantly increased by insulin. Treatment of adipocytes and hSkMC with PEDF induced insulin resistance in adipocytes, skeletal and smooth muscle cells at the level of insulin-stimulated Akt phosphorylation, which was dose-dependent and more prominent in adipocytes. Furthermore, inflammatory NF-κB signaling was induced by PEDF. In hSMC, PEDF induced proliferation (1.7-fold) and acutely activated proliferative and inflammatory signaling pathways (NF-κB, p38 MAPK and mTOR). *Conclusion:* PEDF is one of the most abundant adipokines and its secretion is inversely regulated by insulin and hypoxia. PEDF induces insulin resistance in adipocytes and hSkMC and leads to inflammatory signaling in hSMC. Due to these diverse actions, PEDF is a key adipokine which could play an important role in diabetes and obesity-related disorders.

Keywords: PEDF, adipocytes, insulin resistance, skeletal muscle cells, smooth muscle cells

Introduction

Obesity is increasing dramatically in industrial countries and it is closely associated with the development of chronic diseases including type 2 diabetes and atherosclerosis.[1] It is well established that adipose tissue is not only a storage site for triglycerides but also an important endocrine organ.[2] Adipocytes in expanding fat are capable of releasing a variety of proteins, collectively named adipokines [3] and contributing to a chronic low-grade inflammation state, as well as lipid mediators. One novel adipokine is PEDF, which is a 50 kDa secreted glycoprotein that belongs to the non-inhibitory serpin group.[4] It was first purified from conditioned media of human retinal epithelial cells and identified as a neurotrophic factor able to convert retinoblastoma tumor cells into differentiated non-proliferative neurons.[5] A current study showed that PEDF expression in adipose tissue positively correlates with obesity and insulin resistance in mice.[6] This is in accordance with other studies describing a significant correlation between plasma PEDF and obesity in humans,[7,8] which demonstrates that PEDF secreted from adipose tissue is associated with the metabolic syndrome. The mechanisms how PEDF induces insulin resistance are not fully understood. One factor contributing to insulin resistance is inflammation. PEDF shows pro-inflammatory signaling in several cell types [9] and also correlates with inflammation and vascular dysfunction in type 1 diabetics with microvascular complications in comparison to complication-free patients.[10]

Using 2D-PAGE and MALDI-MS we report here that PEDF is one of the most abundant proteins secreted by *in vitro* differentiated human adipocytes. In this context, the aim of our study was to get insights into the regulation of PEDF in human adipose tissue and its role in inducing insulin resistance and inflammatory signaling in adipocytes, skeletal muscle and smooth muscle cells, and thereby to provide evidence that PEDF could be a key adipokine, playing an important role in diabetes and obesity-related disorders.

Materials and Methods

Materials

Reagents for SDS-PAGE were supplied by Amersham Pharmacia Biotech (Braunschweig, Germany) and by Sigma (München, Germany). Polyclonal antibodies anti-phospho-mTOR (Ser2448), anti-mTOR, anti-phospho-NF-κB (P65) (Ser536), anti-NF-κB (P65), anti-p38 MAPK, anti-phospho-p38 MAPK (Thr180/Tyr182), anti-ATGL were supplied by Cell Signalling Technology (Frankfurt, Germany). Anti-actin antibodies came from Abcam (Cambridge, GB) and anti-PEDF was obtained from Millipore (Schwalbach, Germany). Anti-laminin receptor (H-141) was obtained from Santa Cruz (Heidelberg, Germany). HRP-conjugated goat anti-rabbit and goat anti-mouse IgG antibodies came from Promega (Mannheim, Germany). Collagenase NB4 was obtained from Serva (Heidelberg, Germany). Troglitazone, Cytochalasin B, TNF-α, BSA (fraction V, fatty acid free, low endotoxin) were obtained from Sigma (München, Germany). The Cell Proliferation ELISA (BrdU, chemiluminescent) and Complete protease inhibitor cocktail tablets were from Roche (Mannheim, Germany). FCS was supplied by Gibco (Invitrogen, Carlsbad, CA, USA). Human recombinant PEDF was purchased from Millipore (Schwalbach, Germany). The IKK-inhibitor I229 was kindly provided by Sanofi-Aventis (Frankfurt, Germany).[11] All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma.

Adipocyte isolation and culture

Subcutaneous adipose tissue was obtained from lean or moderately overweight women (n=23, body mass index 28.5 ± 5.7, and aged 42.1 ± 10.8 years) undergoing plastic surgery. The procedure was approved by the ethical committee of the Heinrich-Heine-University (Düsseldorf, Germany). All subjects were healthy, free of medication and had no evidence of diabetes according to routine laboratory tests. Preadipocytes were isolated by collagenase digestion of adipose tissue as previously described by us.[12] Isolated cell pellets were resuspended in Dulbecco's modified Eagles/Hams F12 (DMEM/F12) medium supplemented with 10 % FCS, seeded in 75 cm² culture flasks or six-well culture dishes and maintained at 37° C with 5 % CO_2. After overnight incubation, cultures

were washed and further incubated in an adipocyte differentiation medium (DMEM/F12, 33 µmol/l biotin, 17 µmol/l d-panthothenic-acid, 66 nM insulin, 1 nM triiodo-L-thyronine, 100 nM cortisol, 10 µg/ml apo-transferrin, 50 µg/µl gentamycin, 15 mmol/l HEPES, 14 nmol/l NaHCO3, pH 7.4) for 15 days with medium change every 2-3 days and addition of 5 µM troglitazone for the first 3 days. The degree of differentiation was determined by oil red staining, induction of adiponectin and repression of pref-1. Differentiated adipocytes were used for the generation of adipocyte-conditioned media (CM), as recently described by us.[13] Macrophages were isolated from human adipose tissue, using a method described by Curat et al.[14] For hypoxia treatment, differentiated adipocytes were incubated with a gas mixture containing 1 % O_2, 5 % CO_2 and 94 % N_2 in MIC-101 modular incubator chambers (Billups-Rothenburg, Del Mar, USA) at 37 °C for indicated times.

Culture of human vascular smooth muscle cells (hSMC) and skeletal muscle cells (hSkMC)

Primary human coronary artery SMC and primary human skeletal muscle cells were obtained from PromoCell (Heidelberg, Germany) and Lonza Walkersville Inc. (Cologne, Germany). hSMC from three different donors (Caucasian, male, 21 and 55 years old; female, 56 years old) and skeletal muscle cells from three different donors (Caucasian, female, 21, 25 and 37 years old) were supplied as proliferating cells and kept in culture according to the manufacturer's protocol. For all experiments, subconfluent cells of passage 3 were used. hSMC were characterized by morphologic criteria and by immunostaining with smooth muscle α-actin. Skeletal myoblasts were differentiated and fused by culture in α-modified Eagles medium for 4 days and used for experiments.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and protein identification by MALDI-MS

For sample preparation CM from 4 different donors (200 ml derived from $7x10^7$ adipocytes) were pooled and centrifuged for 20 min at 40,000 × *g* at 4 °C. Subsequently, CM was concentrated using Amicon™ Ultra 15 centrifugal filter devices (Millipore, Billerica, USA) with a cut-off mass of

3,000 Dalton. Protein concentrations were measured using Advanced Protein Assay (Cytoskeleton, Denver, USA). Concentrated CM (4,9 mg/ml) were diluted 1:3 in a buffer containing 25 mM Tris, 4 % CHAPS (w / v), 7 M urea and 2 M thiourea and then separated in the first dimension by isoelectric focusing (IEF) using pH 4 – 7 linear IPG strips performed on a MultiPhor II electrophoresis unit (GE-Healthcare, Freiburg, Germany) and in the second dimension by large format SDS-PAGE (12%) as previously described by us.[15] Directly after electrophoresis, gels were stained with a ruthenium fluorescent stain.[16] Images of protein pattern were acquired by laser scanning of the 2D-gels using blue laser source (457 nm) on a Typhoon 9400 (GE-Healthcare, Freiburg, Germany) laser scanner and a resolution of 100 µm. Detection of protein spots and calculation of relative spot abundances was carried out automatically using Proteomweaver 4.0 image analysis software (BioRad, Munich, Germany).

For the protein identification by MALDI-MS protein spots of interest were excised from the 2D-gels using a Gelpix spot picker (Genetix, Dornach, Germany). After protein digestion with trypsin, extracted peptides were directly applied to a MALDI Pre-spotted AnchorChip target (Bruker Daltonics, Bremen, Germany) according to the manufacturer's instructions. Subsequently, samples were analyzed in a time-of-flight Ultraflex-Tof/Tof mass spectrometer (Bruker Daltonics, Bremen, Germany). Acquired mass spectra were automatically calibrated and annotated using Compass 1.3 software (Bruker Daltonics, Bremen, Germany). Protein identification via peptide mass fingerprinting (PMF) was performed on the fly engaging Biotools 3.3 (Bruker Daltonics, Bremen, Germany). Since all experiments were performed on primary human adipocytes, MS spectra from each individual spot were used to search a human sub-set of Swiss-Prot (Sprot_57.8, 20401 protein entries) non-redundant database using Mascot search engine (Version 2.2, Matrix Science, London, UK) in consideration of the following settings: Mass tolerance 50 ppm, methionine oxidation as variable modification and carbamidomethylation of cystein as fixed modification. As enzyme, trypsin was selected allowing a maximum of one missed cleavage. Using these settings, a mascot score of greater than 70 was taken as significant ($p < 0.01$). Calculated p*I* and molecular mass data were obtained by Mascot. For peptides matching to different isoforms or multiple members of a protein family, we used the following reporting criteria: The experimental

p*I* and molecular mass taken from the 2D-gels were compared with the theoretical data of the different isoforms / protein members. If no conflicts in molecular mass or p*I* were found, the isoform / protein member with the highest mascot score was reported. For verifying the results each protein spot was picked and identified from at least three physically different 2D-gels.

Immunoblotting

All cells were treated as indicated and lysed in a buffer containing 50 mM HEPES, pH 7.4, 1 % TritonX100, Complete protease inhibitor and PhosStop phosphatase inhibitor cocktail. After incubation for 2 h at 4 °C, the suspension was centrifuged at 10.000 x *g* for 15 min. Thereafter, 5 µg protein of hSMC or hSkMC and 10 µg of adipocyte lysates were separated by SDS-PAGE using 10 % horizontal gels and transferred to polyvenylidene fluorid filters in a semidry blotting apparatus.[17] Filters were blocked with Tris-buffered saline containing 0.1 % Tween and 5 % nonfat dry milk and subsequently incubated overnight with a 1:1000 dilution of the appropriate antibodies. After washing, filters were incubated with secondary HRP-coupled antibody and processed for enhanced chemiluminescence detection using Immobilon HRP substrate (Millipore, Billerica, MA, USA). Signals were visualized and evaluated on a LUMI Imager (Boehringer, Mannheim, Germany) or VersaDoc 4000 MP (BioRad, Munich, Germany) work station.

ELISA

PEDF secretion by adipocytes, hSMC, hSkMC and human primary macrophages was determined using ELISA kits purchased from BioVendor GmbH (Heidelberg, Germany). The assays were performed in duplicates according to the manufacturer's instructions.

Proliferation assay for hSMC

To monitor DNA synthesis, hSMC were seeded in 96 well culture dishes and allowed to attach for 24 h, followed by serum starvation for an additional 24 h period. Cells were then stimulated for 24 h with the

different CM in the presence of BrdU (10 µM). The BrdU ELISA Kit from Roche was used to determine proliferation according to the manufacturer's protocol. Signals were visualized and evaluated on a LUMI Imager work station (Boehringer, Mannheim, Germany).

hSMC migration assay

Transwell Cell migration assay was performed using 24-well transwell chambers with 8-µm-pore-size polycarbonat membranes (Cell Biolabs, Inc., San Diego, CA, USA). hSMC were grown to confluence and serum-starved for 24h. 300 µl cell suspension containing 3 x 10^4 detached cells was added to the upper compartment. Serum-free medium, PEDF, CM or FCS were placed in the lower compartment (500 µl/well). In control chambers, 2.5 nM cytochalasin B was added to the upper compartment. The cells were then incubated for 18 h. After removal of non-migratory cells, migratory cells were stained and quantified at 570 nm according to the manufacturer`s protocol.

Presentation of data and statistics

Data are expressed as mean ± SEM. Paired two-tailed Student's t test or one-way ANOVA (post-hoc test: Bonferroni's multiple comparison test) were used to determine statistical significance. All statistical analyses were done using Prism (GraphPad, La Jolla, CA, USA) considering a P value of less than 0.05 as statistically significant. Corresponding significance levels are indicated in the figures.

Results

CM from primary human adipocytes contains high amounts of PEDF

We characterized the secretome of *in vitro* differentiated human adipocytes by 2D-PAGE (Fig. 1A) and MALDI-MS, and found PEDF to be one of the most abundant proteins in CM (45 ng/ml released over 24 h by 350.000 adipocytes). It was identified in 15 spots, which represent 6 % of all calculated spot intensities on the gel. Western blot detection of PEDF

after 2D-PAGE could reproduce the pattern of this adipokine (Fig. 1B). Compared to other adipokines measured by us in earlier studies, the concentration of PEDF in CM was about 1800-fold higher than IL-6, 800-fold higher than IL-8, 20-fold higher than chemerin, and about 7-fold higher than adiponectin (Tab.1). However, in plasma PEDF concentrations are comparable to adiponectin.

PEDF expression and secretion in human primary adipocytes and its regulation

In the light of the high amounts of PEDF found in CM via 2D-PAGE, we analyzed PEDF expression and secretion in human primary adipocytes undergoing differentiation. We found PEDF protein throughout adipocyte differentiation and increasing during this process with a decrease at later time points (Fig. 2A). In comparison, adiponectin expression was induced by differentiation as expected. PEDF secretion increased transiently during differentiation of adipocytes in parallel to its expression (Fig. 2B). Additionally, adipocytes at day 14 secreted PEDF continuously over a period of 48 h (Fig. 2C). As circulating levels of PEDF are high, we tested other primary human cells for their PEDF secretion. As shown in Figure 2D hSkMC, hSMC and human primary macrophages isolated from adipose tissue do secrete PEDF at significantly lower concentrations compared to preadipocytes and adipocytes. Specifically, the secretion of adipose tissue macrophages is very low and it is likely that this cell type does not contribute to the circulating PEDF levels in humans.

Prominent regulators of adipocyte secretory activity such as troglitazone, insulin or TNF-α were tested for their ability to influence PEDF expression and secretion in adipocytes. In addition, as hypoxia is now known to occur in adipose tissue of mice [18-20] and in humans [21] we also tested whether prominent hypoxia of 1 % oxygen has a regulatory function. We found that PEDF expression is significantly reduced after 24 h incubation with TNF-α or under hypoxia whereas troglitazone and insulin have no effect (Fig. 3A). The concentration of PEDF in CM was significantly reduced after troglitazone and hypoxia treatment while insulin increased the secretion of PEDF significantly (Fig. 3B).

Two proteins, namely adipose triglyceride lipase (ATGL) and Laminin-R, were postulated to serve also as receptors for PEDF.[22,23] Preadipocytes

and adipocytes express detectable levels of ATGL and Laminin-R with higher amounts being detected in the fully differentiated state (Fig. 4). We also could detect both receptors in hSMC and hSkMC. In human primary macrophages, Laminin-R is present, whereas ATGL could not be detected.

PEDF action on insulin signaling and inflammatory pathways in adipocytes and hSkMC

Recently is has been put forward that PEDF might be a player in insulin resistance in mice.[6] Therefore, we tested PEDF in vitro on human primary adipocytes and hSkMC for its effects on insulin signaling and the induction of inflammatory stress signaling. PEDF induces insulin resistance in both adipocytes and hSkMC with more prominent effects on adipocytes (Fig. 5 A and C). We also observed a significant induction of NF-κB phosphorylation in adipocytes after 3h-treatment with PEDF and in skeletal muscle cells in a more transient way with a peak after 10 min of PEDF incubation (Fig. 5 B and D). The induction of insulin resistance by PEDF in skeletal muscle cells can be prevented by IKK-inhibition (Fig. 5 E) making it very probable that PEDF affects insulin-stimulated Akt phosphorylation by increasing serine phosphorylation of IRS-1.

PEDF acutely activates multiple signaling pathways and leads to insulin resistance in hSMC

Related to the effects of PEDF on inflammatory signaling and insulin resistance in hSkMC, we further analyzed the impact of PEDF at the level of hSMC. Currently, not much is known about the influence of PEDF on insulin resistance and inflammatory and proliferative signaling pathways in these cells. As shown in Fig. 6A, B and C, we could demonstrate that treatment with PEDF activated NF-κB, p38 MAPK and mTOR significantly after 10 min. In addition, PEDF induces insulin resistance in hSMC at the level of Akt (Ser473) phosphorylation (Fig. 6D).

PEDF induces proliferation of hSMC

To investigate the influence of PEDF on early events in the development of atherosclerosis, we assessed its ability to induce

proliferation of hSMC. Therefore, DNA synthesis was monitored by determining the incorporation of BrdU using a highly sensitive chemiluminescence immunoassay. As presented in Fig. 7A, 10 nM PEDF induced a small but significant increase of hSMC proliferation (1.7-fold). Further, PEDF had no effect on FCS-stimulated proliferation (data not shown). CM alone, which itself contains about 1 nM PEDF, leads to a robust stimulation of proliferation (3-4-fold). Adding PEDF to CM has no additional effect on the CM-induced proliferation. As another readout for early events in the development of atherosclerosis, we determined the effect of PEDF on migration of hSMC. Fig. 7B shows that PEDF alone has no effect on the migration of hSMC. CM leads to a significant increase of hSMC migration, but the combination of CM and PEDF has no further effect on CM-induced migration.

Discussion

In the context of obesity, adipose tissue as an endocrine organ produces a variety of inflammatory adipokines like Il-6, TNF-α or PAI-1. One novel adipokine is PEDF, which has been shown to be expressed and secreted by adipocytes derived from differentiated human mesenchymal stem cells [24] and 3T3-L1 mouse adipocytes.[6,25-27] The expression pattern of PEDF is controversially discussed, as three studies showed a decrease in PEDF mRNA and secretion level during differentiation from preadipocytes to adipocytes in 3T3-L1 cells,[25-27] whereas one study also from 3T3-L1 cells showed a differentiation dependent increase of PEDF protein levels.[6] Additionally, the latter study revealed PEDF as the most abundant adipokine in supernatants of 3T3-L1 adipocytes. Furthermore, two other studies showed a significant higher expression of PEDF in differentiated human adipocytes compared to preadipocytes.[24,28] In our system of *in vitro* differentiated primary human adipocytes, we were able to demonstrate that PEDF is one of the most abundant proteins released by adipocytes, when analyzed by 2D-gelelectrophoresis and MALDI-MS. Compared to other adipokines measured by us in earlier studies, PEDF showed a higher concentration than the new adipokine chemerin and even than adiponectin. Furthermore, we displayed an increased PEDF protein expression and secretion during differentiation. The role of adipose tissue

for PEDF secretion becomes evident since Crowe and colleagues showed that PEDF expression in adipose tissue positively correlates with obesity and insulin resistance in mice.[6] Furthermore, only modest PEDF secretion from hepatocytes and monocytes was detected which may indicate that these tissues do not represent the main source for the circulating levels.[6] We also showed in our study that adipose tissue derived macrophages secrete only small amounts of PEDF, making it likely that adipocytes are the major contributors to PEDF release from adipose tissue. Additionally two other studies describe a significant correlation between plasma PEDF and obesity in humans [7,8] pointing to adipose tissue as the main source of PEDF. However, the relevance of the liver contributing to plasma levels of PEDF in humans remains to be elucidated.

Little is known about the regulation of PEDF expression and secretion. There is evidence that PEDF protein levels are positively correlated with oxygen tension as it is down-regulated in ischemic and hypoxic retinal glial (Mueller) cells as well as in anoxic cardiac myocytes.[29,30] In the present study we could extend the regulation of PEDF by hypoxia to human adipocytes where hypoxia represses PEDF expression and release. This repression occurs not in such a prominent manner as in anoxic cardiac myocytes where a repression of about 40-50% was observed.[30] However, similar to the effects in retinal glial (Müller) cells we also observed a reduction of PEDF release of about 15-20% under 1% oxygen.[29] As PEDF is a substrate for MMP-2 and MMP-9 and MMPs are up-regulated at low oxygen concentrations, reduced levels of PEDF under hypoxia may be due to a post-translational degradation through hypoxia induced MMPs.[31] We also found elevated levels of MMP's after adipocyte treatment with 1 % of oxygen (data not shown) so that the down-regulation found by us may also underlie a mechanism of degradation of PEDF by MMPs. Additionally, we observed a down-regulation of PEDF protein expression under TNF-α treatment without a significant change in PEDF secretion. Yamagishi et al. similarly reported a TNF-α induced reduction of PEDF mRNA in HUVEC.[32] The mechanism of this reduction is not known until now, but it has been proposed that TNF-α-elicited reactive oxygen species (ROS) generation might be involved as it could be demonstrated in another study that angiotensin-II-induced ROS could suppress PEDF mRNA levels.[33] It remains to be elucidated if the reduction of PEDF protein caused by TNF-α treatment occurs by a ROS dependent

mechanism. Additionally it can be noted that hypoxia and TNF-α are both factors which have been shown to regulate some adipokines in a comparable fashion as induction of VEGF and IL-6. Similarly adiponectin is inhibited after 24 h treatment of TNF-α and hypoxia.[34,35] This suggests that PEDF suppression by hypoxia and TNF-α may underlie a common mechanism. We further demonstrate a decrease of PEDF secretion under troglitazone which has also been described for a number of other adipokines *in vivo* and *in vitro* such as PAI-1, TNF-α or chemerin.[36-38] Until now it is not known if TZDs have any regulatory effect *in vivo* on the circulating levels of PEDF. Hyperinsulinemia plays a pivotal role in obesity and type 2 diabetes, and we demonstrate here that insulin treatment of adipocytes led to a significant higher release of PEDF by adipocytes. This might reflect one cause of the elevated plasma levels of PEDF found in type 2 diabetics. This view is supported by Crowe and coworkers who demonstrated elevated PEDF levels in high-fat diet fed mice which were obese and also developed a hyperinsulinemic phenotype.[6] It is assumable that not the obese state alone but the combination with hyperinsulinaemia caused the observed increase in PEDF serum levels.[6] It should be noted that the divergence between PEDF expression and secretion, as it is observed for troglitazone, TNF-α and insulin may be a question of time-course. In the case of TNF-α, PEDF expression is decreased after 24 h, whereas an effect on the secretion of PEDF may only be observable at later time points.

PEDF has been described as a multifunctional protein with different functions in diverse cell types and it is assumed that PEDF acts via multiple receptors and ligands.[9] Until now two distinct proteins were suggested to function as receptors for PEDF, namely ATGL and Laminin-R. In 2006, Notari et al. identified patatin-like phospholipase domain containing protein-(PNPLP)2, also known as ATGL, as a specific receptor for PEDF.[22] There is upcoming evidence that PEDF affects lipolysis in adipose tissue and takes part in hepatic lipid accumulation depending on ATGL.[6,39] It is assumed that PEDF not only affects lipid metabolism but also mediates some of its effects like anti-angiogenic and anti-tumorigenic activities by inducing lipid mediators.[22] The second known receptor of PEDF, Laminin-R, was identified in 2009. This non-integrin receptor is involved in processes like cell adhesion, differentiation, proliferation and migration.[40] All cell types used in the present investigation express both

receptors, however, adipose tissue-located macrophages only have low amounts of Laminin-R and no ATGL. It is known that PEDF can also affect macrophages as it inhibits LPS-induced interleukin (IL)-12 production in a mice macrophage cell line.[41] Further it induces apoptosis in a human monocytic leukemia cell line (THP-1) as well as in human primary monocyte-derived macrophages in a PPAR-γ dependent manner.[42] Until now it is not known which receptor mediates these effects but it is assumable that adipocyte generated PEDF might directly act on adipose tissue located macrophages and is involved in the crosstalk between macrophages and adipocytes.

PEDF could be demonstrated to induce insulin resistance in liver and skeletal muscle in rodents *in vivo*, both after acute and chronic administration.[6] We show here that in primary human adipocytes and skeletal muscle cells PEDF can also induce insulin resistance and activate inflammatory signaling. In this context, adipocytes are more sensitive towards PEDF than skeletal muscle cells. Human adipocytes display significantly decreased insulin-stimulated Akt phosphorylation at 5 to 10 nM PEDF and exhibit prolonged, significant activation of NF-κB. Differently, PEDF-treated skeletal muscle cells are characterized by a modest transient activation of this inflammatory pathway. It should be noted that the used concentrations of PEDF are up to 10-times higher than in CM but also significantly lower than circulating serum levels of this adipokine. The higher sensitivity of adipocytes towards PEDF could be explained by the significantly higher expression of ATGL and Laminin-R as compared to skeletal muscle cells.

Obesity is closely correlated to the development of chronic low-grade inflammation diseases like atherosclerosis.[1] However, in obesity PEDF serum levels are upregulated and consequently more PEDF is affecting the vessel wall. One major cell type in the vessel wall is represented by smooth muscle cells, which in the development of atherosclerosis proliferate and migrate into intima. In this study we could show that in addition to adipocytes and hSkMC, PEDF is also contributing to inflammatory signaling in hSMC. This is in accordance with studies showing that PEDF induces inflammatory signaling in different cell types [9] and correlating PEDF levels to inflammation and vascular dysfunction in type 1 diabetics with microvascular complications.[10] In addition, we could show here for the first time that PEDF also leads to insulin resistance in

hSMC at the level of Akt phosphorylation. PEDF further leads to a slight, but significant induction of proliferation of hSMC. Yamagishi and colleagues showed that PEDF by itself had no effect on SMC proliferation but that it inhibits angiotensin II-induced SMC proliferation (1.3-fold) via suppression of ROS generation.[43] However, in our study we did not observe an anti-proliferative effect, nor with PEDF alone, neither in the combination with CM, which is known to induce ROS production in hSkMC [44] and hSMC (data not shown). CM itself contains 1 nM PEDF. In our experiments we used 10 nM PEDF, suggesting that PEDF itself is not responsible for the CM-induced proliferation. Treating the cells with rapamycin or an IKK-inhibitor, as used by us in a recent study,[11] totally reduced PEDF-induced proliferation to the level of control, suggesting that both mTOR and NF-κB are responsible for the observed proliferative effect of PEDF (data not shown). In accordance to a study from Nakamura et al., we could not observe an effect of PEDF *per se* on the migration of hSMC. However, Nakamura and colleagues showed that PEDF could inhibit the PDGF-induced migration of SMC by blocking ROS generation.[45] In our study we could not detect an inhibitory effect of PEDF on CM-induced migration, despite the fact that CM induced ROS in our cell system.

The role of PEDF is discussed controversially. On the one hand, PEDF has been suggested to have a protective role in atherosclerosis due to its anti-inflammatory, anti-oxidant and anti-thrombotic properties in the vessel wall and platelets.[46] The neuroprotective and anti-angiogenic activities of PEDF make it a promising candidate for a therapeutic agent in the case of proliferative diabetic neuropathy or age-related macular degeneration, where both activities are important for controlling the pathology. PEDF has also emerged as an antitumor agent, which is able to act on many different types of tumors.[47] On the other hand, PEDF leads to insulin resistance and inflammatory signaling in several cell types as shown by us and others.[6,9] These findings point to a yet uncharacterized role of PEDF in the metabolic syndrome, insulin resistance and diabetes. In the light of PEDF as a therapeutic target it should be noticed that PEDF could mediate other unexpected secondary effects in different cell types and tissues. The observation that PEDF is secreted in high amounts by adipocytes and its autocrine action demonstrated here, raise the question which specific role PEDF may play in adipose tissue. Because of its anti-angiogenic properties PEDF is postulated to play a role in maintaining

homoeostasis of expanding adipose tissue by inhibiting excessive vascularization.[6] On the other hand, due to the upcoming evidence that PEDF may play a pivotal role in lipolysis, [6,22,48] PEDF could be involved in lipid homoeostasis of adipose tissue. The autocrine effects described here identify PEDF as a novel player in the induction of adipose tissue inflammation and insulin resistance.

In conclusion, we identified PEDF as one of the most abundant adipokines released by adipocytes concluding adipose tissue represents one of the main sources for circulating levels of PEDF. The different autocrine and paracrine actions mediated by PEDF affecting different cell types and tissues illustrate the complexity of this new adipokine and its role in obesity and obesity-related disorders.

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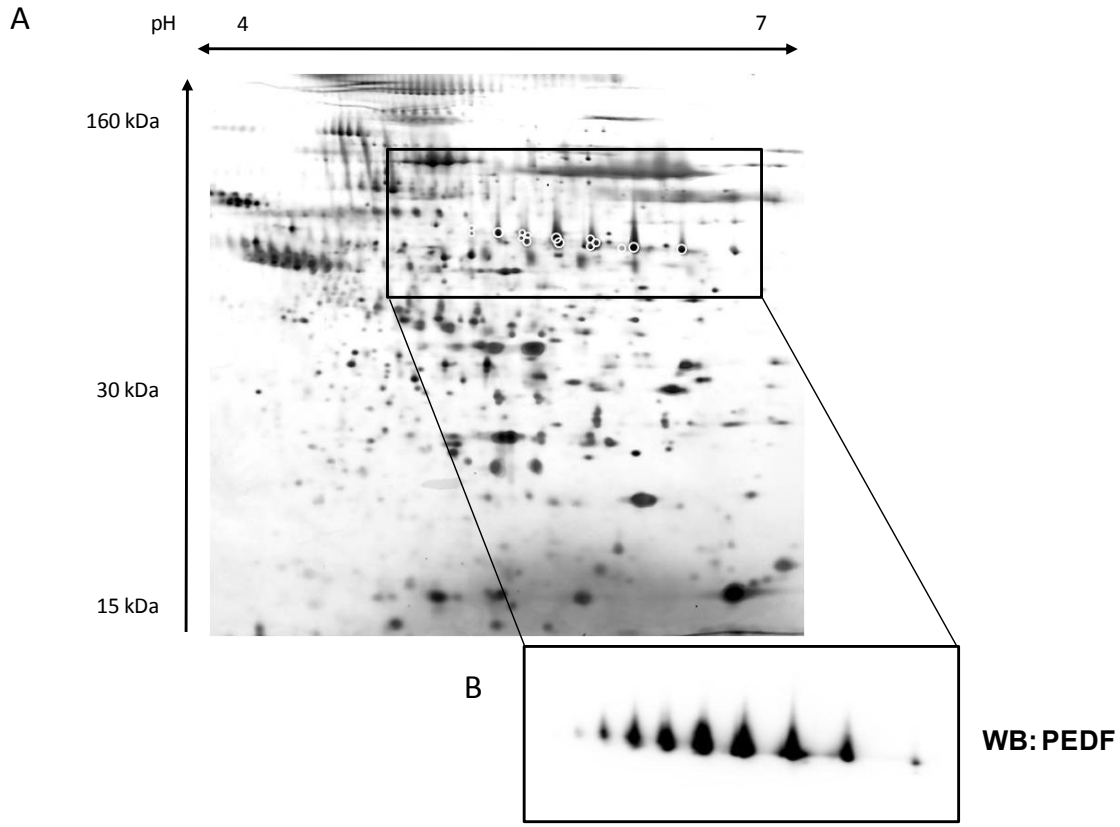

Figure 4.1 2D-pattern of CM from primary human adipocytes and immunoblot of 2D-gel with PEDF-specific antibody.

(A) CM from 4 different donors were generated and concentrated as described in *Materials and Methods* and subsequently separated in the first dimension by IEF (pH 4-7) and in the second dimension by 12 % SDS-PAGE. All PEDF spots identified with MALDI-MS are circled. (B) Western blot of 2D-gel and subsequent detection of PEDF with a PEDF-specific antibody.

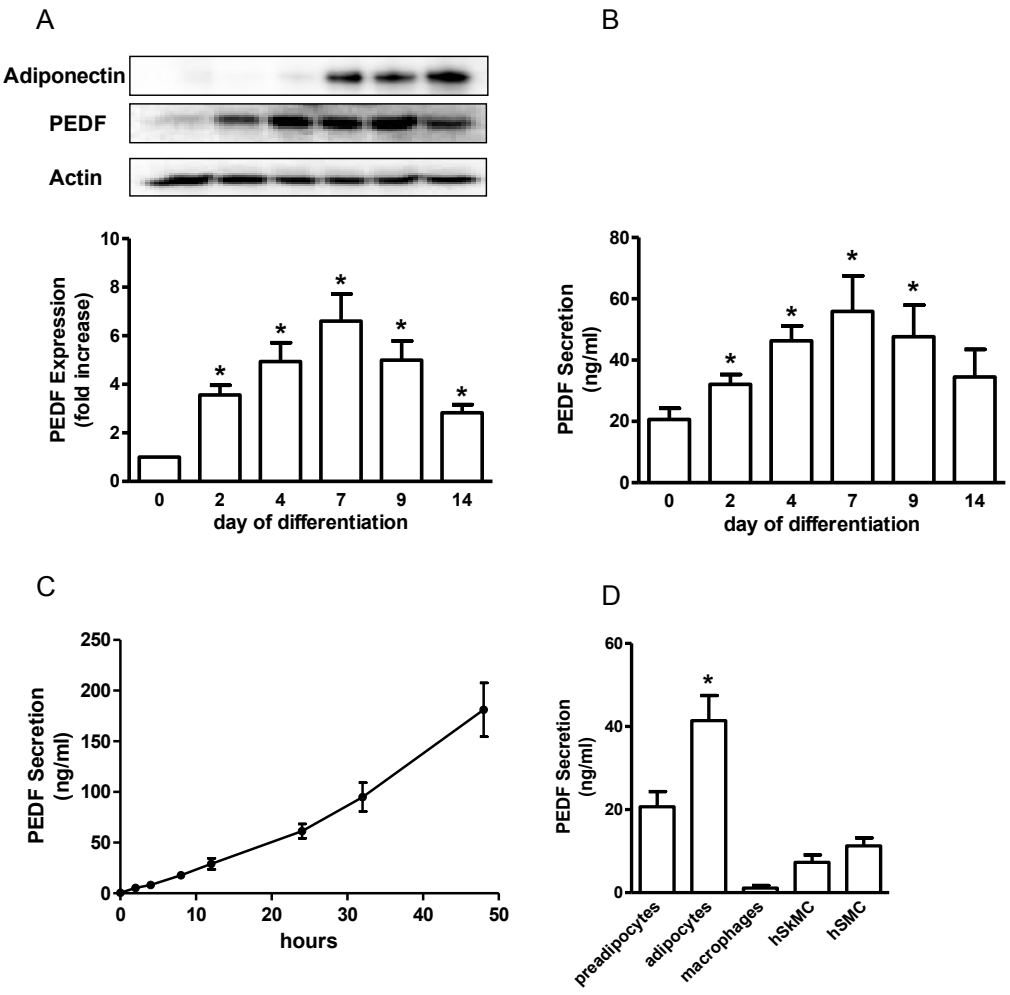

Figure 4.2 Expression and secretion of PEDF during differentiation of primary human adipocytes and in comparison to other primary cells.

Human primary adipocytes from 4 donors were differentiated and on day 0, 2, 4, 7, 9 and 14 of differentiation medium was changed to □-modified Eagles medium for 24 hours. Afterwards CM and cells were harvested. (A) 10 µg of total lysates were resolved by 10% SDS-PAGE and blotted to PDVF membranes. Membranes were blocked with 5% milk in TBS with 0.1% Tween-20. To detect the expression level of PEDF, membranes were incubated overnight with anti-PEDF antibody. After incubation with the appropriate HRP-coupled secondary antibody the signal was detected by ECL. Data were normalized to the level of actin expression and are expressed relative to day 0. Representative blots are shown. Data are mean values ± SEM of four independent experiments. *$p<0.05$ compared to preadipocytes. Secretion of PEDF (B) during adipocyte differentiation, (C) from adipocytes at day 14 over 48 h and (D) of hSkMC, hSMC and human primary macrophages was measured by ELISA. Data are mean values ± SEM of three to seven independent experiments. (A-B) *$p<0.05$ compared to preadipocytes, (D) *$p<0.05$ compared to all other cell types.

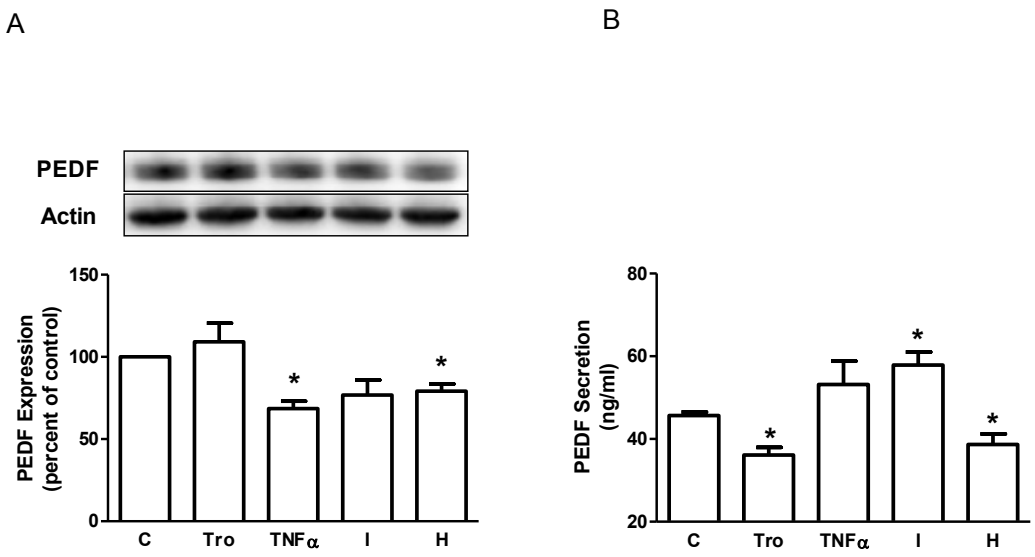

Figure 4.3 Regulation of expression and secretion of PEDF in human primary adipocytes and PEDF secretion of human primary adipocytes in comparison to other human cell types.

Differentiated human primary adipocytes from 3-4 donors were treated with 5 µM trogliazone (Tro), 10 ng TNF-α, 50 nM Insulin (I) or incubated under hypoxic conditions (H) for 24 hours. Afterwards supernatants and cells were harvested. (A) 10 µg of total lysates were analyzed by Western Blot as described in figure 2. Data were normalized to the level of actin expression and are expressed relative to control. Representative blots are shown. Data are mean values ± SEM of four independent experiments. *$p<0.05$ compared to control. (B) PEDF secretion was measured by ELISA. Data are mean values ± SEM of three to four independent experiments. *$p<0.05$ compared to control.

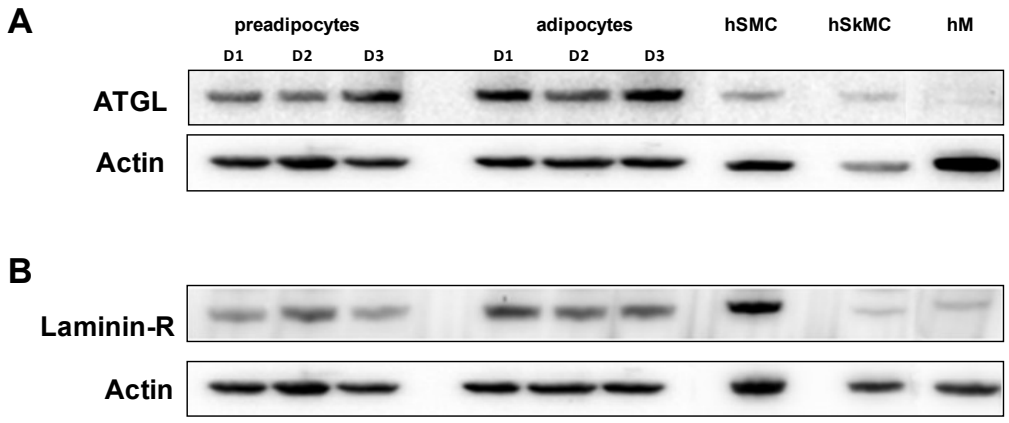

Figure 4.4 Expression of PEDF receptors in different human cell types.

5-10 µg of total lysates of adipocytes, hSMC, hSkMC and human primary macrophages (hM) were analyzed to detect expression levels of ATGL and Laminin-R. Membranes were incubated with the appropriate antibodies as described in *Materials and Methods*. Representative Blots are shown. D1-3: Isolated preadipocytes of three different donors that were *in vitro* differentiated into adipocytes.

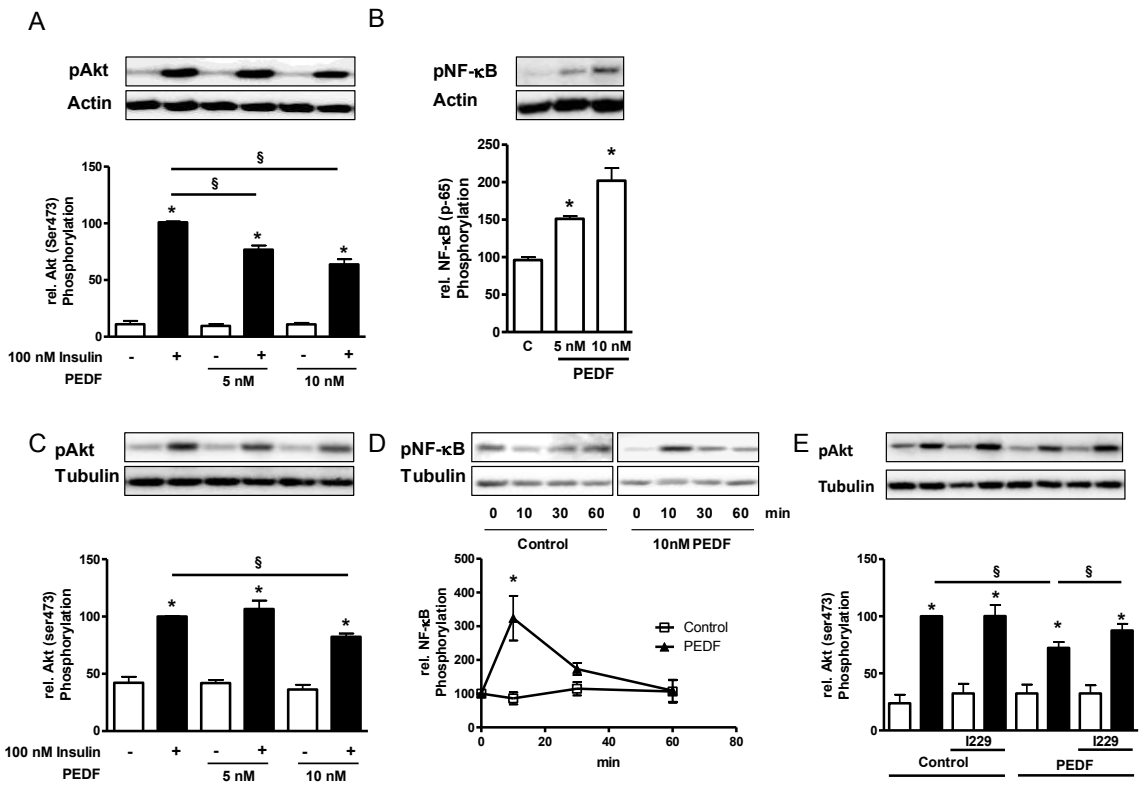

Figure 4.5 Induction of insulin resistance in human primary adipocytes and human skeletal muscle cells by PEDF.

Adipocytes (A, B) and skeletal muscle cells (C, D, E) from different donors were incubated with PEDF for 3 h (A, B), overnight (C, E) and for the indicated times, respectively. The IKK-inhibitor I229 was used as detailed in materials and methods. After insulin stimulation, total cell lysates were resolved by SDS-PAGE and immunoblotted with a phosphospecific antibody for Akt, NF-κB and tubulin or actin for loading control. Representative blots are shown. Data are mean values ± SEM of 3-4 independent experiments. *$p<0.05$ compared to respective basal, § $p<0.05$ compared to respective insulin-stimulated control.

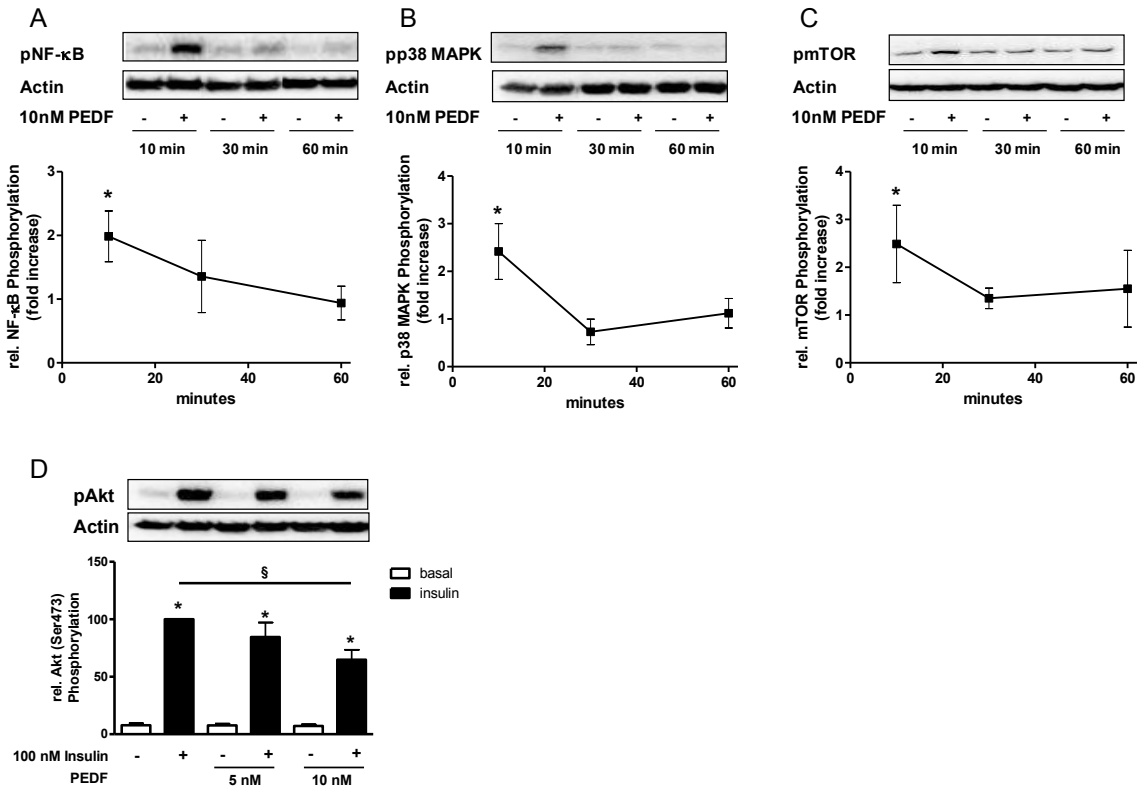

Figure 4.6 PEDF induces proliferative and inflammatory signaling pathways and insulin resistance in hSMC.

(A, B, C) hSMC were serum starved for 24 h and then exposed to PEDF for the indicated time points. Total cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies to phosphorylated and unphosphorylated forms of NF-κB, p38 MAPK or mTOR. (D) Smooth muscle cells were incubated with PEDF for 3 h. After insulin stimulation, total cell lysates were resolved by SDS-PAGE and immunoblotted with a phosphospecific antibody for Akt. Data are mean values ± SEM of three independent experiments. All data were normalized to the level of actin expression and are expressed relative to the control. Representative blots are shown. * $p<0.05$ compared to respective basal, § $p<0.05$ compared to respective insulin-stimulated control.

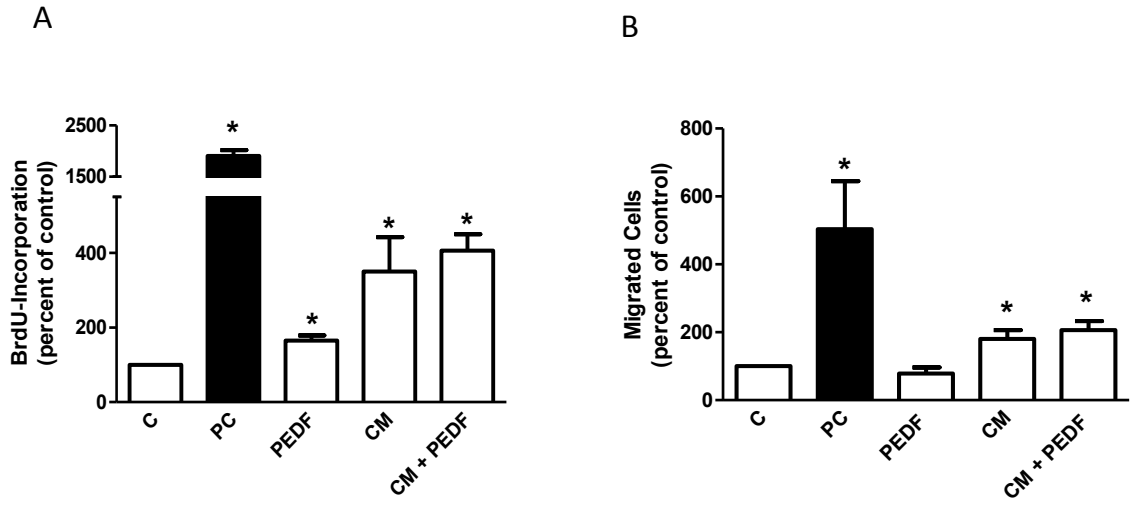

Figure 4.7 Effect of PEDF on hSMC proliferation and migration.

(A) hSMC were serum starved for 24 h and subsequently incubated with BrdU in the absence or presence of PEDF, CM or the combination of both for 24 h. Proliferation was measured as described in Materials and Methods. Data are expressed relative to the basal control value, taken as 100 %. FCS was used as a positive control (PC). Data are mean values ± SEM of three independent experiments. (B) hSMC were serum starved for 24 h, exposed to PEDF or CM for 6 h and migration measured as described in Materials and Methods. FCS was used as positive control (PC). Data are presented as mean ± SEM of four independent experiments using four different CM. *$p<0.05$ compared to control.

Adipokine	Concentration in CM (ng/ml)	Circulating Levels
Adiponectin	6.545 ± 1.46	5-10 µg/ml
Leptin	< 0.001	10 ng/ml
PEDF	**45.7 ± 0.82**	**3-10 µg/ml**
MCP-1	0.54 ± 0.08	100 pg/ml
IL-6	0.025 ± 0.002	1.4 pg/ml
IL-8	0.055 ± 0.013	3-10 pg/ml
VEGF	0.329 ± 0.09	200 pg/ml
Chemerin	2.175 ± 0.294	200 ng/ml

Table 4.1 Adipokines in CM from human primary adipocytes compared to plasma concentrations.

Adipokine concentrations of CM were obtained by ELISA. Plasma concentrations are literature based.

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Chapter 5

Study 4

Hypoxia reduces the response of human adipocytes towards TNF-α resulting in reduced NF-κB signaling and MCP-1 secretion ‡

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Running title: Hypoxia attenuates TNF-α-induced NF-κB signaling

Abbreviations: NF-κB, nuclear factor-kappaB; IL, interleukin; VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor; MCP-1, macrophage chemotactic protein (MCP)-1; Gro-α, growth regulated oncogene alpha; IκBα , inhibitor of NF-κB; IKK, IκB kinase; TNF-R; tumor necrosis factor-receptor

Abstract

Objective: Obesity is associated with adipose tissue hypoxia, and is thought to be linked to the chronic low-grade inflammation of adipose tissue, although the precise mechanism has remained unclear. In this study we investigated the effect of a prominent hypoxia on human primary adipocyte secretion and tumor necrosis factor (TNF)-α-induced nuclear factor-κB (NF-κB) signaling.

Results: Using cytokine array and ELISA analysis we compared the secretion pattern of normoxic and hypoxic (1 % O_2) adipocytes and observed various alterations in adipokine release. We could reproduce known alterations like an induction of interleukin (IL)-6, vascular endothelial growth factor (VEGF), leptin and a reduction in adiponectin release under hypoxia. Interestingly, we observed a significant reduction in the secretion of macrophage chemotactic protein (MCP)-1 and other NF-κB related genes such as growth regulated oncogene (Gro)-α, eotaxin and soluble TNF-Receptor1 (TNF-R1) under hypoxia. TNFα stimulation of hypoxic adipocytes resulted in a significantly reduced phosphorylation of NF-κB and its inhibitor IκBα compared to normoxic cells. Furthermore, chronic treatment of hypoxic adipocytes with TNFα resulted in an expected higher secretion of the chemokines MCP-1 and IL-8, but under hypoxia the secretion level was substantially lower than under normoxia. This reduction in protein release was accompanied by a reduced mRNA expression of MCP-1, whereas IL-8 mRNA expression was not altered. Additionally, we observed a significantly reduced expression of the TNF-receptor TNF-R1, possibly being one cause for the reduced responsiveness of hypoxic adipocytes towards TNFα stimulation.

Conclusion: In conclusion, human primary adipocytes show a basal and TNFα-induced reduction of MCP-1 release under hypoxia. This effect may be due to a reduced expression of TNF-R1 and therefore attenuated TNFα-induced NF-κB signaling. These observations demonstrate a reduced responsiveness of hypoxic adipocytes towards inflammatory stimuli like TNFα, which may represent an adaptation process to maintain adipose tissue function under hypoxia and inflammatory conditions.

Keywords: hypoxia, inflammation, adipokine, chemokine, insulin resistance

Introduction

It is well described that white adipose tissue does not only act as a fuel storage site for triacylglycerides, but also as one of the main endocrine organs.[1] A high number of proteins, the so called adipokines, are released by adipose tissue such as adiponectin, interleukin (IL)-6, vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF)-α or macrophage chemotactic protein (MCP)-1.[2] Adipokines can act in an autocrine manner and are associated with the well described chronic, low grade inflammation of the adipose tissue in obesity, as well as in an endocrine manner, where they contribute to obesity-related disorders such as type 2 diabetes and the metabolic syndrome.[3,4] Current studies are linking hypoxia to adipose tissue inflammation, as various inflammation-related adipokines are inducible by hypoxia.[5] Furthermore, in diverse mouse models of obesity it could be shown that severe adipose tissue hypoxia of about 2% - 2.6% in vivo occurs[6-8], and also a study done in humans showed a mild hypoxia of about 6.1% in adipose tissue of obese patients compared to an oxygen concentration of 7.2% in lean controls.[9]

Hypoxia could be defined as an imbalance between oxygen supply and consumption.[10] The response to hypoxia is a common mechanism that could be observed in tumors, wound healing or ischemia, and there are also some tissues like the thymus or the bone marrow niche, that are described to be relatively hypoxic.[11] However, hypoxia is strongly linked to the inflammatory response like the nuclear factor-κB (NF-κB) signaling, as there is evidence that it induces NF-κB activity.[12,13]

In the present study we established an in vitro model of hypoxia in human primary adipocytes in our laboratory and analyzed alterations in the secretory profile of hypoxic adipocytes. The aim was to assess the effect of a prominent hypoxia (1% O_2) on adipocyte signaling, where we identified a hypoxic regulation of the NF-κB pathway. This study demonstrates that the responsiveness of hypoxia-treated adipocytes towards TNFα stimulation is attenuated and NF-κB related cytokines are down-regulated by a strong hypoxia of 1% oxygen.

Materials and Methods

Materials

Reagents for SDS-PAGE were supplied by Amersham Pharmacia Biotech (Braunschweig, Germany) and by Sigma (Munich, Germany). Human specific anti-phospho-NF-κB (pp65) (Ser536), anti-NF-κB (p65), anti-IκBα and anti-phospho-IκBα (Ser32) antibodies were supplied by Cell Signaling Technology (Frankfurt, Germany). Human anti-HIF-1α was supplied by Novus Biologicals Inc. (Cambridge, GB). Human anti-actin and anti-GLUT1 antibody came from Abcam (Cambridge, GB) and HRP-conjugated goat anti-rabbit and goat anti-mouse IgG antibodies from Promega (Mannheim, Germany). Collagenase NB4 was obtained from Serva (Heidelberg, Germany). FCS was supplied by Gibco (Invitrogen, Carlsbad, CA, USA). Troglitazone and human recombinant TNFα were obtained from Sigma (Munich, Germany). Complete protease inhibitor cocktail tablets were from Roche (Mannheim, Germany). All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma (Munich, Germany) and Applichem Biochemica (Darmstadt, Germany). Gas mixture was supplied by Air Liquide (Düsseldorf, Germany).

Adipocyte isolation and culture

Subcutaneous adipose tissue was obtained from healthy lean or moderately overweight women (n=26, body mass index 27.0 ± 4.4, and aged 43.2 ± 9.6 years) undergoing plastic surgery. The procedure was approved by the ethical committee of the Heinrich-Heine-University (Düsseldorf, Germany). Preadipocytes were isolated by collagenase digestion of adipose tissue as previously described by us.[14] Isolated cell pellets were resuspended in Dulbecco's modified Eagles/Hams F12 (DMEM/F12) medium supplemented with 10 % FCS, seeded in 75 cm² culture flasks or six-well culture dishes and maintained at 37° C with 5 % CO_2. After overnight incubation, cultures were washed and further incubated in an adipocyte differentiation medium (DMEM/F12, 33 µmol/l biotin, 17 µmol/l d-panthothenic-acid, 66 nM insulin, 1 nM triiodo-L-thyronine, 100 nM cortisol, 10 µg/ml apo-transferrin, 50 µg/µl

gentamycin, 15 mmol/l HEPES, 14 nmol/l NaHCO3, pH 7.4) for 14 days with medium change every 2-3 days and addition of 5 µM troglitazone for the first 3 days. The degree of differentiation was determined by oil red staining.

Hypoxia treatment

For hypoxia treatment differentiated adipocytes at day 14 were incubated in α-modified Eagles medium and exposed to a gas mixture containing 1 % O_2, 5 % CO_2 and 94 % N_2 in MIC-101 modular incubator chambers (Billups-Rothenburg, Del Mar, CA, USA) at 37 °C for indicated times.

Immunoblotting

All cells were treated as indicated and lysed in a buffer containing 20 mM MOPS, pH 7.0, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 60 mM β-glycerophosphate, pH 7.2, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 % TritonX100 and Complete protease inhibitor cocktail. Lysates were sonificated at 4 °C and the suspension was centrifuged at 10.000 x *g* for 20 min. Thereafter, 10 µg of protein were separated by SDS-PAGE using 10 % horizontal gels and transferred to polyvenylidenefluorid (PDVF) membrane in a semidry blotting apparatus. Membranes were blocked with Tris-buffered saline containing 0.1 % Tween and 5 % nonfat dry milk or BSA and subsequently incubated overnight with the appropriate antibodies. After washing, membranes were incubated with secondary HRP-coupled antibody and processed for enhanced chemiluminescence detection using Immobilon HRP substrate (Millipore, Billerica, MA, USA). Signals were visualized and evaluated on a VersaDoc 4000 MP work station (BioRad, Munich, Germany).

Cytokine array and ELISA analysis

Conditioned media (CM) from normoxic and hypoxic adipocytes were analyzed by cytokine array and ELISA measurements. Based on former studies[15] CM's were collected over a period of 48 hours to make sure that

differences of low abundant proteins were in the range of array and ELISA sensitivity. RayBio human cytokine arrays C-series 2000 (Raybiotech, Norcross, GA, USA) were performed with undiluted CM according to the manufactures instructions and signals were visualized and evaluated on a LUMI Imager (Boehringer, Mannheim, Germany) work station. Concentration of IL-6, VEGF, MCP-1 and IL-8 secretion was determined using ELISA kits purchased from Gen-Probe (San Diego, CA, USA), Adiponectin concentration was determined with an ELISA kit from Biovendor (Heidelberg, Germany). The assays were performed in duplicates.

RNA-isolation and quantitative real-time PCR

Total RNA was isolated and reverse transcribed using the RNeasy Lipid Tissue and Omniscript Reverse Transcription kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Gene expression was determined by quantitative real-time PCR using QuantiTect primer assays and SYBR green reagents (Qiagen, Hilden, Germany) with 1.25-25ng of generated cDNA on a Step One Plus Cycler (Applied Biosystems, Carlsbad, CA, USA). POLR2A was chosen as a housekeeping gene as it was used as an appropriate reference gene in hypoxia studies of the current literature [16,17]. Expression levels of investigated genes were normalized to POLR2A. Gene expression was analyzed via the ΔΔCt method.

Presentation of data and statistics

Data are expressed as mean ± s.e.m. Paired two-tailed Student's t test or one-way ANOVA (post-hoc test: Bonferroni's multiple comparison test) were used to determine statistical significance. All statistical analyses were done using Prism (GraphPad, La Jolla, CA, USA) considering a p-value of less than 0.05 as statistically significant. Corresponding significance levels are indicated in the figures.

Results

Hypoxia alters the secretory profile of human primary adipocytes

In vitro differentiated human primary adipocytes were incubated under normoxia (21% O_2) or a strong hypoxia of 1% oxygen for up to 24 hours. After 8 hours of hypoxia an accumulation of the transcription factor hypoxia-inducible-factor (HIF)-1α of more than 100 fold could be detected as well as the accumulation of its target gene glucose transporter (GLUT)-1 of about 1.6 fold (Fig.1A). Furthermore, the mRNA level of HIF-1α and GLUT1 after 8 and 24 hours of hypoxia were determined by quantitative real-time PCR and showed the already described reduction in HIF-1α mRNA as well as an elevated GLUT1 mRNA expression[16] (Fig.1B-C).

Subsequently, the secretory products of normoxic and hypoxic adipocytes were collected as adipocyte conditioned medium (CM) for 48 hours. Human cytokine array analysis was used as an initial screening method to detect alterations in the secretory profile due to the hypoxic treatment (Fig.2A). A prominent reduction of monocyte chemotactic protein (MCP)-1 (75%) in CM of hypoxic adipocytes could be observed, as well as reduced concentrations of factors such as growth regulated oncogene (Gro)-α (73 %), eotaxin (37 %) and soluble-TNF-receptor1 (sTNF-R1) (35 %) (Fig.2B). In agreement with earlier reports, reduced adiponectin (61%) and elevated IL-6 (159%), leptin (5672%) and VEGF(139%) secretion could be detected by the cytokine array analysis (Fig.2B). The protein levels of MCP-1, IL-6, leptin, VEGF and adiponectin were further quantified by ELISA measurements (Fig.3A-C). Hereby we could verify a significantly reduced release of MCP-1 by human primary adipocytes under hypoxic conditions of 790 pg/ml vs. 1286 pg/ml under normoxia per 350.000 cells (Fig.3A).

Hypoxia attenuates TNFα-induced NF-κB signaling

To identify signaling pathways, which were involved in the secretory changes, we analyzed normoxic and hypoxic adipocytes (8 hours, 1% oxygen) with a Kinex™ Antibody Microarray as a first screening method. This analysis showed alterations in the expression level of diverse proteins involved in NF-κB signaling. These alterations were validated in further

experiments, and a significant down-regulation of NF-κB (24%) and IκB kinase (IKK)-α (46%) but no significant regulation of the inhibitor of NF-κB (IκBα) and IKKβ could be observed (Fig.4A).

To test whether hypoxia could alter NF-κB signaling in adipocytes, cells were pretreated for 8 hours with normoxia or hypoxia. Thereafter, normoxic and hypoxic adipocytes were stimulated with 50 ng TNFα for 5 to 60 minutes. Hypoxic treatment resulted in a significantly reduced phosphorylation of NF-κB after 5 and 10 minutes of TNFα stimulation, as well as to a significantly reduced phosphorylation of IκBα after 5 minutes (Fig. 4B-C). Furthermore, to test whether this effect was concentration dependent, we used lower TNFα concentrations. Treatment of hypoxic adipocytes with 5 ng TNFα, but not with lower concentrations, for 10 minutes showed a reduction in NF-κB phosphorylation (Fig. 4D).

Hypoxia down-regulates the TNFα-receptor TNF-R1

In order to identify a potential cause for the observed attenuation of the TNFα-induced NF-κB signaling, the expression levels of the TNF-receptors TNF-R1 and -R2 were analyzed. TNF-R1 protein expression was analyzed via Western Blotting and quantitative real-time PCR, whereas TNF-R2 expression was only detectable by quantitative real-time PCR. For TNF-R1 a significant down-regulation of nearly 30% after 8 and 24 hours of 1% hypoxia could be observed at the protein level (Fig.5A), but without an effect at the mRNA level (Fig.5B). In contrast, TNF-R2 showed a significant higher mRNA expression (182%) after 8 hours of hypoxia compared to normoxic adipocytes which normalized after 24 hours (Fig.5C).

Hypoxia reduces the TNFα-induced chemokine secretion

As a consequence of the hypoxia induced down-regulation in NF-κB signaling, we hypothesized that changes in NF-κB related gene expression and secretion would occur. This hypothesis was supported by the reduced amounts of MCP-1, Gro-α, eotaxin and sTNF-R1 in CM of hypoxic adipocytes, all proteins that are NF-κB related genes. After an 8 hour pre-incubation of adipocytes under normoxia or hypoxia, 50 and 5 ng TNFα was added and incubated under normoxia or hypoxia for an additional 18

hours. CM's were collected and analyzed by ELISA for the chemokines MCP-1 and IL-8. Normoxic and hypoxic cells responded to the TNFα stimulation with a significantly enhanced release of MCP-1 and IL-8. However, incubation under hypoxia with and without TNFα led to a significantly lower secretion of MCP-1 compared to the normoxic control, as well as to a decreased IL-8 secretion under TNFα and hypoxia treatment of about 25% (Fig. 6A-B). Furthermore, a significant down-regulation of the MCP-1 mRNA level after treatment with 5 ng TNFα of about 25% could be detected, whereas the mRNA level of IL-8 showed no significant changes.

Discussion

Adipose tissue inflammation is presumed to be the consequence of a dysregulation in adipokine secretion, infiltration of inflammatory cells like macrophages and the occurrence of local tissue hypoxia, which all together contributes to obesity-related disorders such as the metabolic syndrome and insulin resistance.[18-20] Furthermore, hypoxia is shown to alter the secretory profile of adipocytes and especially elevates angiogenic and inflammatory factors.[8,16,21] In our cell culture model of hypoxia, we can reproduce the described effects of elevated levels of leptin, VEGF and IL-6, and reduced adiponectin. Moreover, we can show for the first time in human primary adipocytes that hypoxia leads to a reduction in MCP-1 secretion under basal and TNFα-stimulated conditions. This is a surprising observation as this chemokine acts as an attractant for monocytes[22] with pro-inflammatory properties and it would be expected that hypoxia rather induces it. Additionally, we observed that other adipokines like Gro-α, eotaxin and sTNF-R1 are reduced and it is notable that these factors are all NF-κB regulated genes. To our knowledge this is the first report that these adipokines are less released from adipocytes due to hypoxic treatment. This observation indicates that hypoxia may also exert unexpected beneficial anti-inflammatory effects on adipocyte chemokine expression and release and therefore a possibly altered behavior on immune cell attraction and infiltration into adipose tissue. This effect could be mediated by an altered NF-κB signaling, as we also demonstrate that

hypoxia in adipocytes leads to an attenuation of TNFα-induced NF-κB signaling and eventually a reduced TNFα induced secretion of MCP-1 and IL-8 during chronic hypoxia. This attenuation should not be caused by an enhanced hypoxia induced autocrine TNFα release and a resulting resistance to this cytokine, as we neither observed elevated mRNA levels nor detectable TNFα amounts in CM of hypoxic adipocytes (data not shown). As it is suggested that adipose tissue located macrophages are an important source for various pro-inflammatory cytokines such as TNFα and IL-6[23], it can be assumed that the attenuation of hypoxic adipocytes in NF-κB signaling, and eventually in MCP-1 release, is a protective mechanism towards the paracrine effects of TNFα.

Current studies showed that hypoxia could activate NF-κB activity in various cell lines such as Jurkat, HeLa or 3T3-L1 cells.[8,12,13] In contrast, a recent study on endothelial cells reported a reduction in NF-κB activity and IL-8 protein expression due to hypoxia.[24] Although the effect of hypoxia to induce NF-κB signaling seems to be cell type dependent, our finding of reduced MCP-1 mRNA levels in human primary adipocytes is in accordance with other current studies, that demonstrated as well reduced MCP-1 mRNA expression under hypoxia in various cell types such as macrophages, cardiac cells, ovarian cancer cells and a human adipocyte cell line.[16,25-27] On the other hand, it is also reported that hypoxia could induce MCP-1 expression in human dermal fibroblasts and hypoxic brain.[28,29] However, the mechanism behind the down-regulation of MCP-1 mRNA is not clear, but the group of Safronova et al. suggested a histone deacetylases dependent one.[30] They report that hypoxia induces the deacetylation of histones and therefore repression of MCP-1 mRNA transcription. The group also analyzed the effect on IL-8 mRNA and protein expression, but in contrast to MCP-1 they observed an induction of IL-8. This is in contrast to our observation, as we observed a reduction in IL-8 protein release from TNFα-treated hypoxic adipocytes, whereas we could not detect a significant change in basal as well as TNFα-induced IL-8 mRNA levels. This effect may be caused by a post-transcriptional mechanism like suppression of mRNA translation which is described to occur under hypoxia.[31,32]

Our results suggest that hypoxic adipocytes are less responsive to TNFα treatment. This is supported by the reduced TNFα-induced phosphorylation of IκBα, which is therefore not ubiquitinated and

degraded in the same time-dependent manner as it occurs under normoxic conditions. This effect should result in a decreased release of NF-κB, which is necessary for activation and translocation into the nucleus, and indeed we can show a decreased NF-κB phosphorylation level and consequently activation under hypoxia with direct effects on its target genes. The reduction in MCP-1 mRNA expression and protein secretion displays this direct consequence of the reduced responsiveness of hypoxic adipocytes towards TNFα stimulation and NF-κB signaling.

It is suggested that hypoxia activates NF-κB activity and related gene expression mainly through the canonical pathway.[33] The activation of the canonical pathway depends on IKKβ whereas the non-canonical pathway relies on the function of IKKα.[34] Our expression analysis of the IKK subunits showed an unaltered expression level of IKKβ, whereas IKKα was significantly down-regulated. This implies that the impairment in TNFα-induced NF-κB signaling is not due to a reduced availability of IKKβ and its activity. Regarding the reduced expression level of IKKα it could be assumed that hypoxia also exerts a modulatory effect on the non-canonical pathway in human adipocytes which has to be further investigated.

It is reported that in the majority of cells TNFα signals through the TNF-receptor1 (TNF-R1), which is regarded as the main receptor for canonical NF-κB signaling.[35] Current investigations suggest that activation of TNF-R1 results in an elevated inflammatory state and that it promotes cell death[36-38], whereas TNF-R2 seems to have anti-apoptotic and anti-inflammatory effects as shown in different tissues and cell types.[38-41] In this study we demonstrate that TNF-R1 is less expressed in hypoxic compared to normoxic adipocytes, and this effect cannot be explained by an enhanced membrane shedding, as we also observed reduced sTNF-R1 amounts in CM of hypoxic adipocytes. Regarding the higher mRNA expression of TNF-R2 our data suggests that hypoxia induces a less inflamed state by reducing pro-inflammatory signaling via TNF-R1 and elevated levels of TNF-R2. This switch in inflammatory signaling may explain the observed attenuation of the TNFα-induced NF-κB activation, and the altered expression pattern of the chemokines MCP-1, IL-8, Gro-α and eotaxin.

In summary, our study shows that hypoxia in human primary adipocytes exerts beneficial effects under basal and inflammatory

conditions, which are reflected by reduced basal and TNFα-induced MCP-1 secretion due to an attenuated NF-κB signaling. This effect is possibly mediated by a reduced expression and availability of the receptor TNF-R1. It may be speculated that this unexpected response towards low oxygen concentrations reflects a mechanism to limit inflammation and immune cell infiltration in areas of the adipose tissue, where adipocytes are exposed to hypoxia and high concentrations of inflammatory cytokines like TNFα. Enhanced immune cell infiltration may contribute to the hypoxic and inflammatory environment, as these cells are described to be highly metabolic active with high consumption rates of oxygen and are expected to release high amounts of inflammatory factors like TNFα.[10,21,23] On the other hand, current studies reported that macrophage migration is reduced under hypoxia[27,42] and it is suggested that this inhibition may stimulate the accumulation and repress the egression of macrophages in inflamed regions.[43] However, the repression of chemokine expression and secretion by hypoxia through an attenuated NF-κB signaling should be beneficial for adipose tissue inflammation and homeostasis, especially when adipocytes are affected by macrophage derived TNFα. The direct cross-talk of these less responsive adipocytes with other adipose tissue located cells like macrophages has to be further determined. This study indicates that hypoxia is a complex state that is not only involved in adipose tissue inflammation but may also trigger an attempt to maintain tissue function.

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Conflict of interest statement: There is no conflict of interest to declare.

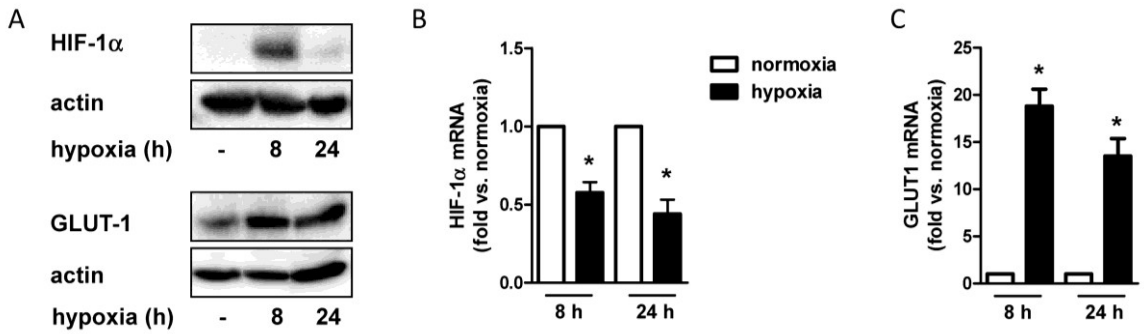

Figure 5.1: Induction of HIF-1α and GLUT1 under hypoxia treatment

In vitro differentiated human primary adipocytes were incubated under normoxia (21% O_2) or in a hypoxia chamber under 1% of oxygen for 8 and 24 hours. Afterwards cells were harvested and directly lysed. (A) Total lysates (10µg) were resolved by 10 % SDS-Page and blotted to PDVF membranes. Membranes were blocked with 5% milk and incubated overnight with anti-HIF-1α or anti-GLUT1 antibody. After incubation with the appropriate horseradish-peroxidase-coupled secondary antibody signals were detected by ECL. Representative blots are shown. (B-C) mRNA was extracted from normoxic and hypoxic cell lysates, reverse transcribed into cDNA and quantitative real-time PCR performed using specific primer sets to detect the expression level of HIF-1α and GLUT1 mRNA. Data were normalized to the mRNA expression level of POLR2A and expressed relative to the normoxic control. Data sets are mean values ± s.e.m. of 4-5 independent experiments. *$p<0.05$ compared to the normoxic control.

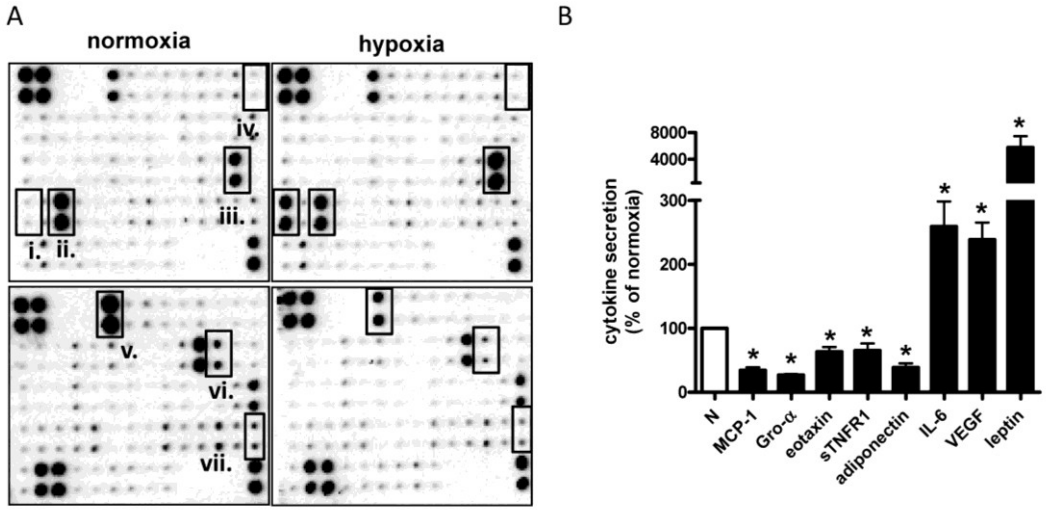

Fig. 5.2: Cytokine array analysis of normoxic and hypoxic CM of human adipocytes.

Secretory products of differentiated human primary adipocytes from four individuals were collected over 48 hours (CM) under normoxia or 1 % hypoxia. (A) Cytokine array membranes were incubated overnight with CM and further processed according to the manufactures instructions. Representative arrays of a normoxic and hypoxic CM from the same individual are shown. i: leptin, ii. MCP-1, iii: IL-6, iv: eotaxin, v: adiponectin, vi: Gro-α, vii: sTNF-R1 (B) Protein signals were quantified, normalized to the positive control of the array and compared with the normoxic control (N). Data sets are mean values ± s.e.m of 4-5 different experiments. *$p<0.05$ compared to the normoxic control.

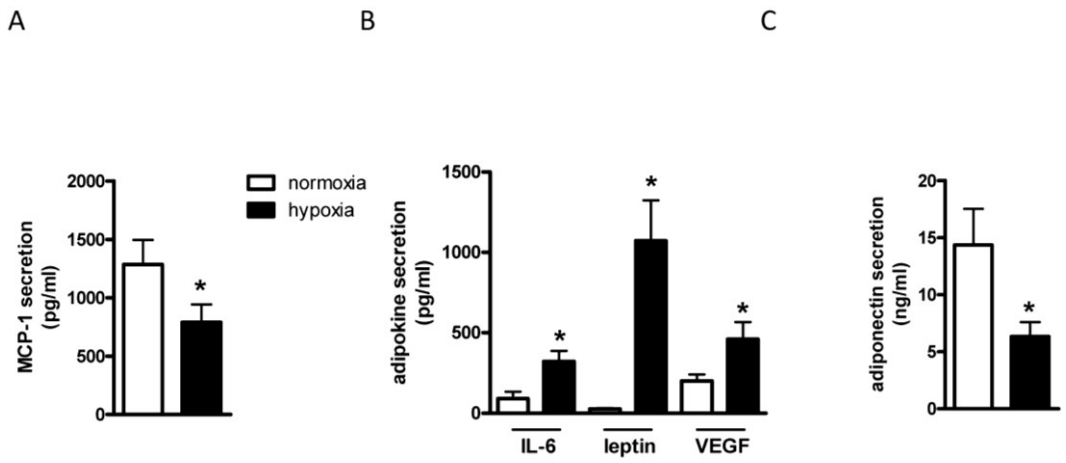

Figure 5.3: Adipokine secretion from normoxic and hypoxic adipocytes

Protein concentration in CM of (A) MCP-1, (B) IL-6, leptin, VEGF and (C) adiponectin were determined by ELISA. Data sets are mean values ± s.e.m. of 4-7 different CM. *$p<0.05$ compared to the normoxic control.

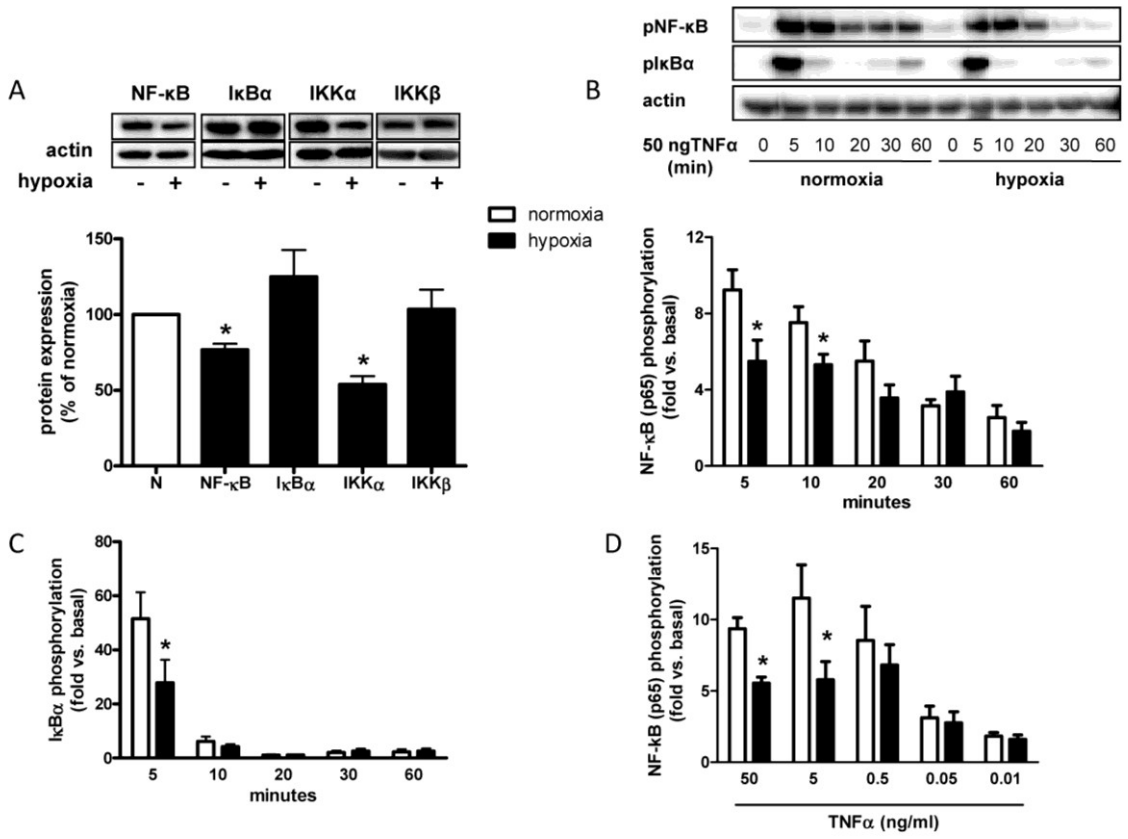

Figure 5.4: Expression of NF-κB signaling proteins under hypoxia and phosphorylation kinetics of NF-κB and IκBα during TNFα stimulation

Differentiated human primary adipocytes were incubated for 8 hours under normoxia or 1% hypoxia. Afterward cells were treated with 50 ng TNFα and directly lysed at the indicated time points. Total lysates (10µg) were analyzed by western blotting as described in Fig.1 (A) Expression level of NF-κB, IκBα, IKKα and IKKβ after 8 hours of normoxia or hypoxia. (B-C) Kinetic of NF-κB and IκBα phosphorylation, shown as the raise according to basal normoxic or hypoxic phosphorylation. (D) Phosphorylation of NF-κB after 10 minutes of stimulation with different concentrations of TNFα. Representative blots are shown. Data sets are mean values ± s.e.m. of 4-10 independent experiments normalized to actin. *p<0.05 compared to the normoxic value.

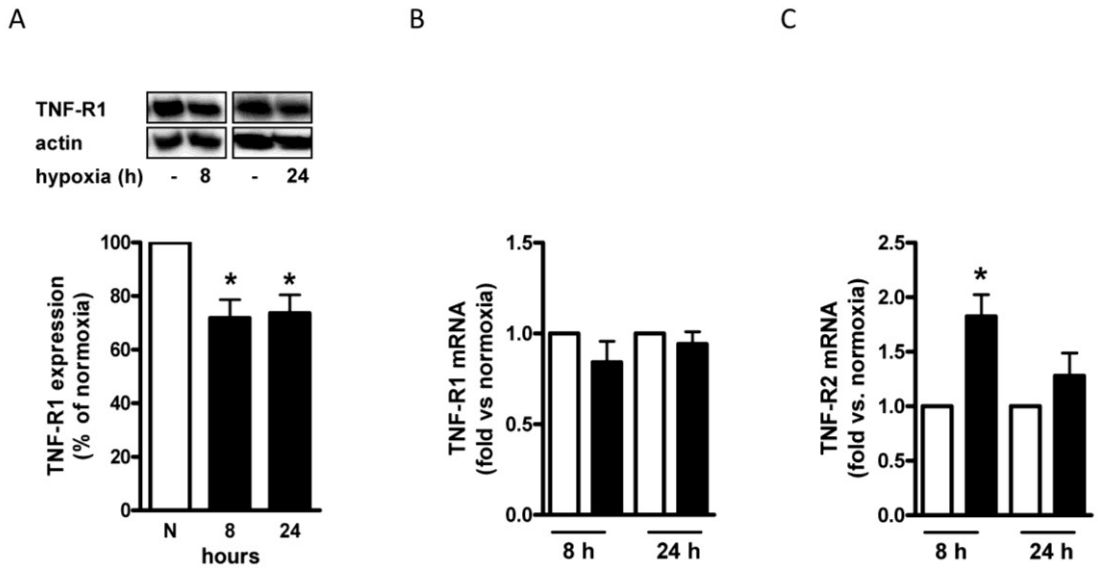

Figure 5.5: Expression of TNF-receptors under hypoxia

Differentiated human primary adipocytes were incubated under hypoxia for 8 and 24 hours. Afterwards cells were harvested and directly lysed. (A) Total lysates (10µg) were analyzed by western blotting as described in Fig.1 and incubated with anti-TNF-R1. Representative Western Blots are shown. (B-C) mRNA was isolated and processed for quantitative real-time PCR as described in Fig.1. Data are mean values ± s.e.m. of 4-6 independent experiments. *$p<0.05$ compared to the normoxic control.

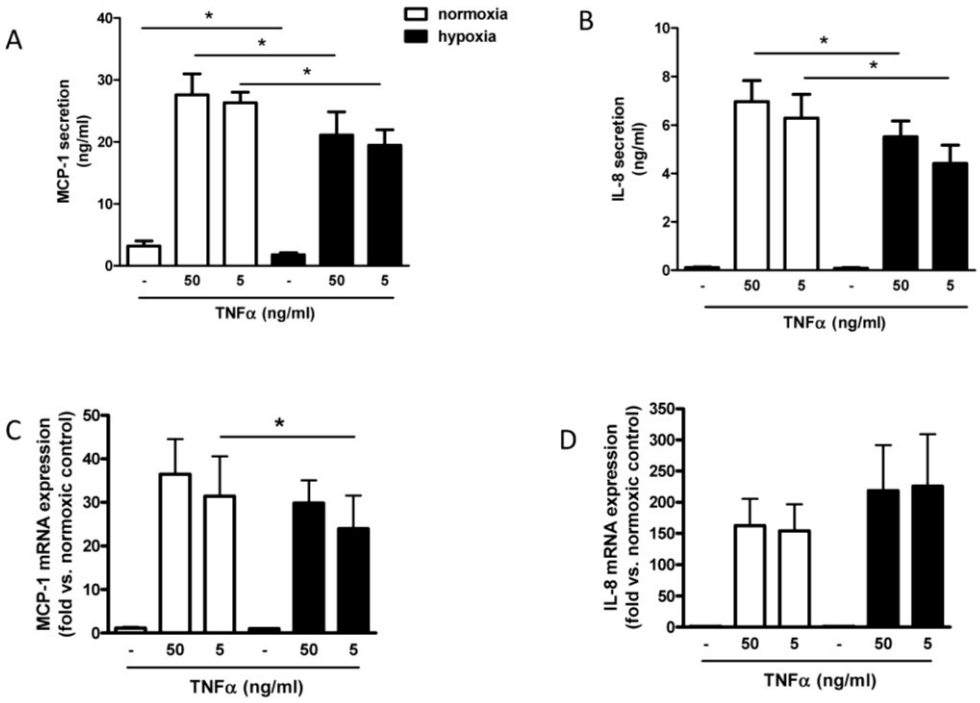

Figure 5.6: TNFα-stimulated chemokine secretion and mRNA expression

Differentiated human primary adipocytes were incubated under normoxia and hypoxia for 8 hours. Afterward 50 or 5 ng TNFα was added and cells further incubated for 18 hours. CM were collected, cells harvested and directly lysed. By ELISA, secreted amounts of (A) MCP-1 and (B) IL-8 were determined. mRNA was isolated and processed for quantitative real-time PCR for (C) MCP-1 and (D) IL-8 mRNA expression level as described in Fig.1. Data sets are mean values ± s.e.m. of 3-8 independent experiments. *$p<0.05$ compared to the normoxic control.

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Chapter 6

Study 5

Differentiation of human adipocytes at physiological oxygen levels results in increased adiponectin secretion and isoproterenol-stimulated lipolysis ǂ

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Running title: Physiologic oxygen tensions affect adiponectin secretion and lipolysis in adipocytes.

Abbreviations: AT, adipose tissue; CM, conditioned medium; SkMC, skeletal muscle cell; SMC, smooth muscle cell; IL-6, interleukin-6; DPP4, dipeptidyl peptidase 4; MCP-1, macrophage chemotactic protein-1; VEGF-A, vascular endothelial factor-A; ATGL, adipose triglyceride lipase; HSL, hormone sensitive lipase; PPARγ, peroxisome proliferator-activated receptor gamma; C/EBPα, CCAAT/enhancer binding protein alpha; hMSC, human bone marrow stromal cells

Abstract

Adipose tissue (AT) hypoxia occurs in obese humans and mice. Acute hypoxia in adipocytes causes dysregulation of adipokine secretion with an increase in inflammatory factors and diminished adiponectin release. O_2 levels in humans range between 3-11 % revealing that conventional in vitro culturing at ambient air and acute hypoxia treatment (1 % O_2) are performed under non-physiological conditions. In this study, we mimicked physiological conditions by differentiating human primary adipocytes under 10 % or 5 % O_2 in comparison to 21 % O_2. Induction of differentiation markers was comparable between all three conditions. Adipokine release by adipocytes differentiated at lower oxygen levels was altered, with a marked upregulation of adiponectin, IL-6 and DPP4 secretion, and reduced leptin levels compared to adipocytes differentiated at 21 % O_2. Isoproterenol-induced lipolysis was significantly elevated in adipocytes differentiated at 10 % and 5 % compared to 21 % O_2. This effect was accompanied by increased protein expression of beta-1 and -2 adrenergic receptor, HSL and perilipin. Conditioned medium (CM) of adipocytes differentiated at the three different conditions was generated for stimulation of human skeletal muscle cells (SkMC) or smooth muscle cells (SMC). CM-induced insulin resistance in SkMC was comparable for the different CMs. However, the SMC proliferative effect of CM from adipocytes differentiated at 10 % O_2 was significantly reduced compared to 21 % O_2. This study demonstrates that oxygen levels during adipogenesis are important factors altering adipocyte functionality such as adipokine release, in particular adiponectin secretion, as well as the hormone-induced lipolytic pathway.

Key Words

Hypoxia, obesity, adipocytes, adipocyte differentiation, lipolysis, adipokines, insulin resistance

Introduction

Adipose tissue (AT) is nowadays established to act as an important endocrine organ and not only as a fuel storage site. Factors released by AT, including fatty acids and a variety of proteins and peptides collectively named adipokines, are associated with the well described chronic low-grade inflammation of enlarged AT, as well as with obesity-related disorders such as insulin resistance, diabetes mellitus and the metabolic syndrome.[1-3]

It is assumed that local areas of hypoxia could occur in expanding AT of obese subjects, contributing to chronic low-grade inflammation of AT.[4] The association of hypoxia and inflammation was shown in diverse obese mouse models, revealing a reduction of the AT oxygen level down to 2 % in obese animals.[5-7] AT oxygenation in lean and obese human subjects is currently described by two groups.[8,9] The first study from Pasarica et al. demonstrated a moderate reduction in AT oxygen level of obese compared to lean patients (6.2 ± 1.4 % vs. 7.3 ± 1.2 % O_2)[8], while the group of Goossens et al. described elevated oxygen levels in obese compared to lean controls (8.9 ± 0.8 % vs. 5.9 ± 0.5 %)[9]. Both studies used different methods of oxygen measurement, thus Pasaricaet al.[8] assessed AT oxygen tension by a Clark electrode, whereas Goossens et al.[9] used continuous measurement based on microdialysis. Furthermore, both studies differ in their study design regarding the subject matching for age, gender, ethnicity and health aspects such as type 2 diabetes. Due to the fact that these studies tested local oxygen concentrations, it could not be excluded that higher or lower level of oxygen in different regions of the AT might occur. However, these studies demonstrated that AT oxygenation could range between 3 % and 11 % O_2.

The role of AT oxygen tension is not completely understood and subject of current investigations. In general, experiments are performed in a non-physiological model using human or murine adipocytes, which were cultured and treated at ambient air, which is about 21 % O_2. In general the experimental settings include differentiation and cultivation at 21 % O_2, which is followed by acute hypoxia induced with 1 % O_2.[10,11] As it is reported that oxygen tensions could range between 3 – 11 % O_2 in human AT[9], this reveals that conventional culture and hypoxic treatment do not reflect physiological conditions of human lean or obese AT. The aim

of the preset study was to mimic more physiological conditions during differentiation of human primary adipocytes and to identify differences in adipocyte secretion and function in comparison to 'standard' conditions. Using two different oxygen tensions normally regarded as hypoxic conditions (10 % and 5 % vs. 21 % O_2), we observed a similar differentiation pattern under all oxygen tensions. However, adipokine release by adipocytes differentiated at more physiological oxygen conditions is altered and in particular adiponectin levels were markedly increased. Furthermore, we could show that isoproterenol-stimulated lipolysis is elevated in adipocytes differentiated under 10 % and 5 % O_2 which might be mediated by elevated beta-adrenergic receptor expression as well as increased HSL and perilipin expression levels. This study shows for the first time that the oxygen level during adipocyte differentiation is important for adipocyte functionality such as adipokine secretion and lipolytic activity.

Materials and Methods

Materials

Reagents for SDS-PAGE were supplied by Amersham Pharmacia Biotech and by Sigma. Human specific anti-phospho-Akt (Ser473) (#9271), anti-phospho-Akt (Thr308) (#9275), anti-Akt (#9272), anti-Glut4 and anti-HSL (#4107) antibodies were supplied by Cell Signaling Technology. Human anti-Glut4 (#MAB1262) was purchased from R&D systems. Human anti-actin (ab6276), anti-adiponectin (ab22554), anti-Glut1 (ab32551), anti-beta 1 (ab77189) and anti-beta 2 adrenergic receptor (ab69598) antibodies came from Abcam. Human anti-ATGL (sc-365278) and HRP-conjugated donkey anti-goat (sc-2020) antibodies were supplied by Santa Cruz biotechnology. Human anti-perilipin antibody (IMG-30288) came from Imgenex and HRP-conjugated goat anti-rabbit (W4011) and goat anti-mouse (W4021) IgG antibodies from Promega. Collagenase NB4 (17465.02) was obtained from Serva (Heidelberg, Germany). FCS (#10270-106), Dulbecco's modified Eagles/HAM F12 (DMEM/F12) medium (#42400-010), α-modified Eagle's (αMEM) medium

(#11900-016) and Ham's F-12 medium (#21700-026) was supplied by Gibco (Invitrogen, Carlsbad, CA, USA). Troglitazone and Oil Red O (O0625) were obtained from Sigma Aldrich. Complete protease inhibitor cocktail tablets (#11697498001) were from Roche. All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma Aldrich or Applichem Biochemica.

Adipocyte isolation and culture

Subcutaneous AT was obtained from healthy lean or moderately overweight women (n=39, body mass index 26.8 ± 3.8, and aged 41.0 ± 12.3 years) undergoing plastic surgery. The procedure was approved by the ethical committee of the Heinrich-Heine-University (Düsseldorf, Germany). Preadipocytes were isolated by collagenase digestion of AT as previously described by us.[12] Isolated cell pellets were resuspended in DMEM/F12 medium supplemented with 10 % FCS, seeded in 75 cm^2 culture flasks, six-well or twelve-well culture dishes and maintained at 37°C with 5 % CO_2. After cells were grown until confluence, cultures were washed and further incubated in an adipocyte differentiation medium (DMEM/F12, 33 µmol/l biotin, 17 µmol/l d-panthothenic-acid, 66 nM insulin, 1 nM triiodo-L-thyronine, 100 nM cortisol, 10 µg/ml apo-transferrin, 50 µg/µl gentamycin, 0.25 µg/ml amphoterecin B, 15 mmol/l HEPES, 14 nmol/l NaHCO3, pH 7.4) for 14 days under normoxia (21% O_2 supplemented with 5 % CO_2) or in an Xvivo hypoxia chamber system (Biospherix, USA) under 10% or 5% O_2 supplemented with 5 % CO_2 and respective concentrations of nitrogen. Medium was changed at the appropriate oxygen tension every 2-3 days with addition of 5 µM troglitazone for the first 3 days. Oil Red O staining was performed at day 14 of differentiation. Therefore, cultures were washed with ice-cold PBS and fixed with a solution containing 71 % picric acid, 24 % acetic acid and 5% formaldehyde for 2 hours. Afterwards, cells were washed with PBS, further incubated with 0.3 % Oil-Red O solution for 10 minutes and washed again with PBS. Subsequently, samples were photographed with a Canon EOS digital camera. To determine lipid droplet size the imaging software ImageJ was used analyzing pictures of 20 fold magnification. Triglyceride content of differentiated adipocytes was determined using a Triglyceride Quantification Kit from Biovision (#K622-100) due to the

manufacturer's instructions. To generate adipocyte conditioned-medium (CM) from adipocytes differentiated at 21 %, 10 % and 5 % O_2, adipocytes were incubated with α-modified Eagles medium (αMEM) at the distinct oxygen levels for 48 hours as described in a previous study.[13]

Immunoblotting

All cells were treated as indicated and lysed in a buffer containing 20 mM MOPS, pH 7.0, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 60 mM β-glycerophosphate, pH 7.2, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol (DTT), 1 % TritonX100 and Complete protease inhibitor cocktail. Lysates were sonificated at 4 °C and the suspension was centrifuged at 10.000 x *g* for 20 min. Protein concentration was determined by using a Bradford protein assay. Thereafter, 10 µg of protein were separated by SDS-PAGE using 10 % horizontal gels and transferred to polyvenylidenefluorid (PDVF) membrane in a semidry blotting apparatus. Membranes were blocked with Tris-buffered saline containing 0.1 % Tween and 5 % nonfat dry milk or BSA and subsequently incubated overnight with the appropriate antibodies. After washing, membranes were incubated with secondary HRP-coupled antibody and processed for enhanced chemiluminescence detection using Immobilon HRP substrate (Millipore, #WBKLS0500). Signals were visualized and evaluated on a BioRad VersaDoc 4000 MP work station.

ELISA analysis

Secretory products from normoxic and hypoxic adipocytes were collected over 24 hours and analyzed by ELISA measurements. Concentration of adiponectin (RD191023100; detection range 1–150 ng/ml) and VEGF-A (RBMS277/2R; detection range 15.6-1000 pg/ml) was determined with ELISA kits from Biovendor, IL-6 (#950.030.192; detection range 6.25-200 pg/ml) and MCP-1 (#650.110.192; detection range 16-1000 pg/ml) was determined using ELISA kits purchased from Gen-Probe and leptin (DLP00; detection range 15.6-1000 pg/ml) and DDP-4 (DC260; detection range 0.31-20 ng/ml) concentrations were analyzed by ELISA kits purchased from R&D systems. If necessary, samples were diluted with a provided dilution buffer of the manufacturer

prior to the assay, which was performed in duplicates due to the manufacturer's instructions.

RNA-isolation and quantitative real-time PCR

Total RNA was isolated and reverse transcribed using the RNeasy Lipid Tissue (Qiagen, #74804) and Omniscript Reverse Transcription kit (Qiagen, #205113) according to the manufacturer's instructions. Gene expression was determined by quantitative real-time PCR using QuantiTect primer assays and SYBR green reagents (Qiagen, POLR2A #QT00033264, CEBPA #QT00203357, PPARG #QT00029841, SYBR Green #204145) with 2.5 – 25ng of generated cDNA on a Step One Plus Cycler (Applied Biosystems). POLR2A was used as a housekeeping gene as done in a previous study by us.[14] Expression levels of investigated genes were normalized to POLR2A. Gene expression was analyzed via the □□Ct method and compared to the designated control.

Measurement of lipolysis

Adipocytes were cultured in twelve-well culture dishes and differentiated at 21 %, 10 % and 5 % of oxygen. Lipolysis experiments were performed under the designated oxygen levels. Therefore, cells were starved 24 hours in αMEM, washed with PBS and incubated for 4 hours in Krebs-Ringer-buffer containing 1 % BSA. Lipolytic stimulation was induced with designated concentrations of isoproterenol. Glycerol release was measured using Glycerol Free reagent (#F6428) from Sigma.

Culture of skeletal muscle cells and smooth muscle cells

Primary human skeletal muscle cells (SkMC) isolated from rectus abdominis muscle of two healthy Caucasian donors (2 females, 33 and 37 of age) were supplied as proliferating myoblasts from PromoCell and cultured as described in our earlier study.[12] For an individual experiment, myoblasts were seeded in six-well culture dishes at a density of 10^5 cells/well and were grown in αMEM/Ham's F-12 medium (PromoCell) containing skeletal muscle cell growth medium supplement pack up to near-confluence. The cells were then differentiated in αMEM for 6 days.

Differentiated cells were incubated for 24 hours with the indicated adipocyte conditioned-medium (CM). Afterwards, cells were stimulated with 100 nmol/l insulin for 10min.

Primary human smooth muscle cells (SMC) from two different donors (Caucasian, male, 58 years old; female, 56 years old) were supplied as proliferating cells from Lonza and PromoCell and kept in culture according to the manufacturer's protocol. SMC were characterized by morphologic criteria and by immunostaining with smooth muscle α-actin.

To analyze SMC proliferation DNA synthesis of proliferating cells was measured by incorporation of 5-bromo-2'-deoxyuridine (BrdU). Therefore, SMC were seeded in 96 well culture dishes and allowed to attach for 24 hours, cultured in Smooth Muscle Cell Growth Medium 2 (#C-22062) supplemented with 5% FCS and an appropriate supplemental mix (PromoCell).Following serum starvation for an additional 24 h period, cells were stimulated for 24 hours with different CM's in the presence of BrdU (10 µM). To determine proliferation the BrdU ELISA Kit (#11669915) from Roche was used according to the manufacturer's protocol. Signals were visualized and evaluated on a LUMI Imager work station (Boehringer, Mannheim, Germany).

Presentation of data and statistics

Data are expressed as mean ± SEM. One-way ANOVA (post-hoc test: Bonferroni's multiple comparison test) was used to determine statistical significance. All statistical analyses were done using Prism (GraphPad) considering a p-value of less than 0.05 as statistically significant. Corresponding significance levels are indicated in the figures.

Results

Differentiation of adipocytes at different oxygen levels affects lipid droplet size and triglyceride content

To mimic *in vitro* more physiological conditions during adipogenesis, we differentiated human primary adipocytes at 10 % or 5 % oxygen and compared these cells with control cells differentiated at 21 % O_2.Oil Red O staining at day 14 of differentiation showed a similar differentiation of adipocytes differentiated at 21 %, 10 % and 5 % of oxygen indicated by lipid droplet accumulation (Fig. 1A). Measurement of lipid droplet size revealed a significant reduction in lipid droplet diameter of adipocytes from 10 % and 5 % $O_2$by about 25 % (Fig. 1B). Adipocytes differentiated at 10 % showed a significant increase in triglyceride content compared to cells from 21 %, whereas no difference in triglyceride content from 5 % differentiated adipocytes could be observed (Fig. 1C).We also tested protein expression of the glucose transporters (GLUT)-4 and -1 but could not detect significant changes between the three oxygen conditions (Fig. 1D).

We further tested the mRNA expression level of the transcription factors PPARγ and C/EBPα which are important for adipogenesis. As shown in Fig. 1E and F, mRNA expression of these transcriptions factors was not significantly altered during differentiation under the three oxygen conditions.

Adipokine secretion is altered in adipocytes differentiated at 10 % and 5 % O_2

As it is known that adipokine secretion is affected by acute hypoxia, we examined the release of distinct adipokines from adipocytes differentiated at 21 %, 10 % and 5 %. The classical adipokine adiponectin showed a marked 3-fold increase in its protein abundance in adipocytes differentiated at 10 % and 5 % compared to 21 % (Fig. 2A). Simultaneously, adiponectin release was increased from adipocytes at 10 % and 5 % O_2 with the highest amounts being secreted by cells at 10 % O_2 (Fig. 2B). For leptin we observed a significant reduction in its secretion from adipocytes differentiated at 10 % compared to the control at 21 %

while a tendency but no significant differences between 5 % and 21 % O_2 was observed (Fig. 2C). We further analyzed the secretion of other hypoxia-related adipokines such as IL-6and VEGF-A. VEGF-A release by adipocytes differentiated at 10 % and 5 % $O_2$was not significantly altered (Fig. 3A). IL-6 concentration in CM of adipocytes differentiated at 10 % $O_2$was substantially elevated, while its release at 5 % O_2 was not significantly altered compared to 21% O_2 (Fig. 3B). Furthermore, we tested the expression and the release of DPP4 that was recently described by our group as a novel adipokine released from adipocytes.[15] As shown in Fig.3C, DPP4 expression was significantly increased by about 2-fold under low oxygen tensions, which was accompanied by an increase in its release (Fig. 3D). Measurement of MCP-1 secretion revealed no differences between the different oxygen conditions (Fig. 3E).

Adipocytes differentiated at lower oxygen tension are lipolytically more active

We tested the lipolytic activity of adipocytes in the basal state as well as after isoproterenol-stimulation. In the basal state adipocytes differentiated at 10 % O_2showed a 2-fold increase in glycerol release compared to the adipocytes differentiated at 21 % O_2as well as to adipocytes differentiated at 5 % O_2 (21 % O_2 3.11 ± 0.34 nM vs. 10 % O_2 10.18 ± 0.92 nM vs. 5 % O_2 4.24 ± 0.86 nM). Stimulation with 1, 10 and 100 nM isoproterenol resulted in a higher glycerol release by the cells at 10 % and 5 % compared to 21 % O_2.When stimulated with 1 nM isoproterenol, glycerol release was also significantly higher from adipocytes differentiated at 10 % compared to 5 % O_2 (Fig. 4A). We further measured protein expression of the two main lipases of adipocytes, namely adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL), via Western Blotting. As demonstrated in Fig. 4B, we observed no significant alteration in ATGL expression at 10 % and 5 % compared to 21 % O_2, but HSL showed a more than 2-fold higher expression in both conditions compared to 21 % O_2 (Fig. 4C). Additionally, the expression of the lipid coating protein perilipin was markedly increased in adipocytes differentiated at 10 % and 5 % compared to 21 % O_2 (Fig. 4D). We further observed a prominent increase in the expression of β1-adrenergic receptor in cells at 5 % O_2 while the β2-adrenergic receptor

was elevated in adipocytes differentiated at 10 % O_2 although this effect was not significant (Fig. 4E-F).

CM of adipocytes reduces insulin-signaling in SkMC irrespective of O_2 during differentiation

As previously shown by our group, adipocyte CM impairs insulin-stimulated Akt phosphorylation in SkMC.[13] To test whether CM from adipocytes differentiated at 21 %, 10 % or 5 % O_2may have a different impact on SkMC insulin signaling, we incubated SkMCs for 24 hours with CM of the different conditions. As shown in Fig. 5A and B, all CM significantly reduced insulin-stimulated Akt phosphorylation on both Ser472 and Thr308 sites with no differences in the potency of the different CM.

CM from adipocytes differentiated at 10 % O_2display a diminished proliferative effect on SMC compared to 21 % and 5 % O_2

We further tested CM-induced SMC proliferation as described by our group in a previous study.[16] Incubation of SMC with CM generated from adipocytes differentiated at 21 %, 10 % or 5 % O_2 significantly enhanced SMC proliferation (Fig. 5C). However, CM from cells of 10 % O_2 showed a significantly reduced potential to induce SMC proliferation compared to the CM from cells differentiated at 21 %, while CM from cells at 5 % O_2 showed a tendency but no significant reduction in its proliferative effect.

Discussion

Oxygen plays a pivotal role in regulating metabolic functions such as glucose and lipid metabolism shown in diverse tissues.[17-19] Oxygen concentration in the human body is much lower than that of ambient air, ranging from 14 % to 4 % in the lung parenchyma and the circulation.[20] Also the oxygen level in distinct tissues is varying, for example, the brain ranges from 0.5 % to 7 % O_2, the eye shows 1 % to 5 % O_2 and the bone marrow niche is nearly anoxic ranging from 0 % to 4 %.[20] Most cell

culture models are performed at ambient air which could be defined rather a 'hyperoxic' than a physiological model and it has been suggested that the physiological oxygen tension could be defined as 'in situ normoxia'.[20] In the present study, we investigated the impact of more physiological oxygen tensions on differentiation and cellular function of primary human adipocytes. We have chosen oxygen levels of 5 % and 10 % O_2, as studies investigating human AT oxygenation reported of O_2 levels ranging between 3-11 %.[8,9] It is shown for 3T3-L1 as well as human bone marrow stromal cells (hMSC), that severe hypoxia of 1 % O_2 inhibit adipocyte differentiation and maintain the cells in a precursor phenotype, while moderate hypoxia of 8 % O_2 could stimulate adipogenesis in murine MSC.[21-23] Our data show that adipocyte differentiation at 5 % and 10 % oxygen is not impaired but comparable to the standard model of cell differentiation at ambient air, as we demonstrate similar lipid droplet accumulation, as well as a similar expression of adipogenesis related factors such as PPARγ, C/EBPα, GLUT1 and GLUT4 in these cells. At 10 % O_2 the cells seem to exert even a higher potential of lipid accumulation, as a slight but significant increase in triglyceride content was observed. However, lipid droplets of adipocytes under more physiological conditions showed a significant lower diameter (Fig. 1B). This effect might directly be mediated by the observed elevated expression level of perilipin, as Sawada et al. recently showed that over-expression of perilipin in 3T3-L1 cells resulted in reduced lipid droplet size and furthermore, mediated a more brown fat-like phenotype of these cells.[24] In our study UCP-1 mRNA expression levels were very low and rather reduced at physiological oxygen conditions (data not shown).

The most intriguing observation in this study is the prominent increase in adiponectin expression and secretion of about 3-fold by adipocytes differentiated under more physiological conditions. Adiponectin is a classical adipokine mainly released by adipocytes that exerts anti-inflammatory, anti-diabetic and anti-atherogenic effects and its serum concentration is negatively associated with obesity and the metabolic syndrome.[25,26] Adiponectin expression and secretion is highly regulated and the underlying mechanisms are intensively investigated but are yet not fully determined.[25] Acute treatment of adipocytes with low oxygen tensions was shown to diminish adiponectin expression and secretion and this effect correlated positively with the oxygen level.[10,14,27] Our finding of

elevated adiponectin release is divergent from the described effects under acute hypoxia and this effect reflects the different cell culture model of our study. Previous studies solely investigated the impact of acute hypoxia treatment on cells differentiated at 21 % O_2, while our study investigated more physiological oxygen conditions throughout differentiation. We suggest that chronic exposure of human primary adipocytes to lower oxygen tensions might result in a kind of reprogramming, altering the secretory behavior of adipocytes. It could be assumed that adiponectin expression and release depend on the oxygen level during adipocyte development. As shown in Fig. 2B, there is a tendency of reduced adiponectin release at 5 % compared to 10 % oxygen. It is conceivable that optimal adiponectin release might be highest from adipocytes developed in an environment of about 10 % O_2while oxygen level above or below might reduce it.

Other adipokines related to hypoxia such as leptin or IL-6, whose release was shown to be induced after acute hypoxia treatment even at 10 % O_2, are altered in a different way in adipocytes differentiated at 10 % and 5 % compared to 21 % O_2. Leptin release is reduced in our cell culture model of adipocyte differentiation under more physiological conditions. IL-6 release shows a different picture under chronic hypoxia. Its release is markedly elevated by about 4 fold at 10 %, but decreased by about 2 fold at 5 % O_2, although this effect is statistical not significant. As Wood et al. demonstrated a straight dose-response correlation of IL-6 release and oxygen tension, the bell-shaped response observed in our study might also underlie the different protocol of adipocyte culture. Regarding the study of Goossens et al. who demonstrated increased oxygen tensions and inflammation in human obese AT, it is likely that physiologically rather higher than lower oxygen concentrations might be the underlying mechanism for the bell-shaped response of IL-6 release. This assumption might also be true for other inflammatory adipokines such as DPP4. Here, we demonstrate an increase in the expression and secretion level of this novel adipokine under more physiological oxygen levels. DPP4 was first identified by our group as a novel adipokine, expressed and released from human primary adipocytes.[15] Moreover, we demonstrated DPP4 to be up-regulated by inflammatory TNFα, and that its release and serum levels in human subjects correlate positively with the BMI and obesity. However, in the latter study we could not demonstrate a

regulatory effect on DPP4 release due to an acute treatment of adipocytes with 1 % O_2. Not much is known about the effect of hypoxia on DPP4 expression and secretion, but it is reported that DPP4 expression could be induced in endothelial cells by acute hypoxia-treatment of about 2.6 % O_2 and also in a HIF-1α-dependent manner in nearly anoxic tumor xenografts.[28,29] As demonstrated in this study, physiologic oxygen tensions do affect DPP4 expression and release, resulting in higher levels of this inflammatory novel adipokine. The role of DPP4 in AT inflammation is underlined by recent reports demonstrating that DPP4 inhibition in obese mice models reduces the expression of inflammatory markers and AT infiltration with inflammatory immune cells.[30,31] As suggested by Goossens et al. physiologically rather increased than decreased AT oxygen levels might trigger an inflammatory response of human adipocytes. The increases in inflammatory IL-6 and DPP4 levels, as well as in triglyceride content and basal lipolysis, events all observable in obesity[1,15], would support this hypothesis. However, in contrast to this hypothesis is the marked increase in anti-inflammatory adiponectin release observed in this study. Moreover, the level of MCP-1 release by adipocytes at physiologic oxygen tensions was not altered, demonstrating that not all inflammatory markers are altered and follow the pathologic situation in the obese state. If the situation of 10 % O_2 in AT would reflect the more inflammatory environment, higher release of MCP-1 and less adiponectin would be expected. It is as well likely that distinct adipokines might play other roles than involvement in inflammation in this context. For example, current studies demonstrated *in vivo* and *in vitro* that IL-6 is a potent inducer of AT lipolysis and increases fatty acid oxidation.[32,33] IL-6 is assumed possibly to play an important role in lipid metabolism[34] and therefore might function as a regulator of adipocyte lipid metabolism. It is conceivable that lipid turnover induced by IL-6 is an oxygen-dependent effect that might be increased at oxygen concentrations around 10 % O_2 while under lower oxygen levels IL-6-induced lipid turnover might be reduced. However, further studies are necessary to evaluate the underlying mechanisms of adipokine regulation, and to elucidate their specific role in this context.

Our cell culture model revealed an altered lipolytic activity of adipocytes under more physiological oxygen tensions. AT lipolysis is regulated by diverse effectors such as cathecholamines, glucocorticoids

and insulin, but also in an autocrine manner by adipokines such as TNF-α and IL-6.[35-37] The lipolytic machinery involves a number of different lipases but it is assumed that TAG hydrolysis is mainly mediated by ATGL and HSL, as a mice model demonstrated that these two lipases account for more than 90 % of lipolytic activity.[38] Lipid droplet coating proteins are as well important regulators of lipolysis as they limit or provide access of lipases to the lipid droplet.[39] In adipocytes the lipid droplet coating protein perilipin plays a pivotal role, as its protein kinase A (PKA)-mediated activation is essential for full HSL activity and indirectly also for ATGL activity.[39] Here we demonstrated a marked up-regulation of isoproterenol-stimulated lipolysis in adipocytes differentiated at lower oxygen tensions compared to cells at ambient air. Our data suggests that this effect on lipolysis is caused by an up-regulation of the hormone sensitive pathway, as we observed increased expression of beta-adrenergic receptors, in particular of the beta1-adrenergic receptor, HSL and perilipin. Basal lipolysis is elevated in cells differentiated at 10 % oxygen compared to cells from 21 % and 5 % O_2. This increase might be mediated by an autocrine mechanism, as adipocytes at 10 % oxygen released substantially more IL-6 (Fig. 2D), that is known to stimulatelipolysis.[36] These alterations in hormone- and adipokine mediated lipolysis seems to be highly regulated by oxygen supply of the environment. There are only a few studies in humans and a mouse model regarding the effect of hypoxia on AT lipolysis. These studies demonstrated, that reduced oxygen supply in the air or in AT could increase adipocyte lipolysis *in vivo* and *in vitro*.[8,40-42] It was suggested by the authors that down-regulation of β-adrenergic stimulation as well as reduced anti-lipolytic activity of insulin on adipocytes was involved in this effect. To our knowledge, the present study is the first to demonstrate a regulatory effect of low oxygen concentrations on the expression level of the lipases HSL and ATGL, as well as the lipid coating protein perilipin in human primary adipocytes.

It is known that basal lipolysis in the obese state is elevated while catecholamine induced lipolysis is reduced.[43,44] As we observed alterations in basal as well as isoproterenol-stimulated lipolysis under physiological conditions, it could be assumed that lipolysis in obese individuals might be affected by oxygen tension. In particular, the reduced lipolytic activity after stimulation with a low concentration of isoproterenol (1 nM) in cells differentiated at 5 % compared to 10 % O_2 might reflect a mechanism of

hypoxia-mediated impairment of cathecholamine-induced lipolysis in the obese state. However, as the current studies on AT oxygenation are conflicting and could not clearly describe if oxygen is reduced or elevated in AT of obese subjects[8,9], we could not classify if the effects of basal and isoproterenol-induced lipolysis might contribute to the dysregulation described for the obese state. Future studies are necessary to elucidate this aspect of AT oxygenation in relationship to defects in lipolysis.

As oxygen tension significantly affects adipokine release, it might be speculated that adipokine signaling to other organs might be altered. Previous studies of our group reported that adipocyte generated CM affect other cell types such as SkMC and SMC. We demonstrated via cross-talk experiments that CM induces insulin-resistance in SkMC as well as proliferation of SMC, whereas SMC proliferation negatively correlates with adiponectin amounts in the CM.[13,16] We therefore assumed that the elevated levels of adiponectin, released into CM of adipocytes differentiated under more physiological conditions, would alter the CM effect on SkMC and SMC. The alterations in CM composition and in particular the increased adiponectin amounts had no positive effect on insulin-sensitivity of CM-treated SkMC. On the other hand, we could show that SMC proliferation is reduced when treated with CM of adipocytes differentiated at 10 % O_2 compared to CM from adipocytes at ambient air. Regarding that adipocytes at 10 % O_2 released the highest amounts of adiponectin, this finding is in accordance to our previous study where we demonstrated that SMC proliferation is dependent on adiponectin concentration in the CM.[16] These cross-talk experiments demonstrate that oxygen dependent alterations in CM composition and in particular elevated adiponectin amounts could not prevent the CM-induced alteration of SkMC insulin-signaling and SMC proliferation.

In conclusion, this study shows that oxygen tension is an important factor affecting adipocyte development. Adipokine expression and secretion is a highly oxygen sensitive mechanism. However, this study revealed that adipocyte culture at 21 % O_2, which could be defined as hyperoxia, seems to impair adipocyte function. This is demonstrated by the reduced lipolytic activity of adipocytes at ambient air. As this study was intended to investigate the role of oxygen during adipogenesis, the specific underlying mechanisms to the observed alterations of adipocyte function have to be revealed in further studies. Future experiments should

take into account that the effects in experiments of the standard culture model might show adverse effects or might be even more pronounced when performed under physiological conditions.

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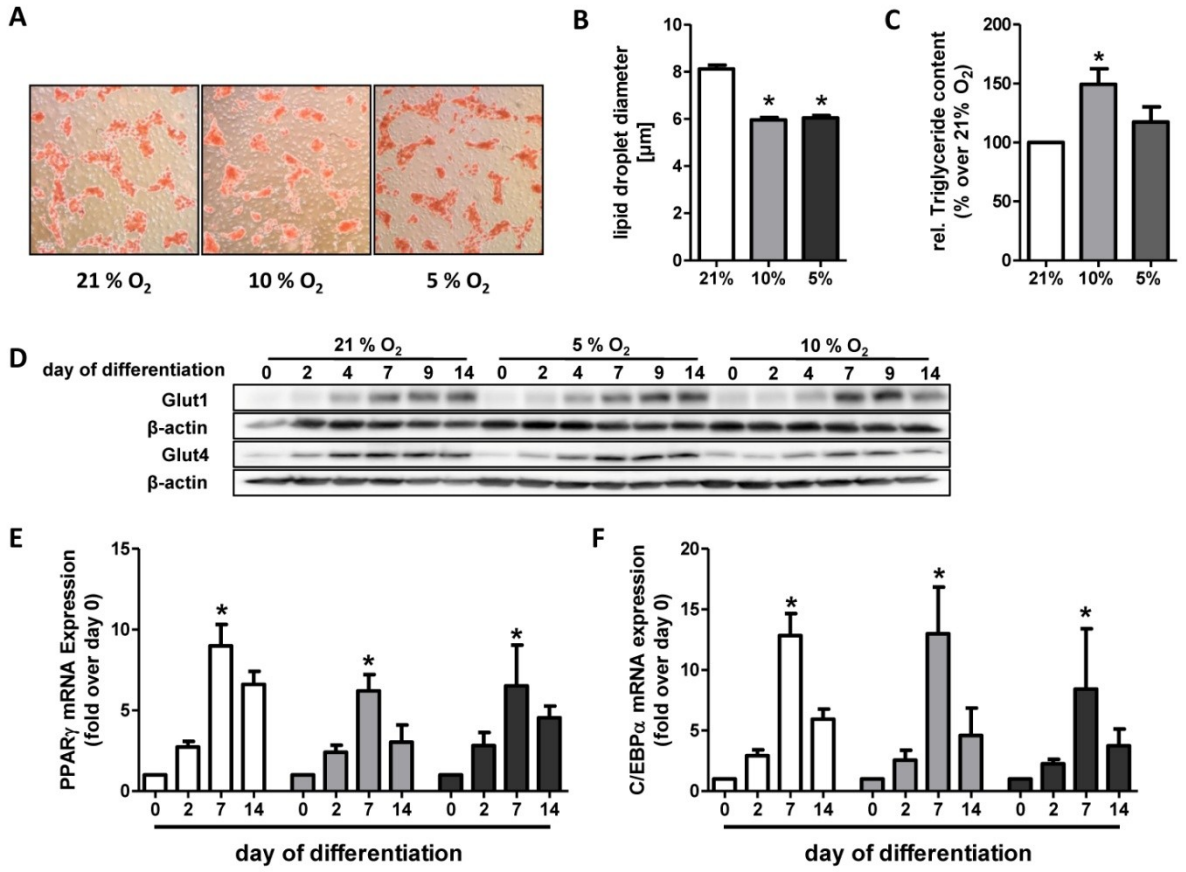

Figure 6.1 Differentiation of human primary adipocytes at low oxygen tensions results in a similar adipogenic profile compared to normoxic cells.

Human primary preadipocytes were differentiated *in vitro* at 21 %, 10 % or 5 % oxygen. (A) Lipid droplet accumulation was analyzed at day 14 of differentiation by Oil Red O staining and digitally documented. Representative pictures at 20 fold magnification are shown. (B) Lipid droplet (LD) size was analyzed using ImageJ software by measuring 200 LDs per situation and individual, using pictures of 20 fold magnification of three individuals. (C) Triglyceride content was measured using a Triglyceride Quantification kit according to the manufacturer's instructions. (D) Preadipocytes before (day 0) and adipocytes at day 2, 4, 7, 9 and 14 after induction of differentiation were harvested at the indicated oxygen concentration and directly lysed. Total lysates (10 µg) were resolved by 10 % SDS-Page and analyzed via Western Blotting on polyvenylidenefluorid (PVDF) membranes and further processed as described in Material and Methods. Representative Western Blots for GLUT1 and GLUT4 are shown. (E-F) mRNA was isolated from preadipocytes before (day 0) and adipocytes at day 2, 7 and 14 after induction of differentiation. mRNA was reverse transcribed into cDNA and analyzed via quantitative real-time PCR using specific primer sets for the genes

PPARγ and C/EBPα. Data were normalized to the mRNA expression level of POLR2A and expressed relative to the control at 21 % $O_2$of day 0. As values were not normally distributed, data was log-transformed prior to statistical analysis. Open bars: 21% O_2, light grey bars: 10% O_2, dark grey bars: 5% O_2. Data sets are mean values± SEM of 3 – 5 independent experiments. *p<0.05 compared with the control at21 %O_2.

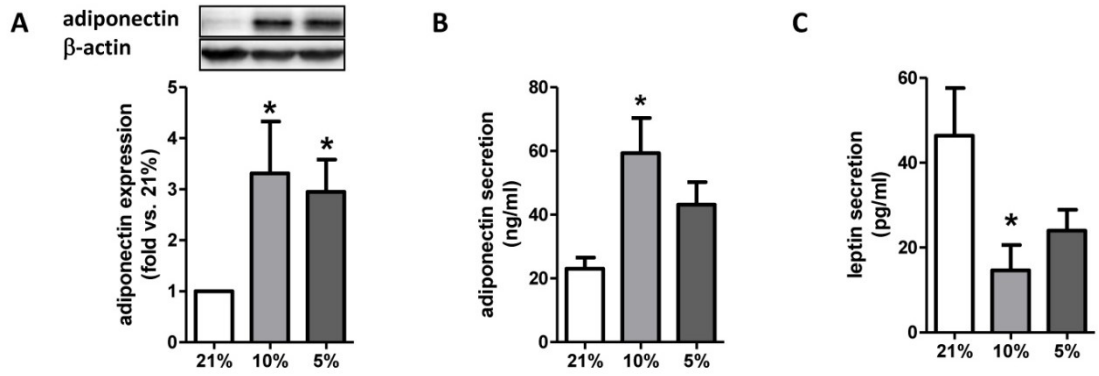

Figure 6.2 Differentiation of human primary adipocytes alters adiponectin and leptin release.

Human primary adipocytes, differentiated at 21 %, 10 % or 5 % O_2, were incubated for 24 hours in αMEM at the same oxygen conditions. Afterwards, supernatants were collected for further ELISA measurements and cells were harvested and directly lysed. Total lysates (10 µg) were resolved by 10 % SDS-Page and analyzed via Western Blotting on polyvenylidenefluorid (PVDF) membranes and further processed as described in Materials and Methods. (A) Protein expression of adiponectin was detected by ECL. Representative blots are shown. Supernatants were analyzed for protein concentration of (B) adiponectin and (C) leptin. Data sets are mean values± SEM of ≥4 independent experiments. *p≤0.05 compared with the control at 21 % O_2.

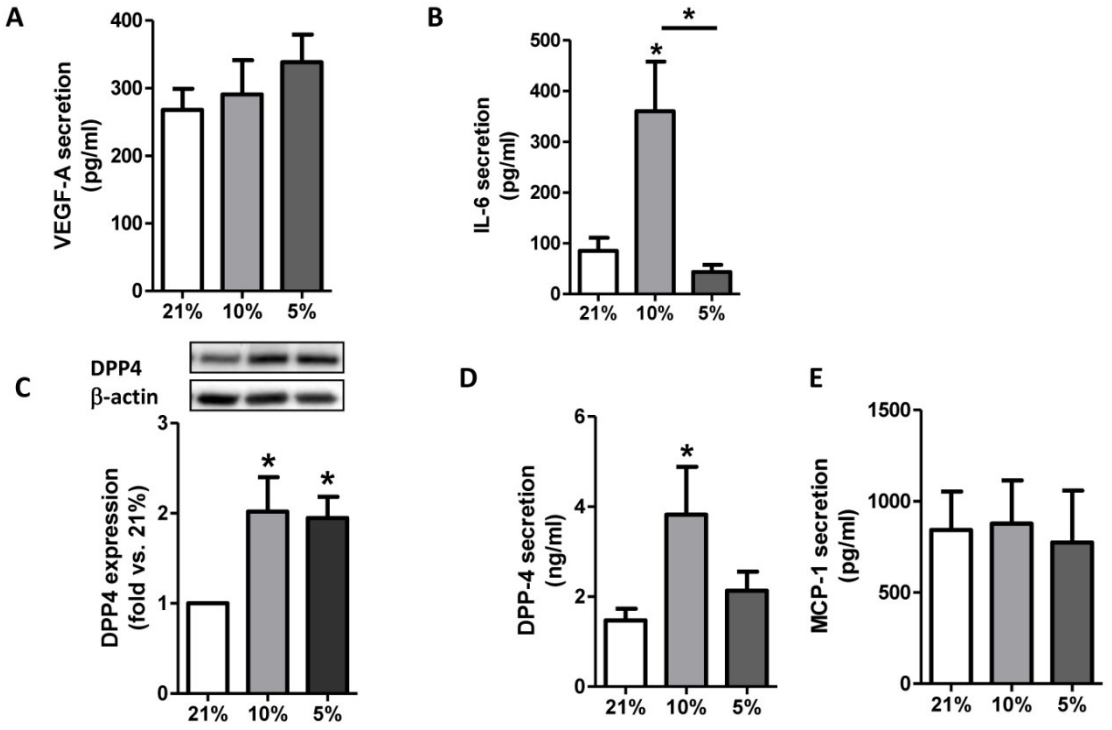

Figure 6.3 Regulation of IL-6, VEGF-A and DPP4 release under lower oxygen tensions.

Supernatants and lysates from human primary adipocytes, differentiated at 21 %, 10 % or 5 % O_2, were obtained and analyzed as described in Fig.2. Protein concentration in the supernatant was determined by ELISA measurement for (A) IL-6, (B) VEGF-A, (D) DPP4 and (E) MCP-1. (C) Protein expression of DPP4 was determined by Western Blotting. A representative blot is shown. Data sets are mean values± SEM of ≥4 independent experiments. *p≤0.05 compared with the control at 21 % O_2.

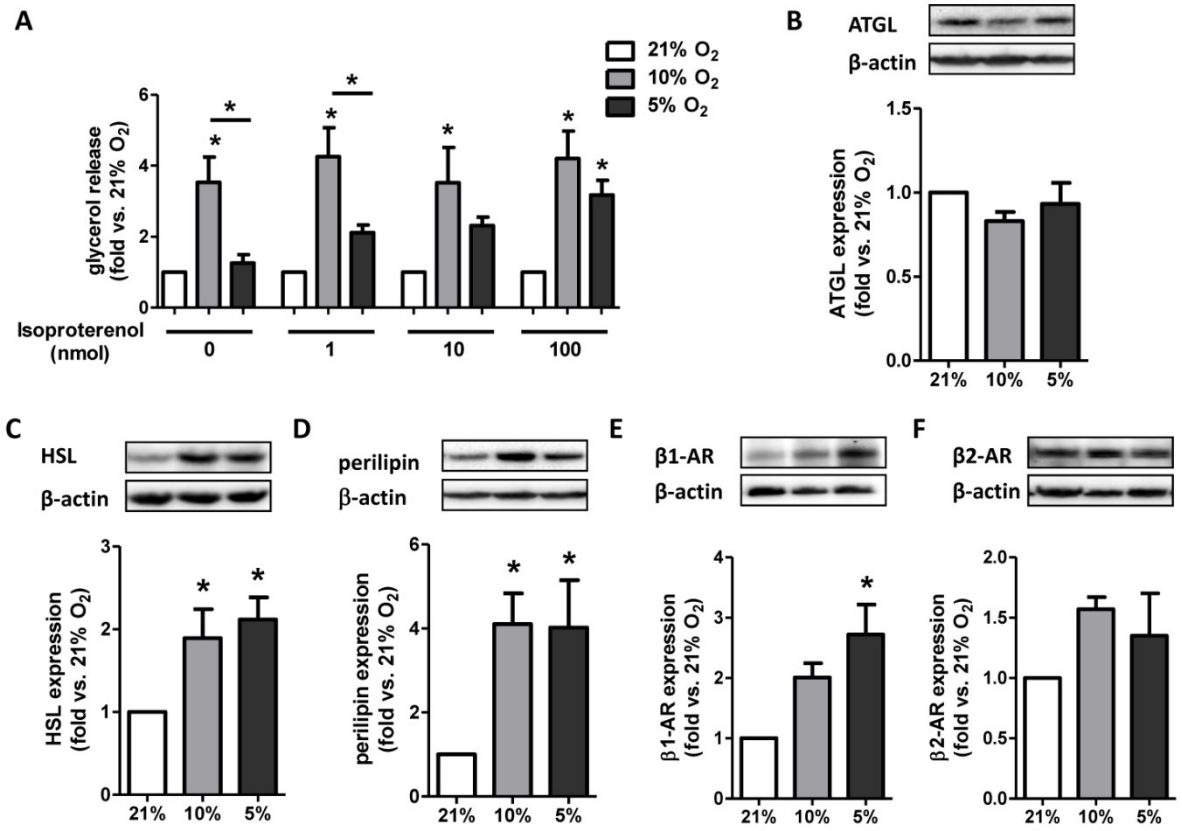

Figure 6.4 Increased lipolysis and expression of proteins of the hormone-sensitive lipolytic pathway at lower oxygen tensions.

(A) For lipolysis experiments adipocytes at the appropriate oxygen condition were starved 24 hours in αMEM. These cells were further incubated in Krebs-Ringer-buffer supplemented with 1 % BSA for 3 hours with or without designated concentrations of isoproterenol. Glycerol release into the supernatant was measured using the Glycerol Free reagent due to the manufacturer's instructions. Cell lysates of unstimulated adipocytes were analyzed via SDS-Page and Western Blotting with appropriate antibodies for (B) ATGL, (C) HSL, (D) perilipin and (E-F) β1- and β2-adrenergic receptors. Representative blots are shown. Data sets are mean values± SEM of 3-8 independent experiments. *$p<0.05$ compared with the control at 21 % O_2.

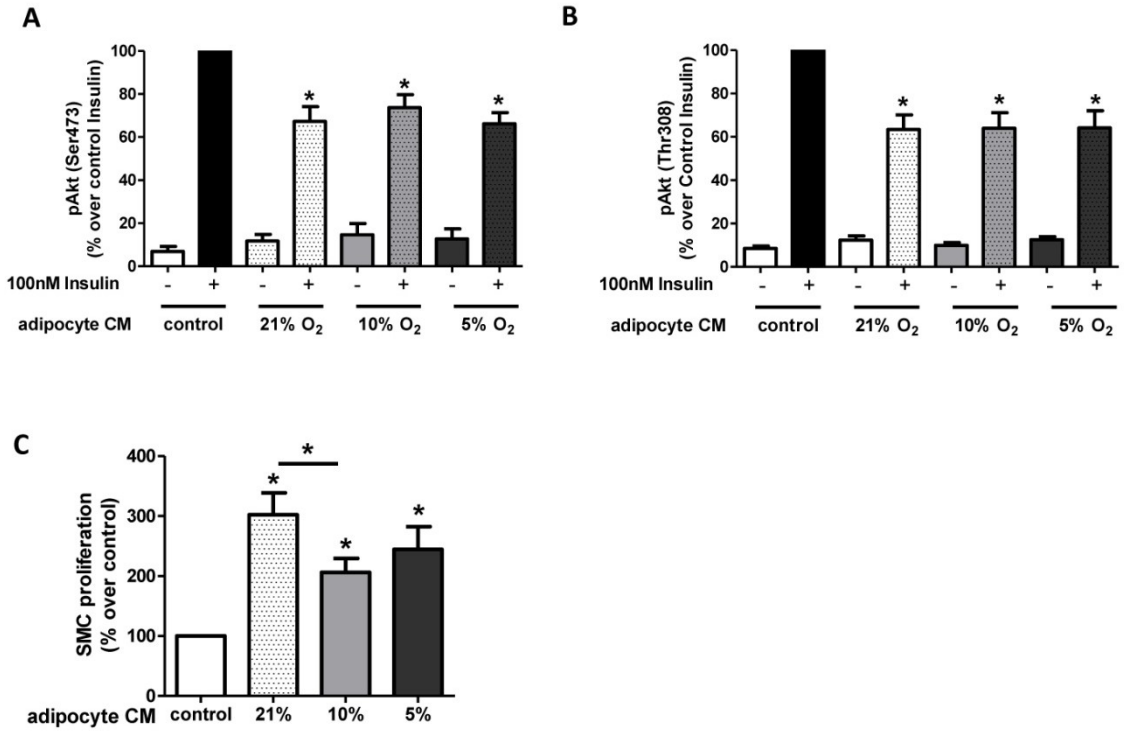

Figure 6.5 Effects of adipocyte-conditioned medium (CM) on human SkMC and SMC.

CMs from adipocytes differentiated at 21 %, 10 % or 5 % O_2 were generated over 48 hours. (A-B) Human SkMCs were incubated for 24 hours with αMEM or CM of the different adipocytes and afterwards stimulated with 100 nM insulin for 10 minutes. Cells were harvested, directly lysed and analyzed via SDS-Page and Western Blotting for the phosphorylation level of Akt (Ser473) and Akt (Thr308). (C) Human SMCs were serum starved for 24 hours and subsequently incubated with BrdU in the absence or presence of CM from the differently differentiated adipocytes for further 24 hours. Proliferation of SMC was measured as described in Materials and Methods. Data sets are mean values± SEM of ≥6 independent experiments. *$p<0.05$ compared to the basal value.

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Chapter 7

General Discussion

7.1. Adipose tissue secretory function – Characterization of novel and high abundant adipokines

Obesity is a hallmark of diverse diseases such as T2D, atherosclerosis and the metabolic syndrome. As outlined in the sections above, expansion of AT mass and its functional dysregulation plays a central role in the development of obesity-related disorders. In particular, the recognition that AT does not only act as an energy storage site but also represents a major endocrine organ, conducted numerous investigations to elucidate the role of AT-derived factors in metabolic disorders. A literature search in the medical database 'pubmed' using the key words 'obesity' and 'adipokines' results in more than 9000 publications since 1995 towards this research topic, demonstrating the importance of AT-derived factors in the context of obesity. Until today, the number of potential adipokines identified in various unbiased, proteomic secretome analysis, which used samples obtained from both rodent and human adipocytes or AT explants, has risen to more than 700 proteins.[55-58,262-266] These experimental approaches demonstrated the secretome of adipocytes and AT as a highly complex mixture of numerous proteins. However, the amount of overlap between the identified adipokines thus far indicates that the secretome of adipocytes remains still incompletely characterized. In regard to the

increasing number of reported potential novel adipokines, only a few are already validated as real adipokines, and further characterized for their function, regulation, and specific role in distinct disorders.

Adipokines are known as important mediators of the inter-organ crosstalk between AT and peripheral tissues such as the skeletal muscle, liver or the brain with AT on top of the crosstalk hierarchy.[267] Thereby, processes including the immune response, blood pressure control, haemostasis, bone mass, reproduction, as well as important metabolic functions such as lipid metabolism, insulin sensitivity and glucose homeostasis are regulated by circulating adipokines.[77] It is apparent that a more detailed characterization of the adipocyte secretome, and in particular of novel adipokines, would improve the current understanding of the distinct role of AT in the development and progression of obesity-related disorders. Moreover, it is of great importance to identify specific biomarkers in this context, either to prevent or delay the development of metabolic pathologies and to improve the therapy of obesity-related diseases such as T2D. Therefore, the first aim of this thesis was the validation and characterization of putative novel adipokines, which were identified from adipocyte-derived CM in a previous extensive proteomic profiling. The novelties of this approach presented in chapter 2, were first the usage of human primary adipocytes, and second the combination of two proteomic methods, namely 1D-gelelectrophoresis with subsequent LC-ESI-MS/MS, and 2D-gelelectrophoresis followed by protein identification via MALDI-MS. This comprehensive proteomic approach identified 347 proteins, of which, after intensive bioinformatics analysis and further literature search, 44 factors were considered as putative novel adipokines. Five of these 44 factors were chosen for further validation due to their possible role in obesity-related AT dysfunctions including oxidative stress, inflammatory response, ECM structure, and metabolic functions. Therefore, the chosen factors were the stress-related heat shock proteins heme-oxygenase-1 (HO-1) and αB-crystallin (CRYAB), complement factor H (CFH), cartilage-immediate layer protein (CILP), and the current drug target for T2D treatment dipeptidyl peptidase 4 (DPP4). These proteins were analysed for their expression profile during adipogenesis and on their release by adipocytes. As AT-located macrophages are important contributors to the secretory output of this organ, we additionally investigated expression and secretion of the novel adipokines in AT-

derived macrophages ($CD14^+$ cells) in comparison to adipocytes. Moreover, prominent regulators of adipocyte secretory activity such as troglitazone, insulin, TNF-α, and hypoxia were used to elucidate the affinity of the novel adipokines to be regulated by obesity-relevant factors.

A second aspect, based on the outcome of the previously described proteomic approach, was the characterization of the protein pigment-epithelial derived factor (PEDF). Although PEDF is already known as an adipokine, the proteomic approach identified this protein as a high abundant adipokine released from human primary adipocytes. The aim was to analyze PEDF expression, secretion, and its regulation in adipocytes. Moreover, the autocrine and endocrine effects on cellular functions such as insulin resistance and inflammation in adipocytes and peripheral tissues, including the skeletal muscle and smooth muscle, were investigated and discussed.

7.1.1. Heme-oxygenase 1 (HO-1)

HO-1, also known as heat shock protein (HSP)-32, is one of two HO isoenzymes in humans and rodents that catabolizes the degradation of heme (iron (Fe) protoporphyrin IX) into labile ferrous iron (Fe^{2+}), biliverdin and carbon monoxide (CO). HO-1 is expressed ubiquitously as an inducible enzyme responsive to cellular stress and oxidative stimuli such as hypoxia, shear stress, UV-light, heme, LPS, hydrogen peroxide, pro-inflammatory cytokines, inflammation, or heavy metals. The second isoenzyme HO-2 is constitutively expressed and not inducible. The metabolites of HO-1 exhibit anti-inflammatory and cytoprotective properties. CO is a gaseous transmitter that acts as vasodilator of blood vessels and exerts additionally anti-apoptotic and anti-inflammatory actions. Biliverdin is quickly converted by the enzyme biliverdin reductase into bilirubin, which act as a potent antioxidant, directly scavenging oxidants such as superoxide anions by inhibition of NADP(H) oxidase.[268,269]

HO-1 is not only a cellular protein as it is detectable in the circulation, although it origin is not known. It was postulated that plasma levels of HO-1 are due to ‘leakage’ from the tissue to the plasma compartments analogous to liver enzymes present in the circulation.[270,271] However, our

data presented in study 1 clearly demonstrated adipocytes to express and secrete HO-1 in a differentiation dependent manner. We also detected HO-1 in serum of lean and morbidly obese patients, with significantly higher levels in the obese subgroup. These serum levels correlated positively with the size of subcutaneous but not visceral adipocytes. As the visceral fat depot is suggested to be the more secretory active and detrimental for metabolic disorders,[35] it is likely that HO-1 might play a more important role in hypertrophic adipocytes of subcutaneous than visceral AT. However, our data also demonstrated increased HO-1 protein expression in subcutaneous and visceral AT of obese subjects, indicating a higher degree of inflammation compared to lean subjects. Evidence for a correlation between circulating HO-1 and inflammatory diseases is given by current reports demonstrating elevated HO-1 plasma or serum level in patients suffering from Parkinson's disease, hemophagocytic syndrome or prostate cancer.[272-274] Also newly diagnosed T2D patients of a Chinese cohort showed increased circulating HO-1 levels compared to non-diabetics.[270]

Recent studies suggested a role for HO-1 in AT adipogenesis and adipokine release. Obese rats and mice presented reduced weight gain and expression levels of TNF-α and IL-6 upon chemical HO-1 induction, while adiponectin levels were increased.[275,276] Moreover, HO-1 improved insulin sensitivity and serum adiponectin levels in Zucker diabetic fat (ZDF) rats, while a decrease in visceral and subcutaneous AT mass could be observed.[275,277] *In vitro* studies revealed reduced adipogenesis in human bone marrow-derive adipocyte stem cells and in murine 3T3-F442A cells upon HO-1 induction.[276,278,279]

As HO-1 is known as an anti-inflammatory factor, it is likely that it exhibits as an adipokine an important regulatory role in AT inflammation. Therefore, our study was the first to demonstrate a negative regulation of HO-1 secretion by TNF-α. This finding is in accordance with current studies reporting a TNF-α-induced reduction of HO-1 in human peripheral monocytes and human chondrocytes.[280,281] On the opposite, HO-1 induction in an animal model leads to the reduction in the circulating TNF-α level.[276] These studies together with our observation point to yet uncharacterized link between HO-1 and TNF-α.

The mechanism by which HO-1 exerts is anti-inflammatory effect is assumed to be mediated by the positive action of its metabolites CO and

bilirubin. A recent study, performed in myeloid cells, demonstrated that HO-1 could also directly bind to the transcription factor IRF3 which is involved in the regulation of specific cytokines necessary for the regulation of a proper innate immune response and autoimmunity.[282] To our current knowledge, the interaction of HO-1 with distinct transcription factors or other cellular proteins in adipocytes is not known. Future investigations should address the question whether adipocyte specific HO-1 expression directly affect adipocyte function and secretion, and the influence of HO-1 on AT inflammation. As we have presented HO-1 as a secretory protein, it is can be assumed that it is not solely an adipokine but might also be released by other cell types such as immune cells, hepatocytes or myotubes. In this study, we detected only low expression of AT-derived macrophages in comparison to adipocytes, which might increase also in this cell type upon inflammation and stress. The function of secreted and circulating HO-1 is currently not known. It could be speculated that anti-inflammatory effects mediated by HO-1 might be caused by a direct interaction of HO-1 with a distinct cell surface receptor or receptor complex.

In conclusion, HO-1 seems not to be only a marker of cellular stress but also acts as an important factor that helps to maintain cellular and tissue homeostasis. Moreover, regarding its release by AT and the elevated circulating levels correlating to obesity and inflammatory factors suggest HO-1 as putative novel biomarker for inflammatory diseases such as T2D.

7.1.2. αB-crystallin

αB-crystallin, also known as heat shock protein B5 (HspB5) or CRYAB, belongs to the family of small heat shock proteins (sHSP's). This sHSP protects again apoptosis and oxidative stress, as well as it acts as a molecular chaperone to counteract aberrantly folded proteins.[283] Our finding is in agreement with the study of Kim et al.[263] who also identified αB-crystallin as an adipokine released from human adipocytes. αB-crystallin is already known as a protein expressed in AT as shown in rodents.[284] Our study demonstrated an increased αB-crystallin expression during adipogenesis, as well as detectable amounts of the protein in

human serum. A recent study reported αB-crystallin release from human retinal pigment epithelial cells via exosomes, demonstrating it as a secretory protein with a yet uncharacterized physiological role outside of cells.[285] Moreover, we observed αB-crystallin expression in both AT depots, subcutaneous and visceral AT, while its expression was markedly increased in the obese state. However, despite we did not observe elevated levels of this adipokine in serum of obese patients, it is conceivable that other tissues than AT might also contribute to its circulating levels.

Formerly, αB-crystallin and its homolog αA-crystallin were assumed to be specific to the ocular lens. While αA-crystallin is still believed as a lens-specific protein, αB-crystallin was demonstrated to be present in tissues with high oxidative capacity, including the heart, skeletal muscle, oxidative regions of the kidney, brain, lungs, and recently AT.[283,284] In the lens, αA-crystallin and αB-crystallin are important factors for the maintenance of lens transparency and refractive properties.[286] In this context, the chaperone activity of α-crystallins prevent protein aggregation and trap denatured or misfolded proteins, until their procession for refolding or degradation by the proteasome.[283] αB-crystallin was shown to be up-regulated and accumulated in inclusion bodies of diverse neurodegenerative diseases, for instance Alzheimer disease, Alexander disease and Parkinson disease.[287] αB-crystallin also participates in cytoskeletal architecture homeostasis, therefore stabilizing intermediate filaments and microtubules. In this context, mutations in its related gene are associated with the development of myofibrillar myopathy, cardiomyopathy and cataract.[283] αB-crystallin seems also to play an important role in multiple sclerosis (MS), as it is the most abundant protein in MS brain lesions, involved in the reduction of astrocyte apoptosis.[288] Moreover, SNP's in the promoter region seem to influence the susceptibility to MS and possibly the MS specific immune response.[289]

A recent study reported an involvement of αB-crystallin in tumour angiogenesis, promoting the survival of endothelial cells of new tumour vessels.[290] This novel role as a possible angiogenic modulator is supported by a recent report demonstrating a direct interaction of αB-crystallin with VEGF-A.[291] The latter demonstrated αB-crystallin as a necessary chaperone for proper VEGF-A-folding under stress conditions, in particular chemical hypoxia. Here, αB-crystallin silencing resulted in VEGF-A

degradation and endothelial apoptosis due to an insufficient VEGF-A-mediated angiogenic response. In regard of this report, it is likely that the increase in αB-crystallin expression in human adipocytes reported by us may also represent an increased demand of this molecular chaperone during adipogenesis. As adipogenesis and angiogenesis are closely related to each other,[118,119] αB-crystallin might reflect an important factor involved in this process helping to maintain proper AT development.

The specific role of αB-crystallin in AT and in particular in the context of obesity related disorders is not characterized yet. A current study conducted in streptozotocin (STZ) rats, which reflects a valid model for the investigation of T1D, demonstrated a diverse expression pattern in different tissues. While αB-crystallin was up-regulated in the lens, heart, muscle, and brain due to oxidative stress, AT-specific expression was reduced at the same time.[284] As the phenotype of T1D differs substantially from T2D, it is difficult to draw a conclusion from this study to obesity-related disorders. However, it is likely that a differential regulation of AT-specific αB-crystallin in comparison to other tissues occurs. Classical regulators of AT did not regulate αB-crystallin in adipocytes, and even severe hypoxia did not affect its expression, although hypoxia and oxidative stress are known to up-regulate αB-crystallin expression in other cell types.[283,292] It is of great interest which factors might influence adipocyte-derived αB-crystallin, in order to shed light on its functional role in AT.

In conclusion, due to its anti-inflammatory properties, it is conceivable that αB-crystallin might play an important role in AT inflammation and obesity-associated inflammatory diseases such as T2D. Moreover, through its possible angiogenic and anti-inflammatory properties, this novel adipokine might be a pivotal player in AT remodelling and homeostasis. Depending on its function, αB-crystallin might not reflect a novel biomarker, but rather a therapeutic target in the treatment of obesity-induced inflammatory diseases.

7.1.3. Cartilage intermediate layer protein (CILP)

Cartilage intermediate layer protein (CILP) is a monomeric glycoprotein residing in the ECM of the middle zone of human articular cartilage.[293] Expression of this protein was found to be age-related, and its enrichment is associated to the early and late stage of osteoarthritis, as well as to rheumatoid arthritis.[293,294] To our knowledge, we were the first to describe CILP being expressed and released from human primary adipocytes. A recent proteomic approach underlined our finding that CILP is not solely specific to the human cartilage, as this study demonstrated CILP as a component of the cardiac ECM in a porcine model.[295]

CILP could directly bind and inhibit TGF-β1, therefore affecting ECM regulation and cartilage homeostasis.[296] Interestingly, TGF-β1 was shown to strongly inhibit adipocyte differentiation[297], as well as several groups reported increased circulating TGF-β1 levels in obesity and related disorders.[298-300] Thereby, inhibition of TGF-β1 or its related Smad3 signalling pathway has been shown to reduce obesity and prevent the development of diabetes and metabolic disorders in a mouse model.[300] Regarding this data, it is likely that adipocyte derived CILP might play a role in counteracting AT-specific TGF-β1 activity, therefore affecting AT mass expansion and homeostasis.

We demonstrated TNF-α-induced down-regulation of adipocyte-specific CILP expression, suggesting that inflammation and inflammatory factors might be involved in the regulation of CILP. This is supported by our findings of reduced CILP serum levels in obesity and the reduced expression in visceral compared to subcutaneous AT. It is likely, that inflammation in the obese state is involved in the down-regulation of CILP. Therefore its inhibitory effect on TGF-β1 would be decreased, leading subsequently an increase in TGF- β1 activity. However, an AT-specific association of TGF-β signalling, inflammation and the role of CILP have to be elucidated in further studies.

7.1.4. Complement factor H (CFH)

Another novel adipokine identified by us, is the glycoprotein complement factor H (CFH), an important factor of the complement system that displays the major non-cellular component of the innate immune system.[301] CFH is one out of three recognition molecules that is involved in the homeostatic regulation of the alternative complement system. In this context, it binds and subsequently inhibits C3 convertase to accelerate the decay of the alternative pathway, therefore preventing unintended injury of host cells.[301] CFH can be found in the circulation at abundant serum levels of 500 µg/ml, although these levels could vary due to genetic and environmental factors.[302] It is constitutively expressed in the liver, as well as locally in cell types including retinal pigment epithelial cells, endothelial cells, epithelial cells, platelets and mesenchymal stem cells (MSC) and others.[301] Our study could extend the list of CFH expressing cells to human adipocytes, which express and release CFH in a differentiation dependent manner, thereby possibly contributing to the high circulating levels. In human adipocytes, CFH expression levels decrease during differentiation, which is in line with two reports performed at the mRNA level in mouse and human adipocytes.[303,304]

In a recent study, CFH serum levels were positively correlated to obesity-associated factors such as BMI, triglycerides, inflammatory parameters, and insulin resistance. Interestingly, insulin resistance correlated only with CFH expression in visceral but not subcutaneous AT, while subcutaneous CFH was negatively associated with age and was generally higher than in visceral AT.[303] Another important aspect correlating CFH to diabetes and the metabolic syndrome is its interaction with adrenomedullin, a protein which is probably ubiquitously expressed.[305] Adrenomedullin was shown to decrease insulin secretion of pancreatic islets and this effect was further increased in the presence of CFH.[306] Adrenomedullin was also characterized as an adipokine, which expression decreases during differentiation in a similar manner as CFH. Moreover, its serum levels are as well elevated in obesity and its expression in adipocytes can be induced by pro-inflammatory factors such as TNF-α and hypoxia. As we demonstrated increased CFH levels due to TNF-α and hypoxia, it is likely that adrenomedullin and CFH expression and secretion correlates to each other, due to their interaction and mutual regulation of their functional biology. In addition, adrenomedullin is

described to act as a vasodilator thereby affecting angiogenesis, as well as it exerts anti-inflammatory, anti-apoptotic and modulatory effects on cytokine production.[305] Regarding the interaction of adrenomedullin and CFH, it is conceivable that both factors together might affect AT homeostasis by regulating AT angiogenesis and counteracting inflammation-induced dysfunction in AT. However, future investigation have to prove this speculative interaction in AT and the functional role of CFH as an adipokine.

7.1.5. Dipeptidyl-peptidase 4 (DPP4)

The previous proteomic analysis of the adipocyte secretome identified DPP4 as a novel adipokine. DPP4 is an ubiquitously expressed transmembrane glycoprotein, belonging to the S9b family of serine proteases. The enzyme activity of this type II cell surface protein cleaves off X-Pro and X-Ala dipeptides from several known substrates including growth hormones, neuropeptides and chemokines. This truncation inhibits the specific biological function and leads to the degradation of its substrates. DPP4 is also known as the cell surface antigen CD26 that can associate with lymphocyte CD45 and adenosine desaminase (ADA), thereby exhibiting a costimulatory effect on the immune response.[307,308] Due to the degradation of the incretin glucagon-like peptide 1 (GLP1), DPP4 has gained considerable interest as a therapeutic target for type 2 diabetes, and a variety of inhibitors that prolong the insulinotrophic effect of GLP1 are now in clinical use.[309] A soluble and active part of DPP4, lacking the cytoplasmic tail and the transmembrane region, can be found at relatively high levels in the circulation and in other body fluids. This soluble DPP4 is gained by proteolytic cleavage of the enzyme from the cell membrane.[310] Although the origin of soluble DPP4 is considered to be most likely from liver epithelium and lymphocytes,[311] the major source for circulating DPP4 and its regulation remains unknown.

Our study revealed DPP4 as an important novel adipokine. The expression and secretion of DPP4 increased substantially during adipocyte differentiation. Comparison to preadipocytes and AT-derived macrophages demonstrated that adipocytes most likely represent the major source of DPP4 released from AT into the circulation. Although it is known that DPP4

expression is regulated by diverse cytokines such as IFNγ, IL-2, IL-12 or IL-15 in a cell-type specific manner,[312-314] regulators of its release in particular from adipocytes are presently unknown. We identified both insulin and TNF-α to augment the CM concentration of DPP4 by about 50% despite an unaltered expression. Thus, factors related to insulin resistance and AT inflammation enhance the shedding of this novel adipokine from the cell surface of human adipocytes. Recent studies suggested a role for DPP4 in AT inflammation, as DPP4 inhibition in obese mice models reduced the expression of inflammatory markers and immune cell infiltration into AT.[315,316] In this context, it is likely that a viscous cycle of TNF-α-mediated DPP4 up-regulation together with DPP4-mediated immune cell attraction might trigger prolonged AT inflammation.

Moreover, the finding of the insulin-induced increase in DPP4 membrane-shedding together with the notion that DPP4 inhibitors in T2D treatment improves insulin sensitivity, points to a functional role of DPP4 in insulin signalling. Moreover, our data obtained from serum analysis of lean and morbidly obese individuals point to a link between hyperinsulinaemia and circulating DPP4, as both parameters correlated positively to each other. To date the direct effects of soluble DPP4 on isolated cells have not been investigated, although it binds to the ECM and may exert signalling functions.[317] We demonstrated for the first time a prominent inhibitory effect of soluble DPP4 on adipocyte insulin signalling, an effect also observable in other cell types such as SkMC and SMC. The used DPP4 concentration is in accordance with circulating levels of lean and morbidly obese individuals. Enzymatic activity of DPP4 appears to be involved in the process of insulin resistance, but DPP4 inhibitors may also affect the binding properties of soluble DPP4 to a putative currently unknown receptor. The underlying mechanisms and the identification of a putative receptor are of major interest and have to be assessed in future studies.

Additionally to its role in insulin resistance and inflammation, DPP4 may also affect AT lipolysis. DPP4 is known as a binding partner for ADA, recruiting it to the cell surface[318] and possibly modulating the well-established anti-lipolytic effects of adenosine. Moreover, DPP4 is a very strong inhibitor of the anti-lipolytic activity of neuropeptide Y (NPY),[319] which is one of the best peptide substrates of the enzyme.[23] Therefore,

enhanced abundance of DPP4 within AT may substantially augment the lipolytic activity of enlarged adipocytes.

In conclusion, our study demonstrated DPP4 as an important novel adipokine that exerts endocrine and autocrine effects on insulin signalling. AT derived DPP4 may represent a major contributor in the pathology of obesity-related T2D and the development of the metabolic syndrome. Moreover, due to its suggested role in AT inflammation it is likely that DPP4 plays a yet undefined functional role in the intra-organ crosstalk between macrophages, adipocytes and other components of the AT, thereby affecting AT homeostasis.

7.1.6. Pigment epithelium-derived factor (PEDF)

Pigment epithelium-derived factor (PEDF) is a 50 kDa glycoprotein, which belongs to the family of non-inhibitory serpines. It was first described as a neurotrophic factor in CM of retinal pigment epithelial cells, which was able to convert Y79 neuroblasoma cells into non-proliferative neurons with a less malignant phenotype.[320] In this context, PEDF possesses numerous biological activities, as it affects cell differentiation and survival of neurons in the brain, eye and spinal cord. Moreover, it represents a potent endogenous anti-angiogenic agent that blocks endothelial proliferation and migration mainly by counteracting the angiogenic activity of VEGF.[321] Decreased levels of PEDF in the eye correlate positively with neovascular and neurodegenerative ocular pathologies.[322,323]

PEDF is known for a decade as an adipokine, expressed and released from adipose-derived stem cells, 3T3-L1 mouse adipocytes and human adipocytes.[58,324-326] However, our study revealed PEDF as one of the most abundant proteins in CM of human primary adipocytes. 2D-gelelectrophoresis and MALDI-MS of adipocyte-generated CM identified this protein within 15 different protein spots, representing 6% of all calculated spot intensities on the 2D-gel. Consistently, a previous study also identified PEDF as a high abundant protein in CM of 3T3-L1 adipocytes.[324] PEDF expression and release increases transiently throughout differentiation of human adipocytes. Moreover, we observed a continuous secretion of PEDF over 48 hours, indicating no self-regulatory effect on its release in human adipocytes. It is reported that PEDF can be

found at high amounts in the circulation and its levels correlate positively with metabolic pathologies such as obesity, insulin resistance, T2D, and the metabolic syndrome.[327-329] The correlation between circulating PEDF levels and obesity suggests AT as an important endocrine organ for its release. Such a role of AT derived PEDF became evident since Crowe and colleagues showed that AT-specific expression of PEDF positively correlated with obesity and insulin resistance in mice.[324] Additionally, the latter study reported of only modest PEDF secretion from hepatocytes and monocytes, making it likely that AT represents the major organ contributing to the high circulating levels. This hypothesis is underlined by our observation that adipocytes release significantly higher amounts of PEDF than AT-derived macrophages, skeletal muscle cells and smooth muscle cells. However, the relevance of the liver contributing to plasma levels of PEDF in humans remains to be elucidated.

PEDF regulation is still incompletely characterized. Reduced oxygen tensions are recognized to down-regulate PEDF expression and secretion levels, as demonstrated in retinal glial (Müller) cells, anoxic cardiac myocytes and retinas of rats, which were exposed to hypobaric hypoxia.[330-332] Consistently, we observed reduced PEDF expression and secretion in human adipocytes under severe hypoxia of 1% O_2. Contrary to these studies, a recent report demonstrated elevated PEDF levels at low oxygen concentrations in retinal glial (Müller) cells.[333] Here, the retinal cells were initially incubated at nearly anoxic conditions of 0.2% O_2 and further cultured at milder oxygen tensions of 2.5%, suggesting that a 'relative hyperoxia' or return to physiological oxygen tensions induces an up-regulation of PEDF. These reports demonstrate that hypoxia-mediated PEDF regulation underlies a complex mechanism and different variations in oxygen tension might result in adverse effects. To our knowledge, our report was the first to demonstrate hypoxia-induced down-regulation of PEDF in human adipocytes. It is likely that also PEDF secretion by AT might vary at alternating cycles of AT oxygenation. Further investigations are necessary to get more detailed insights into oxygen-mediated PEDF regulation in AT.

Furthermore, we demonstrated PEDF to be regulated by classical stimulators of AT, including TNF-α, the PPARγ agonist troglitazone, and insulin. Here, TNF-α and insulin induced an up-regulation of PEDF, while troglitazone reduced its levels. The specific underlying mechanisms are

yet not known. However, Yamagishi et al. reported as well TNF-α induced reduction of PEDF mRNA in HUVEC, an effect possibly mediated by TNF-α-elicited reactive oxygen species (ROS) generation.[334] Such a ROS-mediated mechanism is likely, as it was demonstrated that angiotensin-II-induced ROS is capable to suppress PEDF mRNA levels.[335] Moreover, as hyperinsulinaemia plays a pivotal role in obesity and T2D, insulin-mediated up-regulation of PEDF release by AT might reflect one cause for the elevated plasma levels observed in type 2 diabetics.

A recent study revealed metformin as a putative regulator of PEDF expression and its circulating levels.[336] Newly diagnosed T2D patients treated with metformin demonstrated higher plasma PEDF levels despite weight loss and improvement of insulin sensitivity. This is in contrast to a current report demonstrating reduced PEDF levels after weight loss in addition with improved insulin sensitivity.[337] These differences might be related to the different study designs, as the first used a medication approach to reduce weight and improve insulin sensitivity, and the second was based on caloric restriction. Indeed, Franck et al. suggested caloric intake to regulate PEDF levels, as they reported reduced mRNA levels in AT, and reduced PEDF plasma levels due to a very low caloric diet, while a high caloric diet led to the opposite effect.[338] Moreover, the diet-induced decrease in PEDF plasma levels were reversed after refeeding. These studies emphasise the importance of AT as a source of circulating PEDF implying that nutritional imbalance affects systemic PEDF levels.

Evidence came up for a role of PEDF in lipolysis and fatty acid metabolism. Notari et al. suggested ATGL as a specific receptor for PEDF, indicating a role for PEDF in lipolysis and fatty acid metabolism.[339] As ATGL is an intracellular protein, a possible interaction between PEDF and ATGL had to occur inside of cells. A study performed in hepatocytes reported an uptake of recombinant PEDF and its colocalization with ATGL at lipid droplets, leading to reduced hepatocyte triglyceride content.[340] Moreover, a recent mouse model demonstrated the involvement of PEDF in systemic fatty acid metabolism, as PEDF promoted lipolysis, affected fatty acid oxidation and induced insulin-resistance in an ATGL-dependent manner.[341] Interestingly, in skeletal muscle PEDF reduced fatty acid oxidation and the expression of genes involved in mitochondrial fat oxidation, resulting in TAG accumulation. The same group demonstrated in a previous study that systemic PEDF administration led to insulin

resistance in the liver and skeletal muscle, which they now suggested to be an ATGL-dependent mechanism.[324,341] We also observed an inhibitory effect of PEDF on insulin-stimulated Akt signalling in adipocytes, skeletal and smooth muscle cells, with the most prominent effect on adipocytes. Although ATGL is expressed in skeletal and smooth muscle cells, we demonstrated the highest expression in adipocytes. This expression pattern of ATGL possibly reflects the underlying cause for the stronger effect of PEDF on adipocyte insulin signalling than observed in the other cell types.

In conclusion, we identified PEDF as one of the most abundant adipokines released by adipocytes possibly contributing to its high levels found in the circulation. AT-specific dysregulation of PEDF seems to contribute to obesity-related metabolic dysfunctions. The direct role of PEDF in AT is yet not clear, but a contribution to AT homeostasis and lipolysis is likely.

7.2. Adipose tissue hypoxia

Reduced oxygenation of human tissue's is a natural process that may arise during normal tissue development and wound healing, but also occurs in pathological conditions due to defective vasculature, including T2D, atherosclerosis or cancer progression.[198] As outlined in the previous sections, adaptive mechanisms to reduced oxygenation have developed and are evolutionary highly conserved.[199] These mechanisms are responsible for maintaining cell viability and survival during periods of oxygen deficiency. In general, energy metabolism of hypoxic cells switches from a high oxidative to a glycolytic metabolism, thereby repressing TCA and up-regulating glucose utilization.[198,208] HIF-1α is an important mediator of these processes, as under acute hypoxia it mediates survival and repair processes. However, severe prolonged hypoxia and HIF-1α over-expression have been associated to pathological conditions including impaired wound healing or diabetic complications.[214,220]

AT hypoxia has been suggested as a possible cause and trigger for AT inflammation, as various inflammation-related adipokines are inducible by hypoxia.[52] This hypothesis has gained much attention to diverse *in vivo* studies, mainly performed in obese mouse models. These studies have consistently demonstrated that oxygen levels could decrease down to 2% O_2 in AT of obese mice, and were positively linked to AT inflammation and dysregulation.[236-239] However, assessment of AT oxygenation in human AT have demonstrated a less severe hypoxia than observed in animal models. Here, AT oxygenation have been reported to range between 3-11% O_2, even though these levels also could be correlated with inflammatory responses.[242,243,260]

The aim of the second part of this thesis was to establish and characterize an *in vitro* model of AT hypoxia affecting human primary adipocytes. For this purpose, current knowledge of the hypoxic response was used to validate the effect of hypoxia on human adipocytes. New insights into adipokine dysregulation and its relation to inflammatory responses under acute and severe hypoxia were investigated and will be discussed in the following paragraphs.

A second aspect in this context was the relevance of reduced oxygen tension during adipogenesis. It is apparent that the general cell culture model of human adipocytes at ambient air does not reflect physiological conditions as outlined in chapter 1.3. To this end, adipocyte development under more physiological conditions and its related physiological functions were investigated and compared to the standard cell culture procedure.

7.2.1. Hypoxia-induced adipokine dysregulation and inflammation

Diverse in vitro studies demonstrated hypoxia as a modulator of adipocyte biology. In the light of the established protocols of the current literature[203,247], our cell culture model was based on an acute treatment (8-48 hours) of *in vitro* differentiated human adipocytes under severe hypoxia of 1% O_2. Accumulation of HIF-1α and its target gene GLUT1 represents a classical response to reduced oxygen concentrations, which we could also observe in our cell culture model of hypoxic adipocytes. Moreover, we reproduced known hypoxia-induced effects on distinct adipokines, including reduced adiponectin release, but increased secretion

of leptin, VEGF and IL-6.[203] As we wanted to explore the effect of hypoxia on general adipocyte secretion, we used cytokine arrays evaluating 174 factors as an initial screening method to identify alterations in adipokine content between CMs generated from normoxic and hypoxic adipocytes. Mostly moderate effects could be detected, as from 174 factors just 12 were elevated by more than 50%, and further 9 were reduced by about 50% in comparison to the normoxic control. Among these, mainly already known hypoxia-regulated factors were included, such as VEGF, leptin, IL-6 and adiponectin. However, a very intriguing observation was a prominent suppression of MCP-1 release and other chemokines, including Gro-α, and eotaxin. Since in the obese state AT inflammation has been positively correlated to immune cell infiltration,[68,71] we have rather expected a hypoxia-mediated increase than decrease in the production of immune cell attracting chemokines. Our study was the first to demonstrate suppression of chemokines including MCP-1, Gro-α, and eotaxin under hypoxia in human primary adipocytes.

On purpose to identify signalling pathways, which were involved in the secretory changes, we performed a Kinex™ Antibody Microarray. This multiplex based microarray evaluated the expression and phosphorylation of diverse cellular proteins via 377 pan-specific and 273 phospho-specific antibodies, in lysates of hypoxia-treated adipocytes in comparison to normoxic adipocytes. Here, various signalling pathways were identified by the Kinex™ Antibody Microarray to be affected by hypoxia, including energy metabolism, apoptosis, cell cycle arrest and inflammation. 37 signals showed an up-regulation of at least 50% over the normoxic control, and even 12 signals of more than 100%, while 44 signals were reduced by more than 40%. Among these, the inhibitor of NF-κB, IκBα demonstrated one of the most prominent increases of 152% over the normoxic control. Also other proteins involved in the NF-κB pathway demonstrated altered expression levels, as demonstrated in table 7.1.

Table 7.1 ***Hypoxia-induced changes in protein expression and phosphorylation identified by the*** **Kinex™ Antibody Microarray**

Target protein	expression/phospo-site	% change vs. control
IκBα	Pan-specific	+152
IKKα	Pan / S180/S181	-16 / -24
IKKβ	Pan-specific	-51
IKKγ (NEMO)	Pan-specific	-10
NFκB p65	Pan / S276	20 / 22
PKCβ2	T641	+167
PKA R2β	S114	+137
PI3K	Pan-specific	+ 115
CDK8	Pan-specific	+111
VEGFR2	Y1054+Y1059	+110
Bcl-xL	Pan-specific	+105
JNK1/2/3	Pan-specific	-51
Casp5	Pan-specific	-49
Casp 6	Pan-specific	-47
Casp8	Pan-specific	-42

Since validation of the outcome of this initial screening method was necessary in further experiments, we focused on the NF-κB signalling pathway. To this end, we stimulated human normoxic and hyoxic adipocytes with the pro-inflammatory cytokine TNF-α. It is well described that hypoxia activates NF-κB signalling in diverse cell lines such as Jurkat, HeLa or 3T3-L1 cells.[236,252,254] However, this effect seems to be cell type dependent, as also reduced NF-κB activity under hypoxia in endothelial cells was reported.[342] Importantly, we demonstrated a reduced responsiveness of human adipocytes under acute hypoxia and TNF-α stimulation. This reduced response was reflected by a decreased TNF-α-induced activation of NF-κB in hypoxic adipocytes, an effect possibly facilitated by a reduced inactivation of the NF-κB inhibitor IκBα. As a consequence of this impaired response towards TNF-α, the release of the NF-κB target genes MCP-1 and IL-8 was significantly reduced. Additionally, in the case of MCP-1 also the mRNA expression level was significantly decreased. This finding was in accordance with reports

demonstrating hypoxia to reduce its specific mRNA and protein levels in other cell types.[343-345] However, IL-8 mRNA levels were not affected under these conditions. Moreover, the reduction in TNF-α-induced IL-8 secretion under hypoxia was in contrast to a study performed in HeLa and COS7 cells.[346] The latter study demonstrated elevated IL-8 release from these cell lines due to NF-κB stimulation under hypoxia. The cause for the different outcomes might be due to diverse factors, such as a cell type-specific response in this context. Furthermore, hypoxia has been shown to reduce mRNA translation,[347,348] so that a post-transcriptional mechanism might underlie the observed reduction in IL-8 release. However, further studies have to assess the specific mechanism in this context.

The attenuation of TNF-α-mediated NF-κB signalling might be mediated directly by alterations in the TNF-α-related receptor activity. We observed a slight but significant decrease in TNF-R1 protein expression with no significant regulation on the mRNA level, while for the TNF-R2 an increased mRNA level was detectable. Due to the fact, that we could not assess TNF-R2 on protein level, we could only assume that this receptor is more present on the cell membrane under hypoxia. TNF-R1 is regarded as the main receptor for canonical NF-κB signalling,[349] therefore implicating that NF-κB signalling might be affected upstream of the IKK complex in our study. TNF-R1 signalling relies on the building of specific signalling complexes. Complex I mediate the expression of anti-apoptotic proteins, while complex II triggers cell death processes after the internalization of the receptor. In contrast to TNF-R1, TNF-R2 has no death domain, and is therefore suggested to promote only anti-apoptotic and pro-survival signals, as demonstrated in diverse cell types.[350-353] The role of TNF-R2 is less clear, as several studies point not only to a differently regulation towards TNF-R1, but also to a crosstalk between TNF-R1 and TNF-R2. This crosstalk seems to inhibit the anti-apoptotic pathway and promotes cytotoxicity triggered by TNF-R1.[354,355] However, the signalling complex upstream of IKK is highly complex and dependent on diverse adapter proteins. Depletion or down-regulation of one adapter does not necessarily result in the absence of a specific down-stream signalling event, as rather a balance between anti-apoptotic and apoptotic signals determines the final output of TNF-receptor activation.[350] We could only speculate that our observation of reduced TNF-R1 and increased TNF-R2 expression promotes pro-survival signals, as inflammatory MCP-1 and IL-8 release is

down-regulated, therefore contributing to a less inflammatory response. The specific signalling between the TNF-receptors and NF-κB activation over the IKK complex had to be assessed in future experiments.

Moreover, we assessed only the RelA (p65) subunit of NF-κB, which mediates the predominant activity of the canonical pathway.[146] As the different NF-κB dimers regulate different target genes, it will be of remarkable interest to get more insights how other subunits are regulated under hypoxia and additional TNF-α stimulation. In particular, the activity of the non-canonical pathway had to be investigated, as target genes of this pathway are involved in the activation, maturation and survival of distinct immune cells.[147] Our data supports a regulatory effect of severe hypoxia on this pathway, as we observed a prominent repression of the predominant activator of this pathway IKKα.

Our data suggests an unexpected, but presumably beneficial anti-inflammatory effect of hypoxia on adipocytes. It could be speculated, that these observations reflect a mechanism to limit inflammation and possibly immune cell infiltration into hypoxic regions of AT. Since immune cells such as macrophages produce large amounts of TNF-α,[251,356] which is known to promote adipocyte apoptosis,[110] reduced responsiveness towards this cytokine might be a survival strategy. Such strategies are essential to maintain tissue function and homeostasis and to prevent cell apoptosis and necrosis. However, it was as well demonstrated that hypoxia potently inhibits macrophage migration.[345,357] This mechanism possibly stimulates the accumulation and represses the egression of macrophages in inflamed and hypoxic regions, as it was suggested in the context of atherosclerosis.[358] Moreover, not only cytokines seem to be involved in the attraction of immune cells into AT. A recent study demonstrated that weight loss in obese mice and the related increase in AT lipolysis and lipid fluxes led to an increased recruitment of macrophages to AT.[359] Since hypoxia is known to induce lipolysis, and FFA's were further demonstrated to up-regulate TNF-α secretion by macrophages,[72] both mechanisms together would possibly trigger a high inflammatory state. In this situation, cells would profit from the attenuation of TNF-α-mediated NF-κB signalling, and therefore cell damage might be prevented or delayed by a limited inflammatory response.

7.2.2. Adipocyte function at physiologic oxygen tensions

As outlined in chapter 1.3.1, oxygen tensions in the body are dissimilar from ambient air. For human AT oxygen levels ranging between 3-11% O_2 are reported.[243] Here, the O_2 level is much lower than ambient air that is about 21%. Basically, adipocyte cell culture experiments are performed at ambient air, which does not reflect the physiological situation and could rather be defined as a 'hyperoxic' cell culture model. In study 4, we used this classic culture model, to be in line with current investigations. However, in study 5 we analysed adipocyte function under more physiological conditions after differentiation of human primary adipocytes at reduced oxygen levels. It has been shown for 3T3-L1 as well as human bone marrow stromal cells (hBMSCs), that severe hypoxia of 1% O_2 inhibited adipocyte differentiation and maintained the cells in a precursor phenotype.[360,361] In contrast, moderate hypoxia of 8% O_2 stimulated adipogenesis in murine BMSCs.[362] Due to the latter study and the report of human AT oxygen levels ranging between 3-11% O_2, we considered adipocyte differentiation at 10% and 5% O_2 in comparison to ambient air (21% O_2). We expected similar or even enhanced differentiation, as low oxygen tensions are known to influence the capacity of embryonic stem cell differentiation into divers cell types, including cardiomyocytes, neuronal cells, endothelial cells, or chondrocytes.[363] Finally, adipocyte differentiation at 10% and 5% O_2 was comparable to 21% O_2, as indicated by similar lipid droplet accumulation and expression of distinct differentiation markers. However, physiological oxygen tensions affected adipocyte physiology. Besides reduced lipid droplet size and increased triglyceride content in adipocytes differentiated at 10% O_2, adipokine secretion and energy metabolism was affected. Our results demonstrated that oxygen level is an important molecular regulator of adipocyte development and function. However, the general adipogenic potential of preadipocytes was not impaired in the oxygen range of 5-21% O_2 as it has been observed at 1% O_2.

As mentioned above, adipokine expression and secretion is sensitive towards varying oxygen levels. Wood et al. demonstrated recently that various adipokines are positively or negatively regulated by oxygen in a straight-dose response.[364] In contrast, our study revealed a bell-shaped response in the release of adipokines such as adiponectin and IL-6 from adipocytes differentiated at more physiological oxygen concentrations. To

our knowledge, our study was the first to assess adipokine expression and release from adipocytes with varying O_2 levels during adipogenesis, while prior studies used the standard cell culture model of cell differentiation at ambient air and subsequent acute hypoxia treatment. Therefore, we suggest that the differences observed in our study to the current literature would account to the different protocol of adipocyte differentiation. The bell-shaped response was assessed by three reading points, and a more detailed dose-response would be of remarkable interest. Additional oxygen setpoints would provide more detailed insights into the regulation of adipokine expression and release by oxygen during adipogenesis. Particularly the upper and lower thresholds for the increase or decrease in adipokine secretion would provide a better understanding of AT hypoxia and its role in AT dysregulation and inflammation.

The most intriguing observation was the increased release of adiponectin under physiological oxygen tensions. It is well established that hypoxia inhibits adiponectin expression and secretion.[203,236,237,247,364] This unexpected increase would point to a less inflammatory state in the adipocyte. However, we also detected increased IL-6 and DPP4 levels, which are known to mediate inflammation.[315,316,365,366] This data would support the finding of Goossens et al., who detected increased oxygen concentrations in AT of obese compared to lean individuals, which positively correlated with insulin resistance and AT inflammation.[243] However, Pasarica et al. previously reported rather reduced oxygen levels in obese AT also correlating with inflammatory markers, which is in line with the current hypothesis of AT hypoxia.[242] On the one hand, our observation of elevated inflammatory IL-6 and DPP4, as well as increased triglyceride content and basal lipolysis in adipocytes differentiated at 10% O_2, reflect events in the obese state. This would support the notion that rather increased O_2 levels induce obesity-related AT inflammation and dysregulation in the obese state. On the other hand, we did not observe an up-regulation of all inflammatory markers such as MCP-1, which showed no regulation between the three conditions. More importantly anti-inflammatory adiponectin levels were markedly increased at 10% and tended to decrease at 5% O_2, which would rather support the classic view of reduced AT oxygenation as a trigger of inflammation. Moreover, we demonstrated leptin levels to decrease at 10% compared to 21% O_2. This is in line with a report demonstrating increased circulating leptin levels

and mRNA expression in AT of mice, which were exposed to a hyperoxic environment up to 72 hours.[367] Further studies are necessary to elucidate this complex regulatory role of physiologic oxygen tension during adipogenesis and its effect on AT inflammation.

Our cell culture model further revealed a better lipolytic activity of adipocytes differentiated at physiologic oxygen tensions. In addition to an increase in basal lipolysis of adipocytes differentiated at 10% O_2, we observed an improved glycerol release after isoproterenol-stimulation even at low concentrations of 1-100nM isoproterenol. Elevated glycerol release was also observable for adipocytes differentiated at 5% O_2, although this effect was only significant at higher isoproterenol concentrations of 100nM isoproterenol. Our data supported the notion, that these effects were mediated by an up-regulation of the hormone sensitive pathway. Here, we identified elevated expression levels of HSL, perilipin and β-adrenergic receptors, which represent important mediator of catecholamine-mediated lipolysis. In obesity basal lipolysis is increased while catecholamine-mediated lipolysis is reduced, which has been correlated to reduced expression levels of HSL, ATGL and perilipin in obese AT.[177-181] Insulin resistance has also been assumed as a cause for increased lipolysis in obesity, as diminished anti-lipolytic action was reported for obese rats and women with visceral obesity.[182-184] We observed also an alteration in insulin stimulation of adipocytes differentiated at reduced oxygen levels. Here, we observed a significant decrease in insulin-mediated Akt (Ser473) phosphorylation, of about 35% compared to the normoxic control, while no significant effect on the Akt (Thr308) phosphorylation site could be detected (Fig. 7.1 A-B).

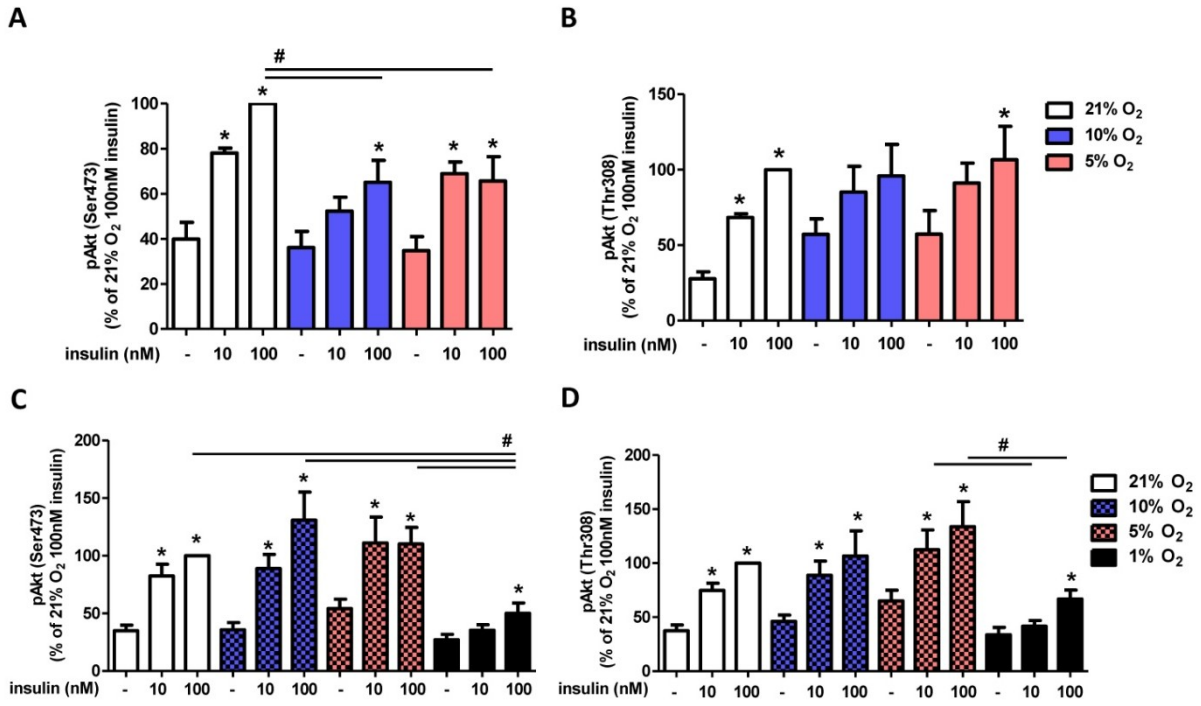

Figure 7.1 Insulin signalling in human adipocytes.

(A-B) Insulin-stimulated Akt (Ser473) and Akt (Thr308) phosphorylation in adipocytes differentiated at 21%, 10% and 5% O_2. *p≤0.05 compared to the respective basal level, #p≤0.05 (C-D) Insulin-stimulated Akt (Ser473) and Akt (Thr308) phosphorylation in adipocytes differentiated at 21% O_2 and further 24h incubation at 10%, 5% or 1% O_2. Data are mean values of ≥4 individual experiments. *p≤0.05 compared to the respective basal level, #p≤0.05

Furthermore, we wanted to elucidate whether the reduction in insulin-stimulated Akt-signalling is a common mechanism due to reduced oxygen levels or dependent on the O_2 level during adipogenesis. For this purpose we measured insulin-stimulated Akt-signalling in adipocytes, which were differentiated at 21% O_2 and subsequently incubated at 10%, 5%, or 1% O_2 for 24 hours. No significant changes in insulin-stimulated phosphorylation of Akt (Ser473) and (Thr308) compared to the control at 21% O_2 (Fig 7.1 D-E) were detectable. However, severe hypoxia of 1% O_2 induced the already described effect of decreased insulin-stimulated Akt phosphorylation on adipocytes differentiated at ambient air.[239,259] This finding is in accordance to the report of Regazzetti et al. who demonstrated in addition to inhibited insulin signalling at 1% O_2 no difference in Akt phosphorylation at 7% O_2, which is similar to our data.[259]

This data clearly demonstrates that the oxygen level during adipogenesis is important for essential signalling pathways such as the insulin dependent one. However, as this data is preliminary and to this time we have not assessed adipocyte glucose uptake under physiologic oxygen conditions, we could not define if glucose metabolism is affected in adipocytes in this state. Akt signalling is involved in numerous cellular processes including glucose metabolism, cell proliferation and cell survival.[138] It is likely that the reduction in insulin-stimulated Akt phosphorylation might not affect glucose metabolism, but is in regard to other cellular functions such as cell cycle arrest and survival. However, since it is known that cells under severe hypoxia switches their metabolism from oxidative to a glucose dependent one,[198,208] enhanced fatty acid and reduced glucose usage as energy source could be assumed under sufficient oxygen supply. Further investigations are necessary to elucidate the downstream targets, which were affected by reduced insulin-stimulated Akt signalling in adipocytes differentiated at physiologic oxygen tensions.

Since it is known that adipokines play a pivotal role in the cross-talk of AT with peripheral tissues, adipokine dysregulation is very well recognized to be involved in the development of pathologies including skeletal muscle insulin resistance and atherosclerosis. It has been demonstrated that adipocyte-generated CM, as well as distinct adipokines, including MCP-1, IL-6, TNF-α, or PEDF, are capable to induce insulin resistance in SkMC. [267,324,368,369] Moreover, induction of SMC proliferation, which represents an important event in the development of atherosclerosis, has as well been reported to be induced by specific adipokines and CM.[370-372] In this context, adiponectin exhibits a specific role. Besides its well known insulin-sensitizing effects,[88] it is also capable to alter the content of adipocyte-generated CM towards reduced amounts of insulin resistance-inducing adipokines[373]. Moreover, CM-induced SMC proliferation has been reported to correlate negatively with the amount of adiponectin in the CM.[372] In this context, we assumed that the elevated levels of adiponectin, released into CM of adipocytes differentiated under more physiological conditions, would alter the effect of CM on SkMC insulin signalling and SMC proliferation. The alterations in CM composition, and particularly the increased adiponectin amounts, could not prevent the inhibitory effect of CM on SkMC insulin signalling. However, SMC

proliferation was reduced when treated with CM of adipocytes differentiated at 10% O_2 compared to CM from adipocytes at ambient air. This finding was in line with the observation that adipocytes at 10% O_2 released the highest amounts of adiponectin, which possibly displays the underlying cause for the reduced proliferation. Nevertheless, both cross-talk experiments demonstrated a pivotal role of adipokines in pathologic processes, as CM induced significantly SkMC insulin resistance and SMC proliferation, independent of oxygen-mediated regulation of adipokine release.

Conclusively, oxygen is still known as an important regulator of adipocyte secretion and function, but this study revealed that adipocyte culture at 21 % O_2, which could be defined as hyperoxia, seem to impair adipocyte function. Since *in vitro* experiments are an approach to mimic physiological events, it should be taken into account that oxygen levels during cell development are an important variable. Investigations on AT hypoxia should rather be performed under physiological oxygen conditions, as distinct effects might be adverse or even more pronounced to observations at ambient air, as demonstrated in this study for adipokine release and the lipolytic activity of adipocytes.

In summary, the presented thesis emphasized the important role of AT as an endocrine organ, which is capable in releasing numerous adipokines involved in AT homeostasis and obesity-related pathologies. Novel adipokines, as well as a high abundant adipokines were validated and characterized (Fig. 7.2). Importantly, DPP4 and PEDF could be demonstrated as potent contributors of autocrine and paracrine insulin resistance. Whether these adipokines exhibit additional roles in AT function and homeostasis, has to be explored in future experiments.

Furthermore, decreases in the oxygen level could be demonstrated to affect AT secretion and function. Acute hypoxia could be shown to possibly reduce adipocyte inflammation, as an attenuated TNF-α-mediated NF-κB signalling was observed, with subsequent reduced secretion of the chemokines MCP-1 and IL-8. In order to mimic more physiologic conditions, prolonged reduction of the oxygen level during adipocyte differentiation caused substantial differences in adipokine release, in

particular increased adiponectin release. Moreover, the lipolytic activity of these adipocytes increased significantly, while insulin signalling was reduced. The precise role of these effects observed under physiological oxygen tensions on AT function and in the context of obesity has to be explored in future investigations.

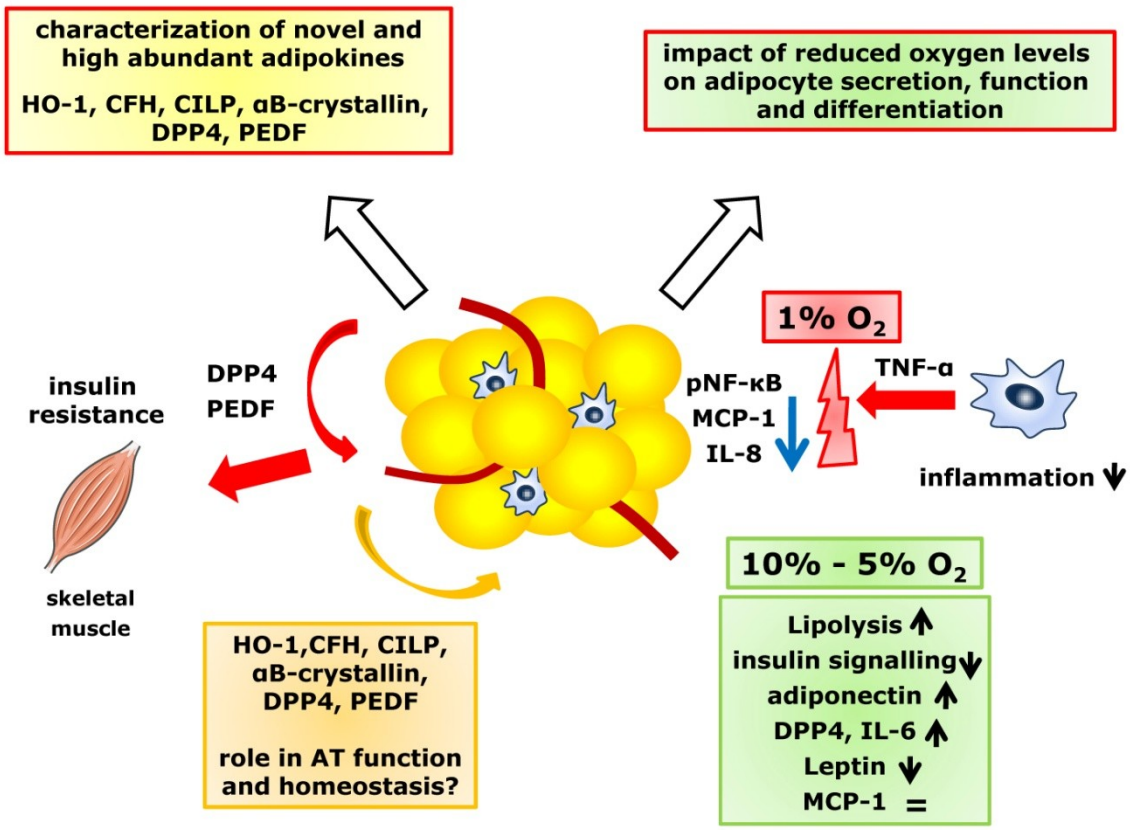

Figure 7.2 Summary of the presented thesis

Validation and characterization of novel and high abundant adipokines as autocrine and endocrine factors; analysis of oxygen tensions on adipocyte secretion and function during acute and severe hypoxia of 1% O_2, as well as chronic exposure to 10% and 5% O_2 during adipogenesis

7.3. Perspectives

The presented thesis met several objectives proposed in the introduction. Proteomic profiling is a powerful technique to reveal novel adipokines and to complement our current knowledge of the outcome of the adipocyte secretome. In this thesis, five out of 44 potential novel adipokines were validated as confirmed adipokines in human primary adipocytes, and further characterized. This analysis suggested specific roles for these adipokines in AT function, including adipogenesis, energy metabolism, and inflammation. However, the precise role of the most factors in obesity-related disorders is still unknown and has to be addressed in future investigations. For DPP4, a prominent role in T2D and insulin resistance has been already appreciated, as this factor is a current drug target in diabetes treatment. The identification of DPP4 as an AT-derived factor together with the observation that DPP4 is a potent inducer of insulin resistance presented more detailed insights into the source and action of DPP4 in metabolic disorders. However, its precise mode of action is still incompletely understood and particularly the identification of potential receptors would be of major interest.

In purpose to identify potential novel biomarkers and important mediators of obesity related disorders, clinical studies should be included in future investigations. The vast number of novel adipokines raises the question of the relevance of these factors in AT physiology and in the context of its dysregulation. In particular, high abundant adipokines such as PEDF, which could be found at high levels in the circulation, underlines the importance of AT as a major endocrine organ. However, the precise role of such adipokines released from AT at substantial amounts into the circulation has to be elucidated, in the light of the cross-talk between AT and peripheral tissues, as well as in comparison to local acting factors such as TNF-α. The knowledge of the endocrine source of important inflammatory and anti-inflammatory factors, and their mode of action might support future drug development and treatment of metabolic pathologies such as T2D.

We further investigated the role of AT hypoxia in the context of adipocyte secretion and inflammation. The current view on the role of hypoxia in adipokine release and dysregulation could be validated and extended. The classic concept of AT hypoxia suggests an inflammatory

role of hypoxia. However, our study contrarily demonstrated a putative anti-inflammatory action in particular on the canonical NF-κB signalling pathway. This was demonstrated by an attenuated TNF-α-induced NF-κB response leading to a reduced release of NF-κB-related chemokines. However, as the NF-κB signalling pathway is very complex and the detailed underlying mechanisms are still unknown, future investigations have to define the modulatory effect of hypoxia on the NF-κB signalling pathway. In this context, various NF-κB dimers are known to target different genes, and until today the regulatory role of hypoxia in the activity of other NF-κB dimers is still unknown. Moreover, as we demonstrated IKKα expression to be down-regulated, and this factor is mainly involved in non-canonical NF-κB signalling, this pathway should as well be investigated to complement the role of hypoxia in AT inflammation.

The oxygen level during adipocyte differentiation was demonstrated in this thesis as an important molecular regulator, inducing alterations in adipocyte function such as adipokine release and lipolysis. The mainly used cell culture model of adipocytes analysed under ambient air is a passable and easy to use *in vitro* model for basic adipocyte investigations, even though it does not reflect the physiologic situation. However, as the presented study was conducted as an initial approach to analyse the effect of oxygen during adipocyte differentiation, the mechanisms for the observed alterations in adipokine secretion and adipocyte signalling are incompletely understood. More detailed insights, in particular a dose-response curve during differentiation, are necessary to emphasise the role of hypoxia in AT dysregulation and inflammation. In addition, also the novel adipokines should be investigated under these conditions, as we demonstrated for DPP4 an even more pronounced release from adipocytes under physiological oxygen tensions. This underlines once more the importance of AT in the contribution to circulating inflammatory factors, which might be regulated by AT oxygen levels.

Contributions

Besides my own contribution, the results published in this thesis involve contributions from colleagues and members of the laboratory of Prof. Eckel. In the following my specific contributions to each publication are outlined.

Chapter 2:

- Culture of adipocytes and generation of lysates and CM for analysis of protein-specific expression and secretion of putative novel adipokines during adipocyte differentiation and after treatment with different stimuli
- Validation and characterization of putative novel adipokines by means of Western Blot and ELISA analysis together with Daniela Lamers
- Isolation of adipose tissue-derived macrophages together with Daniela Lamers
- Literature search for the identification of undescribed factors as putative novel adipokines together with Dr. Henrike Sell, Daniela Lamers, Dr. Kristin Eckardt, Dr. Margriet Ouwens, Dr. Sonja Hartwig and Prof. Eckel
- Preparation of the manuscript together with Dr. Henrike Sell, Daniela Lamers, Dr. Sonja Hartwig, Dr. Stefan Lehr and Prof. Eckel

Chapter 3:

- Culture of adipocytes and generation of lysates and CM for analysis of DPP4-specific expression and secretion during differentiation and after treatment with different stimuli
- Validation of differentiation-dependent expression of DPP4 by means of Western Blot together with Daniela Lamers
- Validation of DPP4 secretion by adipocytes and macrophages by ELISA
- Isolation of adipose tissue-derived macrophages together with Daniela Lamers

- Treatment of adipocytes with DPP4 and insulin for analysis of insulin signalling by means of Western Blot together with Nina Wronkowitz
- Preparation of the manuscript together with Dr. Henrike Sell, Daniela Lamers, Nina Wronkowitz and Prof. Eckel

Chapter 4:

- Culture of adipocytes and generation of lysates and CM for analysis of PEDF-specific expression and secretion during differentiation and after treatment with different stimuli
- Analysis of PEDF expression during differentiation and after treatment with different stimuli by Western Blotting
- Analysis of PEDF secretion during differentiation, after treatment with different stimuli, during a time-course of 48 hours, and in comparison to other cell types by ELISA
- Analysis of insulin-stimulated Akt phosphorylation after treatment with PEDF in adipocytes together with Andrea Cramer and Angelika Horrighs
- Isolation of adipose tissue-derived macrophages together with Daniela Lamers
- Preparation of the manuscript together with Daniela Lamers, Dr. Henrike Sell and Prof. Eckel

Chapter 5:

- Culture and treatment of human primary adipocytes under hypoxic conditions
- Analysis of hypoxia-induced factors HIF-1a and GLUT1 by means of Western Blot and quantitative real-time PCR analysis of mRNA
- Generation of CM from normoxic and hypoxic adipocytes and further adipokine analysis by means of cytokine protein array and ELISA
- Time course of TNF-α-induced NF-κB and IκBα phosphorylation via Western Blotting
- Analysis of protein and mRNA expression of the TNF-R1 and TNF-R2
- ELISA and mRNA analysis of MCP-1 and IL-8 protein release and mRNA expression

- Preparation of the manuscript together with Dr. Henrike Sell and Prof. Eckel

Chapter 6:

- Culture and treatment of human primary adipocytes under low oxygen conditions
- Characterization of adipocytes differentiated at 21%, 10%, and 5% O_2 as means of Oil Red O staining, measurement of lipid droplet size, analysis of triglyceride content, and mRNA analysis of differentiation markers PPARγ and C/EBPα via quantitative real-time PCR
- Analysis of adipokine release and expression by means of ELISA measurements and Western Blot analysis
- Analysis of lipolytic activity by means of glycerol release and analysis of the expression level of proteins involved in adipocyte lipolysis by means of Western Blot
- Analysis of the effect of adipocyte-generated CM on SkMC insulin signalling by means of Western Blot analysis on Akt phosphorylation
- Analysis of the proliferative effect of adipocyte-generated CM on SMC via BrdU proliferation assay together with Raphaela Schlich
- Preparation of the manuscript together with Dr. Henrike Sell and Prof. Eckel

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Eidesstattliche Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf" erstellt worden ist. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

(Susanne Famulla)

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