Identification and Characterization of novel Adipokines

Atherogenic impact of adipocyte-secreted factors on human vascular smooth muscle cells

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Zusammenfassung

Es ist seit einigen Jahren akzeptiert, dass das Fettgewebe neben seiner Funktion als Energiespeicher auch ein endokrines Organ darstellt. Eine Zunahme der Fettgewebsmasse bei Adipositas geht mit der Entstehung von chronischen Krankheiten wie Typ 2 Diabetes und Herz-Kreislauf-Erkrankungen einher. Es wird angenommen, dass diese Korrelation u.a. auf der gesteigerten Sekretion einer Vielzahl von biologisch aktiven Proteinen und Peptiden aus dem Fettgewebe beruht, die auch als Adipokine bezeichnet werden. Kürzlich erschienene Sekretomstudien konnten neue Einblicke in das komplexe Sekretom von Adipozyten geben und lassen vermuten, dass viele Adipokine bis heute noch unentdeckt sind. Deshalb lag das erste Ziel dieser Arbeit in der Sekretomanalyse *in vitro* differenzierter humaner Adipozyten und der anschließenden Validierung und Charakterisierung einiger ausgewählter Proteine. Dieser Ansatz führte zur Identifizierung von insgesamt 347 Proteinen, von denen sich nach bioinformatischer Analyse 263 Proteine als sekretorisch erwiesen. Nach Abgleich mit der Literatur erachten wir 44 Proteine als potentiell neue Adipokine. Die fünf interessantesten Kandidaten, Hämoxygenase 1 (HO-1), αB-Crystallin (CRYAB), Komplement Faktor H (CFH), *cartilage intermediate layer protein* (CILP) und Dipeptidylpeptidase IV (DPP4) wurden validiert und charakterisiert. Mittels Western Blot und/oder ELISAs konnte gezeigt werden, dass alle fünf Proteine von Adipozyten sekretiert werden und eine differenzierungs-abhängige Expression in Adipozyten aufweisen. Der Vergleich der Expression in Adipozyten und primären Makrophagen, die aus dem Fettgewebe isoliert wurden, deutet bei allen fünf Adipokinen darauf hin, dass hauptsächlich Adipozyten den Ursprung für diese Proteine im Fettgewebe darstellen. Die vergleichende Serumanalyse von schlanken und adipösen Probanden zeigte, dass die Menge an HO-1, DPP4 und CFH in adipösen Probanden erhöht ist, wohingegen CILP vermindert ist. Für DDP4 konnte zusätzlich gezeigt werden, dass die Proteinexpression in Fettexplantaten von Adipösen im Vergleich zu Schlanken ansteigt und dass diese Expression in viszeralem Fett höher ist als in subkutanem Fett.

Desweiteren konnten wir durch die Sekretomanalyse *in vitro* differenzierter humaner Adipozyten zeigen, dass der *pigment epithelium-derived factor* (PEDF) eines der abundantesten Proteine im Sekretom der humanen Fettzelle ist. Die Freisetzung von PEDF aus Adipozyten ist signifikant höher als aus anderen primären Zellen wie Makrophagen, was darauf hindeutet, dass Adipozyten für die PEDF Sekretion im Fettgewebe verantwortlich sind. In Adipozyten, Skelettmuskelzellen und glatten Muskelzellen induzierte PEDF Insulinresistenz auf Ebene der Akt Phosphorylierung. In glatten Muskelzellen steigerte PEDF die Proliferation, aber nicht die Migration und

führte zur akuten Aktivierung von proliferativen und inflammatorischen Signalwegen (NF-κB, p38 MAPK und mTOR).

Ein weiterer Themenbereich dieser Arbeit befasste sich mit der atherogenen Wirkung von Adipokinen und freien Fettsäuren auf glatte Muskelzellen. Es ist bekannt, dass die erhöhte Sekretion von Adipokinen und freien Fettsäuren bei der Adipositas eine wichtige Rolle bei der Entstehung der Atherosklerose spielt. Obwohl die Wirkung von einzelnen Adipokinen auf frühe Ereignisse der Atherosklerose, wie der Proliferation und Migration von glatten Muskelzellen, gezeigt werden konnte, ist über die Wirkung von Adipozyten-konditioniertem Medium bisher nur wenig bekannt. In dieser Arbeit wurde gezeigt, dass konditioniertes Medium von humanen *in vitro* differenzierten Adipozyten zur Proliferation und Migration von glatten Muskelzellen führt. Desweiteren erhöhte konditioniertes Medium die Expression des Adhäsionsmoleküls ICAM-1 und aktivierte proliferative und inflammatorische Signalwege (p38 MAPK, mTOR und NF-κB). Die autokrine Wirkung von Adiponectin, bekannt als anti-atherogenes und kardioprotektives Adipokin, hat die vom konditionierten Medium induzierte Proliferation und ICAM-1 Expression fast vollständig aufgehoben. Die Kombination von konditioniertem Medium, das selbst keine freien Fettsäuren enthält, und Ölsäure führte zu einer synergistischen Induktion der Proliferation und Aktivierung von NF-κB im Vergleich zu beiden Faktoren alleine. Die Analyse verschiedener NF-κB Zielgene mittels RT-PCR zeigte, dass die mRNA Expression der zytosolischen Superoxid Dismutase 1 nur in der Kombination von konditioniertem Medium und Ölsäure signifikant vermindert ist. Die Kombination von konditioniertem Medium und Palmitinsäure induzierte synergistisch die mRNA Expression von IL-6 verglichen mit beiden Faktoren alleine. Desweiteren führten sowohl die Kombination von konditioniertem Medium mit Ölsäure, als auch mit Palmitinsäure zu einer verminderten Expression von CIDEA in glatten Muskelzellen.

Zusammenfassend lässt sich sagen, dass die Sekretomanalyse ein wichtiges Werkzeug zur Charakterisierung des Adipozytensekretoms darstellt. Hiermit konnten wir 44 neue Adipokine identifizieren, von denen die fünf interessantesten validiert und charakterisiert wurden. Desweiteren konnte diese Arbeit zeigen, dass die Inkubation von glatten Muskelzellen mit Adipozyten-konditioniertem Medium, mit oder ohne freie Fettsäuren, ein gutes Model darstellt, um den Crosstalk zwischen den beiden Zelltypen im Hinblick auf die Entstehung der Atherosklerose näher zu charakterisieren.

Summary

In recent years it has become well accepted that adipose tissue not only functions as energy storage but can also be viewed as a full endocrine organ. The enlargement of adipose tissue mass in obesity frequently associates with the development of chronic diseases, including type 2 diabetes and cardiovascular disease. This correlation is suggested to be due to an increased release of numerous proteins and bioactive peptides by adipose tissue, known as adipokines, leading to a crosstalk between liver, muscle and fat which underlies the progression of these diseases. In this regard, considerable research in recent years has focused on the characterization of adipose tissue secretome, trying to obtain insight into the complex secretory output and relevant proteins probably involved in obesity-related disorders. Although several proteomic approaches based on adipocytes or adipose tissue explants emphasized the complex nature of the whole secretory output, the adipokinome of human adipocytes remains incompletely characterized. Therefore, one aim of this thesis was to conduct a comprehensive proteomic profiling of conditioned media derived from *in vitro* differentiated human adipocytes. Here, we could identify a total of 347 proteins, with 263 secreted proteins according to bioinformatics analysis. Literature research led to the identification of 44 proteins, which we consider to be potential novel adipokines secreted from primary human adipocytes. Subsequently, the five most interesting target proteins, namely heme oxygenase 1 (HO-1), αB-crystallin (CRYAB), complement factor H (CFH), cartilage intermediate layer protein (CILP), and dipeptidyl peptidase IV (DPP4) were further validated and characterized. By means of immunoblot and/or ELISAs we could validate all five proteins to be secreted by human adipocytes and we could further demonstrate that all candidates are expressed in human adipocytes in a differentiation-dependent manner. Comparing the expression in adipocytes with expression in adipose tissue-derived macrophages indicated that the source of these five novel adipokines in adipose tissue is likely to be represented by adipocytes. Additionally, serum analysis from lean and obese subjects revealed that HO-1, DPP4, and CFH levels are increased in the obese state, whereas the level of CILP is decreasing. For DPP4 we could also show that its protein abundance in fat explants is elevated in obese compared to lean subjects and that this expression level is also increased in visceral vs. subcutaneous adipose tissue.

Secretome analysis of human adipocytes also revealed the pigment epithelium-derived factor (PEDF) as a high abundant adipokine. Adipose tissue-derived macrophages only secrete low amounts of PEDF, pointing to adipocytes as its main source in adipose tissue. In addition, PEDF was shown to induce insulin resistance and inflammatory signaling in adipocytes, skeletal and smooth muscle cells. Furthermore,

PEDF enhances proliferation, but not migration of smooth muscle cells and activates proliferative and inflammatory signaling mediators (mTOR, p38 MAPK, and NF-κB).

A further topic of this work was to address the atherogenic impact of adipokines and free fatty acids on the level at smooth muscle cells. In this context, several studies in humans and animals could show that obesity strongly correlates with the development of atherosclerosis. Although it could be demonstrated that specific adipocyte-derived factors are involved in regulating vascular functions, like smooth muscle cell proliferation and migration, the impact of the whole human adipocyte output on the cross-talk with human smooth muscle cells is only incompletely understood. In the present work, adipocyte-conditioned medium (which does not contain free fatty acids) induced proliferation and migration of human smooth muscle cells. Further, conditioned medium enhanced the expression of ICAM-1 and activated proliferative and inflammatory signaling mediators (p38 MAPK, mTOR, and NF-κB). Autocrine action of adiponectin, which is known as an anti-atherogenic and cardioprotective adipokine, completely abrogated the conditioned medium-induced proliferation and ICAM-1 expression. Combination of conditioned medium with oleic acid synergistically induced proliferation and NF-κB activation in comparison to both factors alone. Investigation of distinct NF-κB target genes revealed that the cytosolic superoxide dismutase 1 is significantly decreased only in the combination of conditioned medium and oleic acid. Furthermore, combination of conditioned medium with palmitic acid synergistically increased IL-6 mRNA expression in comparison to both factors alone. In addition, the combinations of conditioned medium with oleic acid as well as palmitic acid significantly decrease mRNA expression of the lipid droplet coating protein CIDEA in human smooth muscle cells. These findings indicate that the combination of protein factors and lipid mediators augments the deleterious effects in comparison to both conditioned medium and fatty acids alone.

In conclusion, proteomic profiling of conditioned medium of *in vitro* differentiated human adipocytes led to the identification of novel adipokines, which were subsequently validated and characterized. Furthermore, the atherogenic impact of adipocyte-conditioned medium, either alone or in combination with fatty acids, on the level of smooth muscle cells was investigated. In this context the presented work illustrates several novel aspects of the complex crosstalk of adipocytes and smooth muscle cells in the vascular wall.

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List of Abbreviations

1/2D-GE	one/two dimensional gel electrophoresis
ACRP30	adipocyte complement-related protein of 30 kDa
ACE	angiotensin converting enzyme
AdipoR	adiponectin receptor
AMPK	AMP-activated protein kinase
AngII	angiotensin II
ATGL	adipose triglyceride lipase
Apo	apolipoprotein
BMI	body mass index
CAD	coronary artery disease
CFH	complement factor H
CILP	cartilage intermediate-layer protein
CM	adipocyte-conditioned media
CO	carbon monoxide
CRP	c-reactive protein
CRYAB	αB-crystallin
CVD	cardiovascular disease
DAG	diacylglycerol
DPP	dipeptidyl peptidase
EGF	epidermal growth factor
ER	endoplasmatic reticulum
ERK	extracellular signal-regulated kinase
FFA	free fatty acids
FGF	fibroblast growth factor
GH	growth hormone
GLUT	glucose transporter
GLP	glucagon-like peptide
GSK	glycogen synthase kinase
HGF	hepatocyte growth factor
HMW	high molecular weight
HO	heme oxygenase
ICAM	intercellular adhesion molecule
IFN	interferon
IGF	insulin-like growth factor
IKKβ	inhibitor of kappa β kinase

IR	insulin receptor
IRS	insulin receptor substrate
JNK	c-Jun-N-terminal kinase
LC	liquid chromatography
LADA	latent autoimmune diabetes of the adult
LMW	low molecular weight
LPS	lipopolysaccharide
MALDI	matrix-assisted laser desorption/ionization
MAPK	mitogen-activated protein kinase
MCP	monocyte chemotactic protein
MIF	macrophage migration inhibitory factor
MMP	matrix metalloproteinase
MMW	middle molecular weight
MODY	maturity-onset diabetes of the young
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor-kappa B
NO	nitric oxide
OA	oleic acid
PA	palmitic acid
PAI	plasminogen activator inhibitor
PDK	PI3K-dependent serine/threonine kinase
PDGF	platelet-derived growth factor
PEDF	pigment epithelium-derived factor
PGF	placental growth factor
PI	phosphoinositol
PI3K	phosphatidylinositol 3 kinase
PKC	protein kinase C
RANTES	regulated upon activation, normal T-cell expressed, and secreted
RBP	retinol binding protein
ROS	reactive oxygen species
SMC	smooth muscle cells
SOCS	suppressor of cytokine signaling
SOD	superoxide dismutase
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
TNF	tumor necrosis factor
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cells

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CHAPTER 1

General Introduction

1.1 Type 2 diabetes mellitus

The term diabetes mellitus describes a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both. According to the guidelines of the World Health Organization (WHO) and the American Diabetes Association (ADA) diabetes mellitus is diagnosed when 1. the fasting plasma glucose is ≥ 126 mg/dl (7 mmol/l), or 2. symptoms of hyperglycemia occur and the causal plasma glucose is ≥ 200 mg/dl (11.1 mmol/l), or 3. the 2-hour plasma glucose is ≥ 200 mg/dl during an oral glucose tolerance test (OGTT).[1] Diabetes mellitus can be classified into two main forms: type 1 and type 2 diabetes.[2] Type 1 diabetes, accounting for 5-10 % of all diabetic patients, is an autoimmune disorder usually diagnosed in childhood or adolescence, which leads to the destruction of insulin-producing pancreatic β-cells resulting in an absolute insulin deficiency. Therefore, patients with type 1 diabetes, have a lifelong need for insulin treatment.[3] The much more prevalent type 2 diabetes (90-95 % of patients with diabetes) is caused by a combination of resistance to insulin action and an inadequate compensatory insulin secretory response. A degree of hyperglycemia effectual to cause pathological changes in diverse tissues, but without clinical syndromes, can be present for a long period of time before diabetes is diagnosed. Type 2 diabetes patients display a rather relative than absolute insulin deficiency and at least initially do not necessarily need insulin treatment. In some cases adequate glycemic control can be achieved by weight reduction, exercise, as well as oral glucose lowering agents.[4]

Factors that increase the risk for type 2 diabetes as well as for cardiovascular disease are impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT), and are associated with the metabolic syndrome, describing a cluster of several metabolic conditions. The metabolic syndrome (also known as syndrome X or insulin resistance syndrome) is present if a patient displays three out of five defined criteria: 1. abdominal obesity (waist circumference > 88 cm in woman and > 102 cm in men), 2. high

triglyceride levels (more than 150 mg/dl), 3. low high density lipoprotein cholesterol levels (≤ 50 mg/dl in woman and ≤ 40 mg/dl in man), 4. high blood pressure (≥ 135/88 mm Hg), and 5. fasting blood glucose ≥ 110 mg/dl. In the majority of cases, type 2 diabetic patients display a cluster of alterations, such as obesity, dyslipidemia, hypertension and insulin resistance, whereas obesity is the risk factor which is most closely associated with the development of type 2 diabetes.[5]

Worldwide the prevalence and incidence of diabetes is dramatically increasing. In 2007, 246 million adults (5.9 % of adult population) were diagnosed with diabetes,[6] while an estimated 439 million people will have diabetes in 2030 (7.7 %).[7] It is now recognized that it is the developing countries that face the greatest burden of diabetes. In 2007 approximately 80 % of adults with diabetes lived in developing countries and their number will increase from 2010 to 2030 by 69 %, compared to 20 % for developed countries (whereas total adult populations are expected to increase by 36 % and 2 %, respectively). Parts of the world such as Nauru, the United Arab Emirates and Saudi Arabia (30.7 %, 19.5 % and 16.7 % prevalence, respectively) fight diabetes as the major chronic disease of their adult population, while in other regions, like sub-Saharan Africa, malnutrition and HIV are far bigger problems than obesity (Angola 2.7 %, Mozambique 3,1 % prevalence). In Europe, North and Central America there is also a high prevalence of diabetes (Switzerland 11.2 %, Austria 11.1 %, United States 9.2 %, Puerto Rico 12.8 %). According to an estimation of the ADA in 2008,[8] in the United States over 24 million people suffered from diabetes, of which 17.5 million people had diagnosed and additional 6.6 million people undiagnosed diabetes. This implies an enormous economic burden and high costs for society. In the United States the total costs for the treatment of diabetes were 174 billion dollars (2007).

Also in Germany the prevalence of diabetes is raising rapidly and is the highest in Europe with 11.8 % of the adult population in 2007 and estimated 13.3 % in 2025.[6] In 2004, approximately 6.4 million people were treated for diabetes.[9] In addition, studies showed that the prevalence is rising among the elderly population. Whereas 4-10 % of adults aged 40-59 have diabetes, 18-28 % are affected by diabetes among the age of 60 and above.[9] The number of people with diabetes is probably much higher, taking into account that a large proportion of individuals remain unidentified, demonstrated by a study conducted in southern Germany, where 50 % of diabetes cases were undetected in the age group 55-74.[10] Another emerging problem is the increasing number of obese children and adolescents who are diagnosed with type 2 diabetes. In a study with 520 subjects (aged 9-20) 6.7 % displayed an abnormal glucose metabolism (disordered IFG and/or IGT), and already 1.5 % had developed type 2 diabetes.[11] Currently, a few thousands of children and adolescents are diagnosed with type 2 diabetes, but the number is supposed to increase because the prevalence of overweight and obesity is also raising.[9,12]

Type 2 diabetes is a heterogeneous metabolic disorder characterized by chronic hyperglycemia, which in the advanced state of the disease can cause microangiopathies (diabetic nephro-, retino-, and neuropathy) and macroangiopathies (atherosclerosis, stroke).[13] One important pathophysiological feature, namely insulin resistance in peripheral tissues, is an early event in the development of type 2 diabetes and represents a main focus in the attempt to unravel the molecular cause of the disease.[14] Insulin resistance occurring in skeletal muscle, being responsible for 80 % of insulin-stimulated glucose disposal, leads to persisting postprandial hyperglycemia, which can occur 20 years before manifestation of clinical symtoms.[15,16]

It is generally accepted that the development of diabetes can be attributed to a combination of genetic and environmental factors. Regarding the genetic factors of the disease, only a small number of patients have been screened. 5-10 % of these patients display MODY (maturity-onset diabetes of the young) a monogenetic disorder with autosomal dominant inheritance, which is characterized by an early onset and primary defects of insulin secretion.[4,17] Another 2-12 % of all diabetic patients are suggested to develop diabetes because of LADA (latent autoimmune diabetes of the adult). Patients are generally older than 35 years and non-obese but are positive for at least one of the islet autoantibodies.[18-20] However, most of the diabetic patients display polygenic inheritance acting together with environmental factors like overweight or obesity, sedentary life style and smoking.[21]

1.2 Insulin signal transduction

Under normal conditions, plasma glucose remains in a narrow range between 4 and 7 mmol/l. This tight control is regulated by the balance between glucose absorption from the intestine, production by the liver, and uptake and metabolism by peripheral tissues. In this context, insulin acts as the primary regulator of blood glucose concentration by increasing the uptake of glucose in skeletal muscle and adipose tissue and inhibiting hepatic glucose production.[22] Insulin also stimulates cell growth and differentiation, and promotes the storage of energy by enhancing lipogenesis, glycogen, and protein synthesis thereby inhibiting lipolysis, glycogenolysis, and protein breakdown.

Insulin is a polypeptide hormone of 5.8 kDa, which is secreted by pancreatic β-cells. It is synthesized as preproinsulin with 110 amino acids and processed to proinsulin by cleavage of its signal peptide during insertion into the endoplasmatic reticulum (ER). After folding and the formation of disulfide bonds, proinsulin is then transported to the Golgi apparatus and packed into clathrin-coated secretory vesicles. During maturation of these vesicles to secretory granules, proinsulin is processed to insulin and c-peptide by the prohormone convertases 1/3 and 2, which cleave both junction sides of the connecting peptide segment. The mature insulin consist of an α- and a β-chain, and is stored in vesicles and secreted after stimulation of the β-cell by gut hormones such as GLP-1 (incretin effect) and by the postprandial increase of plasma glucose levels.[23]

In insulin-sensitive tissues such as adipose tissue and skeletal muscle, insulin increases glucose uptake by stimulating the translocation of the glucose transporter GLUT4 from intracellular stores to the cell surface. The effects of insulin are mediated by binding to the insulin receptor (IR) and the downstream signaling cascade (figure 1.1). The IR is an integral membrane protein, belonging to the receptor tyrosine kinase family. It consists of two disulfide-linked heterodimers, each of them containing an α- and a β-subunit, which function as allosteric enzymes in which the α-subunit inhibits the tyrosine kinase activity of the β-subunit. Binding of insulin to the α-subunit leads to the derepression of the kinase activity in the β-subunit followed by a transphosphorylation of the β-subunits and a conformational change that further increases kinase activity.[24]

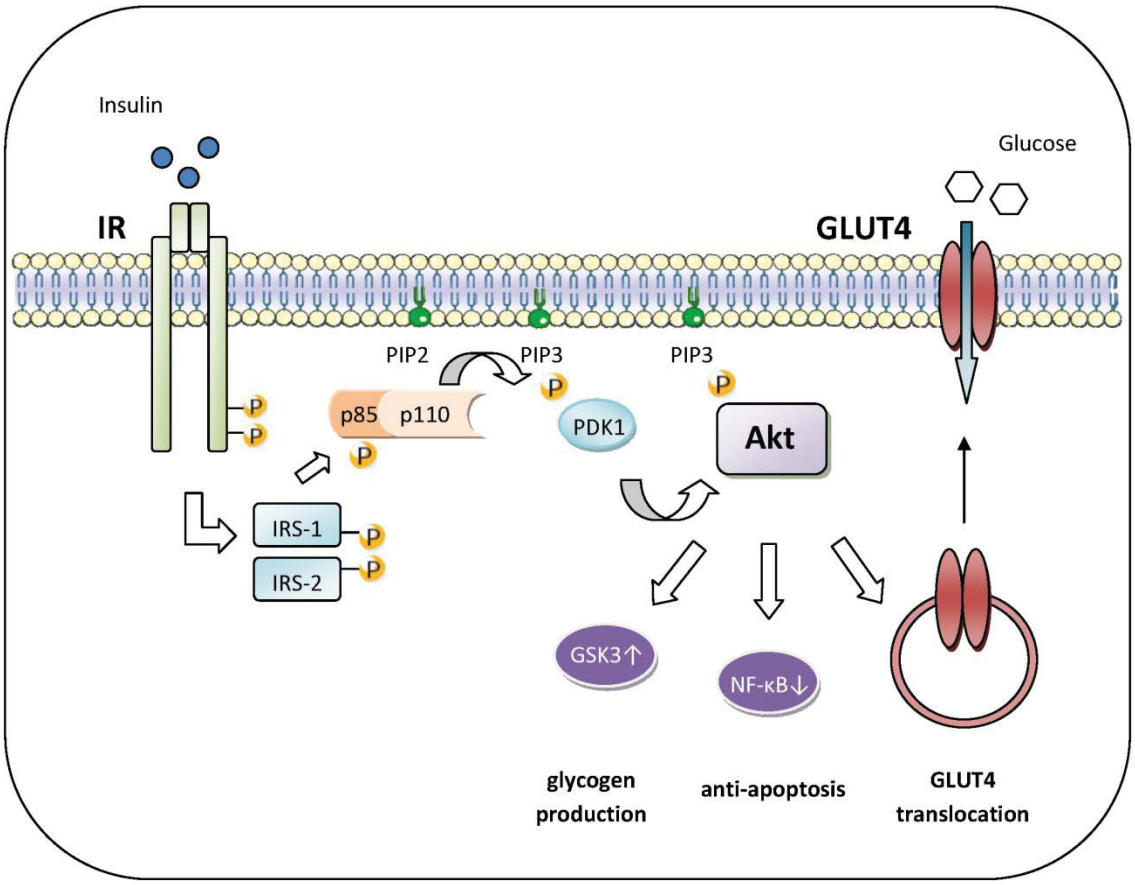

Figure 1.1 Insulin signaling cascade. Binding of insulin to the insulin receptor (IR) leads to the autophosphorylation of IR, which increases its kinase activity and results in tyrosine phosphorylation of downstream interacting proteins including insulin receptor substrate (IRS). Activated IRS phosphorylates the p85 subunit of phosphatidylinositol 3 kinase (PI3K), which stimulates the translocation of PI3K to the plasma membrane, where it phosphorylates phosphoinositol (PI) substrates to generate second messengers (PIP2, PIP3) to recruit the PI3K-dependent serine/threonine kinase (PDK1) and Akt/protein kinase B from the cytoplasm to the plasma membrane. Full activation of Akt by PDK1 regulates the activity of downstream target proteins glycogen synthase kinase (GSK)3 as well as nuclear factor-kappa B (NF-κB) and leads to the translocation of glucose transporter (GLUT)4 containing vesicles to the plasma membrane.

Downstream interacting proteins, including insulin receptor substrate (IRS) 1-4, Cbl, and Shc isoforms are in turn phosphorylated on tyrosines and provide docking sites for Src homology 2 domain containing proteins. The IR and downstream IRS mediate various pathways responsible for metabolism, proliferation, and apoptosis but glucose metabolism is mainly regulated by IRS-1 and IRS-2. In skeletal muscle the two major pathways emanating from activated IRS are the phosphatidylinositol 3 kinase (PI3K)/Akt and the mitogen-activated protein kinase (MAPK) pathways. In adipocytes, insulin activates an alternative pathway via Cap/Cbl,[25-27] however this pathway seems to be less

important in muscle cells. The activation of PI3K is an important step in the insulin-stimulated translocation of GLUT4 containing vesicles to the plasma membrane, which leads to glucose uptake. PI3Ks are heterodimeric cytosolic proteins composed of a catalytic p110-subunit and a regulatory p85-subunit. The p85-subunit stimulates the translocation of PI3Ks to the plasma membrane, where it phosphorylates phosphoinositol (PI) substrates to generate PI(3)P, PI(3,4)P2, and PI(3,4,5)P3, which act as second messengers to recruit the PI3K-dependent serine/threonine kinase (PDK1) and Akt/protein kinase B from the cytoplasm to the plasma membrane. The binding of the second messenger and the recruitment to the plasma membrane lead to conformational changes in Akt, which results in phosphorylation on T308 residue by PDK1.[28] This in turn leads to the full activation of Akt, which regulates the activity of many downstream targets like protein kinase C (PKC) isoforms, nuclear factor-kappa B (NF-κB), and glycogen synthase kinase (GSK)3. GSK3 activates glycogen synthase upon insulin stimulation.[29]

The last step in this signaling cascade is the translocation of GLUT4 containing vesicles along the actin cytoskeleton to the plasma membrane via the interaction of specific SNARE proteins. In this process, SNARE proteins located on the plasma membrane (t-SNARE protein syntaxin 4) interact with SNARE proteins on the vesicles (v-SNARE proteins vesicle associated membrane protein 2 and 3), leading to the fusion of both vesicles and membrane.[30]

1.3 Impact of obesity on diabetes

The primary and classical roles of adipose tissue are to insulate and cushion the body, to store free fatty acids (FFA) after food intake, and to release FFA during the fasting state to ensure sufficient energy status. Beside these functions, adipose tissue also secretes numerous proteins and bioactive peptides, known as adipokines, which in turn act both on the local (autocrine/paracrine) and systemic (endocrine) level.[31] Therefore, adipose tissue is considered as a true endocrine organ. Reasons for an excess in adipose tissue mass are lifestyle changes, like caloric abundance and reduced physical activity, together with genetic factors likely modifying individual susceptibility to environmental factors. However, the alarming increase in worldwide incidence of obesity is also associated with an array of metabolic pathologies like type 2 diabetes, cardiovascular disease, dyslipidemia, fatty liver, Alzheimer's disease, and even some cancers.[32,33]

Obesity develops when energy intake persistently exceeds energy expenditure. The expansion of adipose tissue mass is dependent on several factors like gender, diet, and localization of the adipocyte tissue depot. It is well known that women have more body fat than men with same body mass index (BMI).[34] Men and women also display different distribution of adipose tissue. Whereas men are found to have the largest adipose tissue thickness in the abdominal region, women show a relative preponderance for the gluteal and femoral regions. This difference is mainly due to the local fat cell number between the sexes. In consequence, men are more prone to increased triglyceride, fasting glucose, and insulin levels than adiposity-matched women. Interestingly, a male risk profile was observed in women characterized by abdominal obesity, again indicating that more the abdominal fat depots may be of particular importance for metabolic aberrations, which is due to their unique position and relationship to the portal circulation.[34] A similar observation could be made in another study showing that liposuction of subcutaneous fat in women did not significantly improve obesity-related metabolic abnormalities like insulin resistance.[35] Comparison of diabetic patients to age- and BMI-matched non-diabetic patients revealed that diabetic patients had a significant higher amount of fat in the abdomen, also referred to as visceral fat.[36] All these studies indicate that metabolic disturbances depend more on the adipose tissue location, rather than on its total amount.

Adipose tissue development is a dynamic process which can occur throughout life. The factors determining adipose tissue mass are not fully understood, but increased lipid storage in fully differentiated adipocytes, resulting in enlarged fat cells (hypertrophy), is

well documented and is thought to be the most important mechanism whereby fat depots increase in adults.[37,38] The number of adipocytes represents a major determinant for the fat mass. It is set during childhood and adolescence, and even after weight loss in adulthood and reduced adipocyte volume, the adipose cell number remains the same. Studies could show that there is a remarkable turnover within this population, indicating that adipocyte number is tightly controlled and not influenced by the energy balance.[38] During this continuous turnover new adipocytes are known to differentiate from adipose tissue resident preadipocytes, mesenchymal progenitor cells or bone marrow-derived circulating progenitor cells.[39] In this process, the acquisition of new adipocytes is believed to be irreversible with apoptosis as the only possibility to reduce fat cell number. However, in obesity the number of preadipocytes in the stroma-vascular fraction of abdominal subcutaneous adipose tissue is reduced.[40,41] This observation could be explained by an enhanced apoptosis of preadipocytes or an enhanced preadipocyte differentiation. A recent work revealed that the number of preadipocytes able to differentiate to adipose cells was negatively correlated with both BMI and adipocyte cell size.[42] It is postulated, that chronically elevated tumor necrosis factor (TNF)α levels in the obese condition lead to a complete trans- or dedifferentiation of preadipocytes to another phenotype, including inflammatory cells. In contrast, the pool of potential precursor cells (CD133+) was positively correlated with BMI, suggesting a specific problem for preadipocytes in the abdominal subcutaneous adipose tissue to differentiate and for the commitment of progenitor cells to preadipocytes.

The growth of adipocytes is not only regulated by the intake of nutrients, but also in response to hormones. Aldosterone,[43] thyroid hormones,[44] as well as glucocorticoids[45] are known to promote adipocyte differentiation, whereas growth hormone (GH) inhibits differentiation.[46] Up to date, several transcription factors have been shown to participate in the adipogenic process, notably the peroxisome proliferator-activated receptor (PPAR)γ, several members of the CAAT/enhancer binding proteins (C/EBP) and Krüppel-like factor (KLF) families, signal transducers and activators of transcription (STAT)5, and sterol regulatory element binding protein (SREBP)-1c.[47] Among these, PPARγ is the most important. PPARγ positively regulates adipogenesis and is also involved in lipid and glucose metabolism. Further PPARγ is the molecular target for thiazolidinediones (TZDs) like troglitazone and rosiglitazone, which were studied due to their strong anti-diabetic impact.[48]

As mentioned above, the development of insulin resistance and its complications is predominantly seen with abdominal adipose tissue, which is also associated with fat cell enlargement, rather than peripheral obesity, which is usually associated with recruitment of new preadipocytes and thus small adipose cells.[49,50] Larger adipocytes have greater rate of triglycerol synthesis, lipolysis and therefore greater rates of transmembrane fatty acid flux. They also display a more unfavorable profile of cytokine

secretion,[51] leading to higher amounts of pro-inflammatory cytokines such as TNFα and lesser abundance of the anti-inflammatory adipokine adiponectin.[52]

1.3.1 Adipose tissue dysfunction and induction of insulin resistance

In the context of obesity adipose tissue has to deal with a continual demand on its lipid storage and processing functions. Beyond a critical threshold, adipocytes begin to exhibit several dysfunctions, including apoptotic signaling, disruption of mitochondrial function, production of reactive oxygen species (ROS), endoplasmatic reticulum (ER) stress, increased fatty acid release, altered adipokine production, most of them leading to a chronic low-grade inflammation state.[53,54] In recent years it has become well accepted that expansion of adipose tissue and the concomitant increase in the secretion of pro-inflammatory cytokines like TNFα and MCP-1 leads to the infiltration of immune cells such as macrophages into adipose tissue. Despite the correlation between increased adipose tissue inflammation and metabolic dysfunction, the presence of immune cells is not uniformly detrimental. Macrophages may play a role in the extensive tissue remodeling that occurs during adipose tissue growth.[55,56] Recent work has emphasized the role for a specific activation state of immune cells in determining functional consequences. The classically activated M1 macrophages are associated with an increased pro-inflammatory cytokine release such as TNFα and interleukin (IL)-6,[57] whereas the alternatively activated M2 state plays a role in wound healing, tissue repair, and resolution of acute inflammation via anti-inflammatory signaling.[58,59] In addition to macrophages, recent studies suggest a role for other immune cells in adipose tissue inflammation, such as natural killer (NK) cells,[60] B cells, mast cells, regulatory T cells, T-helper cell type 1 (T_H1), and T-helper cell type 2 (T_H2).[61-63] Like macrophages, T cells can exist in two alternative populations, T_H1 and T_H2, which produce distinct subsets of cytokines with different inflammatory potentials.[59] The pro-inflammatory T_H1 subset has been suggested to be involved in macrophage recruitment and activation of the M1 phenotype,[63] whereas T_H2 T-cell populations, like in adipose tissue, secrete IL-4 and IL-13 and are expected to favor an M2 profile in macrophages.[64]

However, it has become well accepted that changes in inflammatory signaling by adipocytes and infiltration of adipose tissue by distinct immune cells are key features of obesity-induced insulin resistance and associated metabolic diseases in animal models and humans.[57,59,65] In adipose tissue, the increased expression of pro-inflammatory mediators such as TNFα, IL-6, IL-1β, and migration inhibitory factor (MIF) have been shown to disrupt insulin signaling through several mechanisms. This includes the

induction of the suppressors of cytokine signaling (SOCs) family proteins, which inhibit insulin receptor kinase activity, interfere with binding of IRS-1 and IRS-2 to the insulin receptor, and promote IRS degradation.[66,67] In both adipocytes and skeletal muscle, cytokines also activate inflammatory signaling via c-Jun N-terminal kinase (JNK) and inhibitor of kappa β kinase (IKKβ) pathways.[68,69] The activation of JNKs as well as IKKβ have been demonstrated to play a role in the negative regulation of insulin signaling by serine phosphorylation of IRS-1.[70,71] Furthermore, also the extracellular signal-regulated kinase (ERK), which is a member of the MAP kinase family, has been shown to implicated in adipokine-induced insulin resistance in both skeletal muscle and adipose tissue,[72-74] by the negative regulation of IRS production.[73]

Tissues which are responsible for the highest rate of insulin-stimulated glucose uptake and metabolism are skeletal muscle, adipose tissue, and liver. In skeletal muscle, insulin promotes glucose uptake by stimulating translocation of GLUT4 to the plasma membrane and impaired skeletal muscle insulin signaling results in decreased glucose disposal. In the liver, insulin inhibits the expression of key gluconeogenic enzymes and therefore insulin resistance in liver leads to elevated hepatic glucose production. Adipose tissue insulin signaling results in decreased hormone sensitive lipase activity and this anti-lipolytic effect inhibits FFA efflux out of adipocytes. Therefore insulin resistance in adipocytes leads to increased circulating FFA, in turn decreasing insulin sensitivity in nonadipose tissues such as liver, pancreas and skeletal muscle,[75] due to an increase in ectopically stored lipid products.[76] FFA and their mediates are not only substrates in metabolism but can also act as signaling molecules and influence insulin signaling.[77] Here, FFA can activate protein kinase C (PKC)θ, IKK, and JNK, which then inhibit the insulin signaling cascade through IRS-1 serine phosphorylation.[78]

1.3.2 Adipose tissue-derived factors

Enlargement of adipose tissue is associated with adipocyte dysfunction, including altered adipokine production and signaling. There are several adipokines, which are regulated by obesity, and are known to influence insulin action in peripheral tissues like muscle and liver, as well as in adipose tissue itself. Most of the adipokines are significantly increased in obesity and are good predictors of the development of type 2 diabetes,[79] and are correlated to the metabolic and cardiovascular complications observed in the obese state.

The first adipose tissue-derived factor suggested to represent a link between obesity, inflammation, and diabetes was TNFα.[80] The principle source of TNFα are M1 macrophages from the stroma-vascular fraction, but it is also expressed in adipocytes. TNFα is upregulated in several animal models of obesity,[81,82] but its role in human

physiology is still not clear.[83] The targeted deletion of TNFα or its receptors increased insulin sensitivity and glucose tolerance in obese rodents in some,[84] but not all studies.[85] In obese type 2 diabetic humans, TNFα neutralization does not appear to improve glucose tolerance or insulin sensitivity, however in individuals without established type 2 diabetes prolonged treatment does improve insulin sensitivity.[86] The molecular basis for the observed impairment in insulin action involves inhibition of IRS-1 signaling capability through the activation of serine kinases such as JNK or IKKβ and through increased expression of suppressor of cytokine signaling 3 (SOCS3).[87] It is recognized that there is a strong correlation between obesity and TNFα expression in humans,[82] but insulin resistance does not correlate with TNFα plasma levels after adjustment for BMI.[88]

Almost simultaneously to the discovery of adipose tissue-derived TNFα, the leptin gene [89] and its cognate receptor were identified.[90] Leptin plays an important role in the regulation of body weight by controlling food intake and energy expenditure. In the hypothalamus leptin inhibits appetite and weight gain by decreasing orexigenic and increasing anorexigenic peptide expression,[91] whereas it stimulates energy expenditure in peripheral tissues.[92] It also regulates body weight through induction of adipocyte apoptosis.[93] Further, leptin regulates puberty and reproduction, placental and fetal function as well as immune response. The role of leptin in the development of diabetes is ambiguous. Whereas some studies point to the role of leptin treatment in increasing insulin sensitivity and the ability to reverse the obese and diabetic phenotypes of *ob/ob* mice and rats,[94,95] other studies showed that leptin inhibited insulin action in adipocytes and skeletal muscle cells.[96,97] In humans, congenital leptin deficiency causes severe obesity, impaired thermogenesis, and insulin resistance.[98] Contrariwise, high leptin concentrations, as found in the obese state,[91] are reported as negative regulators of insulin sensitivity.[99] Concerning the therapeutic perspective, leptin was first regarded as a promising anti-obesity drug. However, in common obesity leptin plasma levels are already high, in turn leading to leptin resistance. Therefore, leptin treatment appears to be only a valuable option in rare disease states in which levels of this cytokine are low.[100]

Another small polypeptide hormone produced by adipocytes as well as immunocompetent cells, is resistin.[101,102] It was introduced as a novel insulin resistance-inducing adipocytokine in 2001.[103] Later studies revealed that the source of resistin is different between rodents (adipocytes) and humans (macrophages).[101,104] In rodents, resistin seems to be implicated in inducing insulin resistance in liver, skeletal muscle, and adipose tissue,[105] whereof the liver is supposed to be the primary target for resistin.[106] The role of resistin in humans is less clear.[105,107] Here, it is supposed to be involved in the regulation of pro-inflammatory processes, rather than directly in insulin sensitivity.[108] Resistin expression correlates with the release of markers for inflammation such as TNFα and IL-6.[109,110] In human adipocytes and macrophages resistin also induces the secretion of TNFα, further pointing to the pro-inflammatory nature of this adipokine.[111,112]

Monocyte chemotactic protein (MCP)-1 belongs to the family of inducible cytokines, which plays a role in the recruitment of monocytes and T lymphocytes to the site of injury and infection.[113] It is expressed by adipocytes and other cell types including smooth muscle and endothelial cells when exposed to pro-inflammatory stimuli.[113] The link between MCP-1 and obesity was given by the observation that MCP-1 is overexpressed in obese rodents,[114,115] and it also attains significantly higher plasma levels in obese human subjects.[116,117] Expression of MCP-1 was found to be higher in visceral adipose tissue than in subcutaneous adipose tissue, which correlated closely to the number of residing macrophages.[118] Furthermore, clinical data provide good evidence for a relationship between MCP-1 and the development of insulin resistance, as well as type 2 diabetes.[119,120] In addition, *in vitro* studies in adipocytes and in skeletal muscle cells also demonstrated insulin resistance-inducing effects of MCP-1.[114,121] In summary, MCP-1 is not only related to increased adipose tissue mass in obesity, but also seems to play an important role in the recruitment of macrophages into adipose tissue, which is an initial step in the development of adipose tissue inflammation. Therefore, MCP-1 may link inflammation in the obese state and the pathogenesis of type 2 diabetes.[122]

One very important and unique adipokine is adiponectin (also referred to as ACRP30 and AdipoQ). Its expression is largely restricted to adipocytes and it is induced over 100-fold during adipocyte differentiation.[123] It circulates at high concentrations (0.5–30 μg/ml) in plasma under normal physiological conditions, and its levels are maintained within a very narrow range.[124] Adiponectin exerts insulin-sensitizing, anti-inflammatory, and anti-atherogenic properties, and, unlike most other adipokines, circulating levels of adiponectin are reduced in obesity and associated diseases. Human adiponectin is a 244aa/30kDa protein and shares homology with collagen and complement factor C1q. It is composed of four domains: an N-terminal signal peptide, a variable domain which is divergent between species, a collageneous domain, and a globular domain that binds to the adiponectin receptors.[125] The structure of the globular domain has been solved and surprisingly, according to their antagonistic effects, shows significant homology to TNFα.[126] Two adiponectin receptors have been identified, termed AdipoR1 and AdipoR2, which mediate many of adiponectin's pleiotropic effects through activation of intracellular signaling pathways involving PPARα[125] and AMP-activated protein kinase (AMPK), a prime therapeutic target for obesity-related metabolic and cardiovascular diseases.[127-129] AdipoR1 is the most important form mediating adiponectin effects in skeletal muscle while AdipoR2 is proposed to be the predominant player in liver.[130] Adiponectin is synthesized as a single subunit which undergoes multimerisation to form low molecular weight (LMW) (trimers), middle molecular weight (MMW) (hexamers), and high molecular weight (HMW, 12-18-mers) multimers. The HMW oligomer is the major active form mediating the insulin-sensitizing effects of adiponectin, whereas the

central actions of this adipokine are attributed primarily to the hexameric and trimeric oligomers. In patients with type 2 diabetes and coronary heart disease, circulating levels of HMW adiponectin are selectively decreased due to an impaired secretion of this oligomer from adipocytes.[124] Many reports demonstrate a positive correlation between systemic adiponectin concentrations and glucose homeostasis, in part owing to its effects on enhancing insulin sensitivity and glucose uptake.[131] However, recent studies hint at a more complex situation. High circulating levels of total and HMW adiponectin in type 1 diabetic patients are being found, indicating that additional regulatory factors independent of elevated systemic levels of adiponectin might be necessary to modulate hyperglycemia in the absence of insulin.[132] This highlights a possible inverse relationship of plasma insulin and adiponectin levels. There is strong reason to believe that insulin and/or components of the insulin receptor signaling pathway might have an impact on plasma adiponectin levels.[133,134]

One adipocytokine, whose function in obesity-induced insulin resistance is controversially debated at the moment, is interleukin (IL)-6.[135] On the one hand, it is recognized that IL-6 serum levels are increased in obesity and correlate well with the development of type 2 diabetes.[100] The administration of recombinant IL-6 in rodent models and in humans results in hyperglycemia and compensatory hyperinsulinaemia *in vivo*.[136,137] Similarly, blocking IL-6 action via application of neutralizing antibodies improved glucose intolerance and insulin sensitivity in mice.[68] Data from our laboratory could also show that high doses of IL-6 could induce insulin resistance in human skeletal muscle cells.[138] Furthermore, IL-6 downregulates adiponectin [139] and stimulates insulin resistance-inducing MCP-1 in 3T3-L1 adipocytes *in vitro*.[140] On the other hand, IL-6 secretion from muscle is increased after exercise and leads to higher energy expenditure.[141] This acute IL-6 stimulation leads to activation of AMPK, which results in increased glucose uptake and β oxidation.[142] In a more recent study injection of IL-6 increased whole body insulin sensitivity and improved glucose tolerance in rat skeletal muscle cells.[143] However, the observed effects of IL-6 could be related to the time of exposure, since in short-term experiments IL-6 may not be implicated in the induction of insulin resistance,[144] but might rather be more relevant in the study of physical activity. In the case of diabetes and obesity, increased IL-6 levels are chronically exposed to insulin responsive tissues, and could therefore lead to the observed adverse effects resulting in insulin resistance.

1.3.3 Secretome analysis of adipose tissue

Beyond the well-known adipokines described above, considerable research in recent years has attempted to identify the so far unknown adipose tissue-derived factors. In this context, several unbiased proteomic approaches based on isolated adipocytes or adipose tissue explants have emphasized the complex nature of the adipocyte secretome, highlighting that adipocytes secrete a vast number of different proteins.[145-153] Among the older studies, the majority focused on samples from animal origin, whereas few recent studies have reported on the human adipose tissue or adipocyte secretome.[145,147,151,153,154] Concerning the proteomics technology, most of these studies used either one-dimensional SDS-PAGE combined with lipid chromatography (LC)-based workflows and ESI-MS/MS identification of proteins, or two-dimensional SDS-PAGE and subsequent protein identification via MALDI-MS.

Clustering these adipose tissue-derived proteins according to their postulated functions revealed that these adipokines fall into several groups, including adipokines contributing to the extracellular matrix, involved in inflammation, metabolism, oxidative stress, or the control of angiogenesis and blood pressure (figure 1.2). This highlights the important regulatory role of the adipocyte with respect to extracellular matrix components, inflammatory pathways, and angiogenesis. The list of new adipokines is further growing as methods for the analysis of supernatants become more sensitive. Therefore, the total number of confirmed adipokines amounted to 100 in 2008,[155] and added up to more than 250 at the end of 2010.

A novel adipokine, discovered in a proteomic approach in 2004 to be secreted by 3T3-L1 adipocytes, is the pigment epithelium-derived factor (PEDF).[156] PEDF is a 50 kDa glycoprotein that belongs to the non-inhibitory serpin group.[157] It was first purified from conditioned media (CM) of human retinal epithelial cells and identified as a neurotrophic factor able to convert retinoblastoma tumor cells into non-proliferative neurons.[158] A current study could show that PEDF is the most abundant protein in supernatants of 3T3-L1 adipocytes and that the expression in adipose tissue positively correlates with obesity and insulin resistance in mice.[159] This is in accordance with studies describing a significant correlation between plasma PEDF levels and obesity in humans, which demonstrates that PEDF secreted from adipose tissue is associated with the metabolic syndrome.[160,161] The mechanisms by which PEDF induces insulin resistance are not fully resolved. It was demonstrated that PEDF could induce inflammatory signaling in several cell types [162] and also correlates with inflammation and vascular dysfunction in type 1 diabetic patients.[163] A recent study in mice could show that acute PEDF treatment was associated with the activation of ERK and JNK in skeletal muscle and liver, concomitant with a reduction in the insulin-dependent activation of insulin receptor substrate (IRS)-1

and Akt.[159] Furthermore, chronic PEDF treatment was demonstrated to induce insulin resistance by stimulating adipocyte lipolysis and subsequent ectopic diacylglycerol (DAG) and ceramide deposition in muscle and liver.

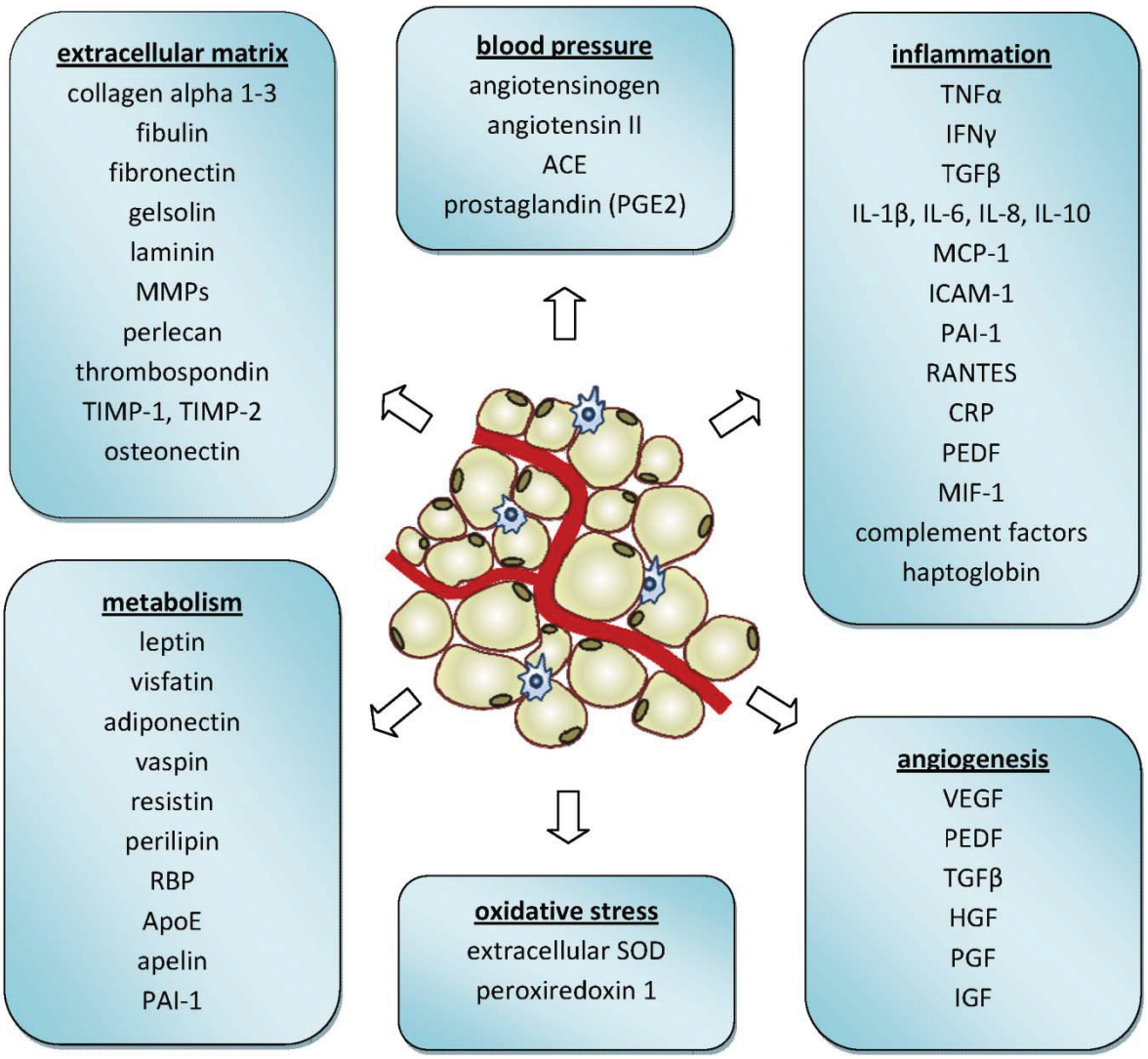

***Figure 1.2 Adipose tissue-derived factors*.** MMPs, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinases; ACE, angiotensin converting enzyme; TNF, tumor necrosis factor; IFN, interferon; TGF, transforming growth factor; IL, interleukin; MCP, monocyte chemotactic protein; ICAM, intercellular adhesion molecule; PAI, plasminogen activator inhibitor; RANTES, regulated upon activation, normal T-cell expressed, and secreted; CRP, c-reactive protein; PEDF, pigment epithelium-derived factor; MIF, macrophage migration inhibitory factor; Apo, apolipoprotein; SOD, superoxide dismutase; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; PGF, placental growth factor; IGF, insulin-like growth factor

However, PEDF is just one out of many novel adipokines or adipose tissue-derived factors that could be identified with current proteomic approaches. PEDF can be quoted as an example how important this technology is for the understanding of adipose tissue as an endocrine organ, possibly providing the initial step in elucidating the implications of novel adipokines in the development and pathophysiology of obesity-related disorders like type 2 diabetes and cardiovascular disease.

1.4 Impact of obesity on atherosclerosis

1.4.1 Pathophysiology of atherosclerosis

Atherosclerosis is a chronic inflammatory process that leads to a group of diseases characterized by a thickening of artery walls. According to the report from the American Heart Association (AHA) in 2010, atherosclerosis causes many deaths from heart attack and stroke and accounts for nearly three fourths of all deaths from CVD.[164] Analysis of data from the REACH Registry showed that atherothrombotic diseases such as CVD, coronary artery disease (CAD), and peripheral artery disease (PAD) are associated with the main causes of death on a worldwide scale.[165]

Atherosclerosis is a disease of large and medium-sized muscular arteries and is characterized by endothelial dysfunction, vascular inflammation, and the buildup of lipids, cholesterol, calcium, and cellular debris within the intima of the vessel wall. This buildup results in plaque formation, vascular remodeling, acute and chronic luminal obstruction, abnormalities of blood flow, and diminished oxygen supply to target organs. A complex and incompletely understood interaction exists between the critical cellular elements of the atherosclerotic lesion. These cellular elements are endothelial cells, smooth muscle cells, platelets, and leucocytes. Vasomotor function, the state of activation of the coagulation cascade, the fibrinolytic system, smooth muscle cell migration and proliferation, and cellular inflammation are complex and interrelated biological processes that contribute to atherogenesis and the clinical manifestations of atherosclerosis.

Over the last century, various hypotheses have been forwarded to explain the initiating events and factors that contribute to the development of atherosclerosis. Among these, the “response-to-injury” is the most accepted, which states that endothelial damage precedes smooth muscle cell migration and proliferation, deposition of intracellular and extracellular lipid, and accumulation of extracellular matrix.[166] Subsequently, this hypothesis was broadened to include endothelial dysfunction as a key event that initiates the inflammatory mechanisms associated with atherosclerosis. The endothelium is a dynamic monolayer lining the blood vessel wall and is responsible for maintaining vascular tone through the production of vasodilators such as nitric oxide (NO), and vasoconstrictors like endothelin-1. It releases several other factors, such as von Willebrand factor, plasminogen inhibitors, and prostacyclin that regulate thrombosis by inhibiting platelet aggregation. The earliest changes that precede the formation of lesions in atherosclerosis include increased endothelial permeability to lipoproteins and other plasma constituents, which is mediated by NO, prostacyclin, platelet-derived

growth factor (PDGF), angiotensin II (AngII), and endothelin-1. Furthermore, rolling and adhesion of monocytes and T cells occurs as a consequence of the upregulation of adhesion molecules on both the endothelium (such as E-selectin, P-selectin, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1) and leukocytes (e.g. L-selectin, and integrins). Another factor contributing to the enhanced recruitment of leukocytes to the vessel wall is oxidized low-density lipoprotein (oxLDL). oxLDL induces endothelial cell and smooth muscle cell activation, secretion of inflammatory mediators, and expression of adhesion molecules, a sequence of steps that culminates in leukocyte accumulation in the subendothelial space.[167] Once in the intima, monocytes become tissue macrophages, which internalize oxLDL particles via scavenger receptors[168] and transform into foam cells (lipid-laden macrophages). This is a prominent feature of the atherosclerotic plaque and the first step in fatty streak formation. Later macrophages are joined by smooth muscle cells, which migrate from the media into the intima. The further progress of the fatty streak to a fibrous plaque is the result of progressive lipid accumulation and the ongoing migration and proliferation of smooth muscle cells. These cells are responsible for the deposition of extracellular connective tissue matrix and form a fibrous cap that overlies a core of lipid-laden macrophages, extracellular lipid, and necrotic cellular debris. In unstable fibrous plaques, the denudation of the overlying endothelium, the rupture of the protective fibrous cap, or ulceration of the fibrous plaque can rapidly lead to thrombosis. This usually occurs at sites of thinning of the fibrous cap, which is apparently due to the continuous influx and activation of macrophages, releasing metalloproteinases and other proteolytic enzymes.[169] These enzymes cause degradation of the matrix, which can lead to hemorrhage from the vasa vasorum or from the lumen of the artery and can result in thrombus formation and occlusion of the artery.

1.4.2 Adipose tissue-derived factors and impact on atherosclerosis

As mentioned above, the metabolic syndrome displays a metabolic and cardiovascular cluster of disorders such as hypertension, glucose intolerance, dyslipidemia, and abdominal obesity, each of which is an independent risk factor for cardiovascular disease (CVD). Among these, obesity is considered as a major determinant of cardiovascular morbidity and mortality, including stroke, congestive heart failure, myocardial infarction, and cardiovascular death.[170,171] Many studies in humans and various animal models have shown that obesity is strongly related to the development of atherosclerosis.[172,173] Adipose tissue plays a prominent role in the

development of a systemic low-grade inflammation state that contributes to obesity-associated vascular dysfunction and cardiovascular risk. In this context, the local secretion of adipokines and FFA by perivascular fat may provide a direct link between obesity and vascular complications.[174]

One important adipocytokine whose expression and circulating levels have been associated with coronary artery disease is IL-6.[175] IL-6 promotes plaque destabilization via the release of proinflammatory cytokines, oxidation of lipoproteins by phospholipases, release of prothrombic mediators, and activation of MMPs.[175,176] Moreover, the increased ROS formation under proinflammatory conditions may play a critical role in the crosstalk between IL-6 and other vasoactive substances, such as AngII and catecholamines.[177]

Also TNFα has been observed to exert effects on the vasculature. Acute administration of TNFα *per se* leads to vasodilatation, but impairs endothelium-dependent vasodilatory effects in response to insulin and acetylcholine in healthy humans.[178,179] Furthermore, TNFα induces inflammatory changes in the vessel wall by activating NF-κB, which in turn increases the expression of ICAM-1 and VCAM-1 as well as the production of MCP-1 from endothelial cells and VSMC.[180]

At present, clinical investigations considered leptin as an independent risk factor for cardiovascular diseases,[181,182] evidencing that its plasma concentration are independently associated with the intima-media thickness of the carotid artery[183] and with the degree of coronary artery calcification in patients with type 2 diabetes.[183,184] Evidences for an involvement of leptin in atherosclerosis have been recently provided by direct leptin administration in apolipoprotein deficient mice and by the finding that *ob/ob* mice which lacked the functional leptin gene, are resistant to atherosclerosis despite the presence of obesity and diabetes.[185,186]

As opposite to the adipokines described above, circulating adiponectin exerts anti-atherogenic properties in the vascular wall.[131] Here, adiponectin reduces the expression of adhesion molecules and scavenger receptors by inhibiting the effects of TNFα and AngII on both endothelial cells and macrophages.[187,188] Furthermore, it was demonstrated that adiponectin suppresses macrophage-to-foam-cell formation[189] and induces the production of anti-inflammatory mediators like IL-10 and IL-1 receptor agonist in primary human monocytes and monocyte-derived macrophages.[190] Endothelium-dependent vasodilatation in response to acetylcholine is significantly reduced in adiponectin-knockout mice when compared to wild type mice.[191] This study suggests that hypoadiponectinemia is associated with endothelium-dependent vasorelaxation and that the measurement of plasma adiponectin levels may be suitable as a marker of endothelial dysfunction. The vasculo-protective effects of adiponectin have been confirmed in clinical studies, which showed that its decreased levels contribute to metabolic and vascular abnormalities in obese subjects.[188,192]

1.4.3 Influence of adipose-tissue derived factors on smooth muscle cells

In physical conditions VSMC play an important role in providing structural integrity of the vessel wall and in controlling vascular tone and blood pressure.[193,194] In particular, this cell type is the main target of the effects of endothelium-released NO, which stimulates the synthesis of cGMP, thus preventing the calcium release from intracellular stores and leading to the relaxation of VSMC.[193-195] The surface complex system, which regulates VSMC responses and modulates the contractile process, involves the expression of receptors for catecholamines, acetylcholine, serotonin, histamine, AngII, neuropeptide Y, and growth factors (e.g. PDGF, epidermal growth factor (EGF), TGFβ, insulin, and insulin-like growth factor (IGF)1). The signal transduction system following membrane activation consists of guanine nucleotide regulatory proteins, phosphoinositide metabolism, cyclic nucleotides (cAMP and cGMP), and calcium.[196,197]

However, following repeated or chronic arterial wall injury, VSMC respond by migration into the intima as well as with increased proliferation. During this process, VSMC undergo a phenotype modulation from a 'contractile' to a 'synthetic' state. There is also evidence that VSMC may take on a 'proinflammatory' phenotype, whereby VSMC secrete cytokines and express cell adhesion molecules, in turn regulating monocyte and macrophage adhesion. Several evidences indicate that adipokines play an important role in inducing this inflammatory phenotype and consequently influence VSMC function. In the case of leptin *in vitro* studies of human aortic SMC could show that it induces migration, proliferation, and expression of MMP-2, by inducing PKC, ERK1/2, and NF-κB signaling pathways.[198] Furthermore, it was observed that leptin may increase cardiovascular risk by promoting osteogenic differentiation and thus vascular calcification.[199] Also resistin is known to induce proliferation of cultured human aortic VSMC through both ERK1/2 and Akt signaling pathways.[200] In addition, resistin expression is increased by hypoxia in cultured rat VSMC.[200] Another adipocytokine, which stimulates VSMC migration[201] and enhances VSMC proliferation, is IL-6.[202,203] Furthermore, proinflammatory mediators and growth factors like IL-2, AngII, and VEGF increase the synthesis and release of IL-6 by VSMC, in turn augmenting VSMC migration and proliferation.[177,204,205] Adiponectin as an anti-atherogenic factor is known to suppress the proliferation and migration of smooth muscle cells by binding directly to and suppressing several growth factors, particularly PDGF-BB, fibroblast growth factor and heparin-binding EGF-like growth factor (HB-EGF).[206,207]

1.4.4 Inflammation, insulin resistance, and atherosclerosis

As previously mentioned the altered pattern of adipocyte-derived cytokines and the increased release of lipid mediators in obesity are substantially involved in the chronic proinflammatory state of obesity and atherosclerosis and are associated with the induction of insulin resistance. A lot of evidence indicates that insulin resistance contributes to the development of CVD.[208-210] Defects in insulin signaling not only impairs glucose utilization, but also exerts several other negative effects on the vessel wall. Under physiological conditions insulin signaling plays a critical role in activating NO synthase, which regulates NO production.[211] NO is a potent vasodilator and anti-atherogenic agent, and its deficiency can result in endothelial dysfunction and accelerated atherosclerosis.[212-214] Furthermore insulin is a potent growth factor, whose growth promoting effects are mediated via the MAPK pathway.[215] Thus, this pathway plays an important role in atherogenesis. Insulin resistance occurring in the PI3K pathway with intact MAPK signaling activates multiple inflammatory pathways, including NF-κB[71] and JNK,[69] which again are implicated in inducing insulin resistance. Due to the insulin signaling defects, impaired glucose utilization causes hyperglycemia, in turn stimulating insulin secretion. As a consequence of the defective IRS1/PI3K pathway, the MAPK pathway is excessively stimulated since it is normally sensitive to insulin. In diabetic and obese patients, continued MAPK stimulation causes smooth muscle cell proliferation, increased collagen formation and excessive production of growth factors and inflammatory cytokines, contributing to accelerated atherosclerosis.[93]

1.5 Objectives

The previous sections could show that there is profound evidence that a negative crosstalk exists between expanded adipose tissue mass in obesity and peripheral tissues and organs like skeletal muscle and the vessel wall. In this context, adipose tissue as an endocrine organ secretes numerous peptides and proteins, which are associated with inflammation, insulin resistance, and atherosclerosis. Although several proteomic approaches based on adipocytes or adipose tissue explants emphasized the complexity of the whole secretory output, the adipokinome of human adipocytes remains incompletely characterized. In that perspective, the identification of novel adipokines could lead to the discovery of new pharmacological targets for the treatment of diabetes or atherosclerosis, or of novel biomarkers indicating a person's individual risk of developing diabetes or atherosclerosis later in life. Therefore the starting point of the presented work is the characterization of the secretome of *in vitro* differentiated human adipocytes.

- It is apparent that adipose tissue as an endocrine organ secretes a variety of bioactive factors. However, the magnitude of the adipocyte secretome is incompletely understood. Therefore, the first aim of the study was to identify so far unknown adipose-tissue derived factors by comprehensive proteomic profiling using both 1D-GE and LC-MS/MS as well as 2D-GE and following protein identification via MALDI-MS/MS. Subsequently, the five most interesting proteins were validated and characterized concerning their expression in adipose tissue and adipose tissue-derived macrophages and their serum concentration in lean and obese subjects.

- The characterization of the adipose tissue secretome identified the dipeptidyl peptidase 4 (DPP4) as novel adipokine. Based on this observation the second objective of this thesis was to investigate the expression and regulation of DPP4 in human adipocytes and adipose-tissue derived macrophages and to detect the expression and secretion of DPP4 in three independent clinical studies performed on lean and obese subjects.

- Secretome analysis of in vitro differentiated adipocytes also revealed the pigment epithelium-derived factor (PEDF) as one of the most abundant adipokines. In this context, the third aim of this thesis was to evaluate 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.

- The expanding fat mass in obesity leads to an increased secretion of adipokines which can negatively affect SMC in the vessel wall. Although effects of specific adipokines on VSMC function have been investigated, effects of the whole secretory output of human adipocytes have not been studied. Therefore the fourth objective of this thesis was to provide insight into the complex cellular mechanisms linking obesity and atherosclerosis by assessing the role of protein factors and lipid mediators in the crosstalk between human VSMC and adipose tissue. In this regard, the role of the autocrine action of adiponectin and its further impact on smooth muscle cell proliferation and expression of adhesion molecules was investigated.

CHAPTER 2

Study 1

Human adipocyte secretome: Identification and validation of novel adipokines[†]

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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 not only an energy depot it rather acts as a major endocrine organ. By releasing a huge diversity of signaling and mediator proteins, termed adipokines, it communicates with other tissues including muscle and liver. Adipokines are involved in the development and progression of diverse disease pattern, including the metabolic syndrome and type 2 diabetes. Although many adipokines have been identified the adipokinome still remains incompletely characterized.

In order to identify novel adipokines, we have utilized protein profiling (1-dimensional SDS-PAGE/LC-ESI-MS/MS and 2-dimensional SDS-PAGE/MALDI-MS) of conditioned medium derived from primary human adipocytes. A total of 347 proteins were identified; 263 were predicted to be secretory proteins and 44 proteins are novel adipokines not been detected before in the secretome of adipose material.

For a first validation of secretion and further investigation of regulation we could confirm expression and release of complement factor H, αB-crystallin, cartilage intermediate-layer protein and heme oxygenase-1 from human adipocytes. Especially heme oxygenase-1 reveals to be a novel adipokine of interest as its expression and release is differentiation-dependent and circulating levels and adipose tissue expression are significantly increased in obese subjects as compared to lean controls. It can be speculated that enlarged adipocytes release higher amounts of this factor that might be involved in the development of obesity-associated metabolic diseases.

Introduction

Obesity has become a critical global health problem and frequently associates with the development of chronic diseases including type 2 diabetes and cardiovascular disease.[1] It is now considered that adipose tissue is one of the major endocrine organs [2,3] and is not only acting as an energy depot. Beside its important function in storage and release of energy, it communicates with other tissues by secreting a huge diversity of signaling and mediator molecules, termed adipokines.[4,5] Recent data indicate that these adipokines create a complex interconnected network mediating the crosstalk between liver, muscle and fat.[6,7] Accordingly, enlargement of adipose tissue potentially leads to a dysregulation of adipokine secretion, which represents a critical pathogenic link between obesity and multifactorial diseases, like insulin resistance and type 2 diabetes.

Because of their relevance in the progression of widespread diseases a rapidly growing number of unbiased, proteomic approaches have addressed the adipocyte secretome.[8-11] Based on isolated adipocytes or adipose tissue explants these studies

have emphasized the complex nature of the adipokinome. Although the list of adipokines is continuously growing the adipokinome still remains incompletely characterized. The majority of the proteomic studies has used samples from animal origin and only very few recent studies have reported on the human adipose tissue or adipocyte secretome.[12-15] Since enlargement of adipose tissue may represent the initial step leading to aberrant adipokine secretion and adipose tissue inflammation,[16] we decided to conduct a comprehensive proteomic profiling of conditioned media derived from differentiated, primary human adipocytes. This resulted in the identification of 347 proteins including 44 putative novel adipokines. The identified proteins are supposed to be involved in various biological processes, highlighting the diversity of the human adipokinome.

For further validation of secretion and regulation we have chosen four putative adipokines, i) complement factor H (CFH), ii) αB-crystallin (CRYAB), iii) cartilage intermediate-layer protein (CILP) and iv) heme oxygenase-1 (HO-1) proposed to have different functions within adipose tissue biology.

Materials and Methods

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, 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). 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.[17] 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 and induction of adiponectin expression. Differentiated adipocytes were used for the generation of adipocyte-conditioned media (CM), as recently described by us.[18] Macrophages were isolated from

human adipose tissue, using a method described by Curat et al.[19] 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 7 x 10^7 adipocytes) were pooled and centrifuged for 20 min at 40,000 × *g* at 4 °C. Subsequently, pooled CM were 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) 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 usage.

SDS-PAGE and protein identification by LC-MS

For LC-MS analysis aliquots of concentrated CM initially were separated by 1-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. 3 µl of 150 mM iodine acetamide were added and 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 cut into 24 equally sized bands, which were chopped into small cubes and washed three times with acetonitrile-water (1:1). Neat 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 12.5 ng/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 % 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 (BrukerDaltoniks, Bremen, Germany) equipped with a nano ESI source (BrukerDaltonics, Bremen, Germany). Samples were introduced by an easy nano LC system (Proxeon, Odense, Dennmark) 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 3.0 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 (BrukerDaltoniks, Bremen, Germany) and xml formatted peaklists were transferred to Proteinscape 2.1 (BrukerDaltoniks, Bremen, Germany).

MASCOT 2.2 (Matrix Science Ltd, London, UK) was used to search a composite decoy database which was built from SwissProt 57.3 (468851 sequences; 166,149,756 residues). The composite database was generated with the Perl script *makeDecoyDB* (BrukerDaltoniks, 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 (BrukerDaltoniks, 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.[20]

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

For 2D-PAGE analysis, aliquots (150 µg) 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.[21] Subsequent to electrophoretic separation, gels were stained with a ruthenium fluorescent stain [22] 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).

For protein identification by MALDI-MS protein spots exceeding intensity levels of 0.2 and matching gel sets of six individual 2D-gels were excised from the 2D-gels 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). Neat 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 and directly applied to a MALDI Pre-spotted AnchorChip target (BrukerDaltoniks, Bremen, Germany) according to the manufacturer's instructions.

Subsequently, samples were analyzed in a time-of-flight Ultraflex-TOF/TOF mass spectrometer (BrukerDaltoniks, Bremen, Germany). Acquired mass spectra were automatically calibrated and annotated using Compass 1.3 software (BrukerDaltoniks, Bremen, Germany) and xml formatted peaklists were transferred to Proteinscape 2.1 (BrukerDaltoniks, Bremen, Germany). Since all experiments were performed on primary human adipocytes, MS spectra from each individual spot were used to search a human subset of Swiss-Prot (Sprot_57.8, 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 using different, independent ways. All of the identified protein sequences consecutively were analysed by:

1. Swiss-Prot/TrEMBL database annotation (http://www.expasy.ch/sprot/)
2. Bio-GPS (http://biogps.gnf.org/#goto=welcome)
3. SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) using the following settings: eukaryote option; searching method neural networks; *D*score cut-off: 0.43
4. SecretomeP 2.0 (http://www.cbs.dtu.dk/services/SecretomeP/) using the following settings: NNscore cut-off: 0.5
5. NCBI Pubmed literature screening (http://www.ncbi.nlm.nih.gov/pubmed)
6. Ingenuity IPA8.5 analysis

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

20 male obese patients and 20 lean age-matched controls were recruited at Gent University. For all patients, anthropometric and routine blood parameters were assessed. Fasting blood samples were collected, adipose tissue biopsies fixed for microscopic evaluation of adipocyte surface area analysis or frozen for protein expression analysis. The study protocol was approved by the local Ethics Committees and all participants gave written, informed consent.

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 polyvenylidenefluorid 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. 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.

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 were used for comparison between groups. Correlations were performed by Pearson or Spearman. 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

In order to identify novel adipokines, we have utilized protein profiling of conditioned medium (CM) derived from primary human adipocytes. Figure 1 gives an overview of the processing steps.

Isolated preadipocytes obtained from five different donors were cultivated and differentiated *in vitro*. A total volume of 200 ml CM was collected, concentrated to a final volume of 200 µl and analyzed by two complementary, orthogonal profiling techniques, i.e. 1-dimensional SDS-PAGE/LC-ESI-MS/MS and 2-dimensional SDS-PAGE/MALDI-MS.

For LC-MS/MS analysis CM was fractionated in a first step by 5-15 % gradient SDS-PAGE in order to reduce complexity and facilitate further analysis. Two replicate gels were cut each into 24 equal slices dividing the mixture according to different molecular weight. Proteins were digested with trypsin and subsequently subjected to LC-ESI-MS/MS analysis (2 x 24 fractions). Keeping the false positive rate below 2 % automated

data processing by Proteinscape™ 2.1 identified 341 unique protein species, consistently within both replicates (TableS1 in Supporting Information).

Complementary to LC-MS based profiling, CM was analyzed using 2D-SDS-PAGE/MS. For 2-dimensional gel electrophoresis samples were separated on large format 2D-gels, covering the pH range of 4-9 (overlapping gel couple pH 4-7 and pH 6-9). Image analysis of the merged gels, reproducible detected more than 1,200 non-redundant protein spots. Consistently detected protein spots were excised from four replicate gel-couples, subjected to in-gel digestion and identified by MALDI-MS. Searching the human subset of Swiss-Prot database, Proteinscape™ 2.1 data processing identified a total of 351 protein spots, at least consistently from three different replicates, which could be assigned to 89 differentprotein species (TableS1 in Supporting Information).

To establish a valid basis for documentation and to ease further analysis all data generated by these two approaches were collected and processed using Proteinscape™ 2.1 data integration system. Overall, the combined MS data resulted in the identification of 347 distinct protein species.

Secretory protein selection and comparison with reported secretomes

To confirm that the identified proteins are putative secretory proteins, we have analyzed all candidates using different web-based bioinformatic tools for prediction (Swiss-Prot/TrEMBL, BioGPS, Ingenuity IPA8.5, SignalP3.0, SecretomeP2.0, NCBI PubMed). Applying these consecutive filter methods 263 proteins (263/347, 76%) were predicted to be or are annotated as secretory proteins (TableS1 in Supporting Information). Comparing our results with identified proteins from previous reported secretome analyses [8,12-15,23] -summarized in table 1- in the field of adipose tissue shows an overlap of 83 % (219/263) (Table S1 in supporting information). Although about 80% of the identified proteins have been reported in these earlier studies, at least 44 proteins are described for the first time to be potential novel adipokines secreted from primary human adipocytes (Table 2).

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

According to the immediate availability of biochemical analysis tools and Gene Onthology (GO) Biological Process and Molecular Function annotations, we selected four putative adipokines, supposed to be engaged in different biological processes, for further validation and investigation of regulation. Namely these are i) CFH being involved in inflammatory processes, ii) CRYAB proposed to regulate apoptosis, iii) CILP participating in extracellular matrix structure and remodeling, and iv) HO-1 described in

the context of oxidative stress response.

All of these candidates, i.e. CFH, CRYAB, HO-1 and CILP were expressed in and secreted by human adipocytes (Fig. 2A-B). It should be noted that CFH and CRYAB were very recently reported as novel adipokines [13,14] but have not been validated so far. Furthermore, all of these factors are more highly expressed in adipocytes as compared to macrophages isolated from human adipose tissue (Fig. 2C).

CFH is more highly expressed in preadipocytes as compared to adipocytes (Fig. 3A) in accordance to the literature available on CFH expression.[24] Its expression is upregulated in adipocytes by troglitazone, TNFα, insulin and hypoxia (Fig. 3B). Furthermore, CFH abundance is significantly higher in serum of obese patients as measured by Western Blot (Fig. 3C) and in accordance with previous findings of CFH being elevated in obesity and associated with insulin resistance.[24]

CRYAB expression is strongly induced during adipogenesis (Fig. 3D) being 10-times higher in adipocytes than in preadipocytes. CRYAB expression is not regulated by any treatment applied in this study (data shown) and the amount of CRYAB detected in serum by Western blotting is not significantly different between lean and obese subjects (Fig. 3E). We also identified CILP as a novel adipokine, so far described as extracellular matrix protein which is highly expressed in the intermediate zone of the human articular cartilage. The 140 kDa monomeric glycoprotein precursor of CILP can be cleaved into two secreted products, a 90 kDa N-terminal CILP-1, and a 62 kDa C-terminal fragment.[25] Using an antibody detecting the N-terminal peptide, we could observe that the abundance of the CILP precursor significantly increases with differentiation reaching about 10-times higher concentrations in adipocytes as compared to preadipocytes (Fig. 4A), Although the same trend could be observed for the abundance of the 90 kDa secreted product, it is not significantly induced during differentiation, which could be due to the release of this secreted form, demonstrated by its high abundance in adipocyte-CM (Fig. 2B). Western blot analysis of the 140 kDa monomeric glycoprotein precursor revealed that it is significantly decreased by troglitazone, whereas the 90 kDa secreted product is significantly inhibited by TNFα (Fig. 4B). Serum analysis of lean and obese man by Western blotting revealed a significantly lower presence of the 90 kDa secreted product of CILP in the obese state (Fig. 4C). The 140 kDa precursor could not be detected in serum. As CILP cannot be measured by ELISA at the moment, the absolute amount of CILP in the circulation remains unknown.

HO-1 expression significantly increases with adipocyte differentiation (Fig. 5A) which is paralleled by an induction of HO-1 secretion (Fig. 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). Circulating levels of HO-1 were measured by ELISA and found to be significantly higher in obese patients as compared to lean controls (Fig. 5D). HO-1 serum concentrations are correlated to the size of subcutaneous adipocytes

making it possible that enlarged adipocytes are characterized by higher HO-1 release (Fig. 5E).

Measurement of CRYAB and HO-1 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. 6). This finding makes them very attractive targets for further characterization in obese patients and patients with the metabolic syndrome.

Discussion

Proteomic profiling of the adipocyte secretome

Nowadays it is generally accepted that adipose tissue is a source to release a huge number of different biological active peptides and proteins, so called adipokines being involved in the development of diverse disease pattern.[1] Therefore, identification of novel protein factors secreted from adipose tissue is necessary to understand the role of adipocytes in disease pathophysiology.

In order to identify novel, disease related biomarkers we conducted an analysis of the adipokinome derived from primary human adipocytes. Proteomic profiling by two complementary, orthogonal techniques, i.e. 1DE-LC-ESI-MS/MS and 2DE-MALDI-MS were used to investigate CM derived from fully differentiated human adipocytes and led to the identification of 347 different proteins. Combining a gel-free and gel-based approach may help to resolve limitations given by each single method and 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 (83/89) were also observed by LC-MS analysis (341 proteins identified), gel based examination afford the opportunity to provide unrivaled additional information. 2DE analysis offer access to analyze protein isoforms or putative posttranslational modifications represented by different localizations within the gel map. This is illustrated impressively 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 p*I*. Using this information very recently, we could demonstrate that pigment epithelium-derived factor (PEDF), indeed an already known adipokine, is one of the most abundant proteins secreted by human adipocytes and induces insulin resistance and inflammatory signaling in muscle and fat cells.[26] 2DE analysis assigned PEDF to 15 different spots, representing 6 % of calculated total spot intensities, highlighting PEDF as a major secretion product of the human adipocyte. Moreover, once established, 2DE protein profiles can serve as reference map for further

quantitative profiling studies using DIGE technology.

A major challenge in secretome analysis is to discriminate between really 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. Therefore, we tested all proteins by hierarchically analysis utilizing expression data screening, signal sequence prediction programs and literature screening to determine supposed sub-cellular localization. As a result we selected 263 proteins to be putative secretory proteins. The high number of identified proteins demonstrates the efficiency of our integrated profiling approach, which is in line with very recently published studies of the human adipocyte secretome.[13,15] Comparison analysis reveals, that 219 (83%) of our putativesecretory proteins arealready reported in previous studies (Table 1), dealing with secretomes derived from different origins, including 3T3 cells, tissue explants or isolated adipocytes. A very recent study conducted by Rosenow et al.,[14] applying an analogous approach to the human SGBS adipocyte cell line, identified only 80 secreted proteins, potentially reflecting the 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. Detection and identification of putative novel adipokines is an irreplaceable basis, but alone will not be sufficient to enhance our current knowledge of the endocrine function of adipose tissue. Therefore, the candidate proteins further have to be validated regarding their expression, secretion and function.

Validation of novel adipokines

To address their putative biological relevance, we have chosen 4 candidate adipokines for further validation experiments. CFH, CRYAB, CILP and HO-1 are representatives of different critical functional groups (inflammation, apoptosis, extracellular matrix structure and oxidative stress) that have been demonstrated to be dysregulated in obesity.[13,27]

Complement factor H (CFH) is an example of the large group of adipocyte-secreted proteins being involved in inflammation. Showing a significant reduction of CFH protein expression during adipogenesis, we corroborate two other studies demonstrating at the mRNA level that this factor is predominantly expressed in preadipocytes while also present in adipocytes.[24,28] In addition, CFH in blood is elevated in insulin-resistant subjects [24] with higher expression in subcutaneous adipose tissue. Consistently, we found higher CFH content in blood of obese men and an upregulation of CFH in adipocytes treated with TNFα, insulin and hypoxia. All together, 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 in obesity remains an open question that is of interest for further analysis. The protection against apoptosis and oxidative stress is a function of αB-crystallin (CRYAB) that we describe to be an adipokine, in agreement with a recent report by Kim et al.[13] It is known that CRYAB is expressed in adipose tissue.[29] However, here we show for the first time that CRYAB is increased during adipogenesis without further regulation by classical adipocyte stimuli (data not shown). Furthermore CRYAB expression is significantly increased in adipose tissue of obese patients as compared to lean controls. The fact that CRYAB concentrations measured by Western blot in serum are not different in lean and obese patients points to a possible contribution of other tissues such as heart and skeletal muscle to circulation levels.[30,31]

CILP is a secreted glycoprotein that resides in the extracellular matrix [32,33] and is thought to play an important role in maintaining cartilage homeostasis.[34] Although CILP expression was claimed to be largely restricted to cartilage,[35-39] we could confirm BioGPS data showing that CILP is highly expressed in adipose tissue, and furthermore demonstrate for the first time that CILP is also an adipokine secreted by *in vitro* differentiated human adipocytes. Several studies could show that CILP dysfunction contributes to various diseases affecting the cartilage. In this regard, the expression of CILP rises substantially in the early stages of osteoarthritis and rheumatoid arthritis.[40] 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.[41,42] Recently it could be shown that CILP acts as negative regulator of TGF-β1 by binding it directly *in vitro,* thereby disrupting normal TGF-β activity.[42] Furthermore, TGF-β was reported to strongly inhibit adipogenesis and the amount of fat in adipocytes.[43] With regard to our study in which we could demonstrate that CILP serum concentration is significantly decreased in obese men, one could speculate that a lower abundance of CILP in the obese state probably leads to a higher TGF-β1 activity, which could result in inhibited adipogenesis. Fain et al. could demonstrate that TGF-β1 release by human adipose tissue is enhanced in obesity.[44] In this context it is suggested to act as an anti-inflammatory cytokine, which could block the effects of inflammatory cytokines like IL-8. The downregulation of CILP could be seen as a counteraction to enhance TGF-β activity, thereby exerting a protective role in obesity. Furthermore we could demonstrate in our study that the abundance of the 90 kDa secreted CILP is decreased by TNFα, which again could be explained as a response to elevated TNFα levels observed in the obese state. In addition, we could observe a decrease of the 140 kDa precursor after treatment with troglitazone, which also has been described for a number of other adipokines such as PAI-1, TNFα or chemerin.[45-47] Until now it is not known whether thiazolidinediones have an regulatory effect on the

level of circulating CILP. However, the role of CILP in adipose tissue biology and its further impact in obesity-related diseases is completely unknown. Function and consequences of CILP secretion by adipocytes and the underlying mechanisms of the reduced CILP serum levels in the obese state remain open questions and should further be investigated.

The novel adipokine 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 and recent studies showed elevated plasma levels in several chronic disorders like Parkinson's disease, 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] However, we are the first to 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 suggests that adipocytes actively secrete HO-1 and possibly release it into the circulation and that this secretory activity is dependent on adipocyte size. It is unlikely that the release of HO-1 is a result of dead cells or leakage as our group could previously show that diverse adipokines like chemerin or PEDF are continuously secreted by human adipocytes.[26,53]
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 [54,55] as well as HO-1 induction in an animal model led to a reduction in the circulating TNFα level,[56] 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.[57] This observations imply that HO-1 seems not to be only a marker of cellular stress [58] but acts also 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 plays an important role in adipose tissue inflammation. Moreover, the elevated plasma levels in obese patients described in this study and concerning the recent study showing elevated levels of circulating HO-1 in newly diagnosed type 2 diabetics [49] HO-1 may be a potent biomarker for obesity and obesity associated disorders like the metabolic syndrome and type 2 diabetes.

Conclusion

The crosstalk between different tissues describes a novel physiological concept which may play an essential role in the pathophysiology of multifactorial diseases like type 2 diabetes and the metabolic syndrome. In this context protein profiling provides a powerful tool to characterize the complex mixtures of biological active proteins released from different tissues comprising various endocrine, autocrine and paracrine functions. Together with the consecutive validation of promising candidate proteins including measurements within clinical studies this opens up the possibility to identify novel disease related biomarkers. Taken together, with this combined proteomic approach, we have identified 4 representative and highly interesting novel adipokines and could describe regulation of these factors during adipogenesis and regulation of their content in blood by body weight. Especially HO-1 exhibiting significantly elevated plasma levels and increased expression in subcutaneous and visceral fat of obese patients may be a potent biomarker candidate for obesity and obesity-associated disorders like the metabolic syndrome and type 2 diabetes. Further studies are necessary for extensive characterization in order to analyze its relevance for clinical purpose.

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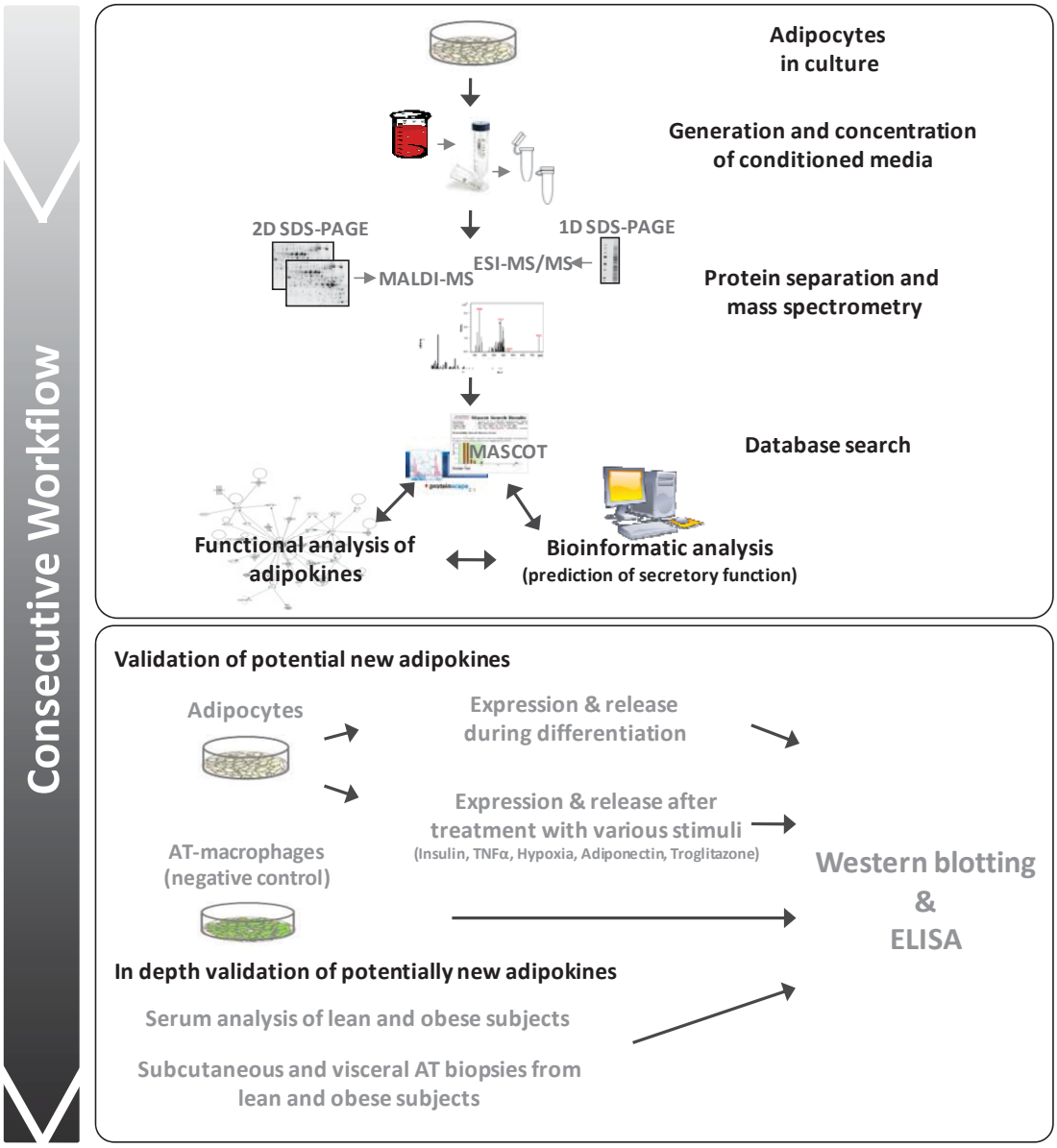

Figure 2.1 Flow chart – work scheme

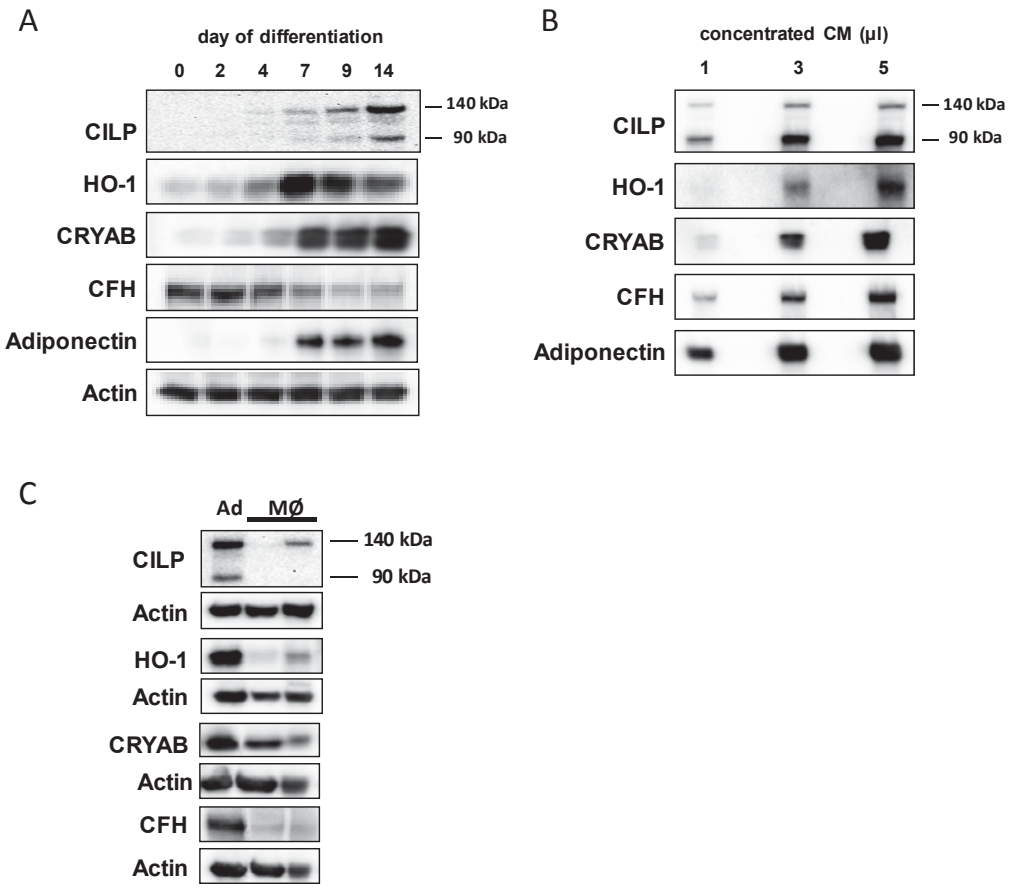

Figure 2.2 Representative Western blots of CILP, HO-1, CRYAB, and CFH protein level and release by human primary adipocytes. Adipocytes were differentiated and concentrated CM was generated as described in materials and methods. (A) Protein level of novel adipokines during adipocyte differentiation. 10 µg of total lysates were analyzed by SDS-PAGE and Western blotting. Signals were detected by ECL. (B) Release of novel adipokines at day 14 of differentiation. 1-5 µl of concentrated CM were analyzed by SDS-PAGE and Western blotting. (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.

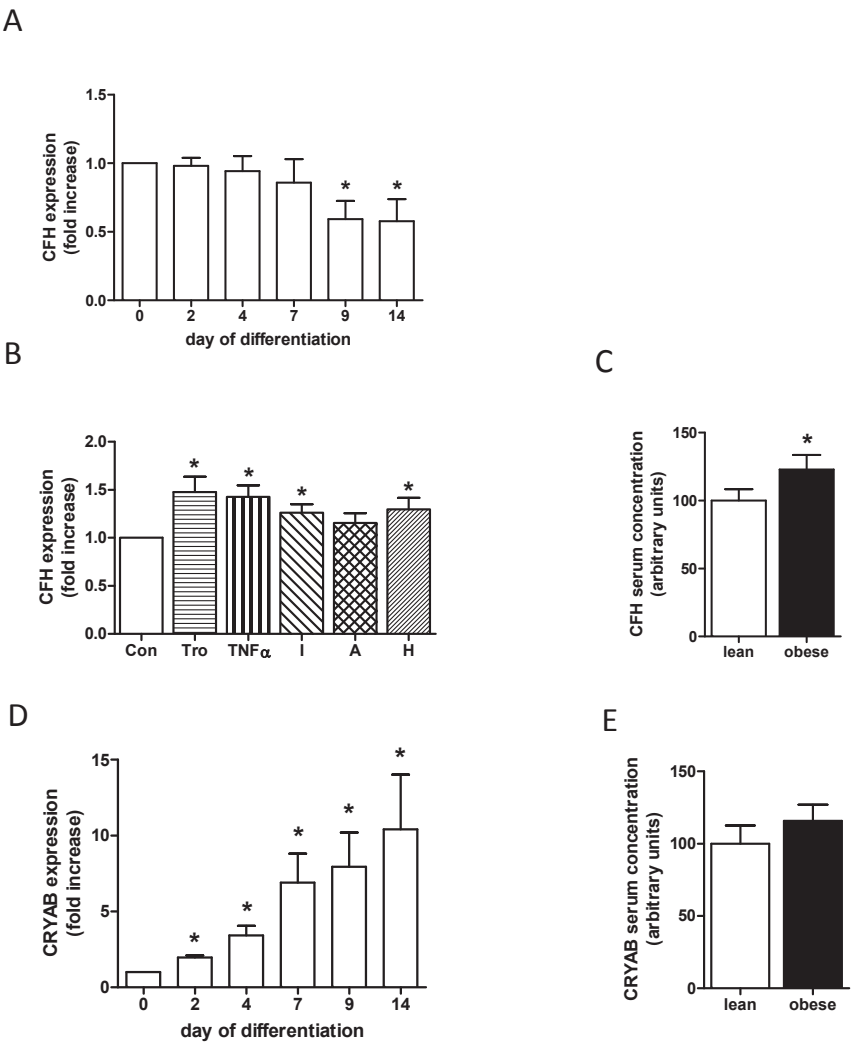

Figure 2.3 Protein level of CFH and CRYAB in adipocytes and relative serum concentrations in lean and obese subjects. Human primary adipocytes were differentiated as described in materials and methods. CFH (A) and CRYAB (D) protein level during differentiation were analyzed by SDS-PAGE and Western blotting. 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), 5 nM adiponectin (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, n≥3, *p<0.05 vs. lean controls.

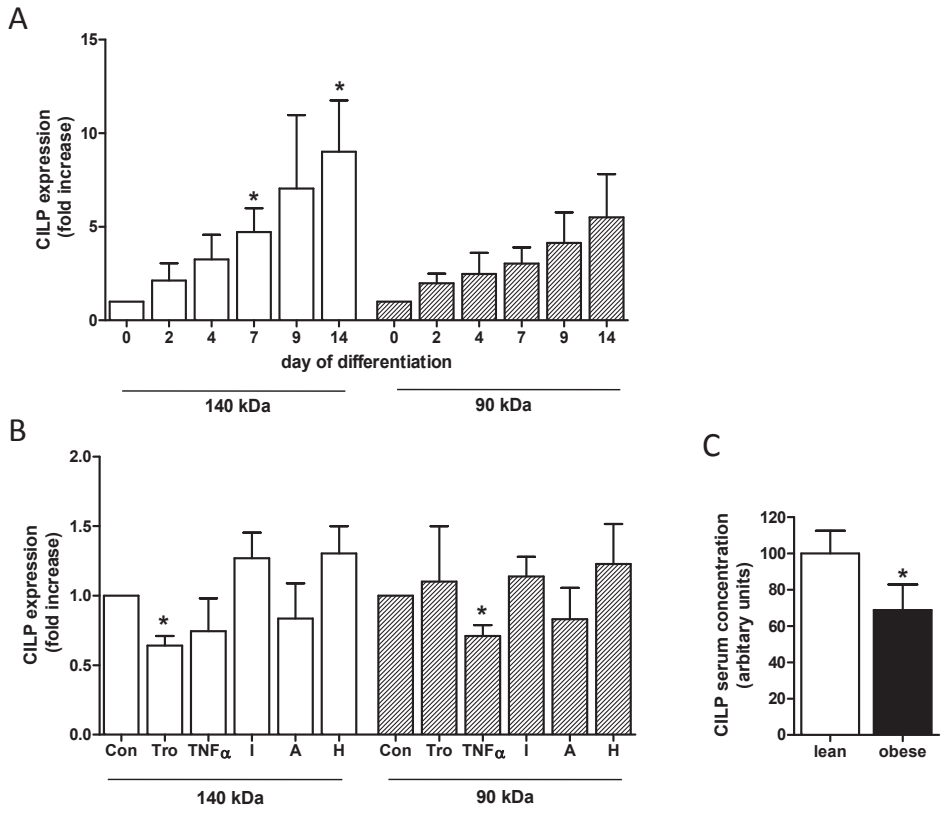

Figure 2.4 Protein level of CILP in adipocytes and relative CILP serum concentration in lean and obese subjects. (A) Human primary adipocytes were differentiated as described in materials and methods. CILP protein level during differentiation was analyzed by SDS-PAGE and Western Blot. Data were normalized to the protein level of actin and are expressed relative to day 0. Data are mean values ± SEM, n≥3, *p<0.05 vs. preadipocytes. (B) Differentiated adipocytes were treated as described in legend to fig. 2.3B. 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) Relative serum concentration of CILP was determined in samples obtained from lean and obese subjects participating in study 1. Sera samples were diluted and analyzed by SDS-PAGE and Western blotting. Data are mean values ± SEM, n≥3, *p<0.05 vs. lean controls.

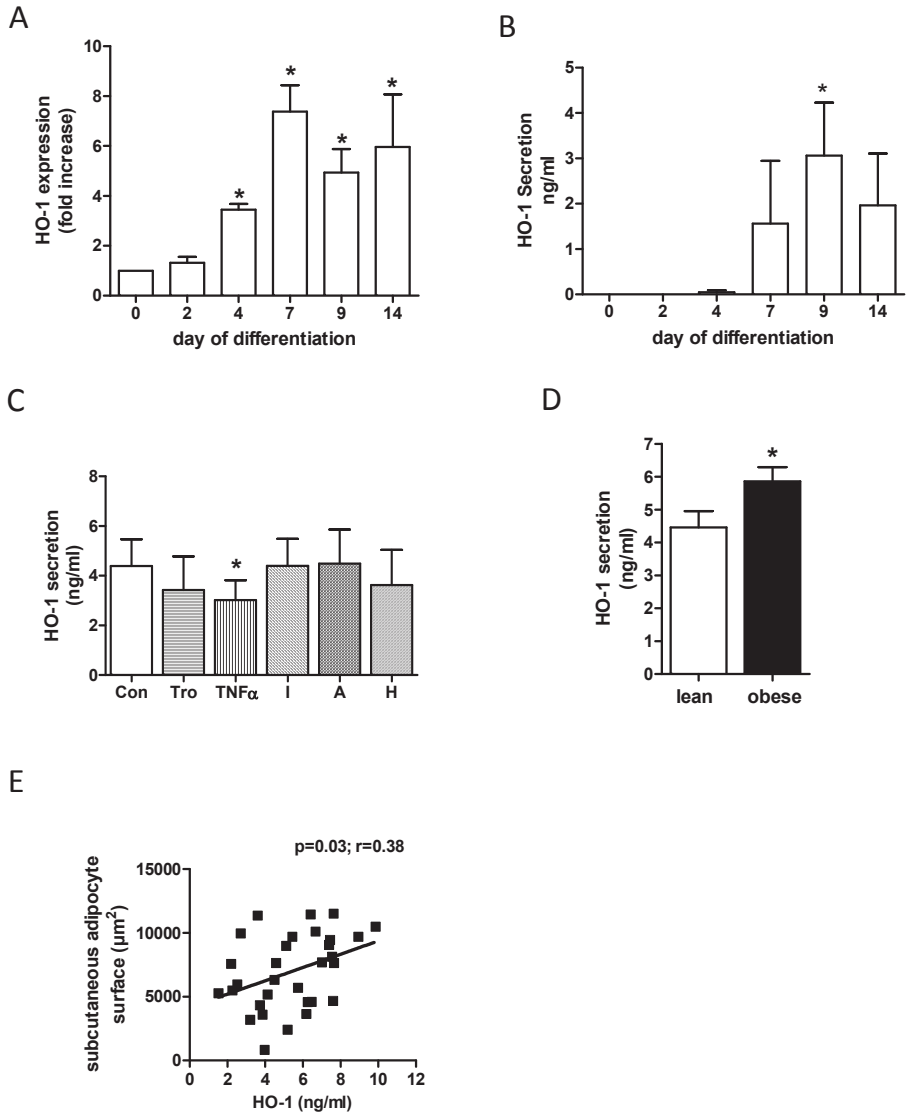

Figure 2.5 HO-1 protein level in adipocytes and relative HO-1 serum concentration in lean and obese subjects. (A) HO-1 protein level during adipocyte differentiation was analyzed by SDS-PAGE and Western blotting. Data were normalized to the protein level of actin and are expressed relative to day 0. Data are mean values ± SEM. n≥5, *p<0.05 vs. preadipocytes. (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. 2.3B. 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 adipocyte (p=0.031; r=0.40).

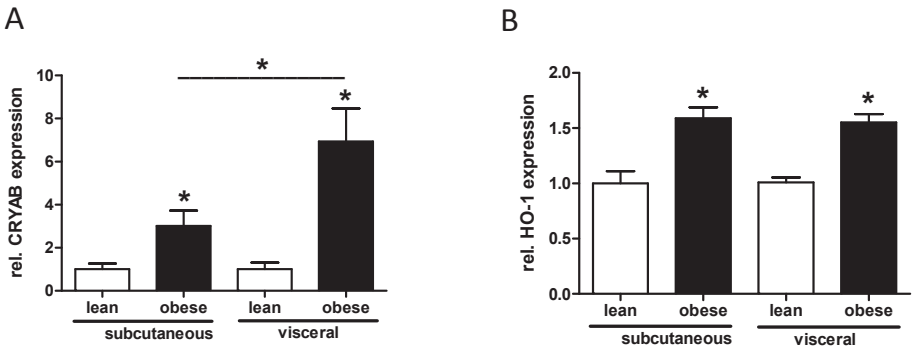

Figure 2.6 CRYAB and HO-1 protein expression in subcutaneous and visceral adipose tissue from lean and obese patients. CRYAB (A) and HO-1 (B) level were determined in adipose tissue lysates from paired subcutaneous and visceral adipose tissue of lean (n=9) and obese (n=15) patients. 5 µg of tissue lysates 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.

Table 1 Literature comparison

study	source	total identified proteins	common with our study identified proteins / predicted secretory proteins
Wang *et al* . Cell Mol. Life Sci. 61(18):2405-2417; (**2004**)	3T3 L1 cells mouse	**41**	**35 / 31**
Chen *et al.* J Proteome Res, 4(2):570-7; (**2005**)	fat pads rat	**183**	**63 / 52**
Alvarez-Llamas *et al.* Mol Cell Proteomics, 6(4):589-600; (**2007**)	visceral tissue explants human	**259**	**149 / 105**
Zvonic *et al* . Mol Cell Proteomics, 6(1):18-28; (**2007**)	huASC human	**101**	**54 / 36**
Molina *et al* . J Proteome Res. 8(1): 48-58; (**2009**)	3T3 L1 cells mouse	**147**	**70 / 47**
Kim *et al.* Proteomics, 10(3): 394-405; (**2010**)	hSVF cells human	**474**	**173 / 129**
Rosenow *et al* . J Proteome Res. Oct 1;9(10):5389-401; (**2010**)	SGBS cells human	**241**	**125 / 90**
Zhong *et al* . J Proteome Res. Oct 1;9(10):5228-38. (**2010**)	adipocytes human	**420**	**195 / 149**

Table 2 Novel Adipokines. All proteins are predicted as potentially secreted by signalP or secretomeP.

Swissprot accession	Name		Detection with LC-MS / MALDI-MS
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	Detection with LC-MS / MALDI-MS	Swissprot accession	Name	Detection with LC-MS / MALDI-MS
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	+/-

References

1. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. *J Clin Invest* 2005; 115/5: 1111-1119.
2. Arner P. The adipocyte in insulin resistance: key molecules and the impact of the thiazolidinediones. *Trends Endocrinol Metab* 2003; 14/3: 137-145.
3. Scherer PE. Adipose tissue: from lipid storage compartment to endocrine organ. *Diabetes* 2006; 55/6: 1537-1545.
4. Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab* 2004; 89/6: 2548-2556.
5. Waki H, Tontonoz P. Endocrine functions of adipose tissue. *Annu Rev Pathol* 2007; 2: 31-56.
6. Breitling R. Robust signaling networks of the adipose secretome. *Trends Endocrinol Metab* 2009; 20/1: 1-7.
7. Sell H, Dietze-Schroeder D, Eckel J. The adipocyte-myocyte axis in insulin resistance. *Trends Endocrinol Metab* 2006; 17/10: 416-422.
8. Chen X, Cushman SW, Pannell LK, Hess S. Quantitative proteomic analysis of the secretory proteins from rat adipose cells using a 2D liquid chromatography-MS/MS approach. *J Proteome Res* 2005; 4/2: 570-577.
9. Klimcakova E, Moro C, Mazzucotelli A, Lolmede K, Viguerie N, Galitzky J, Stich V, Langin D. Profiling of adipokines secreted from human subcutaneous adipose tissue in response to PPAR agonists. *Biochem Biophys Res Commun* 2007; 358/3: 897-902.
10. Kratchmarova I, Kalume DE, Blagoev B, Scherer PE, Podtelejnikov AV, Molina H, Bickel PE, Andersen JS, Fernandez MM, Bunkenborg J, Roepstorff P, Kristiansen K, Lodish HF, Mann M, Pandey A. A proteomic approach for identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes. *Mol Cell Proteomics* 2002; 1/3: 213-222.
11. Tsuruga H, Kumagai H, Kojima T, Kitamura T. Identification of novel membrane and secreted proteins upregulated during adipocyte differentiation. *Biochem Biophys Res Commun* 2000; 272/1: 293-297.
12. Alvarez-Llamas G, Szalowska E, de Vries MP, Weening D, Landman K, Hoek A, Wolffenbuttel BH, Roelofsen H, Vonk RJ. Characterization of the human visceral adipose tissue secretome. *Mol Cell Proteomics* 2007; 6/4: 589-600.
13. Kim J, Choi YS, Lim S, Yea K, Yoon JH, Jun DJ, Ha SH, Kim JW, Kim JH, Suh PG, Ryu SH, Lee TG. Comparative analysis of the secretory proteome of human adipose stromal vascular fraction cells during adipogenesis. *Proteomics* 2010; 10/3: 394-405.
14. Rosenow A, Arrey TN, Bouwman FG, Noben JP, Wabitsch M, Mariman EC, Karas M, Renes J. Identification of novel human adipocyte secreted proteins by using SGBS cells. *J Proteome Res* 2010; 9/10: 5389-5401.
15. Zhong J, Krawczyk SA, Chaerkady R, Huang H, Goel R, Bader JS, Wong GW, Corkey BE, Pandey A. Temporal profiling of the secretome during adipogenesis in humans. *J Proteome Res* 2010; 9/10: 5228-5238.
16. Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin MS. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res* 2005; 46/11: 2347-2355.

17. Dietze-Schroeder D, Sell H, Uhlig M, Koenen M, Eckel J. Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors. *Diabetes* 2005; 54/7: 2003-2011.
18. Sell H, Dietze-Schroeder D, Kaiser U, Eckel J. Monocyte chemotactic protein-1 is a potential player in the negative cross-talk between adipose tissue and skeletal muscle. *Endocrinology* 2006; 147/5: 2458-2467.
19. Curat CA, Miranville A, Sengenes C, Diehl M, Tonus C, Busse R, Bouloumie A. From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. *Diabetes* 2004; 53/5: 1285-1292.
20. Thiele H, Glandorf J, Hufnagel P, Korting G, luggel M. Managing Proteomics Data: From Generation and Data Warehousing to Central Data Repository. Journal of Proteomics and Bioinformatics 1(9), 485-507. 2008.
21. Lehr S, Kotzka J, Avci H, Knebel B, Muller S, Hanisch FG, Jacob S, Haak C, Susanto F, Muller-Wieland D. Effect of sterol regulatory element binding protein-1a on the mitochondrial protein pattern in human liver cells detected by 2D-DIGE. *Biochemistry* 2005; 44/13: 5117-5128.
22. Rabilloud T, Strub JM, Luche S, Van DA, Lunardi J. A comparison between Sypro Ruby and ruthenium II tris (bathophenanthroline disulfonate) as fluorescent stains for protein detection in gels. *Proteomics* 2001; 1/5: 699-704.
23. Molina H, Yang Y, Ruch T, Kim JW, Mortensen P, Otto T, Nalli A, Tang QQ, Lane MD, Chaerkady R, Pandey A. Temporal profiling of the adipocyte proteome during differentiation using a five-plex SILAC based strategy. *J Proteome Res* 2009; 8/1: 48-58.
24. Moreno-Navarrete JM, Martinez-Barricarte R, Catalan V, Sabater M, Gomez-Ambrosi J, Ortega FJ, Ricart W, Bluher M, Fruhbeck G, Rodriguez de CS, Fernandez-Real JM. Complement factor H is expressed in adipose tissue in association with insulin resistance. *Diabetes* 2010; 59/1: 200-209.
25. Lorenzo P, Neame P, Sommarin Y, Heinegard D. Cloning and deduced amino acid sequence of a novel cartilage protein (CILP) identifies a proform including a nucleotide pyrophosphohydrolase. *J Biol Chem* 1998; 273/36: 23469-23475.
26. Famulla S, Lamers D, Hartwig S, Passlack W, Horrighs A, Cramer A, Lehr S, Sell H, Eckel J. 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. *Int J Obes (Lond)* 2010.
27. Henegar C, Tordjman J, Achard V, Lacasa D, Cremer I, Guerre-Millo M, Poitou C, Basdevant A, Stich V, Viguerie N, Langin D, Bedossa P, Zucker JD, Clement K. Adipose tissue transcriptomic signature highlights the pathological relevance of extracellular matrix in human obesity. *Genome Biol* 2008; 9/1: R14.
28. Peake PW, O'Grady S, Pussell BA, Charlesworth JA. Detection and quantification of the control proteins of the alternative pathway of complement in 3T3-L1 adipocytes. *Eur J Clin Invest* 1997; 27/11: 922-927.
29. Kumar PA, Haseeb A, Suryanarayana P, Ehtesham NZ, Reddy GB. Elevated expression of alphaA- and alphaB-crystallins in streptozotocin-induced diabetic rat. *Arch Biochem Biophys* 2005; 444/2: 77-83.
30. Vicart P, Caron A, Guicheney P, Li Z, Prevost MC, Faure A, Chateau D, Chapon F, Tome F, Dupret JM, Paulin D, Fardeau M. A missense mutation in the alphaB-crystallin chaperone gene causes a desmin-related myopathy. *Nat Genet* 1998; 20/1: 92-95.
31. Wang X, Klevitsky R, Huang W, Glasford J, Li F, Robbins J. AlphaB-crystallin modulates protein aggregation of abnormal desmin. *Circ Res* 2003; 93/10: 998-1005.

32. Lorenzo P, Bayliss MT, Heinegard D. A novel cartilage protein (CILP) present in the mid-zone of human articular cartilage increases with age. *J Biol Chem* 1998; 273/36: 23463-23468.
33. Lorenzo P, Aman P, Sommarin Y, Heinegard D. The human CILP gene: exon/intron organization and chromosomal mapping. *Matrix Biol* 1999; 18/5: 445-454.
34. Tsuruha J, Masuko-Hongo K, Kato T, Sakata M, Nakamura H, Nishioka K. Implication of cartilage intermediate layer protein in cartilage destruction in subsets of patients with osteoarthritis and rheumatoid arthritis. *Arthritis Rheum* 2001; 44/4: 838-845.
35. Johnson K, Hashimoto S, Lotz M, Pritzker K, Goding J, Terkeltaub R. Up-regulated expression of the phosphodiesterase nucleotide pyrophosphatase family member PC-1 is a marker and pathogenic factor for knee meniscal cartilage matrix calcification. *Arthritis Rheum* 2001; 44/5: 1071-1081.
36. Lorenzo P, Bayliss MT, Heinegard D. A novel cartilage protein (CILP) present in the mid-zone of human articular cartilage increases with age. *J Biol Chem* 1998; 273/36: 23463-23468.
37. Lorenzo P, Neame P, Sommarin Y, Heinegard D. Cloning and deduced amino acid sequence of a novel cartilage protein (CILP) identifies a proform including a nucleotide pyrophosphohydrolase. *J Biol Chem* 1998; 273/36: 23469-23475.
38. Masuda I, Halligan BD, Barbieri JT, Haas AL, Ryan LM, McCarty DJ. Molecular cloning and expression of a porcine chondrocyte nucleotide pyrophosphohydrolase. *Gene* 1997; 197/1-2: 277-287.
39. Masuda I, Iyama KI, Halligan BD, Barbieri JT, Haas AL, McCarty DJ, Ryan LM. Variations in site and levels of expression of chondrocyte nucleotide pyrophosphohydrolase with aging. *J Bone Miner Res* 2001; 16/5: 868-875.
40. Lorenzo P, Bayliss MT, Heinegard D. A novel cartilage protein (CILP) present in the mid-zone of human articular cartilage increases with age. *J Biol Chem* 1998; 273/36: 23463-23468.
41. Mori M, Nakajima M, Mikami Y, Seki S, Takigawa M, Kubo T, Ikegawa S. Transcriptional regulation of the cartilage intermediate layer protein (CILP) gene. *Biochem Biophys Res Commun* 2006; 341/1: 121-127.
42. Seki S, Kawaguchi Y, Chiba K, Mikami Y, Kizawa H, Oya T, Mio F, Mori M, Miyamoto Y, Masuda I, Tsunoda T, Kamata M, Kubo T, Toyama Y, Kimura T, Nakamura Y, Ikegawa S. A functional SNP in CILP, encoding cartilage intermediate layer protein, is associated with susceptibility to lumbar disc disease. *Nat Genet* 2005; 37/6: 607-612.
43. Locklin RM, Oreffo RO, Triffitt JT. Effects of TGFbeta and bFGF on the differentiation of human bone marrow stromal fibroblasts. *Cell Biol Int* 1999; 23/3: 185-194.
44. Fain JN, Tichansky DS, Madan AK. Transforming growth factor beta1 release by human adipose tissue is enhanced in obesity. *Metabolism* 2005; 54/11: 1546-1551.
45. Gottschling-Zeller H, Rohrig K, Hauner H. Troglitazone reduces plasminogen activator inhibitor-1 expression and secretion in cultured human adipocytes. *Diabetologia* 2000; 43/3: 377-383.
46. Sell H, Laurencikiene J, Taube A, Eckardt K, Cramer A, Horrighs A, Arner P, Eckel J. Chemerin is a novel adipocyte-derived factor inducing insulin resistance in primary human skeletal muscle cells. *Diabetes* 2009; 58/12: 2731-2740.
47. Skurk T, Birgel M, Lee YM, Hauner H. Effect of troglitazone on tumor necrosis factor alpha and transforming growth factor beta expression and action in human adipocyte precursor cells in primary culture. *Metabolism* 2006; 55/3: 309-316.

48. Mandal P, Pritchard MT, Nagy LE. Anti-inflammatory pathways and alcoholic liver disease: role of an adiponectin/interleukin-10/heme oxygenase-1 pathway. *World J Gastroenterol* 2010; 16/11: 1330-1336.
49. Bao W, Song F, Li X, Rong S, Yang W, Zhang M, Yao P, Hao L, Yang N, Hu FB, Liu L. Plasma heme oxygenase-1 concentration is elevated in individuals with type 2 diabetes mellitus. *PLoS One* 2010; 5/8: e12371.
50. Mateo I, Infante J, Sanchez-Juan P, Garcia-Gorostiaga I, Rodriguez-Rodriguez E, Vazquez-Higuera JL, Berciano J, Combarros O. Serum heme oxygenase-1 levels are increased in Parkinson's disease but not in Alzheimer's disease. *Acta Neurol Scand* 2010; 121/2: 136-138.
51. Miyazaki T, Kirino Y, Takeno M, Hama M, Ushihama A, Watanabe R, Takase K, Tachibana T, Matsumoto K, Tanaka M, Yamaji S, Ideguchi H, Tomita N, Fujita H, Ohno S, Ueda A, Ishigatsubo Y. Serum HO-1 is useful to make differential diagnosis of secondary hemophagocytic syndrome from other similar hematological conditions. *Int J Hematol* 2010; 91/2: 229-237.
52. Schipper HM, Chertkow H, Mehindate K, Frankel D, Melmed C, Bergman H. Evaluation of heme oxygenase-1 as a systemic biological marker of sporadic AD. *Neurology* 2000; 54/6: 1297-1304.
53. Sell H, Laurencikiene J, Taube A, Eckardt K, Cramer A, Horrighs A, Arner P, Eckel J. Chemerin is a novel adipocyte-derived factor inducing insulin resistance in primary human skeletal muscle cells. *Diabetes* 2009; 58/12: 2731-2740.
54. Fernandez P, Guillen MI, Gomar F, Alcaraz MJ. Expression of heme oxygenase-1 and regulation by cytokines in human osteoarthritic chondrocytes. *Biochem Pharmacol* 2003; 66/10: 2049-2052.
55. Kirino Y, Takeno M, Murakami S, Kobayashi M, Kobayashi H, Miura K, Ideguchi H, Ohno S, Ueda A, Ishigatsubo Y. Tumor necrosis factor alpha acceleration of inflammatory responses by down-regulating heme oxygenase 1 in human peripheral monocytes. *Arthritis Rheum* 2007; 56/2: 464-475.
56. Kim DH, Burgess AP, Li M, Tsenovoy PL, Addabbo F, McClung JA, Puri N, Abraham NG. Heme oxygenase-mediated increases in adiponectin decrease fat content and inflammatory cytokines tumor necrosis factor-alpha and interleukin-6 in Zucker rats and reduce adipogenesis in human mesenchymal stem cells. *J Pharmacol Exp Ther* 2008; 325/3: 833-840.
57. Li M, Kim DH, Tsenovoy PL, Peterson SJ, Rezzani R, Rodella LF, Aronow WS, Ikehara S, Abraham NG. Treatment of obese diabetic mice with a heme oxygenase inducer reduces visceral and subcutaneous adiposity, increases adiponectin levels, and improves insulin sensitivity and glucose tolerance. *Diabetes* 2008; 57/6: 1526-1535.
58. Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev* 2006; 86/2: 583-650.

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 signaling. 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 signaling. 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 critical 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 liver, muscle and fat 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 signaling 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] Due to the degradation of the incretin glucagon-like peptide (GLP)-1, 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.[7] 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.[8] 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,[9] 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 DPP4 concentration in serum and DPP4 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. 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 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. 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.[10] Thereafter, abdominal subcutaneous adipose tissue biopsies were obtained by needle aspiration as described.[11] One part of the tissue was used for measurements of DPP4 release as described.[12] 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.[13]

Adipocyte isolation and culture

Subcutaneous adipose tissue was obtained from lean or moderately overweight women 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.[14] 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.[14] Macrophages were isolated from human adipose tissue, using a method described by Curat et al.. [15] 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.[14] 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

DPP4 secretion by human primary adipocytes and macrophages was determined using ELISA kits purchased from R&D Systems and Stressgene. 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 modeling 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,[16-18] 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. As shown in Fig. 1C, DPP4 release is significantly upregulated 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 [19] 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 signaling 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 signaling, we determined DPP4-stimulated proliferation and insulin signaling 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 well-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, fat mass, the size of subcutaneous and visceral adipocytes, insulin, leptin and hs-CRP, while a negative correlation with age, HDL-cholesterol and adiponectin could be found (Fig. 5A-J). 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 and visceral adipocytes 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, 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 significantly decrease with an increasing risk score as expected (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,[20] being highly abundant in the kidney, on T lymphocytes and endothelial cells.[19] 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,[21] 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,[7] 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,[22] which may modulate the well-established antilipolytic effects of adenosine. Second, DPP4 is a very strong inhibitor of the anti-lipolytic activity of neuropeptide Y (NPY),[20] which is one of the best peptide substrates of the enzyme.[23] 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,[19] 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 signaling functions.[19] We demonstrate here for the first time that DPP4 consistently impairs insulin signaling 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 signaling, 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 [9]). 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.[20,24] 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, plasma triglycerides, HOMA, 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,[25] 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.[26] Additionally, incretin-based therapies are known to preserve beta-cell function and to exert salutary effects on blood pressure and lipid profile.[27] Second, DPP4 inhibitors are well known to improve glucose tolerance in animal models of obesity.[28] More importantly, chronic DPP4 inhibition in ZDF rats was shown to delay the onset of type 2 diabetes.[28] Finally, preclinical data suggest that GLP-1 is cardioprotective [29] and DPP4 inhibition was shown to improve cardiovascular outcomes in rodents.[30]

In summary, we show here that DPP4 is a novel adipokine that is substantially overexpressed in visceral fat from obese subjects and exhibits an augmented release in obesity. Soluble DPP4 exerts auto- and paracrine effects and impairs insulin signaling. 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 critical link between obesity and the metabolic syndrome.

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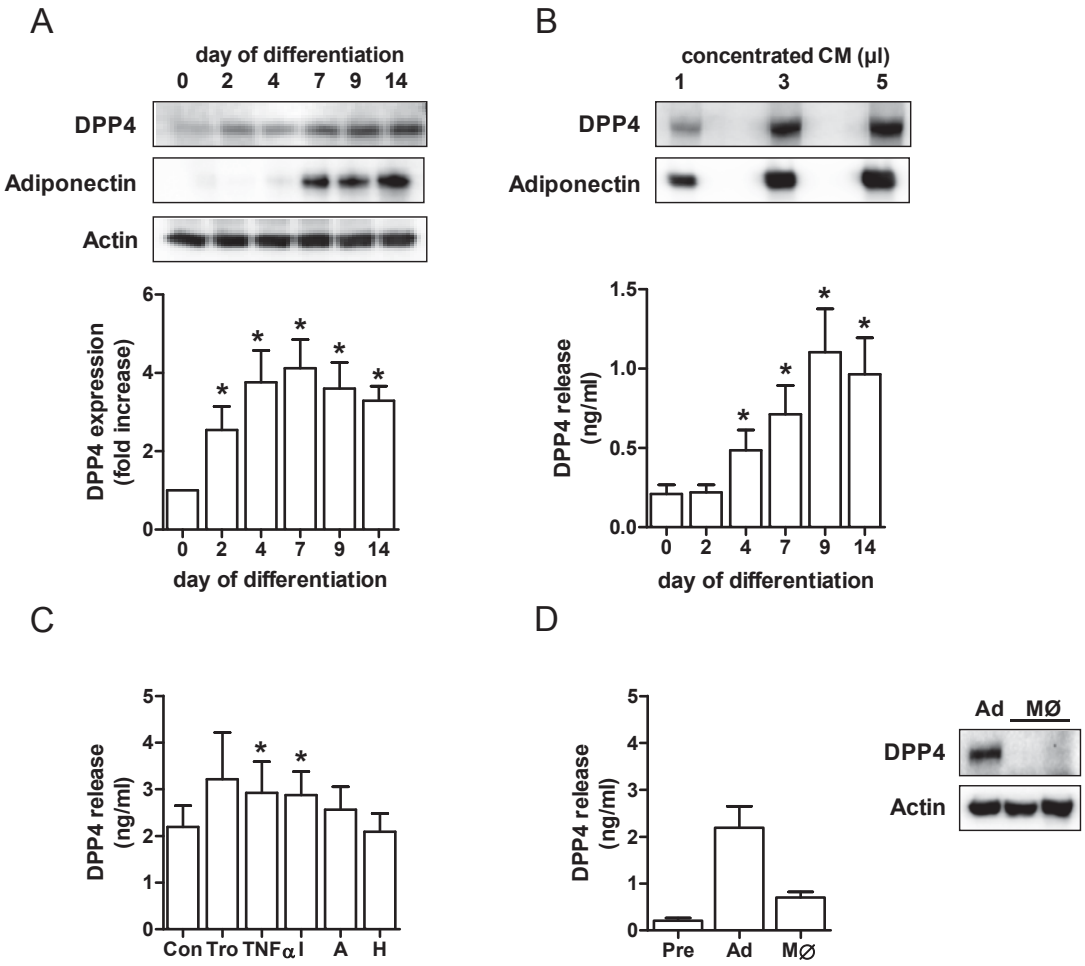

Figure 3.1 DPP4 protein level and release during adipocyte differentiation and after stimulation with different regulatory factors. (A) Human primary adipocytes were differentiated as described in materials and methods, and DPP4 protein level during differentiation was analyzed by SDS-PAGE and Western 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 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.

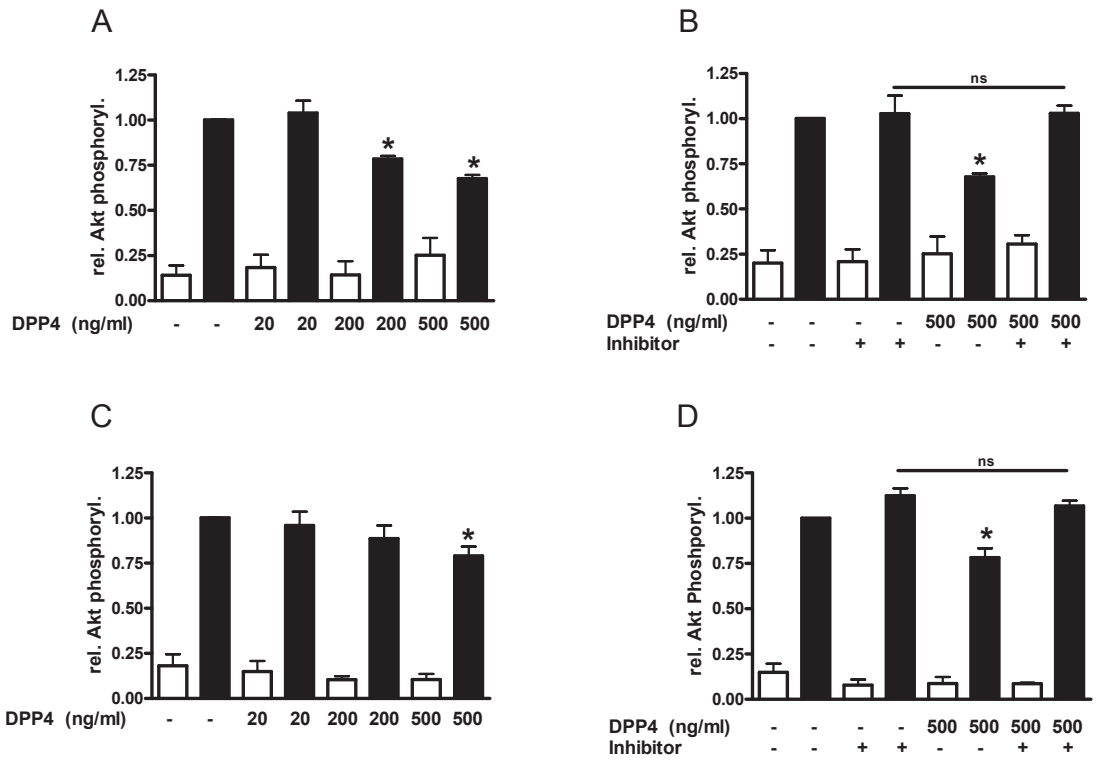

Figure 3.2 Effect of DPP4 on insulin-stimulated Akt phosphorylation in adipocytes and skeletal muscle cells. Differentiated human adipocytes (A-B) and skeletal muscle cells (C-D) were treated with the indicated amounts of DPP4 without and with concomitant administration of a specific DPP4 inhibitor for 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.

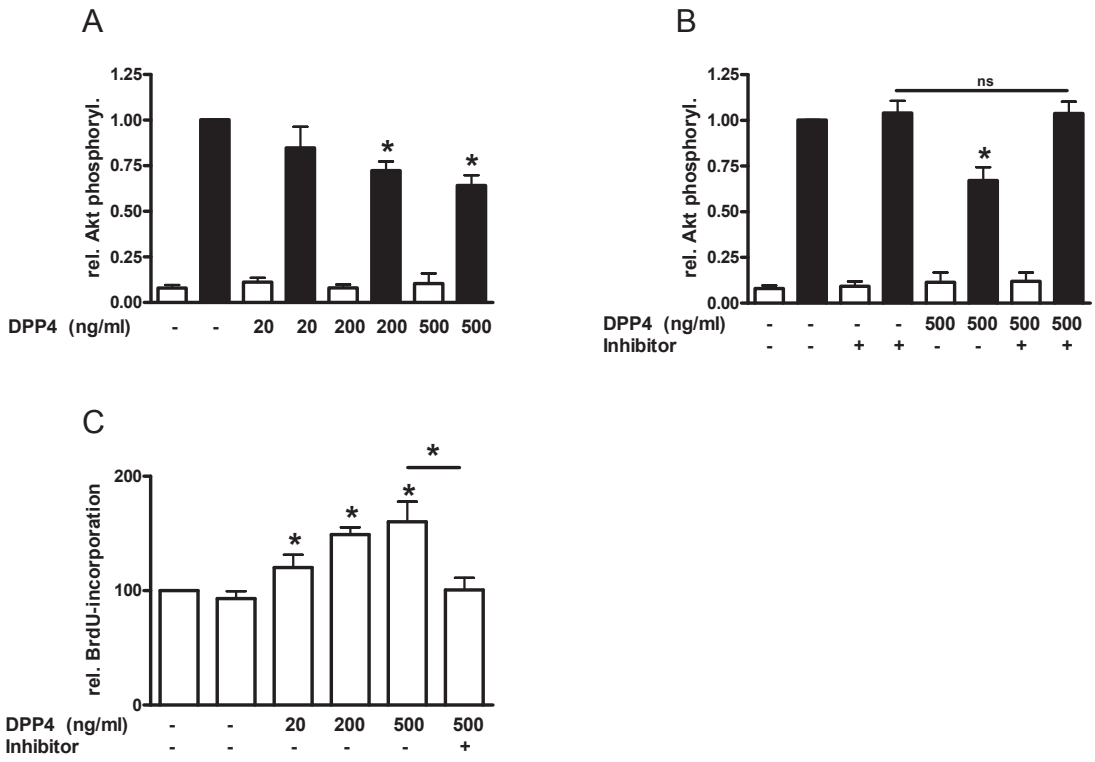

Figure 3.3 Effect of DPP4 on insulin-stimulated Akt phosphorylation and proliferation in smooth muscle cells. (A-B) Smooth muscle cells were treated with the indicated amounts of DPP4 without and with concomitant administration of a specific DPP4 inhibitor for 24 h. After stimulation with insulin (100 nM, 10 min) the cells were lysed and Western Blots performed as indicated in Fig. 3.2. 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.

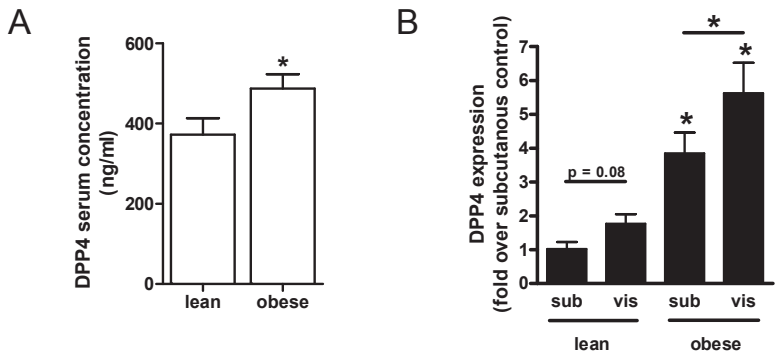

Figure 3.4 DPP4 serum concentration and expression in adipose tissue from lean compared to obese patients (Clinical study 1). (A) Sera from lean (n=20) and morbidly obese men (n=20) were analyzed for their DPP4 concentration by ELISA. Data are mean values ± SEM, *p<0.05 vs. lean group. (B) DPP4 protein 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.

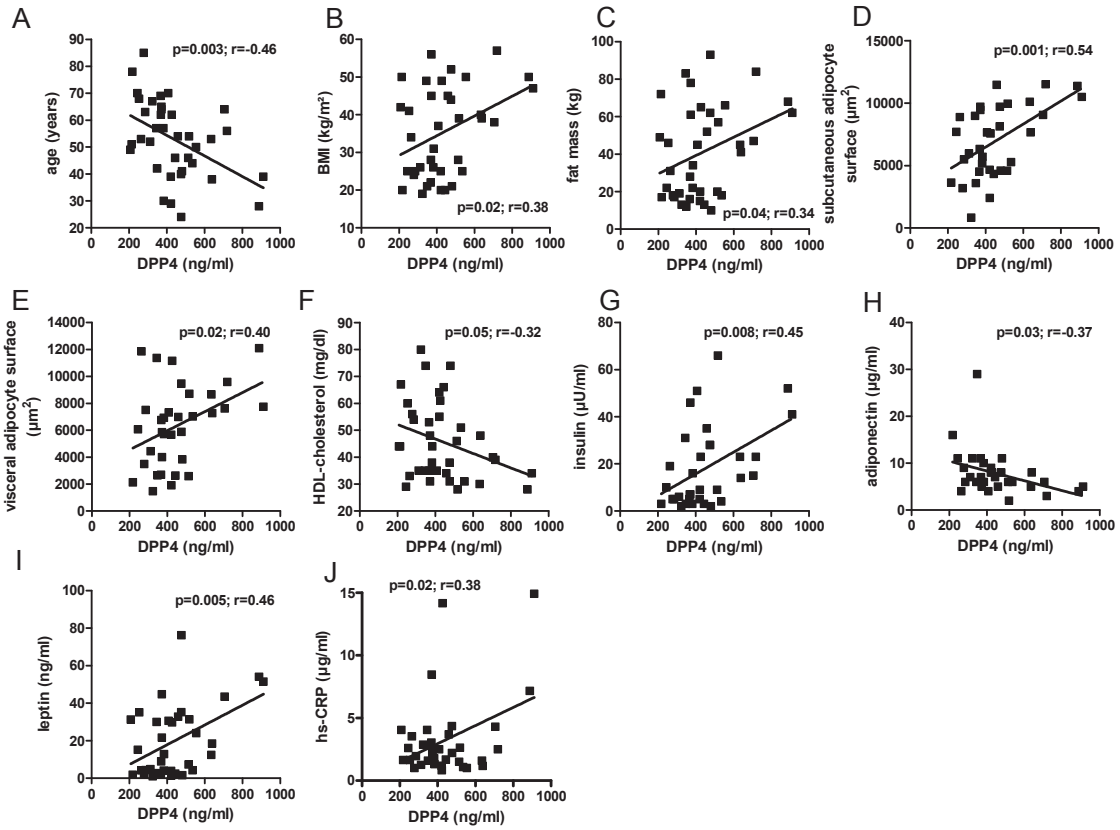

Figure 3.5 DPP4 serum concentrations correlate with various clinical and biochemical parameters (Clinical study 1). Sera from lean (n=20) and morbidly obese men (n=20) were analyzed for their DPP4 concentration by ELISA. Linear regression analysis of DPP4 serum concentration and patient characteristics like age (A), BMI (B), fat mass (C), size of subcutaneous (sc) (D) and visceral (vis) adipocytes (E), HDL-cholesterol concentration (F), insulin concentration (G), adiponectin concentration (H), leptin concentration (I), and concentration of high-sensitive C-reactive protein (hs-CRP) (J).

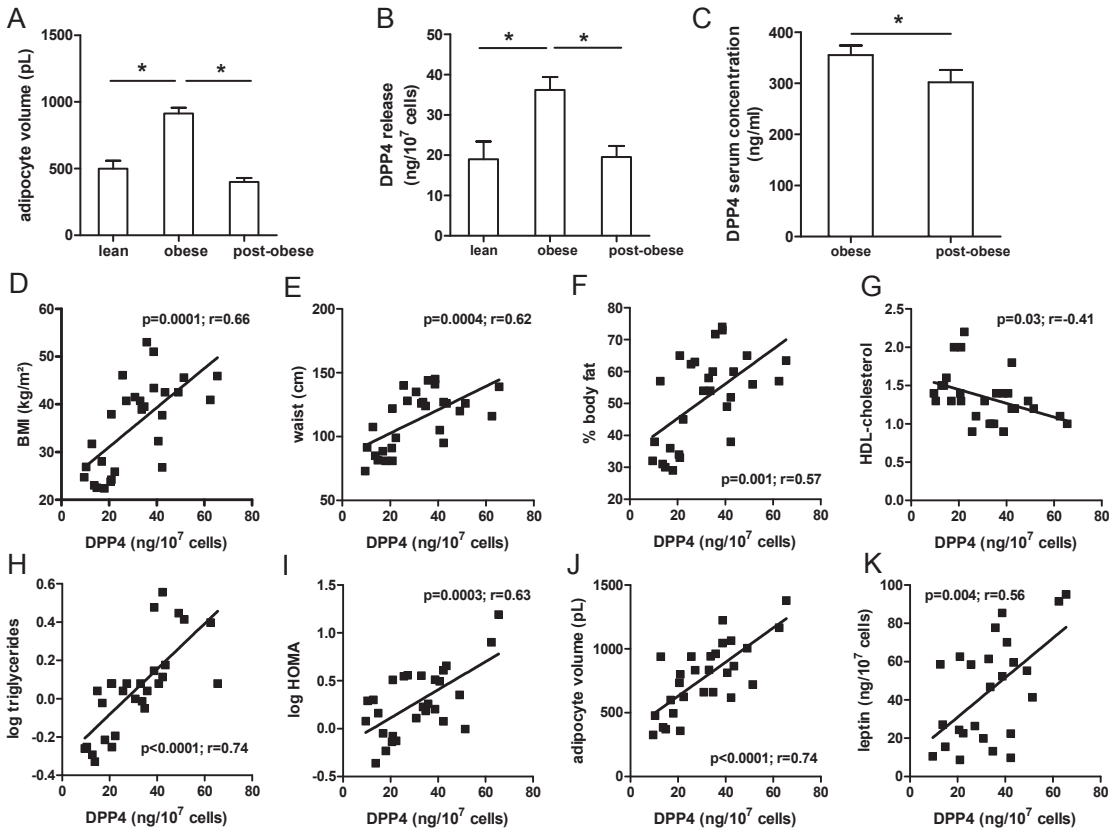

Figure 3.6 DPP4 release of explants obtained from adipose tissue of lean controls and obese patients before and after bariatric surgery as well as linear correlation with various clinical and biochemical parameters (Clinical study 2). (A-B) Samples of adipose tissue were obtained from lean controls (n=10) 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 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). *p<0.05

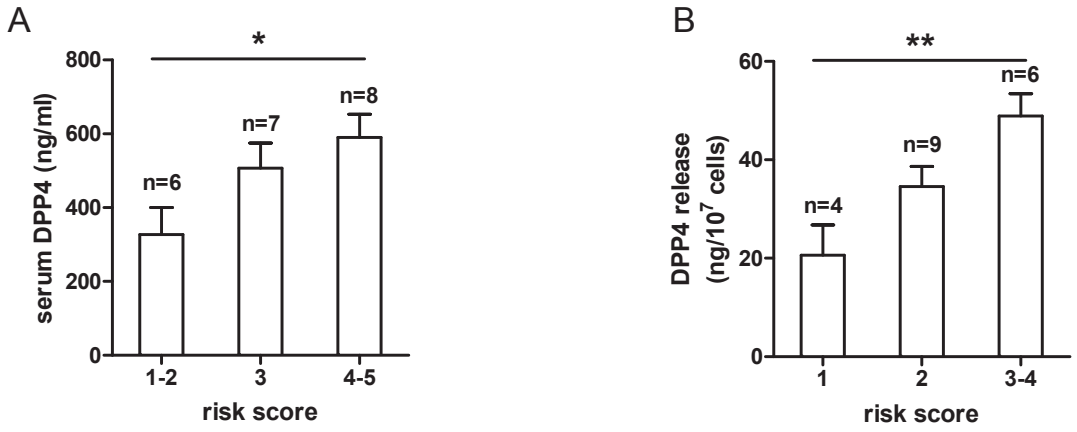

Figure 3.7 DPP4 in serum and release from adipose tissue explants in relation to a risk score for the metabolic syndrome. A risk score for the metabolic syndrome was calculated for all obese subjects from whom serum and adipose tissue explants were analyzed. This score was correlated to the respective DPP4 concentrations in serum or culture media. Data was analyzed using ANOVA and Spearman correlation. *$p<0.05$

References

1. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. *J Clin Invest* 2005; 115/5: 1111-1119.
2. Sell H, Dietze-Schroeder D, Eckel J. The adipocyte-myocyte axis in insulin resistance. *Trends Endocrinol Metab* 2006; 17/10: 416-422.
3. Arner P. The adipocyte in insulin resistance: key molecules and the impact of the thiazolidinediones. *Trends Endocrinol Metab* 2003; 14/3: 137-145.
4. Scherer PE. Adipose tissue: from lipid storage compartment to endocrine organ. *Diabetes* 2006; 55, 1537-45.
5. Breitling R. Robust signaling networks of the adipose secretome. *Trends Endocrinol Metab* 2009; 20, 1-7.
6. Yazbeck R, Howarth GS, Abbott CA. Dipeptidyl peptidase inhibitors, an emerging drug class for inflammatory disease? *Trends Pharmacol Sci* 2009; 30, 600-7.
7. Ahren B. Dipeptidyl peptidase-4 inhibitors: clinical data and clinical implications. *Diabetes Care* 2007; 30, 1344-50.
8. Lambeir AM, Durinx C, Scharpe, S, De Meester I. Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. *Crit Rev Clin Lab Sci* 2003; 40, 209-94.
9. Cordero OJ, Salgado FJ, Nogueira M. On the origin of serum CD26 and its altered concentration in cancer patients. *Cancer Immunol Immunother* 2009; 58, 1723-47.
10. Bonora E, Targher G, Alberiche M, Bonadonna RC, Saggiani F, Zenere MB, Monauni T, Muggeo M. Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. *Diabetes Care* 2000; 23, 57-63.
11. Kolaczynski JW, Morales LM, Moore JH Jr, Considine RV, Pietrzkowski Z, Noto PF, Colberg J, Caro JF. A new technique for biopsy of human abdominal fat under local anaesthesia with Lidocaine. *Int J Obes Relat Metab Disord* 1994; 18, 161-6.
12. Lonnqvist F, Nordfors L, Jansson M, Thorne A, Schalling M, Arner P. Leptin secretion from adipose tissue in women. Relationship to plasma levels and gene expression. *J Clin Invest* 1997; 99, 2398-404.
13. Lofgren P, Hoffstedt J, Naslund E, Wiren M, Arner P. Prospective and controlled studies of the actions of insulin and catecholamine in fat cells of obese women following weight reduction. *Diabetologia* 2005; 48, 2334-42.
14. Dietze-Schroeder D, Sell H, Uhlig M, Koenen M, Eckel J. Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors. *Diabetes* 2005; 54, 2003-2011.
15. Curat CA, Miranville A, Sengenes C, Diehl M, Tonus C, Busse R, Bouloumie A. From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. *Diabetes* 2004; 53, 1285-92.
16. Kim J, Choi YS, Lim S, Yea K, Yoon JH, Jun DJ, Ha SH, Kim JW, Kim JH, Suh PG, Ryu SH, Lee TG. Comparative analysis of the secretory proteome of human adipose stromal vascular fraction cells during adipogenesis. *Proteomics* 2010; 10/3: 394-405.
17. Rosenow A, Arrey TN, Bouwman FG, Noben JP, Wabitsch M, Mariman EC, Karas M, Renes J. Identification of novel human adipocyte secreted proteins by using SGBS cells. *J Proteome Res* 2010; 9/10: 5389-5401.

18. Zhong J, Krawczyk SA, Chaerkady R, Huang H, Goel R, Bader JS, Wong GW, Corkey BE, Pandey A. Temporal profiling of the secretome during adipogenesis in humans. *J Proteome Res* 2010; 9/10: 5228-5238.
19. Gorrell MD. Dipeptidyl peptidase IV and related enzymes in cell biology and liver disorders. *Clin Sci (Lond)* 2005; 108, 277-92.
20. Kos K, Baker AR, Jernas M, Harte AL, Clapham JC, O'Hare JP, Carlsson L, Kumar S, McTernan PG. DPP-IV inhibition enhances the antilipolytic action of NPY in human adipose tissue. *Diabetes Obes Metab* 2009; 11/4: 285-292.
21. Iwaki-Egawa S, Watanabe Y, Kikuya Y, Fujimoto Y. Dipeptidyl peptidase IV from human serum: purification, characterization, and N-terminal amino acid sequence. *J Biochem* 1998; 124/2: 428-433.
22. Schrader WP, West CA, Miczek AD, Norton EK. Characterization of the adenosine deaminase-adenosine deaminase complexing protein binding reaction. *J Biol Chem* 1990; 265/31: 19312-19318.
23. Mentlein R. Dipeptidyl-peptidase IV (CD26)--role in the inactivation of regulatory peptides. *Regul Pept* 1999; 85, 9-24.
24. Bouchard L, Tchernof A, Deshaies Y, Marceau S, Lescelleur O, Biron S, Vohl MC. ZFP36: a promising candidate gene for obesity-related metabolic complications identified by converging genomics. *Obes Surg* 2007; 17/3: 372-382.
25. Reinehr T, Roth CL, Enriori PJ, Masur K. Changes of dipeptidyl peptidase IV (DPP-IV) in obese children with weight loss: relationships to peptide YY, pancreatic peptide, and insulin sensitivity. *J Pediatr Endocrinol Metab* 2010; 23, 101-8.
26. Drucker DJ. The biology of incretin hormones. *Cell Metab* 2006; 3, 153-65.
27. Rizzo M, Rizvi AA, Spinas GA, Rini GB, Berneis K. Glucose lowering and anti-atherogenic effects of incretin-based therapies: GLP-1 analogues and DPP-4-inhibitors. *Expert Opin Investig Drugs* 2009; 18, 1495-503.
28. Sudre B, Broqua P, White RB, Ashworth D, Evans DM, Haigh R, Junien JL, Aubert ML. Chronic inhibition of circulating dipeptidyl peptidase IV by FE 999011 delays the occurrence of diabetes in male zucker diabetic fatty rats. *Diabetes* 2002; 51, 1461-9.
29. Bose AK, Mocanu MM, Carr RD, Brand CL, Yellon DM. Glucagon-like peptide 1 can directly protect the heart against ischemia/reperfusion injury. *Diabetes* 2005; 54, 146-51.
30. Sauve M, Ban K, Momen MA, Zhou YQ, Henkelman RM, Husain M, Drucker DJ. Genetic deletion or pharmacological inhibition of dipeptidyl peptidase-4 improves cardiovascular outcomes after myocardial infarction in mice. *Diabetes* 2010; 59, 1063-73.

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 downregulated 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 downregulation found by us may also underlie a mechanism of degradation of PEDF by MMPs. Additionally, we observed a downregulation 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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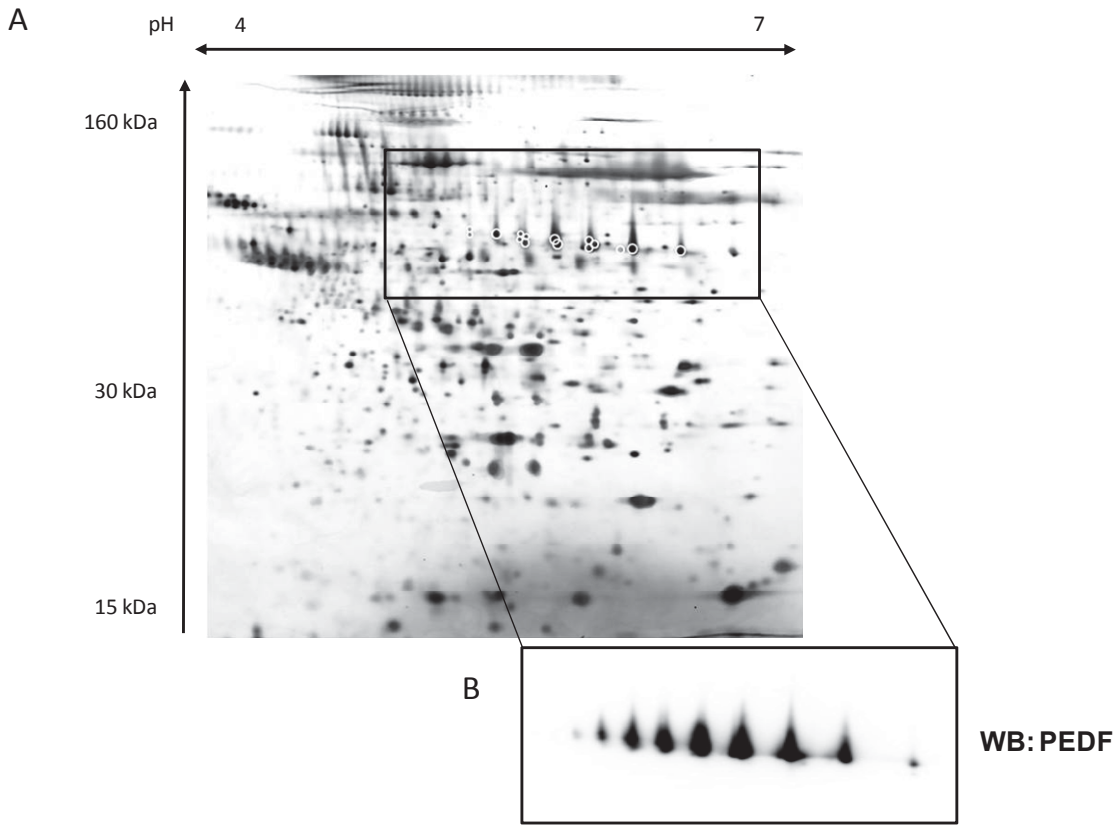

Figure 4.1 2D-pattern of CM from primary human adipocytes and immunoblot of 2D-gel with PEDF-specific antibody. (A) CM from 4 different donors were generated and concentrated as described in *Materials and Methods* and subsequently separated in the first dimension by IEF (pH 4-7) and in the second dimension 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.

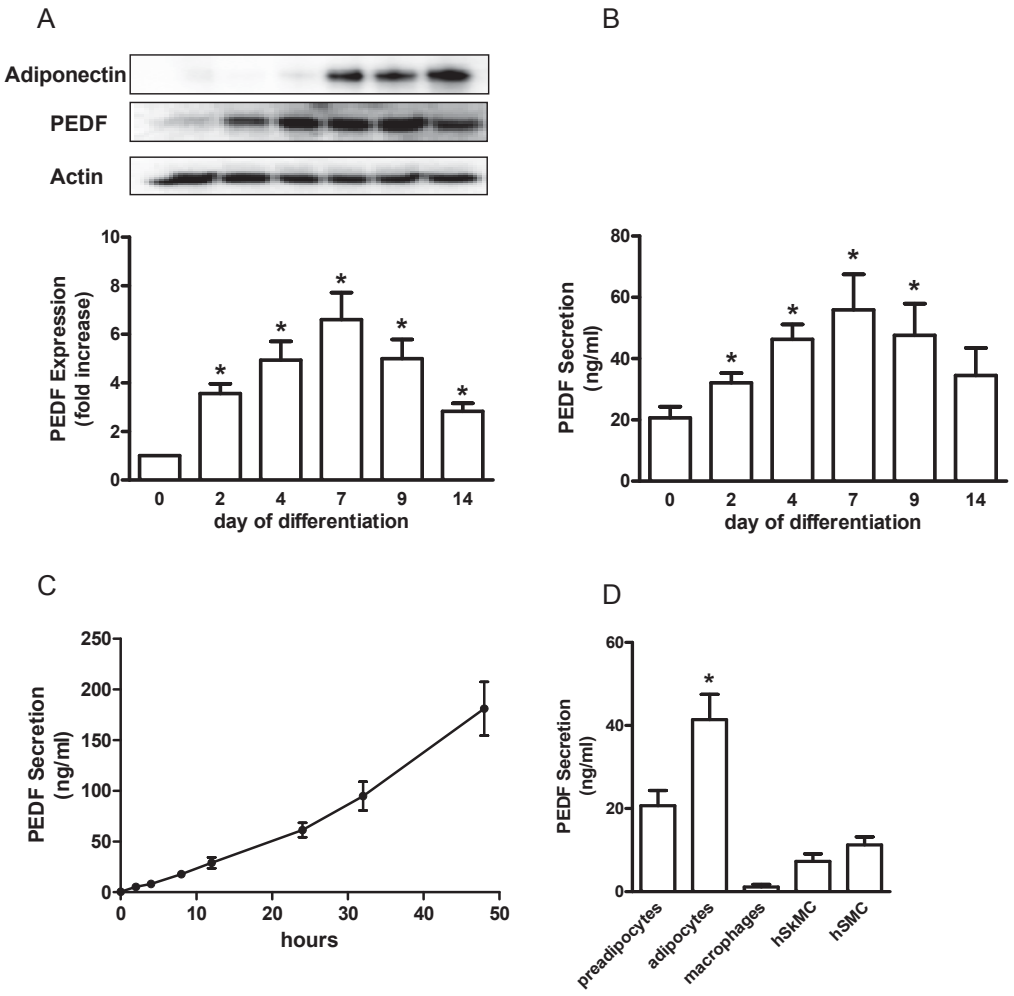

Figure 4.2 Expression and secretion of PEDF during differentiation of primary human adipocytes and in comparison to other primary cells. Human primary adipocytes from 4 donors were differentiated and on day 0, 2, 4, 7, 9 and 14 of differentiation medium was changed to -modified Eagles medium for 24 hours. 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.

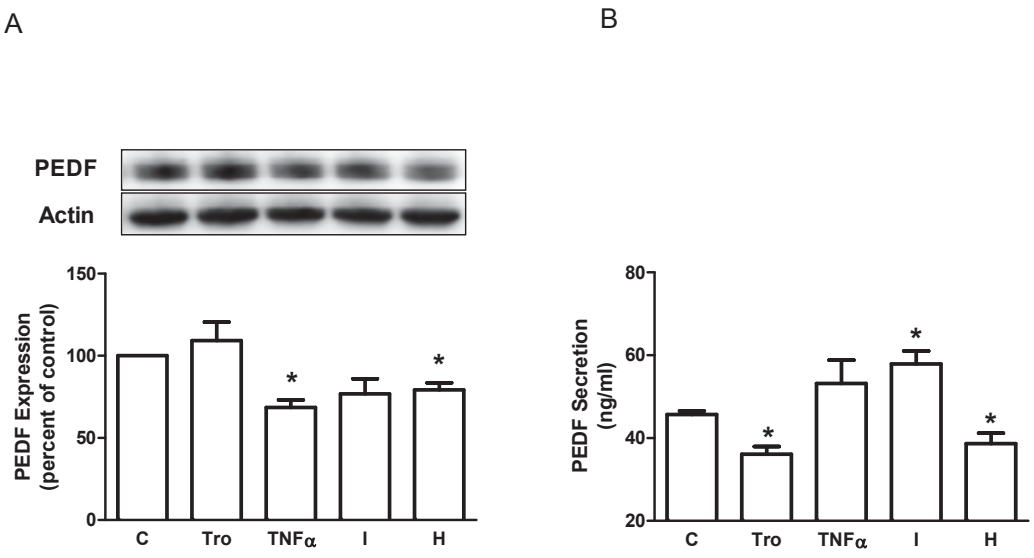

Figure 4.3 Regulation of expression and secretion of PEDF in human primary adipocytes and PEDF secretion of human primary adipocytes in comparison to other human cell types. Differentiated human primary adipocytes from 3-4 donors were treated with 5 µM trogliazone (Tro), 10 ng TNF-α, 50 nM Insulin (I) 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 4.2. 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.

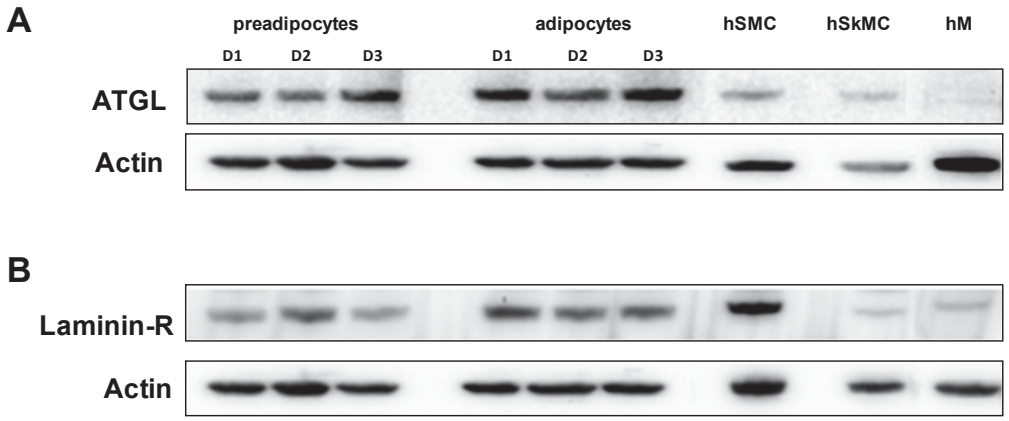

Figure 4.4 Expression of PEDF receptors in different human cell types. 5-10 µg of total lysates of adipocytes, hSMC, hSkMC and human primary macrophages (hM) were analyzed to detect expression levels of ATGL and Laminin-R. Membranes were incubated with the appropriate antibodies as described in *Materials and Methods*. Representative Blots are shown. D1-3: Isolated preadipocytes of three different donors that were *in vitro* differentiated into adipocytes.

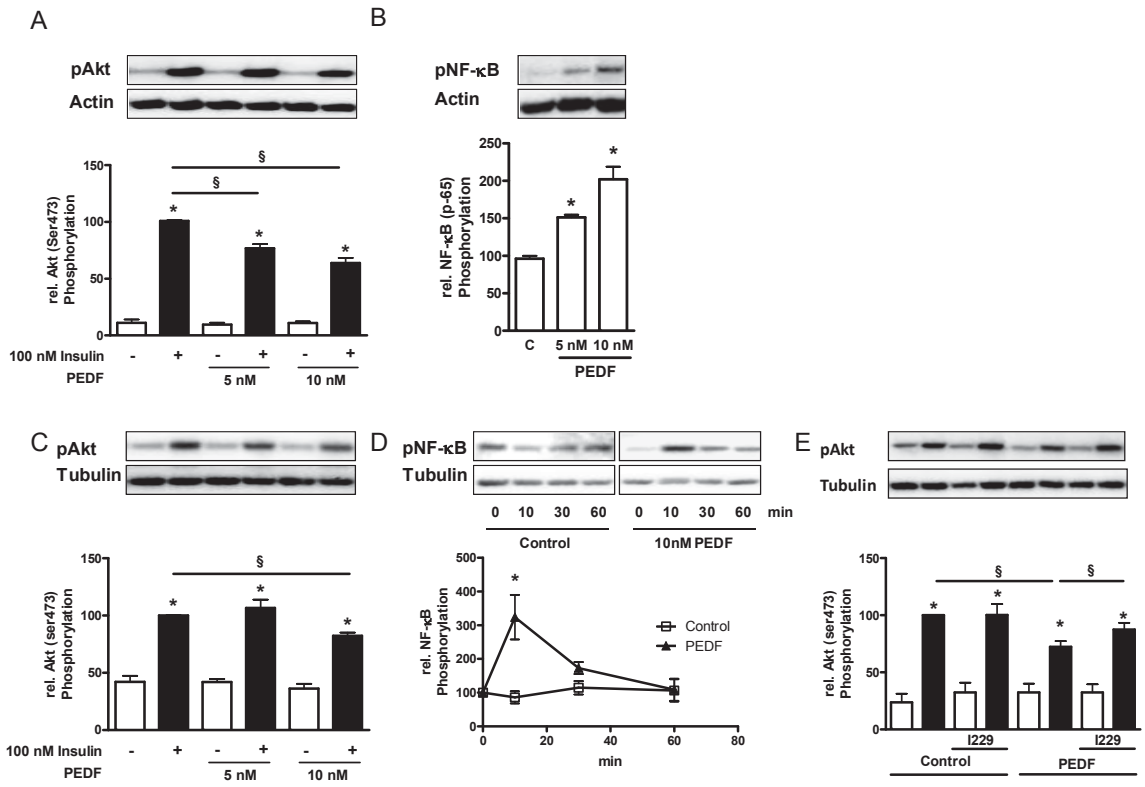

Figure 4.5 Induction of insulin resistance in human primary adipocytes and human skeletal muscle cells by PEDF. Adipocytes (A, B) and skeletal muscle cells (C, D, E) from different donors were incubated with PEDF for 3 h (A, B), overnight (C, E) and for the indicated times, respectively. The IKK-inhibitor 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.

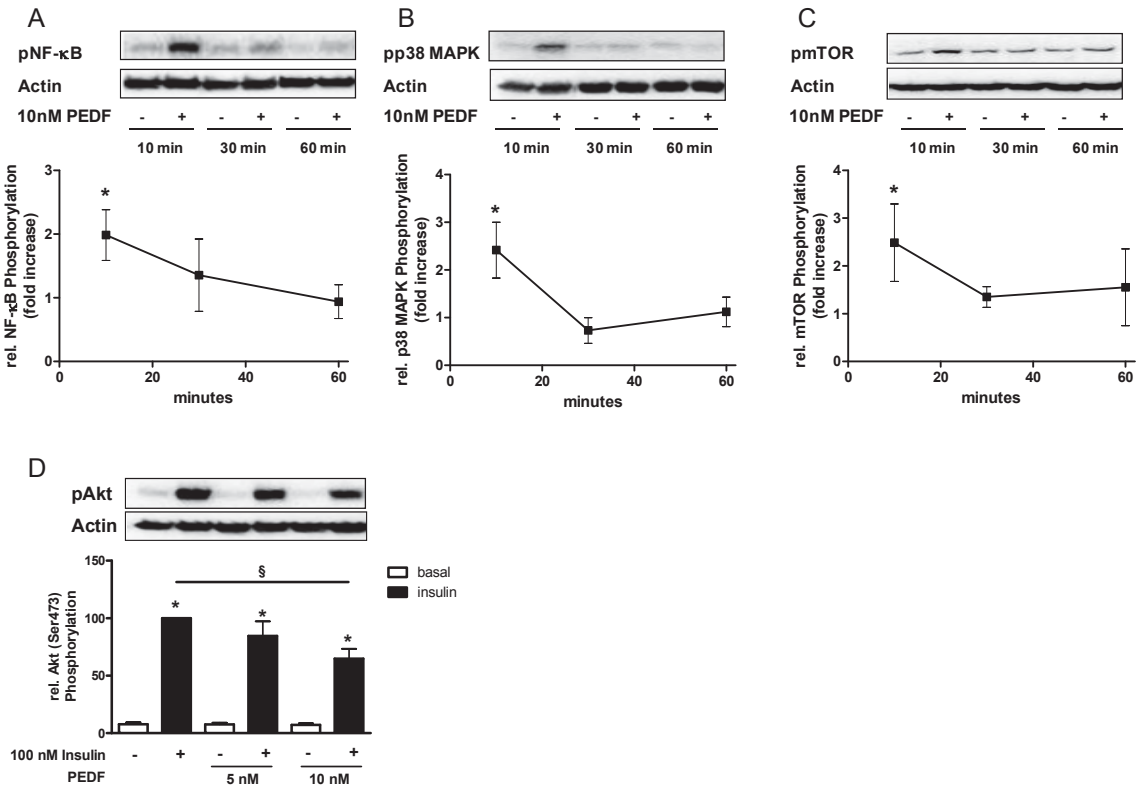

Figure 4.6 PEDF induces proliferative and inflammatory signaling pathways and insulin resistance in hSMC. (A, B, C) hSMC were serum starved for 24 h and then exposed to PEDF for the indicated time points. Total cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies to phosphorylated 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.

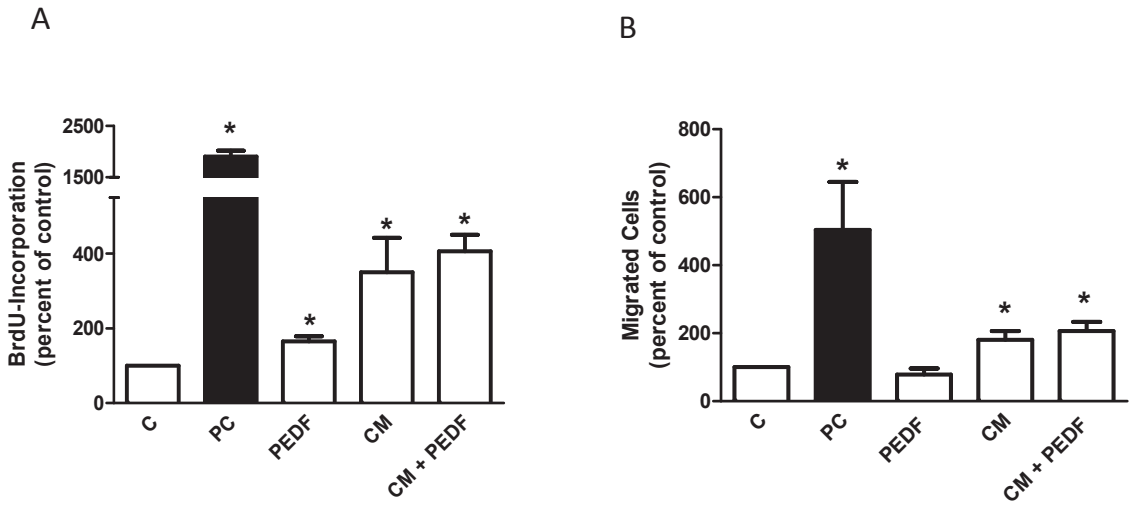

Figure 4.7 Effect of PEDF on hSMC proliferation and migration. (A) hSMC were serum starved for 24 h and subsequently incubated with BrdU in the absence or presence of PEDF, CM or the combination of both for 24 h. Proliferation was measured as described in Materials and Methods. Data are expressed relative 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.

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.

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

References

1. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. *J Clin Invest* 2005; 115/5: 1111-1119.
2. Ahima RS. Adipose tissue as an endocrine organ. *Obesity (Silver Spring)* 2006; 14 Suppl 5: 242S-249S.
3. Trayhurn P. Endocrine and signalling role of adipose tissue: new perspectives on fat. *Acta Physiol Scand* 2005; 184/4: 285-293.
4. Becerra SP. Structure-function studies on PEDF. A noninhibitory serpin with neurotrophic activity. *Adv Exp Med Biol* 1997; 425: 223-237.
5. Tombran-Tink J, Chader GG, Johnson LV. PEDF: a pigment epithelium-derived factor with potent neuronal differentiative activity. *Exp Eye Res* 1991; 53/3: 411-414.
6. Crowe S, Wu LE, Economou C, Turpin SM, Matzaris M, Hoehn KL, Hevener AL, James DE, Duh EJ, Watt MJ. Pigment epithelium-derived factor contributes to insulin resistance in obesity. *Cell Metab* 2009; 10/1: 40-47.
7. Jenkins A, Zhang SX, Gosmanova A, Aston C, Dashti A, Baker MZ, Lyons T, Ma JX. Increased serum pigment epithelium derived factor levels in Type 2 diabetes patients. *Diabetes Res Clin Pract* 2008; 82/1: e5-e7.
8. Yamagishi S, Adachi H, Abe A, Yashiro T, Enomoto M, Furuki K, Hino A, Jinnouchi Y, Takenaka K, Matsui T, Nakamura K, Imaizumi T. Elevated serum levels of pigment epithelium-derived factor in the metabolic syndrome. *J Clin Endocrinol Metab* 2006; 91/6: 2447-2450.
9. Filleur S, Nelius T, de Riese W, Kennedy RC. Characterization of PEDF: a multi-functional serpin family protein. *J Cell Biochem* 2009; 106/5: 769-775.
10. Jenkins AJ, Zhang SX, Rowley KG, Karschimkus CS, Nelson CL, Chung JS, O'Neal DN, Januszewski AS, Croft KD, Mori TA, Dragicevic G, Harper CA, Best JD, Lyons TJ, Ma JX. Increased serum pigment epithelium-derived factor is associated with microvascular complications, vascular stiffness and inflammation in Type 1 diabetes. *Diabet Med* 2007; 24/12: 1345-1351.
11. Lamers D, Schlich R, Greulich S, Sasson S, Sell H, Eckel J. Oleic acid and adipokines synergize in inducing proliferation and inflammatory signaling in human vascular smooth muscle cells. *J Cell Mol Med* 2010.
12. Hauner H, Petruschke T, Russ M, Rohrig K, Eckel J. Effects of tumour necrosis factor alpha (TNF alpha) on glucose transport and lipid metabolism of newly-differentiated human fat cells in cell culture. *Diabetologia* 1995; 38/7: 764-771.
13. Dietze-Schroeder D, Sell H, Uhlig M, Koenen M, Eckel J. Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors. *Diabetes* 2005; 54/7: 2003-2011.
14. Curat CA, Miranville A, Sengenes C, Diehl M, Tonus C, Busse R, Bouloumie A. From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. *Diabetes* 2004; 53/5: 1285-1292.
15. Lehr S, Kotzka J, Avci H, Knebel B, Muller S, Hanisch FG, Jacob S, Haak C, Susanto F, Muller-Wieland D. Effect of sterol regulatory element binding protein-1a on the mitochondrial protein pattern in human liver cells detected by 2D-DIGE. *Biochemistry* 2005; 44/13: 5117-5128.

16. Rabilloud T, Strub JM, Luche S, van Dorsselaer A, Lunardi J. A comparison between Sypro Ruby and ruthenium II tris (bathophenanthroline disulfonate) as fluorescent stains for protein detection in gels. *Proteomics* 2001; 1/5: 699-704.
17. Wichelhaus A, Russ M, Petersen S, Eckel J. G protein expression and adenylate cyclase regulation in ventricular cardiomyocytes from STZ-diabetic rats. *Am J Physiol* 1994; 267/2 Pt 2: H548-H555.
18. Hosogai N, Fukuhara A, Oshima K, Miyata Y, Tanaka S, Segawa K, Furukawa S, Tochino Y, Komuro R, Matsuda M, Shimomura I. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes* 2007; 56/4: 901-911.
19. Rausch ME, Weisberg S, Vardhana P, Tortoriello DV. Obesity in C57BL/6J mice is characterized by adipose tissue hypoxia and cytotoxic T-cell infiltration. *Int J Obes (Lond)* 2008; 32/3: 451-463.
20. Ye J, Gao Z, Yin J, He Q. Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *Am J Physiol Endocrinol Metab* 2007; 293/4: E1118-E1128.
21. Pasarica M, Sereda OR, Redman LM, Albarado DC, Hymel DT, Roan LE, Rood JC, Burk DH, Smith SR. Reduced adipose tissue oxygenation in human obesity: evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response. *Diabetes* 2009; 58/3: 718-725.
22. Notari L, Baladron V, Aroca-Aguilar JD, Balko N, Heredia R, Meyer C, Notario PM, Saravanamuthu S, Nueda ML, Sanchez-Sanchez F, Escribano J, Laborda J, Becerra SP. Identification of a lipase-linked cell membrane receptor for pigment epithelium-derived factor. *J Biol Chem* 2006; 281/49: 38022-38037.
23. Bernard A, Gao-Li J, Franco CA, Bouceba T, Huet A, Li Z. Laminin receptor involvement in the anti-angiogenic activity of pigment epithelium-derived factor. *J Biol Chem* 2009; 284/16: 10480-10490.
24. Chiellini C, Cochet O, Negroni L, Samson M, Poggi M, Ailhaud G, Alessi MC, Dani C, Amri EZ. Characterization of human mesenchymal stem cell secretome at early steps of adipocyte and osteoblast differentiation. *BMC Mol Biol* 2008; 9: 26.
25. Kratchmarova I, Kalume DE, Blagoev B, Scherer PE, Podtelejnikov AV, Molina H, Bickel PE, Andersen JS, Fernandez MM, Bunkenborg J, Roepstorff P, Kristiansen K, Lodish HF, Mann M, Pandey A. A proteomic approach for identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes. *Mol Cell Proteomics* 2002; 1/3: 213-222.
26. Vankoningsloo S, De Pauw A, Houbion A, Tejerina S, Demazy C, De Longueville F, Bertholet V, Renard P, Remacle J, Holvoet P, Raes M, Arnould T. CREB activation induced by mitochondrial dysfunction triggers triglyceride accumulation in 3T3-L1 preadipocytes. *J Cell Sci* 2006; 119/Pt 7: 1266-1282.
27. Wang M, Wang JJ, Li J, Park K, Qian X, Ma JX, Zhang SX. Pigment epithelium-derived factor suppresses adipogenesis via inhibition of the MAPK/ERK pathway in 3T3-L1 preadipocytes. *Am J Physiol Endocrinol Metab* 2009; 297/6: E1378-E1387.
28. Zvonic S, Lefevre M, Kilroy G, Floyd ZE, DeLany JP, Kheterpal I, Gravois A, Dow R, White A, Wu X, Gimble JM. Secretome of primary cultures of human adipose-derived stem cells: modulation of serpins by adipogenesis. *Mol Cell Proteomics* 2007; 6/1: 18-28.
29. Lange J, Yafai Y, Reichenbach A, Wiedemann P, Eichler W. Regulation of pigment epithelium-derived factor production and release by retinal glial (Muller) cells under hypoxia. *Invest Ophthalmol Vis Sci* 2008; 49/11: 5161-5167.

30. Rychli K, Kaun C, Hohensinner P, Dorfner A, Pfaffenberger S, Niessner A, Bauer M, Dietl W, Podesser B, Maurer G, Huber K, Wojta J. The antiangiogenic factor PEDF is present in the human heart and is regulated by anoxia in cardiac myocytes and fibroblasts. *J Cell Mol Med* 2009.
31. Notari L, Miller A, Martinez A, Amaral J, Ju M, Robinson G, Smith LE, Becerra SP. Pigment epithelium-derived factor is a substrate for matrix metalloproteinase type 2 and type 9: implications for downregulation in hypoxia. *Invest Ophthalmol Vis Sci* 2005; 46/8: 2736-2747.
32. Yamagishi S, Inagaki Y, Nakamura K, Abe R, Shimizu T, Yoshimura A, Imaizumi T. Pigment epithelium-derived factor inhibits TNF-alpha-induced interleukin-6 expression in endothelial cells by suppressing NADPH oxidase-mediated reactive oxygen species generation. *J Mol Cell Cardiol* 2004; 37/2: 497-506.
33. Matsui T, Yamagishi S, Nakamura K, Kikuchi S, Inoue H. Azelnidipine, a dihydropyridine-based calcium antagonist, inhibits angiotensin II-induced oxidative stress generation and downregulation of pigment epithelium-derived factor mRNA levels in microvascular endothelial cells. *Drugs Exp Clin Res* 2005; 31/5-6: 215-219.
34. Trayhurn P. Endocrine and signalling role of adipose tissue: new perspectives on fat. *Acta Physiol Scand* 2005; 184/4: 285-293.
35. Wang B, Wood IS, Trayhurn P. Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes. *Pflugers Arch* 2007; 455/3: 479-492.
36. Gottschling-Zeller H, Rohrig K, Hauner H. Troglitazone reduces plasminogen activator inhibitor-1 expression and secretion in cultured human adipocytes. *Diabetologia* 2000; 43/3: 377-383.
37. Skurk T, Birgel M, Lee YM, Hauner H. Effect of troglitazone on tumor necrosis factor alpha and transforming growth factor beta expression and action in human adipocyte precursor cells in primary culture. *Metabolism* 2006; 55/3: 309-316.
38. Sell H, Laurencikiene J, Taube A, Eckardt K, Cramer A, Horrighs A, Arner P, Eckel J. Chemerin is a novel adipocyte-derived factor inducing insulin resistance in primary human skeletal muscle cells. *Diabetes* 2009; 58/12: 2731-2740.
39. Chung C, Doll JA, Gattu AK, Shugrue C, Cornwell M, Fitchev P, Crawford SE. Anti-angiogenic pigment epithelium-derived factor regulates hepatocyte triglyceride content through adipose triglyceride lipase (ATGL). *J Hepatol* 2008; 48/3: 471-478.
40. Nelson J, McFerran NV, Pivato G, Chambers E, Doherty C, Steele D, Timson DJ. The 67 kDa laminin receptor: structure, function and role in disease. *Biosci Rep* 2008; 28/1: 33-48.
41. Zamiri P, Masli S, Streilein JW, Taylor AW. Pigment epithelial growth factor suppresses inflammation by modulating macrophage activation. *Invest Ophthalmol Vis Sci* 2006; 47/9: 3912-3918.
42. Ho TC, Yang YC, Chen SL, Kuo PC, Sytwu HK, Cheng HC, Tsao YP. Pigment epithelium-derived factor induces THP-1 macrophage apoptosis and necrosis by the induction of the peroxisome proliferator-activated receptor gamma. *Mol Immunol* 2008; 45/4: 898-909.
43. Yamagishi S, Matsui T, Nakamura K, Imaizumi T. Pigment epithelium-derived factor (PEDF) inhibits angiotensin II-induced smooth muscle cell proliferation through its anti-oxidative properties. *Protein Pept Lett* 2007; 14/6: 615-617.
44. Sell H, Eckardt K, Taube A, Tews D, Gurgui M, Echten-Deckert G, Eckel J. Skeletal muscle insulin resistance induced by adipocyte-conditioned medium: underlying mechanisms and reversibility. *Am J Physiol Endocrinol Metab* 2008; 294/6: E1070-E1077.

45. Nakamura K, Yamagishi S, Matsui T, Yoshida T, Takenaka K, Jinnouchi Y, Yoshida Y, Ueda S, Adachi H, Imaizumi T. Pigment epithelium-derived factor inhibits neointimal hyperplasia after vascular injury by blocking NADPH oxidase-mediated reactive oxygen species generation. *Am J Pathol* 2007; 170/6: 2159-2170.
46. Rychli K, Huber K, Wojta J. Pigment epithelium-derived factor (PEDF) as a therapeutic target in cardiovascular disease. *Expert Opin Ther Targets* 2009; 13/11: 1295-1302.
47. Fernandez-Garcia NI, Volpert OV, Jimenez B. Pigment epithelium-derived factor as a multifunctional antitumor factor. *J Mol Med* 2007; 85/1: 15-22.
48. Chung C, Doll JA, Stellmach VM, Gonzales J, Surapureddi S, Cornwell M, Reddy JK, Crawford SE. Pigment epithelium-derived factor is an angiogenesis and lipid regulator that activates peroxisome proliferator-activated receptor alpha. *Adv Exp Med Biol* 2008; 617: 591-597.

CHAPTER 5

Study 4

Oleic acid and adipokines synergize in inducing proliferation and inflammatory signaling in human vascular smooth muscle cells†

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Abbreviations: AN, adiponectin; BrdU, bromdesoxyuridin; BSA, bovine serum albumin; CM, adipocyte-conditioned medium; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; FFA, free fatty acids; FGF, fibroblast growth factor; hVSMC, human vascular smooth muscle cells; ICAM, intercellular adhesion molecule; IFN, interferon; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B; NO, nitric oxide; OA, oleic acid; PA, palmitic acid; PDGF, platelet-derived growth factor; PGC, peroxisome proliferator-activated receptor gamma coactivator; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor

Abstract

Objective: In the context of obesity, perivascular fat produces various adipokines and releases free fatty acids, which may induce inflammation and proliferation in the vascular wall. In this study we investigated how adipokines, oleic acid and the combined treatment regulate human vascular smooth muscle cell (hVSMC) proliferation and migration and the underlying signaling pathways. *Methods and Results:* Adipocyte-conditioned media (CM) generated from human adipocytes induces a prominent proliferation and migration of hVSMC. Autocrine action of adiponectin totally abolishes CM-induced proliferation. Furthermore, oleic acid but not palmitic acid induces proliferation of hVSMC. CM itself does not contain fatty acids, but CM in combination with oleic acid markedly enhances proliferation of hVSMC in a synergistic way. Both the NF-κB and the mTOR pathway were synergistically activated under these conditions and found to be essential for hVSMC proliferation. Expression of iNOS and production of NO was only enhanced by combined treatment inducing a marked release of VEGF. Combination of oleic acid and VEGF induces an additive increase of hVSMC proliferation. *Conclusion:* We could show that the combination of CM and OA led to a synergistic proliferation of hVSMC. Expression of iNOS and production of NO were only enhanced under these conditions and were paralleled by a marked release of VEGF. These results suggest that the combined elevated release of fatty acids and adipokines by adipose tissue in obesity might be critically related to hVSMC dysfunction, vascular inflammation, and the development of atherosclerosis.

Keywords: human smooth muscle cells, adipokines, oleic acid, proliferation, inflammation

Introduction

Obesity is a major determinant of mortality of all causes including cardiovascular disease in industrial countries.[1] Adipocytes in expanded fat are active secretory cells capable of releasing lipid mediators and a variety of cytokines, the so-called adipokines.[2] Many studies in humans and in various animal models have shown that obesity is strongly related to the development of atherosclerosis.[3,4] Adipose tissue has a prominent role in the development of a low-grade systemic inflammatory state that contributes to obesity-associated vascular dysfunction and cardiovascular risk.[5] The local secretion of adipokines by perivascular fat may provide a new direct link between obesity and vascular complications.[6] However, the mechanism how perivascular fat

increases the risk of metabolic and cardiovascular disease is not yet fully elucidated. Endothelial cells and, smooth muscle cells (SMC) represent the major cell types of the artery wall preserving vessel wall homeostasis. The migration of vascular smooth muscle cells (VSMC) from the media to intima and their concomitant proliferation occurring in the synthetic state are critical causes of arterial wall thickening. Adipokines such as leptin and resistin have been shown to affect the vasculature by influencing the proliferation and function of SMC.[7,8] Adiponectin (AN) is an anti-atherogenic adipokine and hypoadiponectinemia is not only associated with obesity but also with cardiovascular disease and diabetes.[9] VEGF-induced SMC proliferation and migration is inhibited by AN making it a positive regulator of vascular remodelling.[10] It is apparent that expanded adipose tissue, especially by its secretory output, is a strong risk factor for the development of cardiovascular diseases. The crosstalk of adipose tissue with cells in the arterial wall such as SMC is not yet fully understood. Although effects of specific adipokines on SMC function have been studied, effects of the whole secretory output of human adipocytes have not been investigated. Apart from a single study using CM from mouse cell lines and rat adipose tissue explants [11] showing induction of proliferation in hVSMC, no other data on a direct interaction of adipocytes and SMC exist. Therefore, the main objective of this study was to provide insight into the complex cellular mechanisms linking obesity and atherosclerosis by assessing the role of protein factors and lipid mediators in the crosstalk between human SMC and subcutaneous and perivascular adipocytes.

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-ICAM-1, anti-phospho-NF-κB (P65) (Ser536), anti-NF-κB (P65), anti-p38 MAPK, anti-phospho-p38 MAPK (Thr180/Tyr182), anti- p44/42 MAP Kinase (ERK1/2) and anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) were supplied by Cell Signalling Technology (Frankfurt, Germany). Anti-actin antibodies and anti-iNOS came from Abcam (Cambridge, GB), anti-tubulin from Calbiochem (Merck Biosciences, Schwalbach, Germany) and anti-VCAM-1 from Acris (Herford, 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). Rapamycin and SNAP was obtained from Calbiochem (Merck Biosciences, Schwalbach, Germany). The IKK-Inhibitor I229 was from Sanofi-Aventis (Frankfurt, Germany). I229 has submicromolar activity on the isolated IKK

complex and is highly specific on IKK. Its general structure is described in PCT/EP00/05340. Troglitazone, Cytochalasin B, TNF-α, BSA (fraction V, fatty acid free, low endotoxin), sodium palmitate and sodium oleate were obtained from Sigma (München, Germany). Adiponectin was purchased from BioVendor GmbH (Heidelberg, Germany). The Cell Proliferation ELISA (BrdU, chemiluminescent) and protease inhibitor cocktail tablets were from Roche (Mannheim, Germany). FCS was supplied by Gibco (Invitrogen, Carlsbad, CA, USA). VEGF was purchased from Millipore (Schwalbach, Germany). The Transwell Cell migration assay (8-µm-pore-size, colorimetric) was from Cell Biolabs, Inc. (San Diego, USA). 4-amino-5-methylamino-2´, 7´-difluorofluorescein diacetate was obtained from Molecular Probes (Invitrogen GmbH, Karlsruhe, Germany). 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 26.1 ± 1.1, and aged 36.6 ± 2.0 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 metabolic diseases 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 cm^2 culture flasks 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 $NaHCO_3$, 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-CM, as recently described by us.[13] Briefly, CM was generated by culturing adipocytes for 48 h in SMC basal medium (PromoCell) with addition of 50 ng/ml amphotericin b and 50 µg/ml gentamycin. Each CM was tested for its proliferative effect, the content of adiponectin (negatively correlated to proliferation) and IL-6 (not related to proliferation). A more-detailed characterization of CM was described previously by us.[13] The concentration of FFA in CM was measured with a Fatty Acid Assay Kit from Biovision (Biocat, Heidelberg, Germany) and with HPLC.[14]

Culture of fat explants and preparation of CM

Human epicardial and subcutaneous fat biopsies were obtained from patients without type 2 diabetes undergoing coronary artery bypass surgery (n=3, body mass index 27 ± 0,82, and aged 69 ± 2,6 years). Adipose tissue was collected and used to generate CM as described.[15] Briefly, fat explants were cultured in adipocyte tissue medium (DMEM F12 containing 10 % fetal calf serum, 33 µmol/l biotin, 17 µmol/l panthothenate and antibiotic-antimycotic (Invitrogen, Carlsbad, USA)). After two days, the medium was replaced with adipocyte tissue medium without serum. After 24 h, CM was collected and stored in aliquots at -80°C until further use.

Culture of human vascular smooth muscle cells (hVSMC)

Primary human coronary artery SMC were obtained from PromoCell (Heidelberg, Germany). hVSMC from four different donors (Caucasian, male, 23, 31, 40 years old; female, 56 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. Cells were characterized as hVSMC by morphologic criteria and by immunostaining with smooth muscle α-actin.

Fatty acid treatment of hVSMC

Sodium salts of fatty acids were dissolved in water as a 6 mM stock solution, and were further diluted in sterile serum-free SMC medium containing 4 % (wt/v) BSA. Oleic acid (OA) and palmitic acid (PA) were applied to hVSMC at a final concentration of 100 µmol/l for 18 h. All controls of experiments involving fatty acids were treated with BSA alone.

In vitro analysis of growth promoting activity

To monitor DNA synthesis hVSMC 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). 10.000 hVSMC per 15 mm² well were incubated with the CM of 35.000 adipocytes. The BrdU ELISA Kit 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). Treatment of three different hVSMC donors (F56, M23, M21) with CM from one adipose tissue donor led to a robust and significant stimulation of proliferation (3-4-fold) (Fig. 1A). Fig. 1B shows that the proliferative

capacity of CM is adipocyte-donor dependent. 22 CM were tested for their proliferative activity and the majority induced a 2-4-fold proliferation of hVSMC (14 CM showed a proliferation of 2-fold or higher). Only these CM were used for further experiments. Variations in the potency of CM in inducing proliferation may be explained by the differences in AN content (Fig. 1C). This is in accordance with our view of an autocrine function of AN.[13]

Immunoblotting

hVSMC 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 lysates were separated by SDS-PAGE using 10 % horizontal gels and transferred to polyvenylidene fluorid filters in a semidry blotting apparatus.[16] 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, USA). Signals were visualized and evaluated on a LUMI Imager work station.

ELISA

VEGF secretion by hVSMC and AN release by adipocytes was determined using ELISA kits purchased from BioVendor GmbH (Heidelberg, Germany). The assays were performed in duplicates according to the manufacturer's instructions.

hVSMC migration assay

Transwell Cell migration assay was performed using 24-well transwell chambers with 8-µm-pore-size polycarbonat membranes. hVSMC were grown to confluence and serum-starved for 24 h. 300 µl cell suspension containing 3 x 10^4 detached cells was added to the upper compartment. Serum-free medium, CM, FCS or OA 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 6 h. After removal of non-migratory cells, migratory cells were stained and quantified at 570 nm according to the manufacturer`s protocol. We could demonstrate that hVSMC migrated into the scratch already after incubation with CM for 6 h (Fig. 1D).

A wound scratch assay was used to visualize the effect of CM on hVSMC migration. hVSMC were seeded (2 x 10^5 cells/well) into 6-well culture dishes. After 24 h starvation, the cell monolayers were scratched using a sterile pipette tip, rinsed repeatedly with PBS to remove residual cell debris and then incubated with 5 % FCS (positive control), or with CM for 6 h and photographed under a phase-contrast microscope (Olympus, Hamburg, Germany). Treating hVSMC with CM led to a significant 3-fold increase in the migration of hVSMC compared to untreated cells (Fig. 1E).

Measurement of nitric oxide (NO) production in hVSMC

Treated hVSMC were washed in PBS, incubated with 10 µM 4-amino-5-methylamino-2´, 7´-difluorofluorescein diacetate for 30 min. As a positive control, cells were treated with 500 µM SNAP. Afterward, cells were lysed in the above mentioned lysis buffer and fluorescence measured using excitation wavelength of 485 nm on an Infinte 200 (Tecan, Männersdorf, Germany).

Presentation of data and statistics

Data are expressed as mean ± SEM. Unpaired 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 generated in the presence of AN reduces proliferation of hVSMC

We could show that the treatment with CM led to a robust induction of proliferation (Fig. 1A, B) and migration (Fig. 1D, E) of hVSMC. The proliferative potency of CM is negatively correlated to its AN content (Fig. 1C). To investigate the direct effect of AN on adipocytes we further generated CM in the absence or presence of 10 nM full length AN for 48 h (CMAN) similar to earlier work (Fig. 2).[13] The proliferative effect of CMAN was substantially reduced as compared to CM, whereas AN alone had no effect on hVSMC proliferation (Fig. 2A). AN added freshly to CM (CM+AN) just before incubating with utpatients with atheon the CM-induced proliferation. These findings are in agreement with our view of an autocrine function of AN [13] and suggest that this protein attenuates the release of growth-mediating factors from adipocytes. As another readout for the

pro-atherogenic effects of CM we investigated the expression of the adhesion molecule ICAM-1. We could show that CM led to a significant 2-fold increase in the expression of ICAM-1 (Fig. 2B) that was absent when incubated with CMAN.

Effect of fatty acids and CM on proliferation, migration and the expression of adhesion molecules of hVSMC

In all experiments using fatty acids these were coupled to fatty acid-free BSA, thus BSA was always present under all conditions. Control experiments indicated that it had no effect on the measured parameters. Subsequently CM was applied at a concentration of 50 % (v/v), due to addition of BSA. OA alone induced a significant 3.5-fold increase of hVSMC proliferation. The combination of OA and CM produced a synergistic, substantial stimulation of hVSMC proliferation (7-8-fold) compared to CM and OA alone. In contrast, PA had no proliferative effect on hVSMC. The combination of PA and CM even abrogated the proliferative capacity of CM. Fig. 3B shows the capacity of CM, OA and CMOA to induce migration of hVSMC. While CM induced a robust migration, OA had no effect on hVSMC migration and actually decreased the CM induced migration to the control level. In addition, the increased expression of ICAM-1 in response to CM was prevented by the presence of OA (Fig. 3C). Although CM and OA alone had no effect on the expression of VCAM-1, the combination of both showed a nearly 2-fold increase in the expression of VCAM-1 compared to control (Fig. 3D).

CM and OA activate multiple signaling pathways in hVSMC

CMOA produced a substantial phosphorylation of NF-κB after 5 min, which was much more prominent than the effects of OA and CM alone (Fig. 4A). Treatment with CM alone reached a peak value after 30 min, whereas at later time points NF-κB activation was no longer detectable under all conditions. Activation of p38 MAPK was significantly increased following treatment with CMOA after 10 min, whereas CM alone could activate p38 MAPK more prominently after 60 min (Fig. 4B). No significant activation of p38 MAPK with OA alone was observed throughout the experiment. Furthermore, CMOA induced mTOR phosphorylation after 5, 10 and 30 min, while OA and CM showed no activation of this pathway (Fig. 4C). We did not observe enhanced phosphorylation of ERK after treatment with the combination of CM and OA (data not shown).

mTOR- and IKK-inhibition abolish CM-, OA- and CMOA-induced proliferation of hVSMC

Inhibition of both mTOR by rapamycin and IKK by compound I229 totally abrogated CM- and OA-induced proliferation of hVSMC. Both inhibitors also abolished the synergistic effect of CM and OA and restored the basal proliferation level (Fig. 5 A + B). Compound I229 is a benzimidazole derivative, which showed an IC_{50} value of 1.9 nM against the IKK complex.[17]

CM and OA induce iNOS expression, VEGF release and NO production in hVSMC

We determined iNOS expression in hVSMC after incubation with CM, OA and CMOA for 24 h (Fig. 6A). CM and OA treatment had no significant effect on iNOS expression. However, the combination of both induced a 2.3-fold increase of iNOS expression in SMC. It is well established, that an increased iNOS expression leads to an enhanced VEGF production in different cell types.[18-20] CM alone contains 122 ± 6 pg/ml VEGF (n=16). Both CM and OA increased VEGF concentration in SMC medium 2.1- and 2.3-fold, respectively, taking into account the endogenous VEGF content of CM (Fig. 6B). In addition, CMOA increased VEGF concentration in a synergistic manner (5.5-fold). Concomitantly, a significant increase in NO production by 1.5-fold was observed in hVSMC after incubation with the combination CMOA (Fig. 6C). VEGF treatment showed a significant effect (2.5-fold) on proliferation and the combination of VEGF and OA markedly enhanced the proliferation in an additive way (5-fold) (Fig. 6D). Inhibition of NOS by L-NAME had no effect on the proliferation induced by CM and OA alone, yet it completely abolished the synergistic effect of the two stimuli. Notably, an additive proliferative effect of CM and OA was still observed (Fig. 6E).

Comparison of proliferative capacity between CM from subcutaneous and perivascular adipose tissue

In order to validate our findings obtained with subcutaneous fat, we also assessed the proliferative activity of epicardial fat, which is a perivascular fat depot. CM of both paired subcutaneous and perivascular fat depots were generated from adipose tissue explants from the same patient. Fig. 7 shows, that both subcutaneous and epicardial CM induced a significant 1.6-fold proliferation of hVSMC. These results suggest that secreted factors responsible for the induced proliferation are fat depot-independent.

Discussion

Obesity is associated with an increased risk for cardiovascular diseases such as atherosclerosis.[1] Inflammation in expanded adipose tissue and a concomitant increased release of adipokines and lipid mediators is linked to obesity and might also be a mechanism underlying the development of atherosclerosis. It has been speculated that perivascular adipose tissue releasing various pro-inflammatory adipokines might directly contribute to the pathogenesis of atherosclerosis.[6] Chemotactic adipokines released by perivascular adipose tissue have already been shown to modulate the function of immune cells infiltrating at the interface of adipose tissue and the adventitia of atherosclerotic aortas.[21] As for effects of adipokines on cells of the vascular wall, it is well known that specific adipocyte-derived factors are involved in regulating vascular functions, including hVSMC proliferation and migration.[22,23] We could demonstrate in this study that adipokines secreted from in vitro differentiated human adipocytes also induce proliferation and migration of hVSMC. In our system of primary human SMC CM also induces migration, VEGF secretion and increases the expression of adhesion molecules, which might all be critical features in atherosclerosis development. In atherosclerosis, hVSMC increase the expression of adhesion molecules like ICAM-1, VCAM-1, fractalkine (CX3CL1), which allow them to interact with monocytes, that differentiate into macrophages [24,25] suggesting a role of hVSMC in retaining monocytes and macrophages within the atherosclerotic lesion.[26] We could show in the present study that CM-induced expression of ICAM-1 can be completely inhibited by AN. This new finding is in accordance with data demonstrating that AN is able to suppress the expression of adhesion molecules in endothelial cells.[27]

CM of human adipocytes contains various growth-promoting and migrative factors such as VEGF, fibroblast growth factor (FGF), IGF, PDGF and angiotensin II. In our study, the majority of CM induced a 3-4-fold proliferation of hVSMC while just a few CM lacked this effect. Measuring AN in all used CM, we could demonstrate that a low AN content is associated with high proliferative action of CM. As earlier work demonstrates that AN exerts an autocrine action on adipocytes decreasing the release of various adipokines,[13] we presumed that the AN content in CM might be responsible for the differences in the proliferative potency of CM. Arita and colleagues could show that AN inhibits growth factor-induced proliferation and migration of human aortic SMC by binding these growth factors.[10] Here we can demonstrate that AN added to adipocytes during medium conditioning prevents the proliferative effects of CM. Consistently, AN added to CM after conditioning, and therefore not influencing adipokine release, did not affect CM-induced proliferation. We therefore assume that AN has to be in contact with adipocytes to exert its positive effect on adipokine release rather than binding adipokines and preventing their proliferative effect. The present work emphasizes the importance of the

autocrine function of AN and extends this concept to the control of growth promoting factors released by adipocytes.

It is well established, that OA induces rat VSMC proliferation,[28] migration,[29] and plays a central role in obesity and fatty acid-induced atherosclerosis.[30] In the current study we could reproduce these effects of OA in hVSMC while PA had no effect. This difference between PA and OA are not apoptosis-related as both fatty acids do not induce apoptosis at the concentrations used.[31] However, different effects on VSMC proliferation and migration could be explained by differential activation of PGC-1α expression by OA and PA.[32,33] Zhang and colleagues found that overexpression of PGC-1α blocked OA-induced proliferation, while suppression of PGC-1α expression by siRNA amplified these effects. In contrast, PA markedly induced PGC-1α expression.[32] Our study is the first to test a combination of adipokines and fatty acids for their effects on hVSMC demonstrating a markedly enhanced proliferation of primary hVSMC. Previous studies have shown that OA enhances the mitogenic activity of angiotensin II [34] in rat SMC in a synergistic way similar to the synergy between CM and OA in our study. Furthermore, the combination of CM and OA enhanced the expression of VCAM-1 but not of ICAM-1. VCAM-1 is essential for phenotypic modulation of cultured SMC. Interactions of VCAM-1 and its ligand VLA_4 may influence the phenotype and synthetic capacity of SMC.[35] The different expression of ICAM-1 and VCAM-1 can be explained by the different regulation of these adhesion molecules by mTOR. Minhajuddin et al. showed that mTOR downregulates thrombin-induced ICAM-1 expression,[36] whereas Wood and colleagues could show that inhibition of mTOR decreases VCAM-1 expression.[37] In contrast to proliferation, we could not observe an increase of hVSMC migration after incubation with OA, interestingly OA decreased the CM-induced migration to basal levels. However, other studies in rodents could show that OA induced migration of rat VSMC.[32,33,38] Additional work will be needed to elucidate the precise role of OA in CM-induced hVSMC.

Proliferation of SMC is regulated by different pathways including p38 MAPK, NF-κB and mTOR. p38 MAPK can be activated by stress, inflammatory cytokines and growth factors.[39] CM alone and the combination of CM and OA acutely activated p38 MAPK significantly within 1 h. In contrast, OA alone did not activate p38 MAPK, a finding that confirms similar observations from Lu and colleagues.[34] Proliferation of hVSMC is also regulated by nuclear transcription factors including NF-κB. In SMC cultures, NF-κB is activated by growth stimulants and cytokines.[40-42] In the current study we could show that the combination of CM and OA significantly enhanced NF-κB phosphorylation (5-6-fold) already after 5 min exposure, in comparison to the moderate effects of CM and OA alone (1-2-fold). The inhibition of the IKK complex upstream from NF-κB with the IKK-Inhibitor I229 completely blocked proliferation of hVSMC induced by CM, OA and the combination of both, revealing that NF-κB is an essential pathway for hVSMC

proliferation. An initial screening with the Kinex™ Antibody Microarray revealed that the PI3K-Akt-mTOR-P70S6 kinase pathway is activated in hVSMC after incubation with CM for 24 h (data not shown). In the present study the combination of CM and OA could acutely activate mTOR significantly within 30 min. Inhibition of mTOR with rapamycin reduced CM, OA and CMOA-induced proliferation of hVSMC to the control level (Fig. 6B). Both the IKK-Inhibitor and rapamycin completely abrogated the proliferation of hVSMC, indicating that there is a crosstalk between these pathways. A recent study has shown that the downregulation of PTEN triggered by OA is mediated by a signaling complex made of mTOR and NF-κB in hepatocytes.[43] We therefore suggest that the proliferative potency of the combination of CM and OA could be partly explained by a stronger activation of NF-κB and mTOR.

The expression of iNOS is induced by proinflammatory cytokines such as IL-1β, TNF-α, IFN-γ [44,45] in a number of cell types including SMC. Furthermore Fang et al. have shown that OA induces iNOS expression in human retinal pigment epithelium.[46] In the current study we report that neither CM nor OA alone, but the combination of both significantly enhanced iNOS expression and NO production in hVSMC. In correlation to this finding, CM and OA only moderately induced VEGF secretion by hVSMC, but the combination of both resulted in a markedly stronger effect. Inhibition of iNOS by L-NAME partly inhibited the CMOA-induced hVSMC proliferation, indicating that the synergistic proliferative effect of CM and OA might be due to an enhanced iNOS expression, NO production and VEGF release. The potential mechanisms underlying the NO-induced augmentation of VEGF expression in VSMC are not completely understood. Our results demonstrate that cytokine- and OA-mediated iNOS induction enhanced VEGF secretion. Also the combination of human recombinant VEGF and OA leads to an obvious augmentation of hVSMC proliferation, indicating that the enhanced VEGF secretion after CMOA treatment maybe a responsible factor for the markedly increased proliferative effect of CM and OA. Furthermore, VEGF in CM might be an important candidate for the proliferative effect of this complex mixture of adipokines.

To validate our findings obtained with adipocytes from subcutaneous fat, we also assessed the proliferative activity of epicardial fat, which is a perivascular fat depot. For this purpose we generated CM from paired adipose tissue explants from patients undergoing bypass surgery. Although the release of single adipokines may certainly differ between subcutaneous and epicardial fat, these additional data show that the proliferative effect obtained with subcutaneous adipose tissue can be compared to perivascular fat. Despite the fact that arteries are not surrounded by subcutaneous adipose tissue, we consider that this fat depot might have a strong systemic effect on hVSMC within the vessel wall due to its considerable amount. Unfortunately, the amount of perivascular adipose tissue from surgery is technically restricted and its use only for key experiments is a limitation of this study. It should also be noted that CM

from explants contain secretory products from all cell types present in adipose tissue and is therefore not completely comparable to CM from *in vitro* differentiated adipocytes. However, the mechanisms by which CM induces dysfunction of SMC are certainly similar for perivascular and subcutaneous adipose tissue.

In conclusion, we show here for the first time that lipid mediators and adipokines synergistically disturb SMC function inducing augmented proliferation and inflammatory signaling. Enhanced iNOS expression and VEGF release by SMC may be critically involved in this process. We propose that the combined elevated release of fatty acids and adipokines by adipose tissue in obesity might be a link between adipose dysfunction, SMC dysfunction, vascular inflammation and the development of atherosclerosis.

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The authors confirm that there are no conflicts of interest.

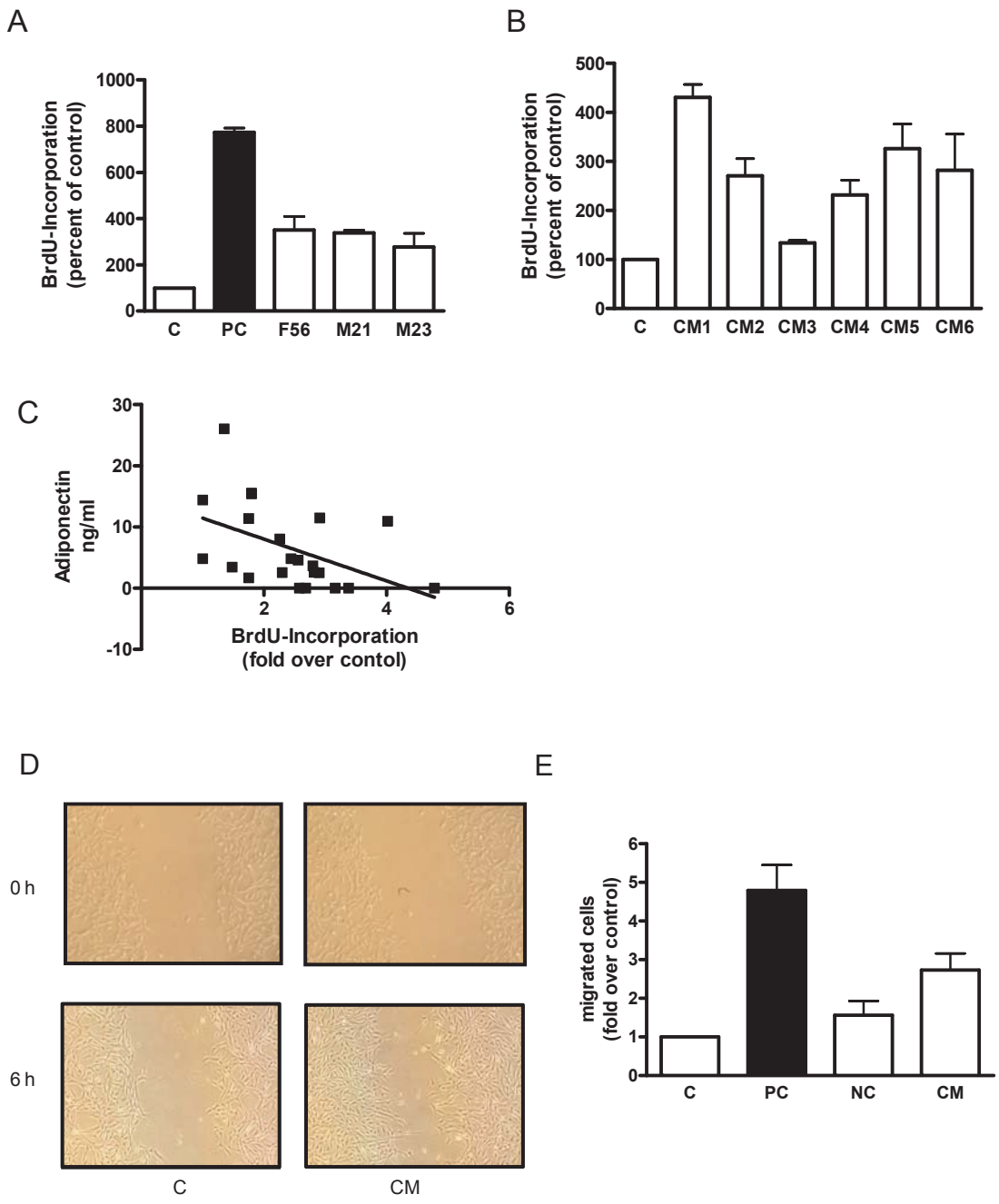

Figure 5.1 Effect of CM on proliferation (A, B, C) and migration (D, E) of hVSMC. The proliferation was determined by measuring the incorporation of BrdU into DNA. Data are expressed relative to the basal control value, taken as 100 %. FCS was used as a positive control (PC). (A) Effect of CM from a single adipose tissue donor on proliferation of 3 different hVSMC donors. Data are mean values ± SEM of three independent experiments using a specific CM. (B) Proliferative effects of CM from 6 different adipose tissue donors determined on one hVSMC donor (F56). Data are mean values ± SEM of three independent experiments. (C) Proliferative effect of 22 different CM correlated to their AN content. Representative micrographs are shown. (D) Effects of CM on migration of hVSMC using an *in vitro* wound scratch assay. (E) Quantitative analysis of hVSMC migration with a Transwell Cell migration assay. 5 % FCS was used as positive control (PC) and in combination with 25 nM cytochalasin B as negative control (NC). Data are

presented as mean ± SEM from four independent experiments using four different CM. *p<0.05 compared to control.

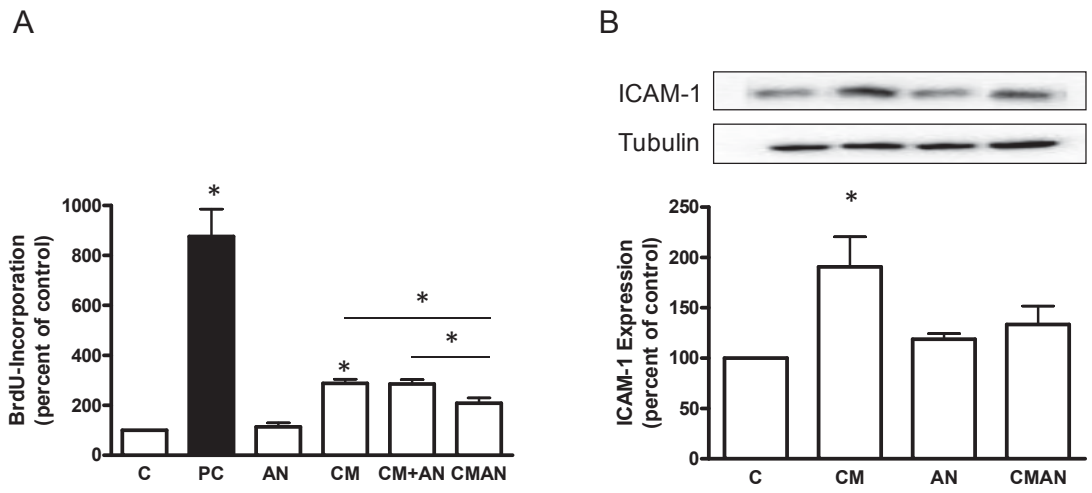

Figure 5.2 Analysis of CM generated in the absence or presence of AN. CM were generated for 48 h with or without 10 nM full length AN (CMAN). Further, AN was added to CM just before the incubation with hVSMC (CM+AN). (A) Effect of AN, CMAN and CM+AN on BrdU incorporation into DNA in hVSMC. Data are expressed relative to the basal control value, which was set as 100 %. (B) Analysis of ICAM-1 expression after 24 h incubation with CMAN. Total cell lysates were resolved by SDS-PAGE and immunoblotted with a specific ICAM-1 antibody. Data are means ± SEM of three independent experiments and three different adipocyte donors. *p<0.05 compared to control.

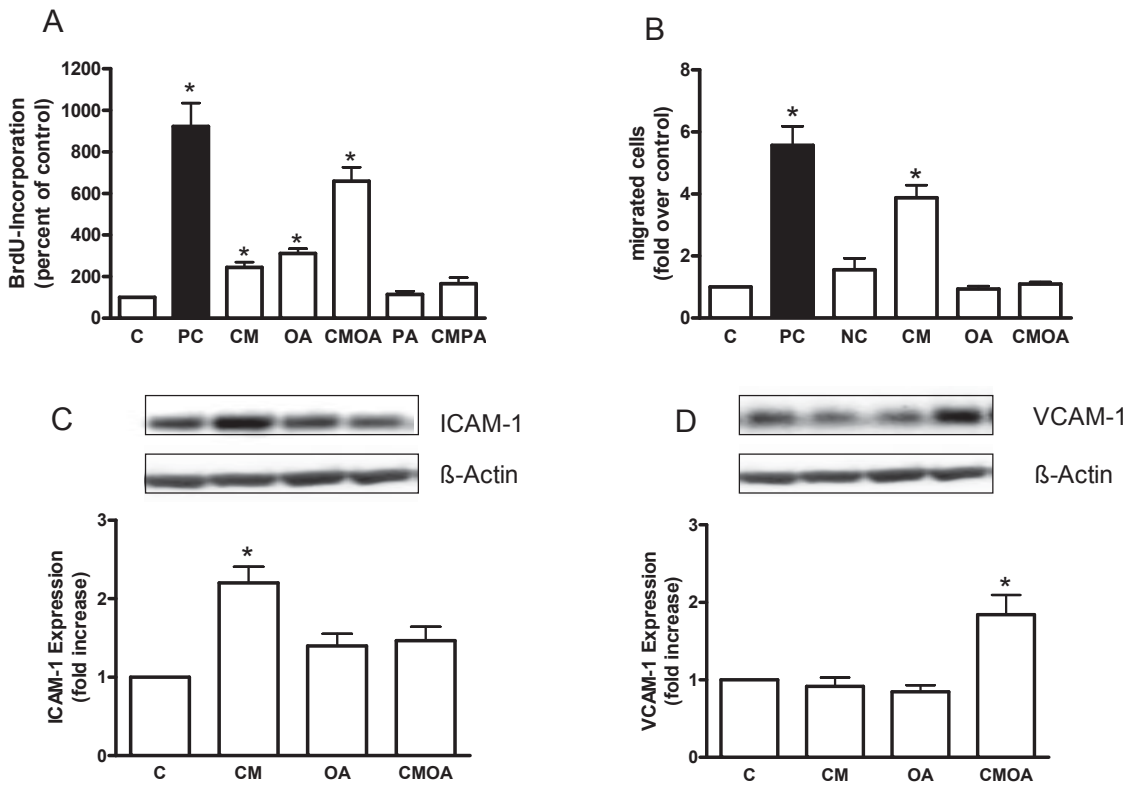

Figure 5.3 Effect of OA and PA (100 µmol/l) on hVSMC proliferation (A), migration (B) and the expression of adhesion molecules ICAM-1 (C) and VCAM-1 (D). (A) For the proliferation assay, hVSMC were serum starved for 24 h and subsequently incubated with BrdU in the absence or presence of CM, OA, PA or the combination of CM with each fatty acid for 18 h. Data are expressed relative to the basal control value, which was set as 100 %. FCS is used as positive control (PC). Data are means ± SEM of eight independent experiments. (B) Effect of CM, OA and the combined treatment on migration of hVSMC. Data are presented as mean ± SEM from four independent experiments. Analysis of ICAM-1 (C) and VCAM-1 (D) expression after 24 h incubation with CM, OA and CMOA. Total cell lysates were resolved by SDS-PAGE and immunoblotted with a specific ICAM-1 or VCAM-1 antibody. Data are mean values ± SEM of three independent experiments. All data were normalized to the level of actin expression and expressed relative to the control. *p<0.05 compared to control.

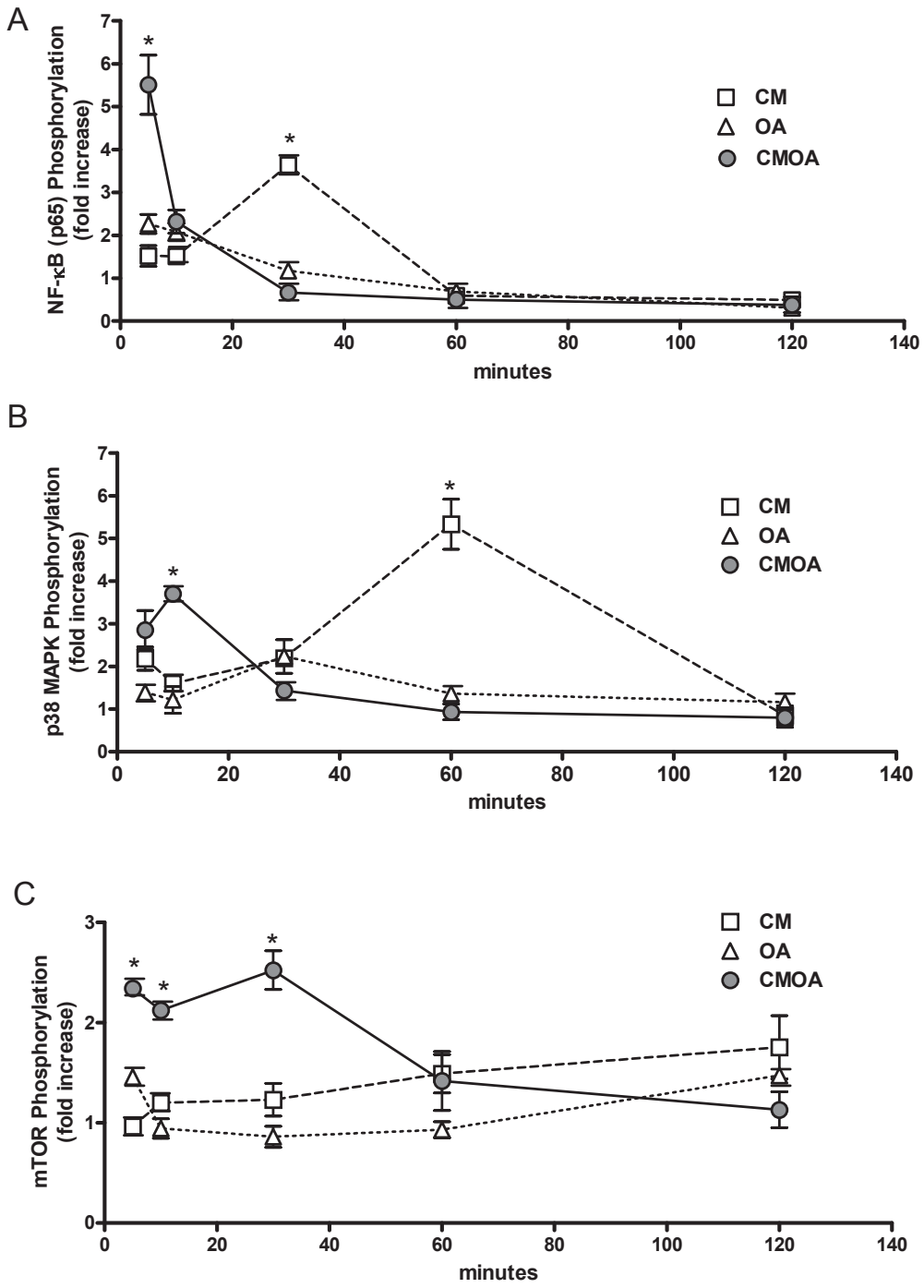

Figure 5.4 CM, OA and the combination of both acutely activate multiple intracellular signaling pathways. hVSMC were serum starved for 24 h and then exposed to CM, 100 µmol/l OA and the combination CMOA for the indicated times. Total cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies to phosphorylated and unphosphorylated forms of NF-κB (A), p38 MAPK (B) and mTOR (C). 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. *p<0.05 compared to control hVSMC. (n = 3-4)

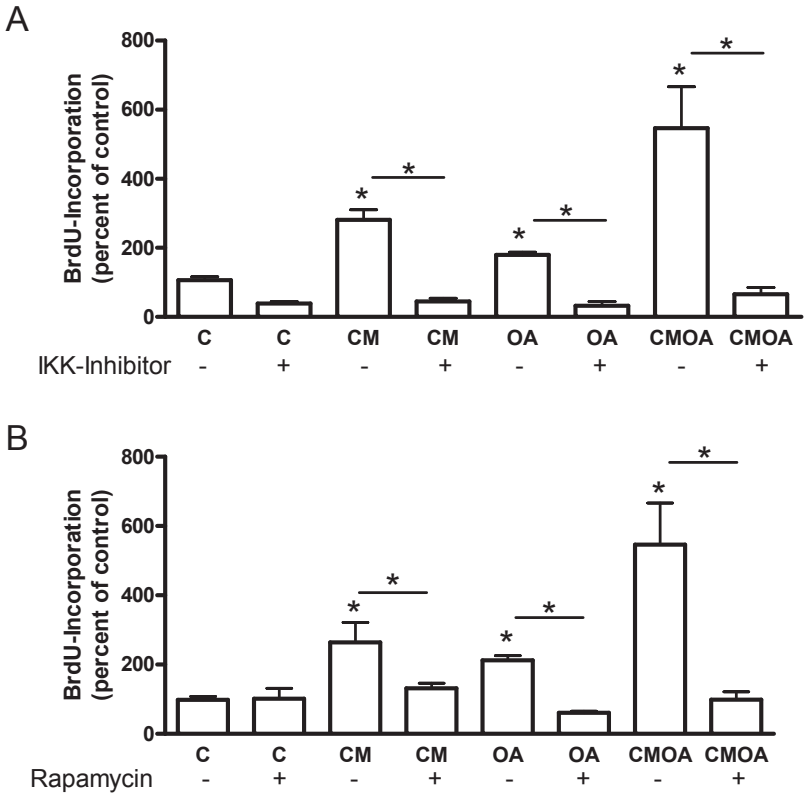

Figure 5.5 Impact of rapamycin and IKK-Inhibitor on the proliferative effect of CM, OA and CMOA in hVSMC. Cells were treated with CM, OA and CMOA as described in the legend to Fig. 5.3, without or with 10 nmol/l rapamycin (A) or 10 µmol/l IKK-Inhibitor (B) for 24 h. Proliferation was measured by the incorporation of BrdU into DNA. Data are expressed relative to the basal control value. *p<0.05 (n = 3-4).

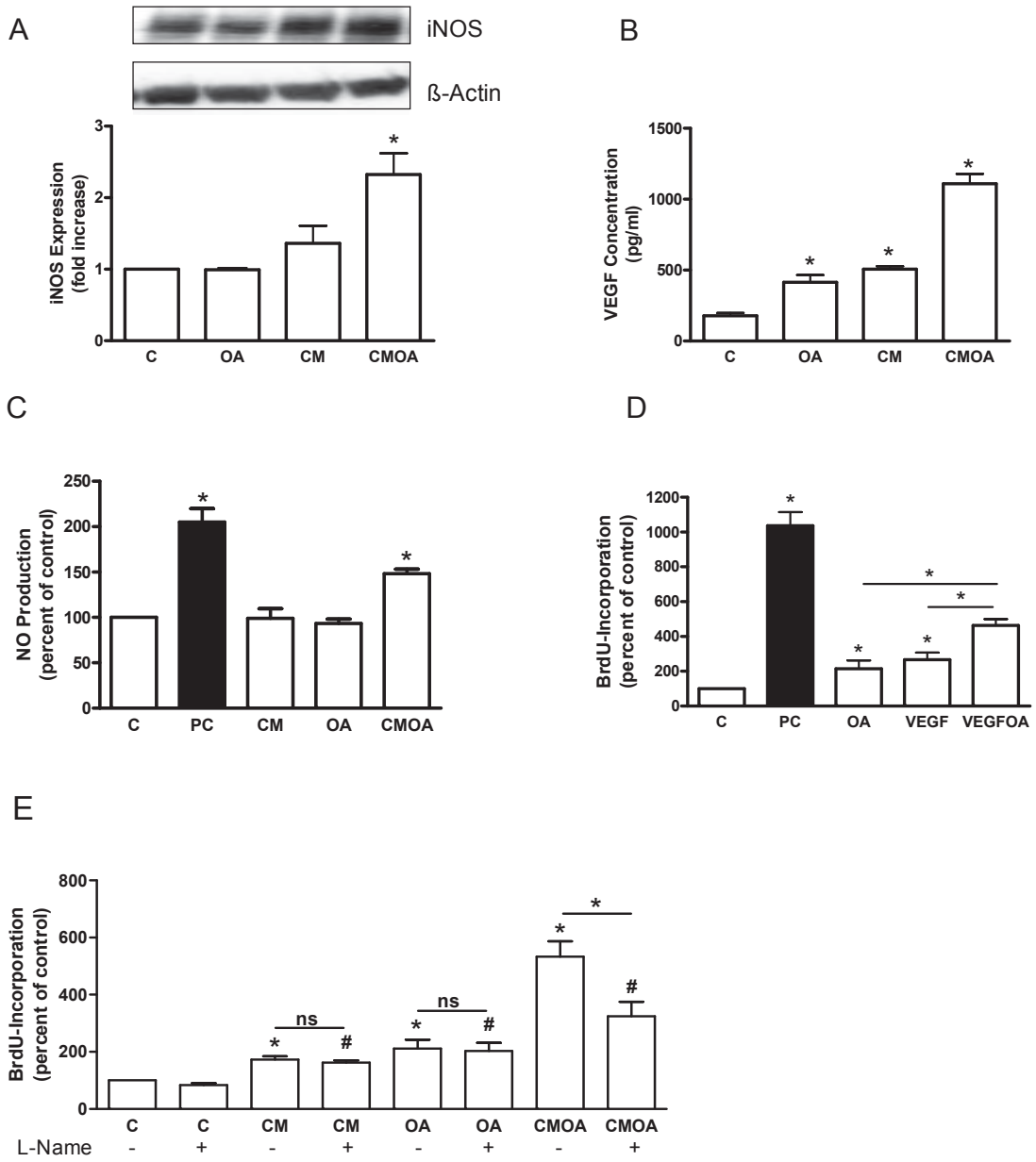

Figure 5.6 Effects of OA, CM and the combination of both on iNOS expression, VEGF concentration and NO production and impact of VEGF and NOS inhibitor L-NAME on proliferation. hVSMC were treated as described in the legend to Fig. 5.3. (A) Total cell lysates were resolved by SDS-PAGE and immunoblotted with a specific iNOS antibody. 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. (B) After 24 h the supernatant were collected and VEGF concentration was measured by ELISA assay. (C) hVSMC were subsequently analyzed for their capacity to produce NO as described in *Materials*. As positive control (PC), cells were treated for 30 min prior the beginning of the experiment with SNAP. (D) Cells were treated with 125 pg VEGF, OA and the combination of VEGF and OA (VEGFOA) for 18 h. Proliferation was measured by the incorporation of BrdU into DNA. (E) Cells were treated with CM, OA and CMOA as described in the legend to Fig. 5.3 with or without 1 mM L-NAME for 24 h. Data are means ± SEM. *p<0.05 compared to untreated hVSMC (n = 3); #p<0.05 compared to L-NAME treated hVSMC (n=3).

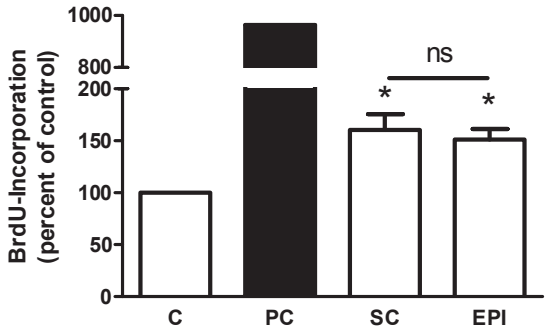

Figure 5.7 Effect of CM from subcutaneous (sc) and epicardial (epi) fat explants on the proliferation of hVSMC. The proliferation was determined by measuring the incorporation of BrdU into DNA. Data are expressed relative to the basal control value, which was set as 100 %. Data are presented as mean ± SEM from three independent experiments using three different CM. Both epicardial and subcutaneous fat were obtained from the same patient. *p<0.05 compared to control.

References

1. Calle EE, Thun MJ, Petrelli JM, Rodriguez C, Heath CW, Jr. Body-mass index and mortality in a prospective cohort of U.S. adults. *N Engl J Med* 1999; 341/15: 1097-1105.
2. Trayhurn P. Endocrine and signalling role of adipose tissue: new perspectives on fat. *Acta Physiol Scand* 2005; 184/4: 285-293.
3. Hu FB, Willett WC, Li T, Stampfer MJ, Colditz GA, Manson JE. Adiposity as compared with physical activity in predicting mortality among women. *N Engl J Med* 2004; 351/26: 2694-2703.
4. van Dam RM, Willett WC, Manson JE, Hu FB. The relationship between overweight in adolescence and premature death in women. *Ann Intern Med* 2006; 145/2: 91-97.
5. Ruan H, Lodish HF. Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-alpha. *Cytokine Growth Factor Rev* 2003; 14/5: 447-455.
6. Iacobellis G, Gao YJ, Sharma AM. Do cardiac and perivascular adipose tissue play a role in atherosclerosis? *Curr Diab Rep* 2008; 8/1: 20-24.
7. Calabro P, Samudio I, Willerson JT, Yeh ET. Resistin promotes smooth muscle cell proliferation through activation of extracellular signal-regulated kinase 1/2 and phosphatidylinositol 3-kinase pathways. *Circulation* 2004; 110/21: 3335-3340.
8. Li L, Mamputu JC, Wiernsperger N, Renier G. Signaling pathways involved in human vascular smooth muscle cell proliferation and matrix metalloproteinase-2 expression induced by leptin: inhibitory effect of metformin. *Diabetes* 2005; 54/7: 2227-2234.
9. Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K, Tobe K. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J Clin Invest* 2006; 116/7: 1784-1792.
10. Arita Y, Kihara S, Ouchi N, Maeda K, Kuriyama H, Okamoto Y, Kumada M, Hotta K, Nishida M, Takahashi M, Nakamura T, Shimomura I, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y. Adipocyte-derived plasma protein adiponectin acts as a platelet-derived growth factor-BB-binding protein and regulates growth factor-induced common postreceptor signal in vascular smooth muscle cell. *Circulation* 2002; 105/24: 2893-2898.
11. Barandier C, Montani JP, Yang Z. Mature adipocytes and perivascular adipose tissue stimulate vascular smooth muscle cell proliferation: effects of aging and obesity. *Am J Physiol Heart Circ Physiol* 2005; 289/5: H1807-H1813.
12. Hauner H, Petruschke T, Russ M, Rohrig K, Eckel J. Effects of tumour necrosis factor alpha (TNF alpha) on glucose transport and lipid metabolism of newly-differentiated human fat cells in cell culture. *Diabetologia* 1995; 38/7: 764-771.
13. Dietze-Schroeder D, Sell H, Uhlig M, Koenen M, Eckel J. Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors. *Diabetes* 2005; 54/7: 2003-2011.
14. Sell H, Eckardt K, Taube A, Tews D, Gurgui M, Echten-Deckert G, Eckel J. Skeletal muscle insulin resistance induced by adipocyte-conditioned medium: underlying mechanisms and reversibility. *Am J Physiol Endocrinol Metab* 2008; 294/6: E1070-E1077.
15. Moro C, Klimcakova E, Lolmede K, Berlan M, Lafontan M, Stich V, Bouloumie A, Galitzky J, Arner P, Langin D. Atrial natriuretic peptide inhibits the production of adipokines and cytokines linked to inflammation and insulin resistance in human subcutaneous adipose tissue. *Diabetologia* 2007; 50/5: 1038-1047.

16. Wichelhaus A, Russ M, Petersen S, Eckel J. G protein expression and adenylate cyclase regulation in ventricular cardiomyocytes from STZ-diabetic rats. *Am J Physiol* 1994; 267/2 Pt 2: H548-H555.
17. Dietze D, Ramrath S, Ritzeler O, Tennagels N, Hauner H, Eckel J. Inhibitor kappaB kinase is involved in the paracrine crosstalk between human fat and muscle cells. *Int J Obes Relat Metab Disord* 2004; 28/8: 985-992.
18. Dulak J, Jozkowicz A, Dembinska-Kiec A, Guevara I, Zdzienicka A, Zmudzinska-Grochot D, Florek I, Wojtowicz A, Szuba A, Cooke JP. Nitric oxide induces the synthesis of vascular endothelial growth factor by rat vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2000; 20/3: 659-666.
19. Frank S, Stallmeyer B, Kampfer H, Kolb N, Pfeilschifter J. Nitric oxide triggers enhanced induction of vascular endothelial growth factor expression in cultured keratinocytes (HaCaT) and during cutaneous wound repair. *FASEB J* 1999; 13/14: 2002-2014.
20. Xiong M, Elson G, Legarda D, Leibovich SJ. Production of vascular endothelial growth factor by murine macrophages: regulation by hypoxia, lactate, and the inducible nitric oxide synthase pathway. *Am J Pathol* 1998; 153/2: 587-598.
21. Henrichot E, Juge-Aubry CE, Pernin A, Pache JC, Velebit V, Dayer JM, Meda P, Chizzolini C, Meier CA. Production of chemokines by perivascular adipose tissue: a role in the pathogenesis of atherosclerosis? *Arterioscler Thromb Vasc Biol* 2005; 25/12: 2594-2599.
22. Berg AH, Scherer PE. Adipose tissue, inflammation, and cardiovascular disease. *Circ Res* 2005; 96/9: 939-949.
23. Correia ML, Haynes WG. Leptin, obesity and cardiovascular disease. *Curr Opin Nephrol Hypertens* 2004; 13/2: 215-223.
24. Braun M, Pietsch P, Schror K, Baumann G, Felix SB. Cellular adhesion molecules on vascular smooth muscle cells. *Cardiovasc Res* 1999; 41/2: 395-401.
25. Huo Y, Ley K. Adhesion molecules and atherogenesis. *Acta Physiol Scand* 2001; 173/1: 35-43.
26. Cai Q, Lanting L, Natarajan R. Growth factors induce monocyte binding to vascular smooth muscle cells: implications for monocyte retention in atherosclerosis. *Am J Physiol Cell Physiol* 2004; 287/3: C707-C714.
27. Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y. Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* 1999; 100/25: 2473-2476.
28. Lu G, Morinelli TA, Meier KE, Rosenzweig SA, Egan BM. Oleic acid-induced mitogenic signaling in vascular smooth muscle cells. A role for protein kinase C. *Circ Res* 1996; 79/3: 611-618.
29. Greene EL, Lu G, Zhang D, Egan BM. Signaling events mediating the additive effects of oleic acid and angiotensin II on vascular smooth muscle cell migration. *Hypertension* 2001; 37/2: 308-312.
30. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993; 362/6423: 801-809.
31. Artwohl M, Lindenmair A, Roden M, Waldhausl WK, Freudenthaler A, Klosner G, Ilhan A, Luger A, Baumgartner-Parzer SM. Fatty acids induce apoptosis in human smooth muscle cells depending on chain length, saturation, and duration of exposure. *Atherosclerosis* 2009; 202/2: 351-362.

32. Zhang Y, Liu C, Zhu L, Jiang X, Chen X, Qi X, Liang X, Jin S, Zhang P, Li Q, Wang D, Liu X, Zeng K, Zhang J, Xiang Y, Zhang CY. PGC-1alpha inhibits oleic acid induced proliferation and migration of rat vascular smooth muscle cells. *PLoS One* 2007; 2/11: e1137.
33. Zhu L, Sun G, Zhang H, Zhang Y, Chen X, Jiang X, Jiang X, Krauss S, Zhang J, Xiang Y, Zhang CY. PGC-1alpha is a key regulator of glucose-induced proliferation and migration in vascular smooth muscle cells. *PLoS One* 2009; 4/1: e4182.
34. Lu G, Meier KE, Jaffa AA, Rosenzweig SA, Egan BM. Oleic acid and angiotensin II induce a synergistic mitogenic response in vascular smooth muscle cells. *Hypertension* 1998; 31/4: 978-985.
35. Duplaa C, Couffinhal T, Dufourcq P, Llanas B, Moreau C, Bonnet J. The integrin very late antigen-4 is expressed in human smooth muscle cell. Involvement of alpha 4 and vascular cell adhesion molecule-1 during smooth muscle cell differentiation. *Circ Res* 1997; 80/2: 159-169.
36. Minhajuddin M, Bijli KM, Fazal F, Sassano A, Nakayama KI, Hay N, Platanias LC, Rahman A. Protein kinase C-delta and phosphatidylinositol 3-kinase/Akt activate mammalian target of rapamycin to modulate NF-kappaB activation and intercellular adhesion molecule-1 (ICAM-1) expression in endothelial cells. *J Biol Chem* 2009; 284/7: 4052-4061.
37. Wood SC, Bushar G, Tesfamariam B. Inhibition of mammalian target of rapamycin modulates expression of adhesion molecules in endothelial cells. *Toxicol Lett* 2006; 165/3: 242-249.
38. Yun MR, Lee JY, Park HS, Heo HJ, Park JY, Bae SS, Hong KW, Sung SM, Kim CD. Oleic acid enhances vascular smooth muscle cell proliferation via phosphatidylinositol 3-kinase/Akt signaling pathway. *Pharmacol Res* 2006; 54/2: 97-102.
39. Omura T, Yoshiyama M, Izumi Y, Kim S, Matsumoto R, Enomoto S, Kusuyama T, Nishiya D, Nakamura Y, Akioka K, Iwao H, Takeuchi K, Yoshikawa J. Involvement of c-Jun NH2 terminal kinase and p38MAPK in rapamycin-mediated inhibition of neointimal formation in rat carotid arteries. *J Cardiovasc Pharmacol* 2005; 46/4: 519-525.
40. Hishikawa K, Oemar BS, Yang Z, Luscher TF. Pulsatile stretch stimulates superoxide production and activates nuclear factor-kappa B in human coronary smooth muscle. *Circ Res* 1997; 81/5: 797-803.
41. Lawrence R, Chang LJ, Siebenlist U, Bressler P, Sonenshein GE. Vascular smooth muscle cells express a constitutive NF-kappa B-like activity. *J Biol Chem* 1994; 269/46: 28913-28918.
42. Obata H, Biro S, Arima N, Kaieda H, Kihara T, Eto H, Miyata M, Tanaka H. NF-kappa B is induced in the nuclei of cultured rat aortic smooth muscle cells by stimulation of various growth factors. *Biochem Biophys Res Commun* 1996; 224/1: 27-32.
43. Vinciguerra M, Veyrat-Durebex C, Moukil MA, Rubbia-Brandt L, Rohner-Jeanrenaud F, Foti M. PTEN down-regulation by unsaturated fatty acids triggers hepatic steatosis via an NF-kappaBp65/mTOR-dependent mechanism. *Gastroenterology* 2008; 134/1: 268-280.
44. Aktan F. iNOS-mediated nitric oxide production and its regulation. *Life Sci* 2004; 75/6: 639-653.
45. Kleinert H, Schwarz PM, Forstermann U. Regulation of the expression of inducible nitric oxide synthase. *Biol Chem* 2003; 384/10-11: 1343-1364.
46. Fang IM, Yang CH, Yang CM, Chen MS. Comparative effects of fatty acids on proinflammatory gene cyclooxygenase 2 and inducible nitric oxide synthase expression in retinal pigment epithelial cells. *Mol Nutr Food Res* 2009; 53/6: 739-750.

CHAPTER 6

General Discussion

6.1 The secretome of human adipocytes

It is well established that in the obese state adipose tissue releases increased amounts of metabolites as well as adipokines. While the release of metabolites, mainly FFA as a primary and classical role of adipocytes during the fasting state had already been recognized, the endocrine role of adipose tissue was first postulated with the discovery of the leptin gene in 1994. In the following years many studies revealed that adipose tissue releases numerous proteins and bioactive peptides acting on both the local and systemic level and making adipose tissue one of the major endocrine organs.[123,216]

As outlined in the previous chapters, obesity is a 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 CVD.[217] It is apparent that a complex inter-organ crosstalk between liver, muscle, and fat underlies the progression of these diseases, with adipose tissue on top of the crosstalk hierarchy.[218] The realization that adipose tissue acts as an endocrine gland affecting whole body energy homeostasis was a major breakthrough towards a better molecular understanding of obesity-related disorders.[219] Recent data could show that the diverse signaling and mediator molecules released by adipose tissue form a complex network of cell-to-cell and organ-to-organ signals. Positive feedback loops in that network can turn into molecular 'vicious cycles' leading to the almost irreversible diabetic state.[220,221] It is argued that a systemic approach to understand the multi-level signaling networks established by the adipose secretome will be crucial for developing efficient treatment of type 2 diabetes and related diseases.[220] Beneath the recent advantages of whole-genome association studies and the concomitant increase in the knowledge of genetic modifiers of diabetes susceptibility, the understanding of adipocyte secretome dynamics is crucial to get more insight into the relevant molecular mechanisms of diabetes development. For this purpose considerable research in recent years has focused on the

characterization of the adipose tissue secretome. Although several unbiased proteomic approaches based on adipocytes or adipose tissue explants have emphasized the complex nature of the whole secretory output,[145-154] the adipokinome of human adipocytes remains incompletely characterized. Among the older studies, the majority of the proteomic studies has used samples from animal origin and only a few recent studies have reported on human adipocyte or adipose tissue secretome.[145,147,151,153,154] Because enlargement of adipose tissue may represent the initial step leading to an altered adipokine secretion and adipose tissue inflammation,[217,222] the first aim of this thesis was to conduct a comprehensive proteomic profiling of conditioned media derived from *in vitro* differentiated, primary human adipocytes. This study represents the first approach using both primary human adipocytes and a combination of two complementary, orthogonal proteomic profiling techniques, namely 1D-GE and LC-ESI-MS/MS as well as 2D-GE followed by protein identification via MALDI-MS. Thereby, we could identify a total of 347 proteins from adipocyte-conditioned medium, with 263 secreted proteins according to bioinformatics analysis. A similar approach was recently conducted in the human SGBS cell line.[151] However, only 80 secreted proteins could be identified with this approach, possibly indicating the limitations of this cell model.

Regarding the technical procedure, the presence of serum proteins and intracellular proteins from damaged cells released during culture displays a major challenge in secretome analysis. To eliminate proteins, which are potential contaminants, we filtered all identified candidates for properties disclosing them as secretory proteins. For this purpose, we tested all proteins by expression data screening, signal sequence prediction programs, and literature research. Applying these constitutive filter methods, 263 out of the 347 originally found proteins (73 %) could be predicted to be or annotated as secretory proteins, demonstrating the high efficiency of this integrated profiling approach. Comparing our results with the identified proteins from the previously reported secretome studies shows an overlap of 219 secretory proteins (83 %), identified in different origins like 3T3-L1 cells, tissue explants, or tissue derived adipocytes. Consequently, our study leads to the identification of 44 proteins which we consider to be potential novel adipokines secreted from primary human adipocytes.

As previously mentioned, clustering adipose tissue-derived factors according to their postulated function reveals that these adipokines fall into several groups (extracellular matrix, inflammation, metabolism, oxidative stress, angiogenesis, and blood pressure; see also figure 1.2). Ingenuity analysis of the function of our identified secretory proteins could show a similar pattern. However, our strategy based on these observations was to validate putative adipokines, which can be included into these important groups. In this regard, we focused on high abundant as well as novel adipokines. Therefore, the following chapter will discuss the role of newly identified proteins such as heme

oxygenase-1 (HO-1), αB-crystallin (CRYAB), complement factor H (CFH), cartilage intermediate-layer protein (CILP), and dipeptidyl peptidase (DPP)4.

Furthermore, we could identify the pigment epithelium-derived factor (PEDF) as one of the most abundant proteins secreted by primary human adipocytes. In this context, another aim of this study was to unravel the expression and regulation of PEDF in adipose tissue and its role in inducing insulin resistance and inflammatory signaling in adipocytes, skeletal muscle, and smooth muscle cells.

6.2 Characterization of novel adipokines

The comprehensive proteomic profiling of primary human adipocytes resulted in the identification of a large number of putative novel adipokines. The verification and characterization of these proteins is very time-consuming and is also associated with high material costs. Therefore, new adipokines were initially subjected to intensive literature research. Subsequently, we focused on five candidates, which we considered to be the most promising adipokines.

6.2.1 Heme oxygenase 1 (HO-1)

One of these novel adipokines is heme oxygenase-1 (HO-1), also known as heat shock protein 32 (HSP-32). HO-1 is one of two heme oxygenase isoenzymes in humans and rodents that catabolizes the degradation of heme, that is iron (Fe) protoporphyrin (IX), into equimolar amounts of labile Fe, carbon monoxide (CO), and biliverdin.[223] HO-1 expression is induced ubiquitously in response to oxidative stress, whereas HO-2 is constitutively expressed and not inducible. Free heme is a reactive Fe compound that can catalyze the formation of cytotoxic hydroxyl radicals ($OH^{\cdot}$) from hydrogen peroxide (H_2O_2) through the Fenton reaction. HO-1 is rate-limiting for pro-oxidative heme and promotes anti-oxidant and anti-apoptotic properties due to its products biliverdin and CO. Biliverdin can be converted by biliverdin reductase into bilirubin, which is a cytoprotective antioxidant. Furthermore, CO has also cytoprotective and anti-inflammatory properties.[223]

Free heme is recognized by immune cells like macrophages and polymorphonuclear (PMN) cells via the pattern recognition receptor (PRR) toll-like receptor (TLR)4 which in turn induces the production of inflammatory cytokines, such as TNFα.[224] It is assumed that limiting the availability of free heme by HO-1 leads to the inhibition of PMN chemotaxis and activation, which in turn results in less oxidative tissue injury.[225]

HO-1 can act on atherosclerosis since its byproducts CO and bilirubin are capable of inhibiting smooth muscle cell proliferation, endothelial apoptosis, macrophage transmigration through the endothelium, and inhibition of inflammatory gene expression.[226] Furthermore, it could be shown in a murine model that HO-1 was able to reverse atherosclerotic lesion progression from a vulnerable to a more stable phenotype.[227]

Additionally it has been shown that HO-1 decreases weight gain, TNFα and IL-6 levels while increasing adiponectin levels in obese rats and diabetic mice.[228,229]

Moreover, induction of HO-1 in Zucker diabetic fat (ZDF) rats improves insulin sensitivity, again accompanied by an increase in serum adiponectin levels together with a decrease in visceral and subcutaneous fat tissue. In addition, it was shown that HO-1 up-regulation reduces adipogenesis in human bone marrow derived stem cells as well as in murine 3T3-F442A cells.[228,230]

Despite these observations demonstrating the potential role of HO-1 in different diseases like obesity, diabetes, or CVD, we are the first who could demonstrate HO-1 to be an adipokine released by human *in vitro* differentiated adipocytes. Furthermore we could show that HO-1 secretion is reduced by TNFα.

Regarding the inflammatory state of adipose tissue in obesity and the concomitant infiltration of distinct immune cells, such as macrophages, it should be noted that there are some studies which provide evidence for a higher contribution of nonadipose cells to adipose tissue secretion.[231,232] The mechanisms by which macrophages are attracted to adipose tissue are versatile (see also chapter 1.3.1). Both paracrine and endocrine signals as well as adipocyte hypertrophy and hyperplasia are thought to contribute to this infiltration. Concerning the role of macrophages in adipose tissue, two major functions are described in literature. First, macrophages accumulate around dead, stressed or aged cells and form so-called 'crown-like structures' with concomitant release of pro-inflammatory cytokines.[222] Second, macrophages are implicated in the neovascularization of expanding adipose tissue,[233] therefore antagonizing hypoxia, thus, playing a beneficial role in adipose tissue biology.

Despite the different roles of macrophages and adipocytes, both cell types have more in common than previously thought. Beneath being present in the same tissue, adipocytes and macrophages share some characteristics such as the potential to secrete TNFα, MCP-1, and IL-8 and their regulation of differentiation and maturation by PPARγ activation.[234] Therefore, it can be assumed that the whole secretory capacity of adipose tissue cannot be attributed to adipocytes alone. To elucidate whether the newly identified adipokines are merely secreted by adipocytes or also by macrophages, we further isolated adipose tissue-resident macrophages (CD14+). Comparison of the HO-1 secretion of adipocytes with the secretion of adipose tissue-derived macrophages revealed that the HO-1 release can be clearly attributed to adipocytes.

In addition, we could show that circulating levels of HO-1 are increased in obese men and correlate with the size of subcutaneous adipocytes. Elevated serum or plasma HO-1 level have also been observed in several chronic disorders such as chronic silicosis,[235] Parkinson's disease,[236] and hemophagocytic syndrome.[237] A recent study could show that plasma HO-1 concentrations were significantly increased in individuals with newly diagnosed type 2 diabetes.[238] The up-regulation of HO-1 can be attributed to the body's attempt to minimize adverse effects of cellular injury in chronic diseases.[239]

Up to now it is not fully elucidated which sources are responsible for the circulating HO-1 levels. It was postulated that plasma HO-1 may represent 'leakage' of the enzyme from tissues to the plasma compartment analogous to the presence of circulating liver enzymes.[239] It was supported by subsequent investigations suggesting that elevated circulating HO-1 levels may derive from lesions in specific tissues.[235,240] Because HO-1 is highly expressed in pancreatic islets, it is further speculated that increased plasma levels of HO-1 may be a result of injured pancreatic islets in individuals with type 2 diabetes.[238] Based on the present study we suggest that adipose tissue could represent a major factor contributing to the circulating HO-1 levels. Here, HO-1 is released in a differentiation-dependant manner, indicating that the HO-1 release is not due to necrotic or injured cells.

6.2.2 αB-crystallin (CRYAB)

The protection against oxidative stress is also a function of αB-crystallin (CRYAB), that we can now describe as an adipokine, in agreement with a recent study of Kim *et al.* (2010).[147] Furthermore, we could show that CRYAB expression is increased during adipocyte differentiation without further regulation by classical adipocyte stimuli. α-crystallin is a predominant protein of the ocular lens and it is composed of two subunits, αA and αB. αA and αB-crystallins belong to the family of small heat shock proteins (sHSP) and share 57% sequence homology.[241] In contrast to αA-crystallin, which is largely lens specific, αB-crystallin has also been shown to be present in non-lenticular tissues like cardiac and skeletal muscle, skin, brain, lungs, and recently in adipose tissue.[242] CRYAB acts as a molecular chaperone that counteracts the formation of aberrantly folded polypeptides and allows their correct folding during stress recovery.[243] Concordantly, it could be observed that CRYAB is constitutively expressed in tissues with a high rate of oxidative metabolism including heart and type I and type IIa skeletal muscle fibers in mice and rats.

Furthermore, it could be shown that two mutations in the CRYAB gene are leading to myofibrillar myopathy associated with cardiomyopathy, confirming the importance of CRYAB in these diseases.[244,245] In addition, increased expression of CRYAB occurs in a multitude of pathologies, especially in degenerative diseases of the central nervous system, wherein oxidative stress is implicated as a major event in their pathogenesis.[246]

However, the role of CRYAB expression and release in the context of obesity and diabetes is only insufficiently resolved. One recent study conducted in streptozotocin (STZ)-induced diabetic rats demonstrated that CRYAB expression is elevated in lens, heart, muscle and brain due to increased oxidative stress. However, in adipose tissue CRYAB expression was decreased. [242] Since this is a common model to study type 1

diabetes, it is difficult to draw a conclusion regarding the role of CRYAB in chronic inflammatory diseases such as type 2 diabetes or atherosclerosis. In the presented study we could also observe a high CRYAB secretion by adipose-tissue derived macrophages, which was comparable to that of adipocytes. The release of CRYAB by these to cell types is an interesting finding, whose role in the context of obesity-related diseases like type 2 diabetes and CVD should further be elucidated.

6.2.3 Complement factor H (CFH)

Complement factor H (CFH) is an example of the large group of adipocyte-secreted proteins involved in inflammation. CFH is an important regulator of complement activation and essential to restrict the action of complement to activating surfaces. CFH is a single polypeptide chain plasma glycoprotein (155 kDa) that binds to C3b, accelerates the decay of the alternative pathway C3-convertase (C3Bb), and acts as a cofactor for the I-mediated proteolytic inactivation of C3b. It regulates complement in fluid-phase and on cellular surfaces. In addition, it has been shown that CFH binds to C-reactive protein (CRP).[247]

CFH is constitutively produced by the liver and is found in human plasma at concentrations of 110-615 µg/ml. In addition, extrahepatic synthesis occurs in a wide variety of cell types. The expression of CFH in adipocytes was first described in 1997.[248] However, we can now show that CFH is also secreted by human adipocytes. Furthermore, we can observe a significant reduction of CFH protein expression during adipogenesis, which is in accordance with two other studies demonstrating at the mRNA level that this factor is predominantly expressed in preadipocytes while also present in adipocytes.[248,249]

A role for CFH in diabetes has been suggested because of its interaction with adrenomedullin, a peptide hormone which induces insulin secretion in the pancreas and is known to be elevated in plasma from patients with pancreatic dysfunctions such as type 1 and type 2 diabetes.[250] Very recently it could be shown that CFH in blood is elevated in insulin-resistant subjects, with higher expression in subcutaneous vs. visceral adipose tissue.[249] Consistently, we found higher CFH content in blood of obese men and an upregulation of CFH in adipocytes after treatment with TNFα, insulin, and hypoxia. Altogether, these data point to a possible higher contribution of adipose tissue to circulating CFH levels in obesity due to adipose tissue inflammation. Whether adipocytes or preadipocytes contribute to increased adipose tissue CFH in obesity remains an open question which is of interest for further analysis.

6.2.4 Cartilage intermediate-layer protein (CILP)

Cartilage intermediate-layer protein (CILP) is an extracellular matrix protein that is highly expressed in the intermediate zone of human articular cartilage where it was shown to function as an IGF-1 antagonist and binding partner for TGFβ.[152,251] Moreover, its expression is enhanced during aging and also in the early stages of human osteoarthritis and rheumatoid arthritis.[252] Here, CILP is expected to act as an autoantigen, which is involved in the pathogenesis of inflammatory joint destruction.[253] In the latter study it could be demonstrated that patients with osteoarthritis and rheumatoid arthritis showed autoimmunity to CILP and that mice immunized with CILP displayed infiltration of mononuclear cells into the synovial membrane and exfoliation and surface irregularity of the cartilage.

In the present study we could show for the first time that the two CILP isoforms are secreted by human adipocytes. Furthermore, CILP expression increases during differentiation with the highest expression in mature adipocytes. In addition, CILP expression is down-regulated by TNFα in adipocytes and its plasma content is significantly lower in obese than lean men.

However, the role of CILP in the context of obesity-related disorders is completely unexplored. The function and consequences of CILP secretion by adipocytes and the outcome of the reduced CILP plasma level in obese men remain open questions and should be further investigated.

6.2.5 Dipeptidyl peptidase IV (DPP4)

Our proteomic approach also identified dipeptidyl peptidase IV (DPP4) as one novel adipokine released by fully differentiated adipocytes. DPP4, which is a member of the S9b serine protease family, is a multifunctional type II integral membrane glycoprotein exhibiting ubiquitous expression in several tissues and cell types, such as kidney, T lymphocytes, endothelial cells,[254] and also adipose tissue.[255] It is also known as the cell surface antigen CD26 associated with CD45 in lymphocytes and a co-stimulatory factor in immune responses. DPP4 cleaves N-terminal dipeptides from a variety of substrates including growth factors and hormones, neuropeptides, and chemokines.[256] Due to the degradation of the incretin glucagon-like peptide (GLP)-1, DPP4 has gained considerable interest as a therapeutic target for type 2 diabetes and a variety of inhibitors that prolong the insulinotrophic effect of GLP-1 are now in clinical use.[257] Substantial DPP4 activity is 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.[258] Both the membrane abundance and the circulating levels have been found to be altered in

several neurological and inflammatory diseases.[256] However, although a fraction of soluble DPP4 most likely originates from cells of the immune system,[259] the major source of circulating DPP4 and its regulation remain unknown. DPP4 can be distinguished from other adipokines by the fact that it is not secreted but released from the plasma membrane as soluble DPP4 subsequently to proteolytic cleavage.[260] Furthermore DPP4 exerts dual functions both as a regulatory protease and a binding protein, besides its established role as a therapeutic target in the treatment of type 2 diabetes.

In the presented study we could demonstrate that DDP4 release is increased substantially during fat cell differentiation. Comparison to preadipocytes and adipose tissue-derived macrophages revealed that adipocytes most likely represent the major source of DPP4 in fat tissue. Up to now, regulators of DPP4 release are unknown, but we show here that both insulin and TNFα augment the shedding of soluble DPP4 by about 50 %, despite an unchanged protein expression level. Both cell surface resident and soluble DPP4 may have multiple auto- and paracrine functional implications in the physiology of adipose tissue. DPP4 recruits adenosine deaminase to the cell surface [261] able to modulate the well-established anti-lipolytic effects of adenosine. In addition, DPP4 is a very strong inhibitor of the anti-lipolytic effect of neuropeptide Y (NPY),[255] which is one of the strongest substrates of this enzyme. As a consequence, increased abundance of resident as well as soluble DPP4 may augment the lipolytic activity of expanded adipose tissue. Furthermore, DPP4 can modulate or inactivate the specificity of several chemokines (e.g. RANTES, MDC, and SDF-I), suggesting a yet undefined role for DPP4 in the crosstalk between adipocytes, immune cells, and other components of the stroma-vascular fraction.

In the present study we could also show, that compared to lean controls morbidly obese men display elevated DPP4 serum concentrations, which can further be correlated to BMI, fat mass, size of adipocytes in subcutaneous and visceral fat, and with adiponectin (negatively) as well as leptin levels. Concerning the protein expression of DPP4, we could observe significant differences both between lean and obese subjects and between their fat depots. With reference to other studies, data about the DPP4 mRNA expression in adipose tissue are contradictory.[255,262] In the present study we clearly observe a prominent induction of DDP expression in subcutaneous as well as visceral adipose tissue, with the highest DPP4 expression in visceral fat from obese subjects. Therefore, we suggest that the expanded visceral fat in obesity may strongly contribute to the circulating level of DPP4.

In conclusion, both circulating levels of DPP4 and released DPP4 by adipose tissue correlate strongly with the number of risk factors for developing the metabolic syndrome, suggesting that DPP4 could be of relevance as a novel biomarker for detection of obese subjects at high risk for obesity-related disorders. Further studies are

needed to investigate the molecular pathways linking the DPP4 release by adipose tissue to the metabolic syndrome and type 2 diabetes.

6.3 PEDF - a high abundant adipokine

As previously described, a novel adipokine discovered in 2004 to be secreted by 3T3-L1 adipocytes is the pigment epithelium-derived factor (PEDF).[156] PEDF, also known as SERPIN F1 or EPC1, is a 50 kDa secreted glycoprotein that is a member of the serpin (serine protease inhibitor) family but lacks a serine reactive loop and thus has no function on protease inhibition.[157]

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-GE and MALDI-MS. It could be identified in 15 different protein spots, which represent 6 % of all calculated spot intensities on the gel. Conversely, a current study could show that PEDF is the most abundant protein in supernatants of 3T3-L1 adipocytes.[159] Compared with other adipokines measured by us in earlier studies, PEDF showed a higher concentration than the new adipokine chemerin and even than adiponectin. Furthermore, we could observe an increased PEDF protein expression and secretion during adipocyte differentiation. The role of adipose tissue PEDF becomes evident since it was recently shown that PEDF expression in fat tissue positively correlates with obesity and insulin resistance in mice.[159] Furthermore, only modest PEDF secretion from hepatocytes and monocytes could be detected, indicating that these cells or tissues most likely do not represent the main source for circulating levels. We could also show 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 by adipose tissue. Additionally two other studies describe a significant correlation between plasma PEDF and obesity in humans,[160,161] further pointing to adipose tissue as a main source of PEDF. However, the relevance of the liver contributing to plasma levels of PEDF in humans remains to be elucidated.

PEDF has been described as a multifunctional protein with different roles in diverse cell types and it is assumed that PEDF functions via multiple receptors and ligands.[263] Until now two different proteins are suggested to act as receptors for PEDF, namely adipose triglyceride lipase (ATGL) and laminin-R. In 2006 the patatin-like phospholipase domain containing protein-2, also known as ATGL, was identified as a specific receptor for PEDF.[264] There is upcoming evidence that PEDF affects lipolysis in adipose tissue and takes part in the hepatic lipid accumulation depending on ATGL.[159,265] PEDF is supposed to affect not only lipid metabolism, but also mediates some of its effects like anti-angiogenic or anti-tumorigenic activities by inducing lipid mediators.[264] The second known receptor for PEDF, laminin-R, was identified in 2009. This non-integrin receptor is involved in processes such as cell adhesion, proliferation, and migration.[266] In this

context, laminin-R may have an important role in angiogenesis. It was shown that laminin-R is expressed by the proliferating retinal vasculature during neovascularization, whereas quiescent vascular cells in normal control retinas showed comparatively low expression levels.[267] Furthermore, laminin-R expression is the highest in rapidly proliferating endothelial cells in culture and declines when cells become contact-inhibited, suggesting that laminin-R levels need to be constantly renewed during the cycles of rounding-up and re-attachment which dividing cells go through during rapid proliferation.[268] All cell types used in our study express both receptors, however, adipose tissue macrophages only display small amounts of laminin-R and no ATGL. It is known that PEDF can also affect macrophages as it inhibits lipopolysaccharide-induced IL-12 production in a mice macrophage cell line.[269] Furthermore, it induces apoptosis in a human monocytic leukemia cell line (THP-1) as well as in human primary monocyte-derived macrophages in a PPARγ-dependent manner.[270] 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 these two cell types.

As previously mentioned, a recent work could demonstrate that PEDF induces insulin resistance in skeletal muscle and liver in rodents *in vivo*, both after acute and chronic administration.[159] In this study we could show 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 to PEDF than skeletal muscle cells. Human adipocytes show significantly decreased insulin-stimulated Akt phosphorylation already after treatment with the lower PEDF concentration (5 nM). In terms of inflammatory signaling, adipocytes exhibit a prolonged activation of NF-κB, whereas skeletal muscle cells are characterized by a modest activation of this inflammatory pathway. These differences could be due to a higher expression of ATGL and laminin-R by adipocytes in comparison to skeletal muscle cells.

Related to the observed effects of PEDF on adipocytes and skeletal muscle cells, we further addressed the role of this adipokine in the development of atherosclerosis at the level of VSMC. Here, we could demonstrate that in addition to adipocytes and skeletal muscle cells, PEDF also leads to acute activation of inflammatory signaling (NF-κB) in VSMC. This is in accordance with studies showing that PEDF induces inflammatory signaling in distinct cell types [162] and correlating PEDF protein levels to inflammation and vascular dysfunction in type 1 diabetics with microvascular complications.[163] The activation of NF-κB in VSMC is comparable to that observed in skeletal muscle cells, but not as prolonged as in adipocytes. This observed effect may be explained by the lesser expression of ATGL in both muscle cell types. In addition, we could demonstrate for the first time that PEDF also leads to the induction of insulin resistance in VSMC at the level of Akt phosphorylation. In this context, VSMC are more sensitive to PEDF than skeletal

muscle cells, but less sensitive than human adipocytes, which can be due to the higher expression of laminin-R in VSMC compared to skeletal muscle cells, and the lesser expression of ATGL in VSMC in contrast to adipocytes.

PEDF further leads to a slight, but significant induction of smooth muscle cell proliferation. A recent study showed that PEDF by itself has no impact on the proliferation of VSMC but that it inhibits angiotensin II-induced VSMC proliferation via suppression of ROS generation.[271] However, in our study we did not observe an anti-proliferative effect of PEDF, neither with PEDF alone nor in combination with CM, which is known to induce ROS production in our system of human skeletal muscle cells[272] and VSMC (preliminary data). CM itself contains 1 nM PEDF. In our experiments we used a concentration of 10 nM, suggesting that PEDF is not responsible for the CM-induced proliferation. Treatment of the cells with rapamycin or an IKK-inhibitor completely reduced PEDF-induced proliferation to the control level, making it likely that both mTOR and NF-κB are responsible for our observed effects. As outlined above, VSMC in our cell system express high amounts of laminin-R compared with skeletal muscle cells and adipocytes. However, several experimental and correlative studies have implicated the role of this receptor in tumor progression[273,274] as well as in angiogenesis, allowing proliferating endothelial cells to attach and form new blood vessels.[273,275,276] Although SMC are known to express laminin-R, its implications in angiogenesis and/or proliferation of this cell type are barely elucidated.[277] Whether the high expression of laminin-R in VSMC is responsible for the PEDF-induced proliferation remains an open question and should further be investigated.

Proliferation of SMC is regulated by different pathways including p38 MAPK, mammalian target of rapamycin (mTOR), and above mentioned NF-κB. p38 MAPK and NF-κB can be activated by stress, inflammatory cytokines, and growth factors.[278-281] Here we could show that PEDF leads to an acute activation of p38 MAPK and NF-κB after 5 min. An initial screening with the Kinex™ antibody microarray revealed that the PI3K-Akt-mTOR-P70S6 kinase pathway is activated in VSMC after incubation with CM for 24 h (table 6.1). In this study we could observe that also PEDF increases the phosphorylation of mTOR significantly within 5 min. In addition, the inhibition of the IKK complex upstream of NF-κB and the inhibition of mTOR activation by rapamycin completely inhibited PEDF-induced proliferation, indicating that both NF-κB and mTOR are responsible for the observed proliferative effect.

Furthermore, we could show that PEDF per se has no effect on the migration of VSMC, which is in accordance with the literature.[282] However, it could be shown that PEDF inhibits the PDGF-induced migration of SMC by blocking ROS generation. 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.

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

6.4 The crosstalk between adipocytes and vascular smooth muscle cells

As outlined previously, obesity is considered as a major determinant of cardiovascular morbidity and mortality.[170,171] Several studies in humans and various animal models have demonstrated that obesity strongly correlates with the development of atherosclerosis.[172,173] Increased secretion of adipokines and FFA by enlarged fat tissue plays an important role in the development of a chronic low-grade inflammation state, associated with vascular dysfunction and cardiovascular risk. However, almost all blood vessels are surrounded by perivascular adipose tissue, which initially has been considered merely a structural support for vasculature. Recent studies revealed that perivascular fat produces and releases various bioactive substances, playing a role in vascular function [283] and providing a direct link between obesity and vascular complications.[174,284] It was already shown that chemotactic factors released by perivascular adipose tissue modulate the function of infiltrating leukocytes at the interface between human perivascular fat and the adventitia of atherosclerotic aortas.[285]

6.4.1 Role of adipokines

Regarding the effects of adipokines, it is well-known that specific adipocyte-derived factors such as leptin, IL-6, and resistin are involved in regulating vascular functions, including VSMC proliferation and migration.[198,200,206] However, the crosstalk of adipose tissue with cells of the arterial wall is not yet fully understood. Although effects of specific adipokines on SMC function have been studied, the impact of the whole secretory output of adipocytes has not been investigated. Apart from one single study using CM from mouse cell lines and rat adipose tissue explants showing induction of proliferation in human VSMC,[286] no further data on a direct interaction of adipocytes and SMC exist. In the present study, we could demonstrate that adipocyte-conditioned medium (CM) from *in vitro* differentiated human adipocytes induces proliferation and migration of human VSMC. In this context, CM of human adipocytes contains various growth-promoting and migrative factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), IGF, PDGF and AngII, which can be responsible for these observed effects. In our study, the majority of CM induced a 3-4-fold proliferation of VSMC, whereas only a few lacked this effect. It should be noted that the release of FFA by *in vitro* differentiated adipocytes is very low and barely detectable by high

performance liquid chromatography, so that is can be presumed that FFA do not have an important role in our setting. Measuring the amount of adiponectin in all used CM revealed that low adiponectin content is associated with high proliferative action of CM. With respect to an earlier study performed by our working group demonstrating that adiponectin exerts an autocrine effect on adipocytes decreasing the release of various adipokines,[287] we presumed that the adiponectin content in CM might be responsible for the differences in the proliferative potency of CM. In contrast to the majority of adipokines, adiponectin has a particular role in the cardiovascular system by exerting anti-inflammatory, anti-atherogenic, and cardioprotective effects on multiple target tissues and organs like the heart and blood vessels.[288] Many adipokines are positively correlated with adiposity, but adiponectin levels are negatively regulated by accumulation of body fat and visceral fat in particular.[289] In this regard, plasma adiponectin levels are low in obese individuals.[290,291] Clinical studies implicate hypoadiponectinemia in the pathogenesis of type 2 diabetes,[292,293] coronary artery disease (CAD),[294-296] and hypertension.[297] Regarding the role of adiponectin in atherosclerosis, it could be demonstrated that administration of adenovirus-mediated adiponectin reduces atherosclerosis lesion size in ApoE knockout mice, accompanied by reductions in the expression of class A scavenger receptor, TNFα, and VCAM-1.[298] ApoE/adiponectin double deficient mice have accelerated T-lymphocyte accumulation in atheromata and augmented atherosclerosis.[299] Consistent with this anti-atherogenic actions, *in vivo* experiments showed that adiponectin reduces TNFα-stimulated expression of VCAM-1 in endothelial cells by suppressing the activation of NF-κB.[187,192] Adiponectin also reduces lipopolysaccharide (LPS)-stimulated TNFα production[189] and increases the anti-inflammatory cytokine IL-10 in human macrophages.[300]

Concerning the actions of adiponectin on the level of smooth muscle cells, it could be shown that adiponectin strongly suppressed PDGF-BB-induced proliferation and migration of VSMC through the direct association with this growth factor.[206] In the present study we could demonstrate that adiponectin added to adipocytes during medium conditioning prevents the proliferative effect of CM. Consistently, adiponectin added to CM after conditioning, and therefore not influencing adipokine release, did not affect CM-induced proliferation. We therefore assume that adiponectin has to be in contact with adipocytes to exert its positive effects on adipokine release rather than binding adipokines and preventing their proliferative effect.

Proliferation of SMC can be regulated by several distinct pathways. To elucidate which pathways are involved in CM-induced proliferation in particular we conducted an initial screening with the Kinex™ antibody microarray. This microarray permits a screening of SMC lysates with 377 pan-specific and 273 phospho-site-specific antibodies. After 24 h incubation with adipocyte-CM, 87 proteins show an upregulation (≥ +50 %

compared to control) and 58 proteins a downregulation (≥ -50 % compared to control). Table 6.1 shows the most interesting signaling molecules involved in SMC proliferation.

Table 6.1 Conditioned medium-induced changes of protein expression and/or phosphorylation-sites of different signaling mediators in vascular smooth muscle cells

Target protein	Full target protein name	Phospho-site	% change vs. control cells
mTOR	Mammalian target of rapamycin	Pan-specific/ S2448	-11/ +134
S6Ka (p70/p85 S6Ka)	p70/ p85 ribosomal protein-serine S6 kinase alpha	Pan-specific/ T229	-42/ +110
p38a MAPK	Mitogen-activated protein-serine kinase p38 alpha	T180+Y182	+77
NF-κB p65	NF-kappaB p65 nuclear transcription factor	S267	+59
Rac1/cdc42	Ras-related C3 botolinum toxin substrate 1	S71	+242
FAK	Focal adhesion protein-tyrosine kinase	Pan-specific/ S723/ Y397	-23/ +210/ +89
STAT1	Signal transducer and activator of transcription 1	S727	+52
PKA Ca/b	cAMP dependent protein serine kinase (catalytic subunit	Pan-specific/ T197	+260/ +91
PKA 2b	cAMP dependent protein serine kinase (regulatory subunit)	Pan-specific/ S114	+11/ +256
EGFR	Epidermal growth factor receptor-tyrosine kinase	Pan-specific	+130
PDGFRa/b	Platelet-derived growth factor receptor kinase alpha/beta	Y572+Y574/ Y579+Y581	+57

In the following we focused on three of these proteins, namely p38 MAPK, NF-κB, and mTOR. With Western blotting we could show that CM significantly increases the phosphorylation of all three signaling mediators after short time incubation. Both the IKK inhibitor and rapamycin completely abrogated the CM-induced proliferation, indicating that there is a crosstalk between these pathways. In this context, a recent study has shown that the down-regulation of PTEN in hepatocytes is mediated by a signaling complex made of mTOR and NF-κB.[301] Therefore, it is likely that both mTOR and NF-κB are responsible for the CM-induced proliferation.

Furthermore, we could demonstrate in this study that CM induces the expression of ICAM-1. Several studies during the last years have shown that, in addition to endothelial cells, SMC can also express a number of cellular adhesion molecules such as ICAM-1, VCAM-1, selectins, or fractalkine (CX3CL1).[302] Regarding the expression of ICAM-1, increased amounts of this adhesion molecule could be detected in SMC in the intima of atherosclerotic lesions compared to normal SMC in the adult aorta.[303,304] In addition, ICAM-1 expression was occasionally seen in medial smooth muscle cells adjacent to atherosclerotic plaques.[305] The expression of adhesion molecules by SMC allows them to interact with monocytes, which concomitantly differentiate into macrophages.[302,306] The correlation between the extent of adhesion molecules in the atherosclerotic vessel wall and mononuclear cell infiltration suggests a potential role of SMC in retaining monocytes and macrophages within the atherosclerotic lesion,[307] representing a marker of the phenotypic change of SMC from the contractile to the synthetic state. Several studies investigated the effects of single cytokines and growth factors on the expression of ICAM-1 in cultured human smooth muscle cells. It could be shown that TNFα, IL-1β as well as IFNγ can induce ICAM-1 in several, but not all studies, depending on the different vascular regions SMC were isolated from.[302,308,309]

Regarding the effect of adiponectin on the expression of adhesion molecules, it could be shown that this adipokine can reduce VCAM-1 expression in atherosclerotic lesions of ApoE knockout mice.[298] Furthermore, adiponectin treatment decreases the amount of ICAM-1, VCAM-1, and E-selectin in human aortic endothelial cells.[296] In our system of VSMC we could demonstrate that adiponectin added to adipocytes during medium conditioning, referring to the autocrine action of adiponectin, completely inhibits the CM-induced ICAM-1 expression, again demonstrating that adiponectin has to be in contact with adipocytes to exert its protective functions. In this regard, the presented work emphasizes the importance of the autocrine function of adiponectin and extents this concept to the control of growth- and inflammation-promoting factors released by adipocytes.

To validate our findings obtained with adipocytes from subcutaneous fat, we also assessed the proliferative capacity of epicardial fat, which is a perivascular fat depot. For this purpose we generated CM from paired adipose tissue explants from patients undergoing bypass surgery. We could show that CM generated from both subcutaneous and epicardial fat induces a significant 1.5-fold proliferation of VSMC compared to control media. Although the release of single adipokines by these two fat depots may certainly differ, these data show that the proliferative effect of subcutaneous fat is comparable to that observed with epicardial fat. Despite the fact that arteries are not in direct contact to subcutaneous adipose tissue, we suggest that this fat depot may exert a strong systemic effect on SMC in the vessel wall due to its considerable amount in the obese state. Since the amount of perivascular adipose tissue from surgery is technically

restricted, it could only be used for key experiments. It should be noted that CM from explants contains secretory products from all cell types present in adipose tissue and is therefore not completely comparable to CM of *in vitro* differentiated adipocytes. However, we suggest that the mechanisms are certainly similar for perivascular and subcutaneous adipose tissue.

In conclusion, we could show for the first time that CM from *in vitro* differentiated human adipocytes induces proliferation, migration, and the expression of adhesion molecule ICAM-1 in human VSMC. Furthermore, autocrine action of adiponectin entirely abolished VSMC proliferation and expression of ICAM-1. In addition, CM induces proliferative and inflammatory signaling in VSMC (p38 MAPK, mTOR and NF-κB). We therefore propose that the elevated release of adipokines in the obese state might be a critical link between adipose tissue dysfunction, SMC dysfunction, vascular inflammation and the development of atherosclerosis.

6.4.2 Role of FFA

Beneath the increased secretion of adipokines by enlarged fat tissue, also elevated levels of FFA play an important role in the development of vascular dysfunction and cardiovascular risk. It is well-established that adipose tissue and adipocytes are secretory active from a metabolic point of view by releasing FFA into circulation. Although this FFA release is very low in our model system of *in vitro* differentiated adipocytes, it plays a crucial role in human physiology. In obesity, the regulation of fat storage and energy supply by adipose tissue is impaired so that plasma FFA levels become elevated and excessive metabolism of FFA including storage of triglycerides and high levels of FFA metabolites occurs in non-adipose tissue like liver, muscle, pancreas, heart, and cells of the blood vessels such as SMC.[75,310] These ectopic fat stores exert lipotoxic effects on peripheral tissues since these organs are not capable of storing large amounts of lipids. In the context of atherosclerosis development, the accumulation of fat around blood vessels can directly affect vascular function in a paracrine manner by the release of secretory products, such as proatherogenic adipokines including smooth muscle cell growth factors.[310] Furthermore, high amounts of perivascular fat could mechanistically contribute to the increased vascular stiffness seen in obesity. However, FFA can affect smooth muscle cells in the vessel wall either in a paracrine way, released by perivascular adipose tissue, or in an endocrine manner by its circulating plasma levels mainly released by subcutaneous fat.

Since CM in our system of *in vitro* differentiated adipocytes contains no detectable amounts of FFA, we also incubated VSMC with oleic acid (OA), a monounsaturated FA (18:1n-9), or palmitic acid (PA), a saturated FA (16:0), either alone or in combination

with adipocyte-CM. Among the different FFA in plasma, OA is the most abundant unsaturated FA and PA the most abundant saturated FA, which is due to their occurrences in food.[311] Regarding the role of OA, it was shown that obese hypertensive subjects display elevated plasma FFA, particularly OA,[312] which promotes the transition of VSMC from the contractile to the synthetic type and contributes to the formation of organized atherosclerotic plaques.[313] It is also well established that OA induces rat VSMC proliferation as well as migration and plays a central role in obesity and FA-induced atherosclerosis.[313-315] Here, we could reproduce the effect of OA on VSMC proliferation, whereas PA had no effect. The differences between OA and PA are not due to apoptosis as both FA do not induce apoptosis at the concentrations used.[316] However, different effects on VSMC proliferation and migration could be explained by a differential activation of PGC-1α expression by OA and PA.[317,318] It could be demonstrated that overexpression of PGC-1α blocked OA-induced proliferation, whereas suppression of PGC-1α by siRNA amplified these effects. By contrast, PA markedly induced PGC-1α expression.[317]

Furthermore, this study is the first to test a combination of adipokines and FA for their effects on human VSMC. Interestingly, this combination of CM and OA led to a synergistic effect of VSMC proliferation in comparison to OA and CM alone. Previous studies have shown that OA enhances the mitogenic activity of angiotensin II in rat VSMC in a synergistic way similar to the effects of CM and OA in our study.[319] In addition we could show that the combined treatment with CM and OA significantly enhanced NF-κB activation (5-6-fold) already after 5 min exposure, in comparison to the moderate effects of CM and OA alone (1-2-fold). Since NF-κB is an important transcription factor involved in the regulation of various genes responsible for its pro-inflammatory and proliferative effects, we have begun to elucidate the effects of this synergistic up-regulation by investigating the impact of CM, OA, PA, and the combination CMOA and CMPA on NF-κB target genes, namely cytosolic superoxide dismutase (SOD)-1, cell death-inducing DNA fragmentation factor-α like effector A (CIDEA), and IL-6 (Figure 6.1). Preliminary data demonstrate that only the combination of CMOA significantly decreases SOD-1 mRNA expression in comparison to CM and OA alone. SOD-1 is a homodimeric enzyme that decreases the intracellular concentration of superoxide radicals by catalyzing their dismutation to O_2 and H_2O_2, thereby serving a key anti-oxidative role.[320] A growing amount of evidence indicates that oxidative stress, characterized by an imbalance of endogenous ROS and antioxidants, contributes markedly to endothelial dysfunction and increases VSMC proliferation. [177,321] Future work will be needed to elucidate if the observed decrease of SOD-1 will result in an enhanced ROS generation, which in turn could explain the synergistic effect of CMOA on the proliferation of VSMC in our cell system.

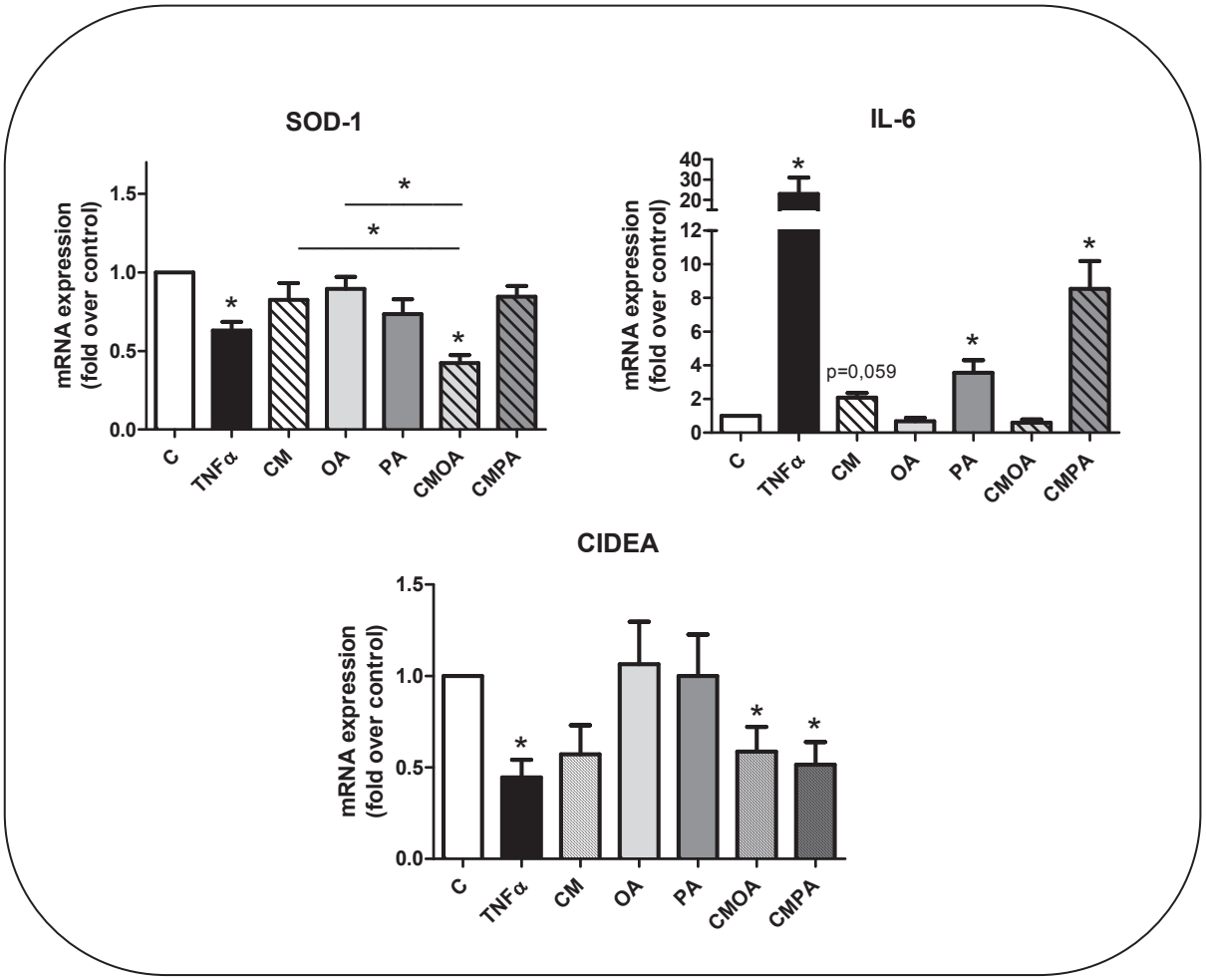

Figure 6.1 Effect of adipocyte-conditioned medium (CM), oleic acid (OA), palmitic acid (PA), and the combinations CMOA and CMPA on the mRNA expression of NF-κB target genes in human vascular smooth muscle cells (VSMC). VSMC were serum starved for 24 h and then exposed to CM, 100 µmol/l OA, 100 µmol/l PA, and the combinations CMOA and CMPA for 4 h. Total RNA was isolated with QIAcube and the RNeasy Mini Kit (QIAGEN) according to the manufacturer`s instructions and subsequently converted into cDNA using Omniscript RT Kit (QIAGEN). For RT-PCR, primers for superoxide dismutase (SOD)-1, interleukin (IL)-6, and cell death-inducing DNA fragmentation factor-α like effector A (CIDEA) were obtained from Qiagen. Samples were run in triplicates using StepOne plus (Applied Biosystems). Amplification was done using SYBR Green technology (Applied Biosystems). TNFα was used as positive control. Data are mean values ± SEM of four independent experiments and were normalized to the level of actin expression. *p≤0.05 compared to control or indicated data.

Furthermore we could show that PA increases IL-6 mRNA expression (3.5-fold) in VSMC. This is in accordance with a study showing that PA induces IL-6 mRNA and protein expression in human endothelial and smooth muscle cells.[322] Interestingly, the combination of CMPA increases IL-6 mRNA levels in a synergistic way (8.5-fold) in comparison to CM and PA alone. Since the combination of CMPA does not induce a

synergistic activation of NF-κB (data not shown), it can be suggested that IL-6 expression is mediated by distinct mechanisms. In this context, it could recently be observed that TGFβ triggers inflammatory responses e.g. the induction of IL-6 release in airway SMC by a yet unknown mechanism.[323] Furthermore, it could be demonstrated that IL-6 release of aortic SMC after exposure to lysophosphatidic acid (LPA), produced by activated platelets and formed in oxidized LDL, is due to PKC-induced p38 MAPK activation.[324] Therefore, further studies have to elucidate the precise mechanisms responsible for the synergistic effect of CMPA on the expression of IL-6. Regarding the known effects of IL-6 on the induction of VSMC migration,[201] it would be interesting to elucidate the effects of CMPA in our cell system.

In addition, we could show that the combined treatment with CMOA and CMPA significantly decreases the expression of CIDEA mRNA levels in comparison to CM, OA, and PA alone. CIDEA is a lipid droplet coating protein, which colocalizes with perilipin, a regulator of lipolysis.[325] In mice CIDEA expression was found largely to be restricted to brown adipose tissue,[326] whereas in humans CIDEA is highly expressed in white adipose tissue.[327,328] A previous study could show that the expression of CIDEA in adipose tissue inversely correlated with whole-body insulin resistance in lean versus obese subjects.[327] In this context, CIDEA is suggested to enhance the storage of triglycerides in lipid droplets of adipose tissue, decreasing FA levels in the circulation, thereby protecting muscle and liver from high FFA levels that impair insulin sensitivity.[325] However, CIDEA expression in SMC has never been investigated. We could show that only the combination of CMOA enhances the accumulation of lipids in VSMC (preliminary data). Here, the downregulation of CIDEA would enhance the release of ectopically stored lipids, suggesting a role in decreasing the amount of lipids in these stores. However, the consequences of CIDEA downregulation in smooth muscle cell biology, also with regard to other known lipid droplet coating proteins in this complex setting (e.g. perilipin, CIDEC, ADRP), is only incompletely understood and should be further investigated.

To summarize the presented work, combined proteomic profiling of *in vitro* differentiated human adipocytes resulted in the identification of 44 novel adipokines, which were predicted to be secreted by expression data screening, signal sequence prediction programs, and literature research. Among these, the five most interesting candidates, namely HO-1, CRYAB, CFH, CILP, and DPP4 (figure 6.2) were further validated and characterized concerning their expression in adipose tissue and adipose tissue-derived macrophages and their serum concentration in lean and obese subjects. In case of DPP4 also the protein expression in subcutaneous vs. visceral adipose tissue was investigated.

Furthermore, proteomic profiling revealed PEDF as a high abundant adipokine. Here we could show that PEDF induces inflammatory signaling and insulin resistance in adipocytes as well as skeletal and smooth muscle cells.

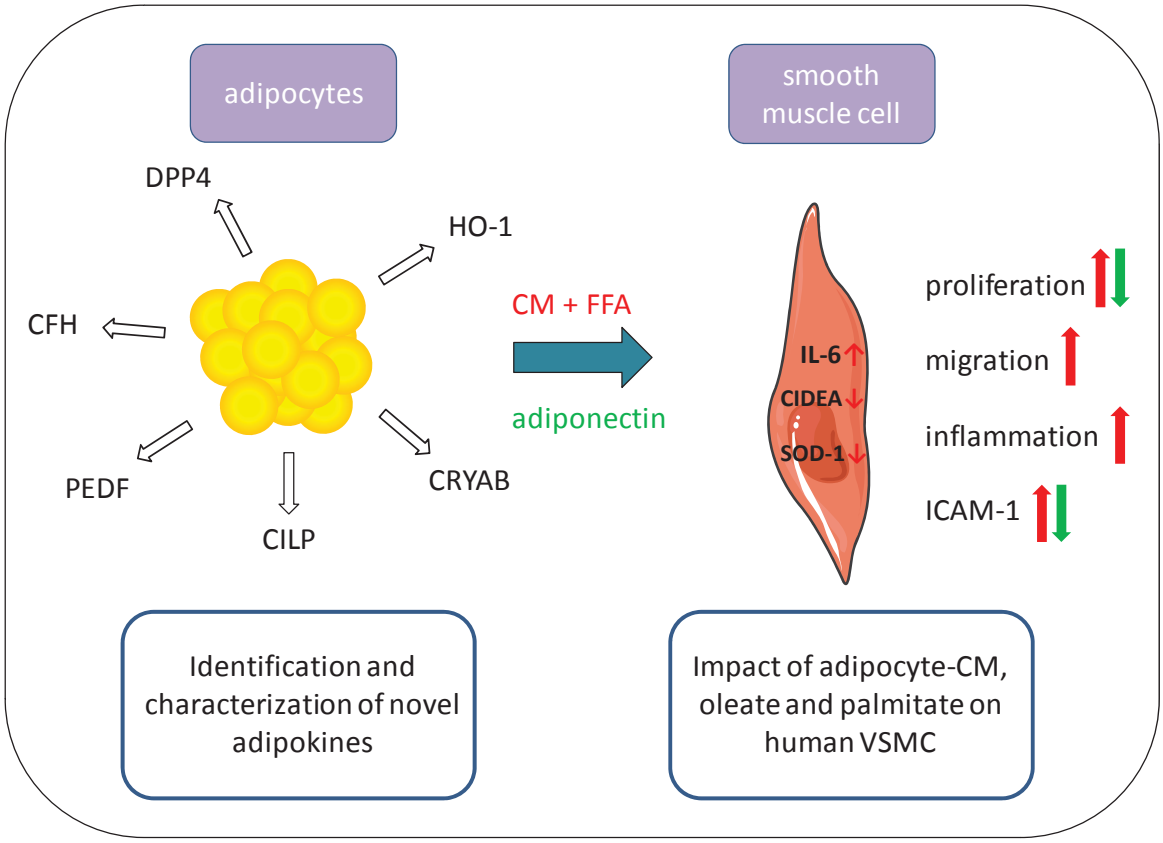

Figure 6.2 Summary of the presented work. DPP4, dipeptidyl peptidase 4; CFH, complement factor H; PEDF, pigment epithelium-derived factor; CILP, cartilage intermediate layer protein; CRYAB, αB-crystallin; HO-1, heme oxygenase 1; CM, adipocyte-conditioned medium; FFA, free fatty acids; IL-6, interleukin-6; SOD-1, superoxide dismutase 1; CIDEA, cell death-inducing DNA fragmentation factor-α like effector A; ICAM-1, intercellular adhesion molecule-1

Investigating the crosstalk of adipocytes and VSMC in the vascular wall, the whole secretory output of adipocytes in terms of CM induces proliferation and migration of VSMC. Furthermore, CM activates proliferative and inflammatory signaling mediators like mTOR, p38 MAPK, and NF-κB and enhances the expression of the adhesion molecule ICAM-1. We could further demonstrate that autocrine action of adiponectin inhibits CM-induced proliferation and ICAM-1 expression. The combined treatment of CM and OA augments proliferation and NF-κB activation in a synergistic way. RT-PCR profiling of distinct NF-κB target genes revealed that CMOA significantly reduced SOD-1 and CIDEA

mRNA levels in comparison to CM and OA alone, whereas the combination of CMPA increases the expression of IL-6 in a synergistic way.

6.5 Perspectives

The dissertation presented here met several objectives put forward in the introduction. With combined proteomic profiling using CM of *in vitro* differentiated human adipocytes, we could identify 44 novel adipokines, of which the five most interesting candidates were further validated and characterized. Furthermore, secretome analysis revealed one adipokine to be high abundantly expressed and secreted by human adipocytes. Proteomic profiling represents a crucial method to unravel the complexity of the whole secretory output of adipocytes or adipose tissue. Consequently, future work has to elucidate the precise role of novel or high abundant adipokines regarding their impact on obesity-related disorders like type 2 diabetes and CVD. In this context, the established *in vitro* model system of our working group using primary skeletal or smooth muscle cells provides an important tool to study the crosstalk between fat, either in terms of single adipokines or adipocyte-CM, and peripheral tissues like skeletal muscle or cells of the vascular wall. In this regard, the effects of novel adipokines on insulin signaling or inflammatory pathways in skeletal and smooth muscle cells should further be investigated. Additionally, future work should rule out the impact of these adipokines on atherogenic processes like proliferation and migration of smooth muscle cells.

In the other part of this work, it was shown that proliferation and migration was induced by adipocyte-CM, representing the whole secretory output of adipocytes. Here, we could also demonstrate that CM enhances ICAM-1 expression and inflammatory signaling in vascular smooth muscle cells. The autocrine action of adiponectin entirely abrogated the CM-induced proliferation and ICAM-1 expression. Several studies could show that beside the increased release of adipokines also the enhanced release of FFA is associated with cardiovascular risk. In this regard, the absence of FFA in CM in our model system provides a good tool to study the effects of adipokines and lipid mediators either separately or in combination. The combined treatment of CM and OA synergistically induced proliferation and activation NF-κB in VSMC in comparison to CM and OA alone. The underlying mechanisms responsible for these observed effects are not yet elucidated. The investigation of distinct NF-κB target genes revealed that the expression of cytosolic SOD-1 mRNA levels are only significantly decreased after treatment with the combination of CM and OA, probably leading to the enhanced production of ROS, which could explain the synergistic effect of CMOA on proliferation. In this context, further studies have to investigate the mechanisms leading to these adverse observed effects. Furthermore, this model system provides a promising tool to study the effects of several

other FFA and to further assess the effects of a combination of specific FFA on human VSMC, which have not been investigated so far.

Bibliography

1. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 1998; 15/7: 539-553.
2. Kerner W, Brückel J, Böhm BO. Definition, Klassifikation und Diagnostik des Diabetes mellitus. *Evidenzbasierte Leitlinie DDG* 2004.
3. Bluestone JA, Herold K, Eisenbarth G. Genetics, pathogenesis and clinical interventions in type 1 diabetes. *Nature* 2010; 464/7293: 1293-1300.
4. American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2010; 33 Suppl 1: S62-S69.
5. Zimmet P, Magliano D, Matsuzawa Y, Alberti G, Shaw J. The metabolic syndrome: a global public health problem and a new definition. *J Atheroscler Thromb* 2005; 12/6: 295-300.
6. Multiple authors. *Diabetes Atlas (International Diabetes Federation, Brussels) 3rd edn* 2007.
7. Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res Clin Pract* 2010; 87/1: 4-14.
8. American Diabetes Association. Economic costs of diabetes in the U.S. In 2007. *Diabetes Care* 2008; 31/3: 596-615.
9. Hauner H. Diabetesepidemie und Dunkelziffer. *Deutscher Gesundheitsbericht - Diabetes* 2007.
10. Rathmann W, Haastert B, Icks A, Lowel H, Meisinger C, Holle R, Giani G. High prevalence of undiagnosed diabetes mellitus in Southern Germany: target populations for efficient screening. The KORA survey 2000. *Diabetologia* 2003; 46/2: 182-189.
11. Wabitsch M, Hauner H, Hertrampf M, Muche R, Hay B, Mayer H, Kratzer W, Debatin KM, Heinze E. Type II diabetes mellitus and impaired glucose regulation in Caucasian children and adolescents with obesity living in Germany. *Int J Obes Relat Metab Disord* 2004; 28/2: 307-313.
12. Moss A, Wabitsch M, Kromeyer-Hauschild K, Reinehr T, Kurth BM. [Prevalence of overweight and adiposity in German school children]. *Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz* 2007; 50: 1424-1431.
13. Coccheri S. Approaches to prevention of cardiovascular complications and events in diabetes mellitus. *Drugs* 2007; 67/7: 997-1026.
14. Koistinen HA, Zierath JR. Regulation of glucose transport in human skeletal muscle. *Ann Med* 2002; 34/6: 410-418.
15. DeFronzo RA, Tripathy D. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. *Diabetes Care* 2009; 32 Suppl 2: S157-S163.
16. Krook A, Bjornholm M, Galuska D, Jiang XJ, Fahlman R, Myers MG, Jr., Wallberg-Henriksson H, Zierath JR. Characterization of signal transduction and glucose

transport in skeletal muscle from type 2 diabetic patients. *Diabetes* 2000; 49/2: 284-292.
17. Velho G, Robert JJ. Maturity-onset diabetes of the young (MODY): genetic and clinical characteristics. *Horm Res* 2002; 57 Suppl 1: 29-33.
18. Naik RG, Palmer JP. Latent autoimmune diabetes in adults (LADA). *Rev Endocr Metab Disord* 2003; 4/3: 233-241.
19. Urakami T, Miyamoto Y, Matsunaga H, Owada M, Kitagawa T. Serial changes in the prevalence of islet cell antibodies and islet cell antibody titer in children with IDDM of abrupt or slow onset. *Diabetes Care* 1995; 18/8: 1095-1099.
20. Naik RG, Brooks-Worrell BM, Palmer JP. Latent autoimmune diabetes in adults. *J Clin Endocrinol Metab* 2009; 94/12: 4635-4644.
21. Howard BV, Rodriguez BL, Bennett PH, Harris MI, Hamman R, Kuller LH, Pearson TA, Wylie-Rosett J. Prevention Conference VI: Diabetes and Cardiovascular disease: Writing Group I: epidemiology. *Circulation* 2002; 105/18: e132-e137.
22. Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 2001; 414/6865: 799-806.
23. Zhu X, Orci L, Carroll R, Norrbom C, Ravazzola M, Steiner DF. Severe block in processing of proinsulin to insulin accompanied by elevation of des-64,65 proinsulin intermediates in islets of mice lacking prohormone convertase 1/3. *Proc Natl Acad Sci U S A* 2002; 99/16: 10299-10304.
24. White MF. The insulin signalling system and the IRS proteins. *Diabetologia* 1997; 40 Suppl 2: S2-17.
25. Rudich A, Klip A. Push/pull mechanisms of GLUT4 traffic in muscle cells. *Acta Physiol Scand* 2003; 178/4: 297-308.
26. Watson RT, Khan AH, Furukawa M, Hou JC, Li L, Kanzaki M, Okada S, Kandror KV, Pessin JE. Entry of newly synthesized GLUT4 into the insulin-responsive storage compartment is GGA dependent. *EMBO J* 2004; 23/10: 2059-2070.
27. Zorzano A, Palacin M, Guma A. Mechanisms regulating GLUT4 glucose transporter expression and glucose transport in skeletal muscle. *Acta Physiol Scand* 2005; 183/1: 43-58.
28. Czech MP, Corvera S. Signaling mechanisms that regulate glucose transport. *J Biol Chem* 1999; 274/4: 1865-1868.
29. Ueki K, Yamamoto-Honda R, Kaburagi Y, Yamauchi T, Tobe K, Burgering BM, Coffer PJ, Komuro I, Akanuma Y, Yazaki Y, Kadowaki T. Potential role of protein kinase B in insulin-induced glucose transport, glycogen synthesis, and protein synthesis. *J Biol Chem* 1998; 273/9: 5315-5322.
30. Ishiki M, Klip A. Minireview: recent developments in the regulation of glucose transporter-4 traffic: new signals, locations, and partners. *Endocrinology* 2005; 146/12: 5071-5078.
31. Trayhurn P, Beattie JH. Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. *Proc Nutr Soc* 2001; 60/3: 329-339.
32. Gesta S, Tseng YH, Kahn CR. Developmental origin of fat: tracking obesity to its source. *Cell* 2007; 131/2: 242-256.

33. de Ferranti S, Mozaffarian D. The perfect storm: obesity, adipocyte dysfunction, and metabolic consequences. *Clin Chem* 2008; 54/6: 945-955.
34. Krotkiewski M, Bjorntorp P, Sjostrom L, Smith U. Impact of obesity on metabolism in men and women. Importance of regional adipose tissue distribution. *J Clin Invest* 1983; 72/3: 1150-1162.
35. Klein S, Fontana L, Young VL, Coggan AR, Kilo C, Patterson BW, Mohammed BS. Absence of an effect of liposuction on insulin action and risk factors for coronary heart disease. *N Engl J Med* 2004; 350/25: 2549-2557.
36. Rattarasarn C, Leelawattana R, Soonthornpun S, Setasuban W, Thamprasit A. Gender differences of regional abdominal fat distribution and their relationships with insulin sensitivity in healthy and glucose-intolerant Thais. *J Clin Endocrinol Metab* 2004; 89/12: 6266-6270.
37. Bjorntorp P. Effects of age, sex, and clinical conditions on adipose tissue cellularity in man. *Metabolism* 1974; 23/11: 1091-1102.
38. Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, Blomqvist L, Hoffstedt J, Naslund E, Britton T, Concha H, Hassan M, Ryden M, Frisen J, Arner P. Dynamics of fat cell turnover in humans. *Nature* 2008; 453/7196: 783-787.
39. Crossno JT, Jr., Majka SM, Grazia T, Gill RG, Klemm DJ. Rosiglitazone promotes development of a novel adipocyte population from bone marrow-derived circulating progenitor cells. *J Clin Invest* 2006; 116/12: 3220-3228.
40. Permana PA, Nair S, Lee YH, Luczy-Bachman G, Vozarova De Court, Tataranni PA. Subcutaneous abdominal preadipocyte differentiation in vitro inversely correlates with central obesity. *Am J Physiol Endocrinol Metab* 2004; 286/6: E958-E962.
41. Tchoukalova Y, Koutsari C, Jensen M. Committed subcutaneous preadipocytes are reduced in human obesity. *Diabetologia* 2007; 50/1: 151-157.
42. Isakson P, Hammarstedt A, Gustafson B, Smith U. Impaired preadipocyte differentiation in human abdominal obesity: role of Wnt, tumor necrosis factor-alpha, and inflammation. *Diabetes* 2009; 58/7: 1550-1557.
43. Rondinone CM, Rodbard D, Baker ME. Aldosterone stimulated differentiation of mouse 3T3-L1 cells into adipocytes. *Endocrinology* 1993; 132/6: 2421-2426.
44. Jiang W, Miyamoto T, Kakizawa T, Sakuma T, Nishio S, Takeda T, Suzuki S, Hashizume K. Expression of thyroid hormone receptor alpha in 3T3-L1 adipocytes; triiodothyronine increases the expression of lipogenic enzyme and triglyceride accumulation. *J Endocrinol* 2004; 182/2: 295-302.
45. Hauner H, Schmid P, Pfeiffer EF. Glucocorticoids and insulin promote the differentiation of human adipocyte precursor cells into fat cells. *J Clin Endocrinol Metab* 1987; 64/4: 832-835.
46. Richelsen B. Action of growth hormone in adipose tissue. *Horm Res* 1997; 48 Suppl 5: 105-110.
47. White UA, Stephens JM. Transcriptional factors that promote formation of white adipose tissue. *Mol Cell Endocrinol* 2010; 318/1-2: 10-14.

48. Knouff C, Auwerx J. Peroxisome proliferator-activated receptor-gamma calls for activation in moderation: lessons from genetics and pharmacology. *Endocr Rev* 2004; 25/6: 899-918.
49. Bjorntorp P. Fat cell distribution and metabolism. *Ann N Y Acad Sci* 1987; 499: 66-72.
50. Kissebah AH, Vydelingum N, Murray R, Evans DJ, Hartz AJ, Kalkhoff RK, Adams PW. Relation of body fat distribution to metabolic complications of obesity. *J Clin Endocrinol Metab* 1982; 54/2: 254-260.
51. Smith J, Al Amri M, Dorairaj P, Sniderman A. The adipocyte life cycle hypothesis. *Clin Sci (Lond)* 2006; 110/1: 1-9.
52. Gustafson B, Hammarstedt A, Andersson CX, Smith U. Inflamed adipose tissue: a culprit underlying the metabolic syndrome and atherosclerosis. *Arterioscler Thromb Vasc Biol* 2007; 27/11: 2276-2283.
53. Gregor MG, Hotamisligil GS. Adipocyte stress: The endoplasmic reticulum and metabolic disease. *J Lipid Res* 2007.
54. Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell* 2010; 140/6: 900-917.
55. Bourlier V, Zakaroff-Girard A, Miranville A, De Barros S, Maumus M, Sengenes C, Galitzky J, Lafontan M, Karpe F, Frayn KN, Bouloumie A. Remodeling phenotype of human subcutaneous adipose tissue macrophages. *Circulation* 2008; 117/6: 806-815.
56. Pang C, Gao Z, Yin J, Zhang J, Jia W, Ye J. Macrophage infiltration into adipose tissue may promote angiogenesis for adipose tissue remodeling in obesity. *Am J Physiol Endocrinol Metab* 2008; 295/2: E313-E322.
57. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003; 112/12: 1796-1808.
58. Odegaard JI, Ricardo-Gonzalez RR, Goforth MH, Morel CR, Subramanian V, Mukundan L, Red EA, Vats D, Brombacher F, Ferrante AW, Chawla A. Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. *Nature* 2007; 447/7148: 1116-1120.
59. Olefsky JM, Glass CK. Macrophages, inflammation, and insulin resistance. *Annu Rev Physiol* 2010; 72: 219-246.
60. Ohmura K, Ishimori N, Ohmura Y, Tokuhara S, Nozawa A, Horii S, Andoh Y, Fujii S, Iwabuchi K, Onoe K, Tsutsui H. Natural killer T cells are involved in adipose tissues inflammation and glucose intolerance in diet-induced obese mice. *Arterioscler Thromb Vasc Biol* 2010; 30/2: 193-199.
61. Duffaut C, Galitzky J, Lafontan M, Bouloumie A. Unexpected trafficking of immune cells within the adipose tissue during the onset of obesity. *Biochem Biophys Res Commun* 2009; 384/4: 482-485.
62. Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A, Lee J, Goldfine AB, Benoist C, Shoelson S, Mathis D. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat Med* 2009; 15/8: 930-939.

63. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, Otsu M, Hara K, Ueki K, Sugiura S, Yoshimura K, Kadowaki T, Nagai R. CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nat Med* 2009; 15/8: 914-920.
64. Kang K, Reilly SM, Karabacak V, Gangl MR, Fitzgerald K, Hatano B, Lee CH. Adipocyte-derived Th2 cytokines and myeloid PPARdelta regulate macrophage polarization and insulin sensitivity. *Cell Metab* 2008; 7/6: 485-495.
65. Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. *J Clin Invest* 2006; 116/7: 1793-1801.
66. Howard JK, Flier JS. Attenuation of leptin and insulin signaling by SOCS proteins. *Trends Endocrinol Metab* 2006; 17/9: 365-371.
67. Lebrun P, Van Obberghen E. SOCS proteins causing trouble in insulin action. *Acta Physiol (Oxf)* 2008; 192/1: 29-36.
68. Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, Shoelson SE. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* 2005; 11/2: 183-190.
69. Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS. A central role for JNK in obesity and insulin resistance. *Nature* 2002; 420/6913: 333-336.
70. Hotamisligil GS. Role of endoplasmic reticulum stress and c-Jun NH2-terminal kinase pathways in inflammation and origin of obesity and diabetes. *Diabetes* 2005; 54 Suppl 2: S73-S78.
71. Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M, Shoelson SE. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. *Science* 2001; 293/5535: 1673-1677.
72. Bandyopadhyay GK, Yu JG, Ofrecio J, Olefsky JM. Increased p85/55/50 expression and decreased phosphotidylinositol 3-kinase activity in insulin-resistant human skeletal muscle. *Diabetes* 2005; 54/8: 2351-2359.
73. Jager J, Gremeaux T, Cormont M, Marchand-Brustel Y, Tanti JF. Interleukin-1beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression. *Endocrinology* 2007; 148/1: 241-251.
74. Sell H, Dietze-Schroeder D, Kaiser U, Eckel J. Monocyte chemotactic protein-1 is a potential player in the negative cross-talk between adipose tissue and skeletal muscle. *Endocrinology* 2006; 147/5: 2458-2467.
75. Assimacopoulos-Jeannet F. Fat storage in pancreas and in insulin-sensitive tissues in pathogenesis of type 2 diabetes. *Int J Obes Relat Metab Disord* 2004; 28 Suppl 4: S53-S57.
76. Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW, Shulman GI. Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J Biol Chem* 2002; 277/52: 50230-50236.

77. Belfort R, Mandarino L, Kashyap S, Wirfel K, Pratipanawatr T, Berria R, DeFronzo RA, Cusi K. Dose-response effect of elevated plasma free fatty acid on insulin signaling. *Diabetes* 2005; 54/6: 1640-1648.
78. Gao Z, Zhang X, Zuberi A, Hwang D, Quon MJ, Lefevre M, Ye J. Inhibition of insulin sensitivity by free fatty acids requires activation of multiple serine kinases in 3T3-L1 adipocytes. *Mol Endocrinol* 2004; 18/8: 2024-2034.
79. Festa A, D'Agostino R, Jr., Tracy RP, Haffner SM. Elevated levels of acute-phase proteins and plasminogen activator inhibitor-1 predict the development of type 2 diabetes: the insulin resistance atherosclerosis study. *Diabetes* 2002; 51/4: 1131-1137.
80. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 1993; 259/5091: 87-91.
81. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am J Physiol Endocrinol Metab* 2001; 280/5: E745-E751.
82. Moller DE. Potential role of TNF-alpha in the pathogenesis of insulin resistance and type 2 diabetes. *Trends Endocrinol Metab* 2000; 11/6: 212-217.
83. Halse R, Pearson SL, McCormack JG, Yeaman SJ, Taylor R. Effects of tumor necrosis factor-alpha on insulin action in cultured human muscle cells. *Diabetes* 2001; 50/5: 1102-1109.
84. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* 1997; 389/6651: 610-614.
85. Schreyer SA, Chua SC, Jr., LeBoeuf RC. Obesity and diabetes in TNF-alpha receptor- deficient mice. *J Clin Invest* 1998; 102/2: 402-411.
86. Tam LS, Tomlinson B, Chu TT, Li TK, Li EK. Impact of TNF inhibition on insulin resistance and lipids levels in patients with rheumatoid arthritis. *Clin Rheumatol* 2007; 26/9: 1495-1498.
87. Steinberg GR, McAinch AJ, Chen MB, O'Brien PE, Dixon JB, Cameron-Smith D, Kemp BE. The suppressor of cytokine signaling 3 inhibits leptin activation of AMP-kinase in cultured skeletal muscle of obese humans. *J Clin Endocrinol Metab* 2006; 91/9: 3592-3597.
88. Bluher M, Kratzsch J, Paschke R. Plasma levels of tumor necrosis factor-alpha, angiotensin II, growth hormone, and IGF-I are not elevated in insulin-resistant obese individuals with impaired glucose tolerance. *Diabetes Care* 2001; 24/2: 328-334.
89. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994; 372/6505: 425-432.
90. Chen H, Charlat O, Tartaglia LA, Woolf EA, Weng X, Ellis SJ, Lakey ND, Culpepper J, Moore KJ, Breitbart RE, Duyk GM, Tepper RI, Morgenstern JP. Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell* 1996; 84/3: 491-495.

91. Ahima RS, Flier JS. Leptin. *Annu Rev Physiol* 2000; 62: 413-437.
92. Havel PJ. Control of energy homeostasis and insulin action by adipocyte hormones: leptin, acylation stimulating protein, and adiponectin. *Curr Opin Lipidol* 2002; 13/1: 51-59.
93. Della-Fera MA, Qian H, Baile CA. Adipocyte apoptosis in the regulation of body fat mass by leptin. *Diabetes Obes Metab* 2001; 3/5: 299-310.
94. Muzzin P, Eisensmith RC, Copeland KC, Woo SL. Correction of obesity and diabetes in genetically obese mice by leptin gene therapy. *Proc Natl Acad Sci U S A* 1996; 93/25: 14804-14808.
95. Yuan CS, Attele AS, Zhang L, Lynch JP, Xie JT, Shi ZQ. Leptin reduces body weight gain in neonatal rats. *Pediatr Res* 2000; 48/3: 380-383.
96. Cohen B, Novick D, Rubinstein M. Modulation of insulin activities by leptin. *Science* 1996; 274/5290: 1185-1188.
97. Sweeney G, Keen J, Somwar R, Konrad D, Garg R, Klip A. High leptin levels acutely inhibit insulin-stimulated glucose uptake without affecting glucose transporter 4 translocation in l6 rat skeletal muscle cells. *Endocrinology* 2001; 142/11: 4806-4812.
98. Campfield LA, Smith FJ, Burn P. The OB protein (leptin) pathway--a link between adipose tissue mass and central neural networks. *Horm Metab Res* 1996; 28/12: 619-632.
99. Zierath JR, Frevert EU, Ryder JW, Berggren PO, Kahn BB. Evidence against a direct effect of leptin on glucose transport in skeletal muscle and adipocytes. *Diabetes* 1998; 47/1: 1-4.
100. Fasshauer M, Paschke R. Regulation of adipocytokines and insulin resistance. *Diabetologia* 2003; 46/12: 1594-1603.
101. Kim KH, Lee K, Moon YS, Sul HS. A cysteine-rich adipose tissue-specific secretory factor inhibits adipocyte differentiation. *J Biol Chem* 2001; 276/14: 11252-11256.
102. Patel L, Buckels AC, Kinghorn IJ, Murdock PR, Holbrook JD, Plumpton C, Macphee CH, Smith SA. Resistin is expressed in human macrophages and directly regulated by PPAR gamma activators. *Biochem Biophys Res Commun* 2003; 300/2: 472-476.
103. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA. The hormone resistin links obesity to diabetes. *Nature* 2001; 409/6818: 307-312.
104. Savage DB, Sewter CP, Klenk ES, Segal DG, Vidal-Puig A, Considine RV, O'Rahilly S. Resistin / Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans. *Diabetes* 2001; 50/10: 2199-2202.
105. Satoh H, Nguyen MT, Miles PD, Imamura T, Usui I, Olefsky JM. Adenovirus-mediated chronic "hyper-resistinemia" leads to in vivo insulin resistance in normal rats. *J Clin Invest* 2004; 114/2: 224-231.
106. Muse ED, Obici S, Bhanot S, Monia BP, McKay RA, Rajala MW, Scherer PE, Rossetti L. Role of resistin in diet-induced hepatic insulin resistance. *J Clin Invest* 2004; 114/2: 232-239.
107. Kralisch S, Klein J, Bluher M, Paschke R, Stumvoll M, Fasshauer M. Therapeutic perspectives of adipocytokines. *Expert Opin Pharmacother* 2005; 6/6: 863-872.

108. Lehrke M, Reilly MP, Millington SC, Iqbal N, Rader DJ, Lazar MA. An inflammatory cascade leading to hyperresistinemia in humans. *PLoS Med* 2004; 1/2: e45.
109. Gambino R, Bo S, Musso G, Uberti B, Alemanno N, Ghione F, Guidi S, Tiozzo E, Chiusano V, Gentile L, Durazzo M, Pagano G, Cassader M. Microsomal triglyceride transfer protein 493-T variant is associated with resistin levels and C-reactive protein. *Clin Biochem* 2007; 40/16-17: 1219-1224.
110. Shetty GK, Economides PA, Horton ES, Mantzoros CS, Veves A. Circulating adiponectin and resistin levels in relation to metabolic factors, inflammatory markers, and vascular reactivity in diabetic patients and subjects at risk for diabetes. *Diabetes Care* 2004; 27/10: 2450-2457.
111. Kusminski CM, da Silva NF, Creely SJ, Fisher FM, Harte AL, Baker AR, Kumar S, McTernan PG. The in vitro effects of resistin on the innate immune signaling pathway in isolated human subcutaneous adipocytes. *J Clin Endocrinol Metab* 2007; 92/1: 270-276.
112. Silswal N, Singh AK, Aruna B, Mukhopadhyay S, Ghosh S, Ehtesham NZ. Human resistin stimulates the pro-inflammatory cytokines TNF-alpha and IL-12 in macrophages by NF-kappaB-dependent pathway. *Biochem Biophys Res Commun* 2005; 334/4: 1092-1101.
113. Baggiolini M. Chemokines and leukocyte traffic. *Nature* 1998; 392/6676: 565-568.
114. Sartipy P, Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc Natl Acad Sci U S A* 2003; 100/12: 7265-7270.
115. Takahashi K, Mizuarai S, Araki H, Mashiko S, Ishihara A, Kanatani A, Itadani H, Kotani H. Adiposity elevates plasma MCP-1 levels leading to the increased CD11b-positive monocytes in mice. *J Biol Chem* 2003; 278/47: 46654-46660.
116. Christiansen T, Richelsen B, Bruun JM. Monocyte chemoattractant protein-1 is produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects. *Int J Obes (Lond)* 2005; 29/1: 146-150.
117. Kim CS, Park HS, Kawada T, Kim JH, Lim D, Hubbard NE, Kwon BS, Erickson KL, Yu R. Circulating levels of MCP-1 and IL-8 are elevated in human obese subjects and associated with obesity-related parameters. *Int J Obes (Lond)* 2006; 30/9: 1347-1355.
118. Bruun JM, Lihn AS, Pedersen SB, Richelsen B. Monocyte chemoattractant protein-1 release is higher in visceral than subcutaneous human adipose tissue (AT): implication of macrophages resident in the AT. *J Clin Endocrinol Metab* 2005; 90/4: 2282-2289.
119. Herder C, Baumert J, Thorand B, Koenig W, de Jager W, Meisinger C, Illig T, Martin S, Kolb H. Chemokines as risk factors for type 2 diabetes: results from the MONICA/KORA Augsburg study, 1984-2002. *Diabetologia* 2006; 49/5: 921-929.
120. Piemonti L, Calori G, Mercalli A, Lattuada G, Monti P, Garancini MP, Costantino F, Ruotolo G, Luzi L, Perseghin G. Fasting plasma leptin, tumor necrosis factor-alpha receptor 2, and monocyte chemoattracting protein 1 concentration in a population of glucose-tolerant and glucose-intolerant women: impact on cardiovascular mortality. *Diabetes Care* 2003; 26/10: 2883-2889.

121. Sell H, Dietze-Schroeder D, Kaiser U, Eckel J. Monocyte chemotactic protein-1 is a potential player in the negative cross-talk between adipose tissue and skeletal muscle. *Endocrinology* 2006; 147/5: 2458-2467.
122. Sell H, Eckel J. Monocyte chemotactic protein-1 and its role in insulin resistance. *Curr Opin Lipidol* 2007; 18/3: 258-262.
123. Scherer PE. Adipose tissue: from lipid storage compartment to endocrine organ. *Diabetes* 2006; 55/6: 1537-1545.
124. Wang Y, Lam KS, Yau MH, Xu A. Post-translational modifications of adiponectin: mechanisms and functional implications. *Biochem J* 2008; 409/3: 623-633.
125. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M, Murakami K, Ohteki T, Uchida S, Takekawa S, Waki H, Tsuno NH, Shibata Y, Terauchi Y, Froguel P, Tobe K, Koyasu S, Taira K, Kitamura T, Shimizu T, Nagai R, Kadowaki T. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 2003; 423/6941: 762-769.
126. Shapiro L, Scherer PE. The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor. *Curr Biol* 1998; 8/6: 335-338.
127. Tsao TS, Tomas E, Murrey HE, Hug C, Lee DH, Ruderman NB, Heuser JE, Lodish HF. Role of disulfide bonds in Acrp30/adiponectin structure and signaling specificity. Different oligomers activate different signal transduction pathways. *J Biol Chem* 2003; 278/50: 50810-50817.
128. Wu X, Motoshima H, Mahadev K, Stalker TJ, Scalia R, Goldstein BJ. Involvement of AMP-activated protein kinase in glucose uptake stimulated by the globular domain of adiponectin in primary rat adipocytes. *Diabetes* 2003; 52/6: 1355-1363.
129. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, Kadowaki T. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 2002; 8/11: 1288-1295.
130. Kadowaki T, Yamauchi T, Kubota N. The physiological and pathophysiological role of adiponectin and adiponectin receptors in the peripheral tissues and CNS. *FEBS Lett* 2008; 582/1: 74-80.
131. Hopkins TA, Ouchi N, Shibata R, Walsh K. Adiponectin actions in the cardiovascular system. *Cardiovasc Res* 2007; 74/1: 11-18.
132. Leth H, Andersen KK, Frystyk J, Tarnow L, Rossing P, Parving HH, Flyvbjerg A. Elevated levels of high-molecular-weight adiponectin in type 1 diabetes. *J Clin Endocrinol Metab* 2008; 93/8: 3186-3191.
133. Lihn AS, Pedersen SB, Richelsen B. Adiponectin: action, regulation and association to insulin sensitivity. *Obes Rev* 2005; 6/1: 13-21.
134. Semple RK, Soos MA, Luan J, Mitchell CS, Wilson JC, Gurnell M, Cochran EK, Gorden P, Chatterjee VK, Wareham NJ, O'Rahilly S. Elevated plasma adiponectin in humans with genetically defective insulin receptors. *J Clin Endocrinol Metab* 2006; 91/8: 3219-3223.

135. Pedersen BK, Febbraio MA. Point: Interleukin-6 does have a beneficial role in insulin sensitivity and glucose homeostasis. *J Appl Physiol* 2007; 102/2: 814-816.
136. Stith RD, Luo J. Endocrine and carbohydrate responses to interleukin-6 in vivo. *Circ Shock* 1994; 44/4: 210-215.
137. Tsigos C, Papanicolaou DA, Kyrou I, Defensor R, Mitsiadis CS, Chrousos GP. Dose-dependent effects of recombinant human interleukin-6 on glucose regulation. *J Clin Endocrinol Metab* 1997; 82/12: 4167-4170.
138. Dietze D, Ramrath S, Ritzeler O, Tennagels N, Hauner H, Eckel J. Inhibitor kappaB kinase is involved in the paracrine crosstalk between human fat and muscle cells. *Int J Obes Relat Metab Disord* 2004; 28/8: 985-992.
139. Fasshauer M, Kralisch S, Klier M, Lossner U, Bluher M, Klein J, Paschke R. Adiponectin gene expression and secretion is inhibited by interleukin-6 in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* 2003; 301/4: 1045-1050.
140. Fasshauer M, Klein J, Kralisch S, Klier M, Lossner U, Bluher M, Paschke R. Monocyte chemoattractant protein 1 expression is stimulated by growth hormone and interleukin-6 in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* 2004; 317/2: 598-604.
141. Wallenius K, Jansson JO, Wallenius V. The therapeutic potential of interleukin-6 in treating obesity. *Expert Opin Biol Ther* 2003; 3/7: 1061-1070.
142. Carey AL, Steinberg GR, Macaulay SL, Thomas WG, Holmes AG, Ramm G, Prelovsek O, Hohnen-Behrens C, Watt MJ, James DE, Kemp BE, Pedersen BK, Febbraio MA. Interleukin-6 increases insulin-stimulated glucose disposal in humans and glucose uptake and fatty acid oxidation in vitro via AMP-activated protein kinase. *Diabetes* 2006; 55/10: 2688-2697.
143. Holmes AG, Mesa JL, Neill BA, Chung J, Carey AL, Steinberg GR, Kemp BE, Southgate RJ, Lancaster GI, Bruce CR, Watt MJ, Febbraio MA. Prolonged interleukin-6 administration enhances glucose tolerance and increases skeletal muscle PPARalpha and UCP2 expression in rats. *J Endocrinol* 2008; 198/2: 367-374.
144. Weigert C, Hennige AM, Brodbeck K, Haring HU, Schleicher ED. Interleukin-6 acts as insulin sensitizer on glycogen synthesis in human skeletal muscle cells by phosphorylation of Ser473 of Akt. *Am J Physiol Endocrinol Metab* 2005; 289/2: E251-E257.
145. Alvarez-Llamas G, Szalowska E, de Vries MP, Weening D, Landman K, Hoek A, Wolffenbuttel BH, Roelofsen H, Vonk RJ. Characterization of the human visceral adipose tissue secretome. *Mol Cell Proteomics* 2007; 6/4: 589-600.
146. Chen X, Cushman SW, Pannell LK, Hess S. Quantitative proteomic analysis of the secretory proteins from rat adipose cells using a 2D liquid chromatography-MS/MS approach. *J Proteome Res* 2005; 4/2: 570-577.
147. Kim J, Choi YS, Lim S, Yea K, Yoon JH, Jun DJ, Ha SH, Kim JW, Kim JH, Suh PG, Ryu SH, Lee TG. Comparative analysis of the secretory proteome of human adipose stromal vascular fraction cells during adipogenesis. *Proteomics* 2010; 10/3: 394-405.

148. Klimcakova E, Kovacikova M, Stich V, Langin D. Adipokines and dietary interventions in human obesity. *Obes Rev* 2010; 11/6: 446-456.
149. Kratchmarova I, Kalume DE, Blagoev B, Scherer PE, Podtelejnikov AV, Molina H, Bickel PE, Andersen JS, Fernandez MM, Bunkenborg J, Roepstorff P, Kristiansen K, Lodish HF, Mann M, Pandey A. A proteomic approach for identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes. *Mol Cell Proteomics* 2002; 1/3: 213-222.
150. Molina H, Yang Y, Ruch T, Kim JW, Mortensen P, Otto T, Nalli A, Tang QQ, Lane MD, Chaerkady R, Pandey A. Temporal profiling of the adipocyte proteome during differentiation using a five-plex SILAC based strategy. *J Proteome Res* 2009; 8/1: 48-58.
151. Rosenow A, Arrey TN, Bouwman FG, Noben JP, Wabitsch M, Mariman EC, Karas M, Renes J. Identification of novel human adipocyte secreted proteins by using SGBS cells. *J Proteome Res* 2010; 9/10: 5389-5401.
152. Tsuruga H, Kumagai H, Kojima T, Kitamura T. Identification of novel membrane and secreted proteins upregulated during adipocyte differentiation. *Biochem Biophys Res Commun* 2000; 272/1: 293-297.
153. Zhong J, Krawczyk SA, Chaerkady R, Huang H, Goel R, Bader JS, Wong GW, Corkey BE, Pandey A. Temporal profiling of the secretome during adipogenesis in humans. *J Proteome Res* 2010; 9/10: 5228-5238.
154. Peinado JR, Jimenez-Gomez Y, Pulido MR, Ortega-Bellido M, Diaz-Lopez C, Padillo FJ, Lopez-Miranda J, Vazquez-Martinez R, Malagon MM. The stromal-vascular fraction of adipose tissue contributes to major differences between subcutaneous and visceral fat depots. *Proteomics* 2010; 10/18: 3356-3366.
155. Halberg N, Wernstedt-Asterholm I, Scherer PE. The adipocyte as an endocrine cell. *Endocrinol Metab Clin North Am* 2008; 37/3: 753-7xi.
156. Wang P, Mariman E, Keijer J, Bouwman F, Noben JP, Robben J, Renes J. Profiling of the secreted proteins during 3T3-L1 adipocyte differentiation leads to the identification of novel adipokines. *Cell Mol Life Sci* 2004; 61/18: 2405-2417.
157. Becerra SP. Structure-function studies on PEDF. A noninhibitory serpin with neurotrophic activity. *Adv Exp Med Biol* 1997; 425: 223-237.
158. Tombran-Tink J, Chader GG, Johnson LV. PEDF: a pigment epithelium-derived factor with potent neuronal differentiative activity. *Exp Eye Res* 1991; 53/3: 411-414.
159. Crowe S, Wu LE, Economou C, Turpin SM, Matzaris M, Hoehn KL, Hevener AL, James DE, Duh EJ, Watt MJ. Pigment epithelium-derived factor contributes to insulin resistance in obesity. *Cell Metab* 2009; 10/1: 40-47.
160. Jenkins A, Zhang SX, Gosmanova A, Aston C, Dashti A, Baker MZ, Lyons T, Ma JX. Increased serum pigment epithelium derived factor levels in Type 2 diabetes patients. *Diabetes Res Clin Pract* 2008; 82/1: e5-e7.
161. Yamagishi S, Adachi H, Abe A, Yashiro T, Enomoto M, Furuki K, Hino A, Jinnouchi Y, Takenaka K, Matsui T, Nakamura K, Imaizumi T. Elevated serum levels of pigment epithelium-derived factor in the metabolic syndrome. *J Clin Endocrinol Metab* 2006; 91/6: 2447-2450.

162. Filleur S, Nelius T, de Riese W, Kennedy RC. Characterization of PEDF: a multi-functional serpin family protein. *J Cell Biochem* 2009; 106/5: 769-775.
163. Jenkins AJ, Zhang SX, Rowley KG, Karschimkus CS, Nelson CL, Chung JS, O'Neal DN, Januszewski AS, Croft KD, Mori TA, Dragicevic G, Harper CA, Best JD, Lyons TJ, Ma JX. Increased serum pigment epithelium-derived factor is associated with microvascular complications, vascular stiffness and inflammation in Type 1 diabetes. *Diabet Med* 2007; 24/12: 1345-1351.
164. Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, Ferguson TB, Ford E, Furie K, Gillespie C, Go A, Greenlund K, Haase N, Hailpern S, Ho PM, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, Marelli A, McDermott MM, Meigs J, Mozaffarian D, Mussolino M, Nichol G, Roger VL, Rosamond W, Sacco R, Sorlie P, Roger VL, Thom T, Wasserthiel-Smoller S, Wong ND, Wylie-Rosett J. Heart disease and stroke statistics--2010 update: a report from the American Heart Association. *Circulation* 2010; 121/7: e46-e215.
165. Steg PG, Bhatt DL, Wilson PW, D'Agostino R, Sr., Ohman EM, Rother J, Liau CS, Hirsch AT, Mas JL, Ikeda Y, Pencina MJ, Goto S. One-year cardiovascular event rates in outpatients with atherothrombosis. *JAMA* 2007; 297/11: 1197-1206.
166. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med* 1999; 340/2: 115-126.
167. Witztum JL, Steinberg D. Role of oxidized low density lipoprotein in atherogenesis. *J Clin Invest* 1991; 88/6: 1785-1792.
168. Libby P. Inflammation in atherosclerosis. *Nature* 2002; 420/6917: 868-874.
169. Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest* 1994; 94/6: 2493-2503.
170. Kenchaiah S, Evans JC, Levy D, Wilson PW, Benjamin EJ, Larson MG, Kannel WB, Vasan RS. Obesity and the risk of heart failure. *N Engl J Med* 2002; 347/5: 305-313.
171. Lakka HM, Lakka TA, Tuomilehto J, Salonen JT. Abdominal obesity is associated with increased risk of acute coronary events in men. *Eur Heart J* 2002; 23/9: 706-713.
172. Hu FB, Willett WC, Li T, Stampfer MJ, Colditz GA, Manson JE. Adiposity as compared with physical activity in predicting mortality among women. *N Engl J Med* 2004; 351/26: 2694-2703.
173. van Dam RM, Willett WC, Manson JE, Hu FB. The relationship between overweight in adolescence and premature death in women. *Ann Intern Med* 2006; 145/2: 91-97.
174. Ruan H, Lodish HF. Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-alpha. *Cytokine Growth Factor Rev* 2003; 14/5: 447-455.
175. Schuett H, Luchtefeld M, Grothusen C, Grote K, Schieffer B. How much is too much? Interleukin-6 and its signalling in atherosclerosis. *Thromb Haemost* 2009; 102/2: 215-222.

176. Yudkin JS, Kumari M, Humphries SE, Mohamed-Ali V. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis* 2000; 148/2: 209-214.
177. Wang Z, Castresana MR, Newman WH. Reactive oxygen and NF-kappaB in VEGF-induced migration of human vascular smooth muscle cells. *Biochem Biophys Res Commun* 2001; 285/3: 669-674.
178. Aljada A, Ghanim H, Assian E, Dandona P. Tumor necrosis factor-alpha inhibits insulin-induced increase in endothelial nitric oxide synthase and reduces insulin receptor content and phosphorylation in human aortic endothelial cells. *Metabolism* 2002; 51/4: 487-491.
179. Rask-Madsen C, Dominguez H, Ihlemann N, Hermann T, Kober L, Torp-Pedersen C. Tumor necrosis factor-alpha inhibits insulin's stimulating effect on glucose uptake and endothelium-dependent vasodilation in humans. *Circulation* 2003; 108/15: 1815-1821.
180. Landry DB, Couper LL, Bryant SR, Lindner V. Activation of the NF-kappa B and I kappa B system in smooth muscle cells after rat arterial injury. Induction of vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1. *Am J Pathol* 1997; 151/4: 1085-1095.
181. Lieb W, Sullivan LM, Harris TB, Roubenoff R, Benjamin EJ, Levy D, Fox CS, Wang TJ, Wilson PW, Kannel WB, Vasan RS. Plasma leptin levels and incidence of heart failure, cardiovascular disease, and total mortality in elderly individuals. *Diabetes Care* 2009; 32/4: 612-616.
182. Wannamethee SG, Tchernova J, Whincup P, Lowe GD, Kelly A, Rumley A, Wallace AM, Sattar N. Plasma leptin: associations with metabolic, inflammatory and haemostatic risk factors for cardiovascular disease. *Atherosclerosis* 2007; 191/2: 418-426.
183. Wolk R, Berger P, Lennon RJ, Brilakis ES, Johnson BD, Somers VK. Plasma leptin and prognosis in patients with established coronary atherosclerosis. *J Am Coll Cardiol* 2004; 44/9: 1819-1824.
184. Qasim A, Mehta NN, Tadesse MG, Wolfe ML, Rhodes T, Girman C, Reilly MP. Adipokines, insulin resistance, and coronary artery calcification. *J Am Coll Cardiol* 2008; 52/3: 231-236.
185. Bodary PF, Gu S, Shen Y, Hasty AH, Buckler JM, Eitzman DT. Recombinant leptin promotes atherosclerosis and thrombosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 2005; 25/8: e119-e122.
186. Hasty AH, Shimano H, Osuga J, Namatame I, Takahashi A, Yahagi N, Perrey S, Iizuka Y, Tamura Y, Amemiya-Kudo M, Yoshikawa T, Okazaki H, Ohashi K, Harada K, Matsuzaka T, Sone H, Gotoda T, Nagai R, Ishibashi S, Yamada N. Severe hypercholesterolemia, hypertriglyceridemia, and atherosclerosis in mice lacking both leptin and the low density lipoprotein receptor. *J Biol Chem* 2001; 276/40: 37402-37408.
187. Kobashi C, Urakaze M, Kishida M, Kibayashi E, Kobayashi H, Kihara S, Funahashi T, Takata M, Temaru R, Sato A, Yamazaki K, Nakamura N, Kobayashi M. Adiponectin inhibits endothelial synthesis of interleukin-8. *Circ Res* 2005; 97/12: 1245-1252.

188. Ouchi N, Walsh K. Adiponectin as an anti-inflammatory factor. *Clin Chim Acta* 2007; 380/1-2: 24-30.
189. Ouchi N, Kihara S, Arita Y, Nishida M, Matsuyama A, Okamoto Y, Ishigami M, Kuriyama H, Kishida K, Nishizawa H, Hotta K, Muraguchi M, Ohmoto Y, Yamashita S, Funahashi T, Matsuzawa Y. Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation* 2001; 103/8: 1057-1063.
190. Wolf AM, Wolf D, Rumpold H, Enrich B, Tilg H. Adiponectin induces the anti-inflammatory cytokines IL-10 and IL-1RA in human leukocytes. *Biochem Biophys Res Commun* 2004; 323/2: 630-635.
191. Ouchi N, Ohishi M, Kihara S, Funahashi T, Nakamura T, Nagaretani H, Kumada M, Ohashi K, Okamoto Y, Nishizawa H, Kishida K, Maeda N, Nagasawa A, Kobayashi H, Hiraoka H, Komai N, Kaibe M, Rakugi H, Ogihara T, Matsuzawa Y. Association of hypoadiponectinemia with impaired vasoreactivity. *Hypertension* 2003; 42/3: 231-234.
192. Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, Hotta K, Nishida M, Takahashi M, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y. Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway. *Circulation* 2000; 102/11: 1296-1301.
193. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev* 2004; 84/3: 767-801.
194. Yoshida T, Owens GK. Molecular determinants of vascular smooth muscle cell diversity. *Circ Res* 2005; 96/3: 280-291.
195. Tsai EJ, Kass DA. Cyclic GMP signaling in cardiovascular pathophysiology and therapeutics. *Pharmacol Ther* 2009; 122/3: 216-238.
196. Azar ZM, Mehdi MZ, Srivastava AK. Insulin-like growth factor type-1 receptor transactivation in vasoactive peptide and oxidant-induced signaling pathways in vascular smooth muscle cells. *Can J Physiol Pharmacol* 2007; 85/1: 105-111.
197. Berk BC. Vascular smooth muscle growth: autocrine growth mechanisms. *Physiol Rev* 2001; 81/3: 999-1030.
198. Li L, Mamputu JC, Wiernsperger N, Renier G. Signaling pathways involved in human vascular smooth muscle cell proliferation and matrix metalloproteinase-2 expression induced by leptin: inhibitory effect of metformin. *Diabetes* 2005; 54/7: 2227-2234.
199. Zeadin M, Butcher M, Werstuck G, Khan M, Yee CK, Shaughnessy SG. Effect of leptin on vascular calcification in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 2009; 29/12: 2069-2075.
200. Calabro P, Samudio I, Willerson JT, Yeh ET. Resistin promotes smooth muscle cell proliferation through activation of extracellular signal-regulated kinase 1/2 and phosphatidylinositol 3-kinase pathways. *Circulation* 2004; 110/21: 3335-3340.
201. Wang Z, Newman WH. Smooth muscle cell migration stimulated by interleukin 6 is associated with cytoskeletal reorganization. *J Surg Res* 2003; 111/2: 261-266.

202. Ikeda U, Ikeda M, Oohara T, Oguchi A, Kamitani T, Tsuruya Y, Kano S. Interleukin 6 stimulates growth of vascular smooth muscle cells in a PDGF-dependent manner. *Am J Physiol* 1991; 260/5 Pt 2: H1713-H1717.
203. Nabata T, Morimoto S, Koh E, Shiraishi T, Ogihara T. Interleukin-6 stimulates c-myc expression and proliferation of cultured vascular smooth muscle cells. *Biochem Int* 1990; 20/3: 445-453.
204. Kranzhofer R, Schmidt J, Pfeiffer CA, Hagl S, Libby P, Kubler W. Angiotensin induces inflammatory activation of human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 1999; 19/7: 1623-1629.
205. Norioka K, Hara M, Harigai M, Kitani A, Hirose T, Hirose W, Suzuki K, Kawakami M, Kawagoe M, Nakamura H. Pretreatment of human vascular smooth muscle cells with interleukin-1 enhances interleukin-6 production and cell proliferation (action of IL-1 on vascular smooth muscle cells). *Autoimmunity* 1990; 7/1: 41-50.
206. Arita Y, Kihara S, Ouchi N, Maeda K, Kuriyama H, Okamoto Y, Kumada M, Hotta K, Nishida M, Takahashi M, Nakamura T, Shimomura I, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y. Adipocyte-derived plasma protein adiponectin acts as a platelet-derived growth factor-BB-binding protein and regulates growth factor-induced common postreceptor signal in vascular smooth muscle cell. *Circulation* 2002; 105/24: 2893-2898.
207. Wang Y, Lam KS, Xu JY, Lu G, Xu LY, Cooper GJ, Xu A. Adiponectin inhibits cell proliferation by interacting with several growth factors in an oligomerization-dependent manner. *J Biol Chem* 2005; 280/18: 18341-18347.
208. Bonora E, Formentini G, Calcaterra F, Lombardi S, Marini F, Zenari L, Saggiani F, Poli M, Perbellini S, Raffaelli A, Cacciatori V, Santi L, Targher G, Bonadonna R, Muggeo M. HOMA-estimated insulin resistance is an independent predictor of cardiovascular disease in type 2 diabetic subjects: prospective data from the Verona Diabetes Complications Study. *Diabetes Care* 2002; 25/7: 1135-1141.
209. Ferrannini E, Balkau B, Coppack SW, Dekker JM, Mari A, Nolan J, Walker M, Natali A, Beck-Nielsen H. Insulin resistance, insulin response, and obesity as indicators of metabolic risk. *J Clin Endocrinol Metab* 2007; 92/8: 2885-2892.
210. Hanley AJ, Williams K, Stern MP, Haffner SM. Homeostasis model assessment of insulin resistance in relation to the incidence of cardiovascular disease: the San Antonio Heart Study. *Diabetes Care* 2002; 25/7: 1177-1184.
211. Steinberg HO, Brechtel G, Johnson A, Fineberg N, Baron AD. Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *J Clin Invest* 1994; 94/3: 1172-1179.
212. Behrendt D, Ganz P. Endothelial function. From vascular biology to clinical applications. *Am J Cardiol* 2002; 90/10C: 40L-48L.
213. Cersosimo E, DeFronzo RA. Insulin resistance and endothelial dysfunction: the road map to cardiovascular diseases. *Diabetes Metab Res Rev* 2006; 22/6: 423-436.
214. Hsueh WA, Lyon CJ, Quinones MJ. Insulin resistance and the endothelium. *Am J Med* 2004; 117/2: 109-117.

215. Wang CC, Goalstone ML, Draznin B. Molecular mechanisms of insulin resistance that impact cardiovascular biology. *Diabetes* 2004; 53/11: 2735-2740.
216. Arner P. The adipocyte in insulin resistance: key molecules and the impact of the thiazolidinediones. *Trends Endocrinol Metab* 2003; 14/3: 137-145.
217. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. *J Clin Invest* 2005; 115/5: 1111-1119.
218. Sell H, Dietze-Schroeder D, Eckel J. The adipocyte-myocyte axis in insulin resistance. *Trends Endocrinol Metab* 2006; 17/10: 416-422.
219. Trujillo ME, Scherer PE. Adipose tissue-derived factors: impact on health and disease. *Endocr Rev* 2006; 27/7: 762-778.
220. Breitling R. Robust signaling networks of the adipose secretome. *Trends Endocrinol Metab* 2009; 20/1: 1-7.
221. Kitano H, Oda K, Kimura T, Matsuoka Y, Csete M, Doyle J, Muramatsu M. Metabolic syndrome and robustness tradeoffs. *Diabetes* 2004; 53 Suppl 3: S6-S15.
222. Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin MS. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res* 2005; 46/11: 2347-2355.
223. Gozzelino R, Jeney V, Soares MP. Mechanisms of cell protection by heme oxygenase-1. *Annu Rev Pharmacol Toxicol* 2010; 50: 323-354.
224. Figueiredo RT, Fernandez PL, Mourao-Sa DS, Porto BN, Dutra FF, Alves LS, Oliveira MF, Oliveira PL, Graca-Souza AV, Bozza MT. Characterization of heme as activator of Toll-like receptor 4. *J Biol Chem* 2007; 282/28: 20221-20229.
225. Soares MP, Marguti I, Cunha A, Larsen R. Immunoregulatory effects of HO-1: how does it work? *Curr Opin Pharmacol* 2009; 9/4: 482-489.
226. Wang CY, Chau LY. Heme oxygenase-1 in cardiovascular diseases: molecular mechanisms and clinical perspectives. *Chang Gung Med J* 2010; 33/1: 13-24.
227. Cheng C, Noordeloos AM, Jeney V, Soares MP, Moll F, Pasterkamp G, Serruys PW, Duckers HJ. Heme oxygenase 1 determines atherosclerotic lesion progression into a vulnerable plaque. *Circulation* 2009; 119/23: 3017-3027.
228. Kim DH, Burgess AP, Li M, Tsenovoy PL, Addabbo F, McClung JA, Puri N, Abraham NG. Heme oxygenase-mediated increases in adiponectin decrease fat content and inflammatory cytokines tumor necrosis factor-alpha and interleukin-6 in Zucker rats and reduce adipogenesis in human mesenchymal stem cells. *J Pharmacol Exp Ther* 2008; 325/3: 833-840.
229. Li M, Kim DH, Tsenovoy PL, Peterson SJ, Rezzani R, Rodella LF, Aronow WS, Ikehara S, Abraham NG. Treatment of obese diabetic mice with a heme oxygenase inducer reduces visceral and subcutaneous adiposity, increases adiponectin levels, and improves insulin sensitivity and glucose tolerance. *Diabetes* 2008; 57/6: 1526-1535.
230. Tanaka H, Nakamura S, Onda K, Tazaki T, Hirano T. Sofalcone, an anti-ulcer chalcone derivative, suppresses inflammatory crosstalk between macrophages

and adipocytes and adipocyte differentiation: implication of heme-oxygenase-1 induction. *Biochem Biophys Res Commun* 2009; 381/4: 566-571.

231. Bruun JM, Lihn AS, Madan AK, Pedersen SB, Schiott KM, Fain JN, Richelsen B. Higher production of IL-8 in visceral vs. subcutaneous adipose tissue. Implication of nonadipose cells in adipose tissue. *Am J Physiol Endocrinol Metab* 2004; 286/1: E8-13.
232. Fain JN, Madan AK, Hiler ML, Cheema P, Bahouth SW. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology* 2004; 145/5: 2273-2282.
233. Khmelewski E, Becker A, Meinertz T, Ito WD. Tissue resident cells play a dominant role in arteriogenesis and concomitant macrophage accumulation. *Circ Res* 2004; 95/6: E56-E64.
234. Lehrke M, Lazar MA. Inflamed about obesity. *Nat Med* 2004; 10/2: 126-127.
235. Sato T, Takeno M, Honma K, Yamauchi H, Saito Y, Sasaki T, Morikubo H, Nagashima Y, Takagi S, Yamanaka K, Kaneko T, Ishigatsubo Y. Heme oxygenase-1, a potential biomarker of chronic silicosis, attenuates silica-induced lung injury. *Am J Respir Crit Care Med* 2006; 174/8: 906-914.
236. Mateo I, Infante J, Sanchez-Juan P, Garcia-Gorostiaga I, Rodriguez-Rodriguez E, Vazquez-Higuera JL, Berciano J, Combarros O. Serum heme oxygenase-1 levels are increased in Parkinson's disease but not in Alzheimer's disease. *Acta Neurol Scand* 2010; 121/2: 136-138.
237. Miyazaki T, Kirino Y, Takeno M, Hama M, Ushihama A, Watanabe R, Takase K, Tachibana T, Matsumoto K, Tanaka M, Yamaji S, Ideguchi H, Tomita N, Fujita H, Ohno S, Ueda A, Ishigatsubo Y. Serum HO-1 is useful to make differential diagnosis of secondary hemophagocytic syndrome from other similar hematological conditions. *Int J Hematol* 2010; 91/2: 229-237.
238. Bao W, Song F, Li X, Rong S, Yang W, Zhang M, Yao P, Hao L, Yang N, Hu FB, Liu L. Plasma heme oxygenase-1 concentration is elevated in individuals with type 2 diabetes mellitus. *PLoS One* 2010; 5/8: e12371.
239. Schipper HM. Heme oxygenase expression in human central nervous system disorders. *Free Radic Biol Med* 2004; 37/12: 1995-2011.
240. Kirino M, Kirino Y, Takeno M, Nagashima Y, Takahashi K, Kobayashi M, Murakami S, Hirasawa T, Ueda A, Aihara M, Ikezawa Z, Ishigatsubo Y. Heme oxygenase 1 attenuates the development of atopic dermatitis-like lesions in mice: implications for human disease. *J Allergy Clin Immunol* 2008; 122/2: 290-7, 297.
241. de Jong WW, Caspers GJ, Leunissen JA. Genealogy of the alpha-crystallin--small heat-shock protein superfamily. *Int J Biol Macromol* 1998; 22/3-4: 151-162.
242. Kumar PA, Haseeb A, Suryanarayana P, Ehtesham NZ, Reddy GB. Elevated expression of alphaA- and alphaB-crystallins in streptozotocin-induced diabetic rat. *Arch Biochem Biophys* 2005; 444/2: 77-83.
243. Arrigo AP, Simon S, Gibert B, Kretz-Remy C, Nivon M, Czekalla A, Guillet D, Moulin M, Diaz-Latoud C, Vicart P. Hsp27 (HspB1) and alphaB-crystallin (HspB5) as therapeutic targets. *FEBS Lett* 2007; 581/19: 3665-3674.

244. Inagaki N, Hayashi T, Arimura T, Koga Y, Takahashi M, Shibata H, Teraoka K, Chikamori T, Yamashina A, Kimura A. Alpha B-crystallin mutation in dilated cardiomyopathy. *Biochem Biophys Res Commun* 2006; 342/2: 379-386.
245. Vicart P, Caron A, Guicheney P, Li Z, Prevost MC, Faure A, Chateau D, Chapon F, Tome F, Dupret JM, Paulin D, Fardeau M. A missense mutation in the alphaB-crystallin chaperone gene causes a desmin-related myopathy. *Nat Genet* 1998; 20/1: 92-95.
246. Horwitz J. Alpha-crystallin. *Exp Eye Res* 2003; 76/2: 145-153.
247. Rodriguez dC, Esparza-Gordillo J, Goicoechea dJ, Lopez-Trascasa M, Sanchez-Corral P. The human complement factor H: functional roles, genetic variations and disease associations. *Mol Immunol* 2004; 41/4: 355-367.
248. Peake PW, O'Grady S, Pussell BA, Charlesworth JA. Detection and quantification of the control proteins of the alternative pathway of complement in 3T3-L1 adipocytes. *Eur J Clin Invest* 1997; 27/11: 922-927.
249. Moreno-Navarrete JM, Martinez-Barricarte R, Catalan V, Sabater M, Gomez-Ambrosi J, Ortega FJ, Ricart W, Bluher M, Fruhbeck G, Rodriguez dC, Fernandez-Real JM. Complement factor H is expressed in adipose tissue in association with insulin resistance. *Diabetes* 2010; 59/1: 200-209.
250. Martinez A, Pio R, Lopez J, Cuttitta F. Expression of the adrenomedullin binding protein, complement factor H, in the pancreas and its physiological impact on insulin secretion. *J Endocrinol* 2001; 170/3: 503-511.
251. Seki S, Kawaguchi Y, Chiba K, Mikami Y, Kizawa H, Oya T, Mio F, Mori M, Miyamoto Y, Masuda I, Tsunoda T, Kamata M, Kubo T, Toyama Y, Kimura T, Nakamura Y, Ikegawa S. A functional SNP in CILP, encoding cartilage intermediate layer protein, is associated with susceptibility to lumbar disc disease. *Nat Genet* 2005; 37/6: 607-612.
252. Lorenzo P, Bayliss MT, Heinegard D. A novel cartilage protein (CILP) present in the mid-zone of human articular cartilage increases with age. *J Biol Chem* 1998; 273/36: 23463-23468.
253. Tsuruha J, Masuko-Hongo K, Kato T, Sakata M, Nakamura H, Nishioka K. Implication of cartilage intermediate layer protein in cartilage destruction in subsets of patients with osteoarthritis and rheumatoid arthritis. *Arthritis Rheum* 2001; 44/4: 838-845.
254. Gorrell MD. Dipeptidyl peptidase IV and related enzymes in cell biology and liver disorders. *Clin Sci (Lond)* 2005; 108/4: 277-292.
255. Kos K, Baker AR, Jernas M, Harte AL, Clapham JC, O'Hare JP, Carlsson L, Kumar S, McTernan PG. DPP-IV inhibition enhances the antilipolytic action of NPY in human adipose tissue. *Diabetes Obes Metab* 2009; 11/4: 285-292.
256. Yazbeck R, Howarth GS, Abbott CA. Dipeptidyl peptidase inhibitors, an emerging drug class for inflammatory disease? *Trends Pharmacol Sci* 2009; 30/11: 600-607.
257. Ahren B. Dipeptidyl peptidase-4 inhibitors: clinical data and clinical implications. *Diabetes Care* 2007; 30/6: 1344-1350.

258. Lambeir AM, Durinx C, Scharpe S, De M, I. Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. *Crit Rev Clin Lab Sci* 2003; 40/3: 209-294.
259. Cordero OJ, Salgado FJ, Nogueira M. On the origin of serum CD26 and its altered concentration in cancer patients. *Cancer Immunol Immunother* 2009; 58/11: 1723-1747.
260. Iwaki-Egawa S, Watanabe Y, Kikuya Y, Fujimoto Y. Dipeptidyl peptidase IV from human serum: purification, characterization, and N-terminal amino acid sequence. *J Biochem* 1998; 124/2: 428-433.
261. Schrader WP, West CA, Miczek AD, Norton EK. Characterization of the adenosine deaminase-adenosine deaminase complexing protein binding reaction. *J Biol Chem* 1990; 265/31: 19312-19318.
262. Bouchard L, Tchernof A, Deshaies Y, Marceau S, Lescelleur O, Biron S, Vohl MC. ZFP36: a promising candidate gene for obesity-related metabolic complications identified by converging genomics. *Obes Surg* 2007; 17/3: 372-382.
263. Filleur S, Nelius T, de Riese W, Kennedy RC. Characterization of PEDF: a multi-functional serpin family protein. *J Cell Biochem* 2009; 106/5: 769-775.
264. Notari L, Baladron V, Aroca-Aguilar JD, Balko N, Heredia R, Meyer C, Notario PM, Saravanamuthu S, Nueda ML, Sanchez-Sanchez F, Escribano J, Laborda J, Becerra SP. Identification of a lipase-linked cell membrane receptor for pigment epithelium-derived factor. *J Biol Chem* 2006; 281/49: 38022-38037.
265. Chung C, Doll JA, Gattu AK, Shugrue C, Cornwell M, Fitchev P, Crawford SE. Anti-angiogenic pigment epithelium-derived factor regulates hepatocyte triglyceride content through adipose triglyceride lipase (ATGL). *J Hepatol* 2008; 48/3: 471-478.
266. Nelson J, McFerran NV, Pivato G, Chambers E, Doherty C, Steele D, Timson DJ. The 67 kDa laminin receptor: structure, function and role in disease. *Biosci Rep* 2008; 28/1: 33-48.
267. Stitt AW, McKenna D, Simpson DA, Gardiner TA, Harriott P, Archer DB, Nelson J. The 67-kd laminin receptor is preferentially expressed by proliferating retinal vessels in a murine model of ischemic retinopathy. *Am J Pathol* 1998; 152/5: 1359-1365.
268. Donaldson EA, McKenna DJ, McMullen CB, Scott WN, Stitt AW, Nelson J. The expression of membrane-associated 67-kDa laminin receptor (67LR) is modulated in vitro by cell-contact inhibition. *Mol Cell Biol Res Commun* 2000; 3/1: 53-59.
269. Zamiri P, Masli S, Streilein JW, Taylor AW. Pigment epithelial growth factor suppresses inflammation by modulating macrophage activation. *Invest Ophthalmol Vis Sci* 2006; 47/9: 3912-3918.
270. Ho TC, Yang YC, Chen SL, Kuo PC, Sytwu HK, Cheng HC, Tsao YP. Pigment epithelium-derived factor induces THP-1 macrophage apoptosis and necrosis by the induction of the peroxisome proliferator-activated receptor gamma. *Mol Immunol* 2008; 45/4: 898-909.

271. Yamagishi S, Matsui T, Nakamura K, Imaizumi T. Pigment epithelium-derived factor (PEDF) inhibits angiotensin II-induced smooth muscle cell proliferation through its anti-oxidative properties. *Protein Pept Lett* 2007; 14/6: 615-617.
272. Sell H, Eckardt K, Taube A, Tews D, Gurgui M, Echten-Deckert G, Eckel J. Skeletal muscle insulin resistance induced by adipocyte-conditioned medium: underlying mechanisms and reversibility. *Am J Physiol Endocrinol Metab* 2008; 294/6: E1070-E1077.
273. Grant DS, Kibbey MC, Kinsella JL, Cid MC, Kleinman HK. The role of basement membrane in angiogenesis and tumor growth. *Pathol Res Pract* 1994; 190/9-10: 854-863.
274. Montuori N, Sobel ME. The 67-kDa laminin receptor and tumor progression. *Curr Top Microbiol Immunol* 1996; 213 (Pt 1): 205-214.
275. Schnaper HW, Kleinman HK, Grant DS. Role of laminin in endothelial cell recognition and differentiation. *Kidney Int* 1993; 43/1: 20-25.
276. Vacca A, Ribatti D, Roncali L, Lospalluti M, Serio G, Carrel S, Dammacco F. Melanocyte tumor progression is associated with changes in angiogenesis and expression of the 67-kilodalton laminin receptor. *Cancer* 1993; 72/2: 455-461.
277. Hinek A. Biological roles of the non-integrin elastin/laminin receptor. *Biol Chem* 1996; 377/7-8: 471-480.
278. Hishikawa K, Oemar BS, Yang Z, Luscher TF. Pulsatile stretch stimulates superoxide production and activates nuclear factor-kappa B in human coronary smooth muscle. *Circ Res* 1997; 81/5: 797-803.
279. Lawrence R, Chang LJ, Siebenlist U, Bressler P, Sonenshein GE. Vascular smooth muscle cells express a constitutive NF-kappa B-like activity. *J Biol Chem* 1994; 269/46: 28913-28918.
280. Obata H, Biro S, Arima N, Kaieda H, Kihara T, Eto H, Miyata M, Tanaka H. NF-kappa B is induced in the nuclei of cultured rat aortic smooth muscle cells by stimulation of various growth factors. *Biochem Biophys Res Commun* 1996; 224/1: 27-32.
281. Omura T, Yoshiyama M, Izumi Y, Kim S, Matsumoto R, Enomoto S, Kusuyama T, Nishiya D, Nakamura Y, Akioka K, Iwao H, Takeuchi K, Yoshikawa J. Involvement of c-Jun NH2 terminal kinase and p38MAPK in rapamycin-mediated inhibition of neointimal formation in rat carotid arteries. *J Cardiovasc Pharmacol* 2005; 46/4: 519-525.
282. Nakamura K, Yamagishi S, Matsui T, Yoshida T, Takenaka K, Jinnouchi Y, Yoshida Y, Ueda S, Adachi H, Imaizumi T. Pigment epithelium-derived factor inhibits neointimal hyperplasia after vascular injury by blocking NADPH oxidase-mediated reactive oxygen species generation. *Am J Pathol* 2007; 170/6: 2159-2170.
283. Gao YJ. Dual modulation of vascular function by perivascular adipose tissue and its potential correlation with adiposity/lipoatrophy-related vascular dysfunction. *Curr Pharm Des* 2007; 13/21: 2185-2192.
284. Mahabadi AA, Reinsch N, Lehmann N, Altenbernd J, Kalsch H, Seibel RM, Erbel R, Mohlenkamp S. Association of pericoronary fat volume with atherosclerotic

plaque burden in the underlying coronary artery: a segment analysis. *Atherosclerosis* 2010; 211/1: 195-199.

285. Henrichot E, Juge-Aubry CE, Pernin A, Pache JC, Velebit V, Dayer JM, Meda P, Chizzolini C, Meier CA. Production of chemokines by perivascular adipose tissue: a role in the pathogenesis of atherosclerosis? *Arterioscler Thromb Vasc Biol* 2005; 25/12: 2594-2599.
286. Barandier C, Montani JP, Yang Z. Mature adipocytes and perivascular adipose tissue stimulate vascular smooth muscle cell proliferation: effects of aging and obesity. *Am J Physiol Heart Circ Physiol* 2005; 289/5: H1807-H1813.
287. Dietze-Schroeder D, Sell H, Uhlig M, Koenen M, Eckel J. Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors. *Diabetes* 2005; 54/7: 2003-2011.
288. Shibata R, Ouchi N, Murohara T. Adiponectin and cardiovascular disease. *Circ J* 2009; 73/4: 608-614.
289. Ryo M, Nakamura T, Kihara S, Kumada M, Shibazaki S, Takahashi M, Nagai M, Matsuzawa Y, Funahashi T. Adiponectin as a biomarker of the metabolic syndrome. *Circ J* 2004; 68/11: 975-981.
290. Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 1999; 257/1: 79-83.
291. Cnop M, Havel PJ, Utzschneider KM, Carr DB, Sinha MK, Boyko EJ, Retzlaff BM, Knopp RH, Brunzell JD, Kahn SE. Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex. *Diabetologia* 2003; 46/4: 459-469.
292. Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, Matsuzawa Y. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol* 2000; 20/6: 1595-1599.
293. Spranger J, Kroke A, Mohlig M, Bergmann MM, Ristow M, Boeing H, Pfeiffer AF. Adiponectin and protection against type 2 diabetes mellitus. *Lancet* 2003; 361/9353: 226-228.
294. Kumada M, Kihara S, Sumitsuji S, Kawamoto T, Matsumoto S, Ouchi N, Arita Y, Okamoto Y, Shimomura I, Hiraoka H, Nakamura T, Funahashi T, Matsuzawa Y. Association of hypoadiponectinemia with coronary artery disease in men. *Arterioscler Thromb Vasc Biol* 2003; 23/1: 85-89.
295. Otsuka F, Sugiyama S, Kojima S, Maruyoshi H, Funahashi T, Sakamoto T, Yoshimura M, Kimura K, Umemura S, Ogawa H. Hypoadiponectinemia is associated with impaired glucose tolerance and coronary artery disease in non-diabetic men. *Circ J* 2007; 71/11: 1703-1709.

296. Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y. Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* 1999; 100/25: 2473-2476.
297. Iwashima Y, Katsuya T, Ishikawa K, Ouchi N, Ohishi M, Sugimoto K, Fu Y, Motone M, Yamamoto K, Matsuo A, Ohashi K, Kihara S, Funahashi T, Rakugi H, Matsuzawa Y, Ogihara T. Hypoadiponectinemia is an independent risk factor for hypertension. *Hypertension* 2004; 43/6: 1318-1323.
298. Okamoto Y, Kihara S, Ouchi N, Nishida M, Arita Y, Kumada M, Ohashi K, Sakai N, Shimomura I, Kobayashi H, Terasaka N, Inaba T, Funahashi T, Matsuzawa Y. Adiponectin reduces atherosclerosis in apolipoprotein E-deficient mice. *Circulation* 2002; 106/22: 2767-2770.
299. Okamoto Y, Folco EJ, Minami M, Wara AK, Feinberg MW, Sukhova GK, Colvin RA, Kihara S, Funahashi T, Luster AD, Libby P. Adiponectin inhibits the production of CXC receptor 3 chemokine ligands in macrophages and reduces T-lymphocyte recruitment in atherogenesis. *Circ Res* 2008; 102/2: 218-225.
300. Kumada M, Kihara S, Ouchi N, Kobayashi H, Okamoto Y, Ohashi K, Maeda K, Nagaretani H, Kishida K, Maeda N, Nagasawa A, Funahashi T, Matsuzawa Y. Adiponectin specifically increased tissue inhibitor of metalloproteinase-1 through interleukin-10 expression in human macrophages. *Circulation* 2004; 109/17: 2046-2049.
301. Vinciguerra M, Veyrat-Durebex C, Moukil MA, Rubbia-Brandt L, Rohner-Jeanrenaud F, Foti M. PTEN down-regulation by unsaturated fatty acids triggers hepatic steatosis via an NF-kappaBp65/mTOR-dependent mechanism. *Gastroenterology* 2008; 134/1: 268-280.
302. Braun M, Pietsch P, Schror K, Baumann G, Felix SB. Cellular adhesion molecules on vascular smooth muscle cells. *Cardiovasc Res* 1999; 41/2: 395-401.
303. Davies MJ, Gordon JL, Gearing AJ, Pigott R, Woolf N, Katz D, Kyriakopoulos A. The expression of the adhesion molecules ICAM-1, VCAM-1, PECAM, and E-selectin in human atherosclerosis. *J Pathol* 1993; 171/3: 223-229.
304. Printseva OY, Peclo MM, Gown AM. Various cell types in human atherosclerotic lesions express ICAM-1. Further immunocytochemical and immunochemical studies employing monoclonal antibody 10F3. *Am J Pathol* 1992; 140/4: 889-896.
305. Poston RN, Haskard DO, Coucher JR, Gall NP, Johnson-Tidey RR. Expression of intercellular adhesion molecule-1 in atherosclerotic plaques. *Am J Pathol* 1992; 140/3: 665-673.
306. Huo Y, Ley K. Adhesion molecules and atherogenesis. *Acta Physiol Scand* 2001; 173/1: 35-43.
307. Cai Q, Lanting L, Natarajan R. Growth factors induce monocyte binding to vascular smooth muscle cells: implications for monocyte retention in atherosclerosis. *Am J Physiol Cell Physiol* 2004; 287/3: C707-C714.
308. Braun M, Pietsch P, Felix SB, Baumann G. Modulation of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 on human coronary smooth muscle cells by cytokines. *J Mol Cell Cardiol* 1995; 27/12: 2571-2579.

309. Couffinhal T, Duplaa C, Moreau C, Lamaziere JM, Bonnet J. Regulation of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 in human vascular smooth muscle cells. *Circ Res* 1994; 74/2: 225-234.
310. Montani JP, Carroll JF, Dwyer TM, Antic V, Yang Z, Dulloo AG. Ectopic fat storage in heart, blood vessels and kidneys in the pathogenesis of cardiovascular diseases. *Int J Obes Relat Metab Disord* 2004; 28 Suppl 4: S58-S65.
311. Katan MB, Zock PL, Mensink RP. Effects of fats and fatty acids on blood lipids in humans: an overview. *Am J Clin Nutr* 1994; 60/6 Suppl: 1017S-1022S.
312. Egan BM, Lu G, Greene EL. Vascular effects of non-esterified fatty acids: implications for the cardiovascular risk factor cluster. *Prostaglandins Leukot Essent Fatty Acids* 1999; 60/5-6: 411-420.
313. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993; 362/6423: 801-809.
314. Greene EL, Lu G, Zhang D, Egan BM. Signaling events mediating the additive effects of oleic acid and angiotensin II on vascular smooth muscle cell migration. *Hypertension* 2001; 37/2: 308-312.
315. Lu G, Morinelli TA, Meier KE, Rosenzweig SA, Egan BM. Oleic acid-induced mitogenic signaling in vascular smooth muscle cells. A role for protein kinase C. *Circ Res* 1996; 79/3: 611-618.
316. Artwohl M, Lindenmair A, Roden M, Waldhausl WK, Freudenthaler A, Klosner G, Ilhan A, Luger A, Baumgartner-Parzer SM. Fatty acids induce apoptosis in human smooth muscle cells depending on chain length, saturation, and duration of exposure. *Atherosclerosis* 2009; 202/2: 351-362.
317. Zhang Y, Liu C, Zhu L, Jiang X, Chen X, Qi X, Liang X, Jin S, Zhang P, Li Q, Wang D, Liu X, Zeng K, Zhang J, Xiang Y, Zhang CY. PGC-1alpha inhibits oleic acid induced proliferation and migration of rat vascular smooth muscle cells. *PLoS One* 2007; 2/11: e1137.
318. Zhu L, Sun G, Zhang H, Zhang Y, Chen X, Jiang X, Jiang X, Krauss S, Zhang J, Xiang Y, Zhang CY. PGC-1alpha is a key regulator of glucose-induced proliferation and migration in vascular smooth muscle cells. *PLoS One* 2009; 4/1: e4182.
319. Lu G, Meier KE, Jaffa AA, Rosenzweig SA, Egan BM. Oleic acid and angiotensin II induce a synergistic mitogenic response in vascular smooth muscle cells. *Hypertension* 1998; 31/4: 978-985.
320. Forstermann U. Oxidative stress in vascular disease: causes, defense mechanisms and potential therapies. *Nat Clin Pract Cardiovasc Med* 2008; 5/6: 338-349.
321. Madamanchi NR, Moon SK, Hakim ZS, Clark S, Mehrizi A, Patterson C, Runge MS. Differential activation of mitogenic signaling pathways in aortic smooth muscle cells deficient in superoxide dismutase isoforms. *Arterioscler Thromb Vasc Biol* 2005; 25/5: 950-956.
322. Staiger H, Staiger K, Stefan N, Wahl HG, Machicao F, Kellerer M, Haring HU. Palmitate-induced interleukin-6 expression in human coronary artery endothelial cells. *Diabetes* 2004; 53/12: 3209-3216.

323. Michaeloudes C, Sukkar MB, Khorasani NM, Bhavsar PK, Chung KF. TGF-{beta} regulates Nox4, MnSOD and catalase expression and IL-6 release in airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2010.
324. Hao F, Tan M, Wu DD, Xu X, Cui MZ. LPA induces IL-6 secretion from aortic smooth muscle cells via an LPA1-regulated, PKC-dependent, and p38alpha-mediated pathway. *Am J Physiol Heart Circ Physiol* 2010; 298/3: H974-H983.
325. Puri V, Ranjit S, Konda S, Nicoloro SM, Straubhaar J, Chawla A, Chouinard M, Lin C, Burkart A, Corvera S, Perugini RA, Czech MP. Cidea is associated with lipid droplets and insulin sensitivity in humans. *Proc Natl Acad Sci U S A* 2008; 105/22: 7833-7838.
326. Zhou Z, Yon TS, Chen Z, Guo K, Ng CP, Ponniah S, Lin SC, Hong W, Li P. Cidea-deficient mice have lean phenotype and are resistant to obesity. *Nat Genet* 2003; 35/1: 49-56.
327. Nordstrom EA, Ryden M, Backlund EC, Dahlman I, Kaaman M, Blomqvist L, Cannon B, Nedergaard J, Arner P. A human-specific role of cell death-inducing DFFA (DNA fragmentation factor-alpha)-like effector A (CIDEA) in adipocyte lipolysis and obesity. *Diabetes* 2005; 54/6: 1726-1734.
328. Su AI, Cooke MP, Ching KA, Hakak Y, Walker JR, Wiltshire T, Orth AP, Vega RG, Sapinoso LM, Moqrich A, Patapoutian A, Hampton GM, Schultz PG, Hogenesch JB. Large-scale analysis of the human and mouse transcriptomes. *Proc Natl Acad Sci U S A* 2002; 99/7: 4465-4470.

Contributions to Chapter 2-5

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

Chapter 2

- Validation and characterization of putative novel adipokines by means of Western blotting and ELISAs together with Susanne Famulla
- Isolation of adipose tissue-resident macrophages
- Literature research together with Dr. Henrike Sell, Susanne Famulla, Dr. Kristin Eckardt, Dr. Sonja Hartwig, Dr. Margriet Owens, and Prof. Jürgen Eckel
- Preparation of manuscript together with Dr. Henrike Sell, Dr. Sonja Hartwig, Dr. Stefan Lehr, Susanne Famulla and Prof. Jürgen Eckel

Chapter 3

- Differentiation-dependant protein expression of DPP4 in *in vitro* differentiated human adipocytes together with Susanne Famulla
- Analysis of DPP4 secretion in adipocyte-conditioned medium
- Analysis of DPP4 expression level in visceral and subcutaneous adipose tissue explants of lean and obese subjects by Western blotting
- Isolation of adipose tissue-resident macrophages

Chapter 4

- Analysis of PEDF secretion in adipocyte-conditioned medium by means of 2D-PAGE
- Effect of PEDF on proliferation and migration of vascular smooth muscle cells
- Effect of PEDF on inflammatory signaling pathways (p38 MAPK, NF-κB, mTOR) in smooth muscle cells
- Analysis of insulin-stimulated Akt phosphorylation after treatment with PEDF
- Isolation of adipose tissue-resident macrophages

- Preparation of manuscript together with Susanne Famulla, Dr. Henrike Sell, and Prof. Jürgen Eckel

Chapter 5

- Effect of adipocyte-conditioned medium on proliferation and migration of smooth muscle cells
- Generation of adipocyte-CM in absence or presence of adiponectin and analysis of the autocrine effect of adiponectin on smooth muscle cell proliferation and expression of ICAM-1
- Analysis of proliferative effect of CM from epicardial and subcutaneous fat explants on vascular smooth muscle cells together with Raphaela Schlich
- Preparation of manuscript together with Raphaela Schlich, Dr. Henrike Sell, and Prof. Jürgen Eckel

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Eidesstattliche Erklärung

Die vorliegende Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

(Daniela Lamers)

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