Proatherogenic crosstalk between adipose tissue and smooth muscle cells

Identification of pivotal mechanisms and mediators

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„Es gibt zwei Arten, sein Leben zu leben. Entweder so, als wäre nichts ein Wunder, oder so, als wäre alles eines. Ich glaube an Letzteres."

-Albert Einstein-

Zusammenfassung

Adipositas ist ein wichtiges Kennzeichen des metabolischen Syndroms und stellt weltweit eines der größten gesundheitlichen Probleme dar, welches häufig mit der Entwicklung von chronischen Erkrankungen, wie Typ 2 Diabetes und kardiovaskulären Erkrankungen, assoziiert ist. Interessanterweise unterliegt der Verlauf dieser Krankheiten einem komplexen Zusammenspiel zwischen einzelnen Organen, hierzu gehören die Leber, die Muskulatur, die Blutgefäße und das Fettgewebe, wobei das Fettgewebe an oberster Stelle dieser Hierarchie steht. Heutzutage wird das Fettgewebe, da es eine große Vielfalt von Proteinen und bioaktiven Peptiden, auch Adipokine genannt, sekretiert, als eines der größten endokrinen Organe angesehen. Desweiteren konnten mehrere Studien zeigen, dass bei Adipositas neben der erhöhten Sekretion von Adipokinen auch die gesteigerte Freisetzung von freien Fettsäuren mit dem Risiko für kardiovaskuläre Erkrankungen und der Entstehung von Atherosklerose assoziiert sind. In diesem Zusammenhang, untersuchten in den letzten Jahren immer mehr Studien den Einfluss von einzelnen Adipokinen mit und ohne freie Fettsäuren auf die Proliferation, Migration und Signalwege von glatten Muskelzellen, um somit neue Einblicke in die komplexen zellulären Mechanismen von Adipositas und kardiovaskuläre Erkrankungen zu erhalten. Obwohl eine Studie die Effekte von konditionierten Medien von Mauszelllinien und Ratten-Fettgewebsexplantaten untersuchte, ist der Einfluss des gesamten humanen Adipozyten-Sekretoms auf humane glatte Muskelzellen nur unvollständig entschlüsselt. Darum war das Hauptziel dieser Arbeit, den atherogenen Einfluss von Adipokinen und freien Fettsäuren auf der Ebene der humanen glatten Muskelzellen zu untersuchen. Hier konnten wir zeigen, dass konditionierte Medien von *in vitro* differenzierten humanen Adipozyten, welches selbst keine freien Fettsäuren enthält, Proliferation und Migration von vaskulären glatten Muskelzellen induzierte. Desweiteren steigerte konditioniertes Medium die Expression des Adhäsionsmoleküls ICAM-1 und aktivierte proliferative und inflammatorische Signalwege (p38 MAPK, NF-κB, und mTOR). Die Kombination von konditionierten Medien mit Ölsäure induzierte die Proliferation synergistisch und steigerte die oben genannten Signalwege stärker im Vergleich zu beiden Faktoren alleine. Außerdem erhöhte nur die gemeinsame Behandlung die Expression von iNOS und NO-Produktion, wodurch die verstärkte Freisetzung von VEGF aus den humanen vaskulären glatten Muskelzellen induziert wurde.

Interessanterweise korrelierte der proliferative Einfluss von verschiedenen konditionierten Medien hochsignifikant mit ihrem Gehalt an VEGF. Humanes rekombinantes VEGF alleine steigerte auch die Proliferation von glatten Muskelzellen und die Kombination von VEGF und Ölsäure induzierte die Proliferation auf additive Weise.

Die Blockierung von VEGF mit einem spezifischen Antikörper reduzierte signifikant die Proliferation unter allen Bedingungen. Desweiteren erhöhte die Behandlung von glatten Muskelzellen mit Ölsäure alleine oder in Kombination mit konditionierten Medien den Triglyzeridgehalt in glatten Muskelzellen signifikant. Außerdem konnten wir hier beobachten, dass konditionierte Medien die Expression des Fettsäuretransporters CD36 induzierte. Silencing von CD36 mit einer spezifischen siRNA reduzierte auch die Proliferation der glatten Muskelzellen nach Behandlung mit Ölsäure, konditionierten Medien und VEGF alleine oder in Kombination. Interessanterweise verminderte das Silencing von CD36 den durch VEGF induzierten ERK Signalweg ohne die Expression der VEGF Rezeptoren zu beeinflussen. In diesem Zusammenhang setzten perivaskuläre Fettgewebsexplantate von Diabetikern signifikant höhere Mengen an VEGF frei und induzierten eine stärkere Proliferation der glatten Muskelzellen im Vergleich zu subkutanem Fett desgleichen Patienten oder beiden Fettdepots von Nicht-Diabetikern, parallel zu eine verstärkten Induktion von CD36 und VEGF Rezeptoren.

Die Kombination von konditionierten Medien und Ölsäure induzierte die Aktivierung von NF-κB synergistisch. Die Analyse verschiedener NF-κB Zielgene zeigte unterschiedliche Ergebnisse nach der Behandlung von glatten Muskelzellen mit der einfach ungesättigten Fettsäure Ölsäure bzw. der gesättigten Fettsäure Palmitinsäure in Kombination mit konditionierten Medien. Während die Kombination von konditionierten Medien mit Ölsäure die mRNA Level der zytosolischen Superoxid Dismutase 1, der Matrix Metalloproteinase 1 und von Activin A verminderte, erhöhte die Kombination von konditionierten Medien mit Palmitinsäure die mRNA Expression und Sekretion von IL-6, die Produktion von ROS und die Aktivität der Matrix Metalloproteinase 1 im Vergleich zu den jeweiligen Einzelfaktoren. Außerdem führte sowohl die Kombination von konditionierten Medien mit Ölsäure, als auch mit Palmitinsäure zu einer signifikanten Reduktion der mRNA Expression von Angiopoietin-1 und CIDEA in glatten Muskelzellen.

Zusammenfassend lässt sich sagen, dass Adipokine und freie Fettsäuren einzeln oder in Kombination die Proliferation von humanen glatten Muskelzellen induzierten, wobei VEGF und CD36 eine entscheidende Rolle für den synergistischen Effekt von Adipokinen und Ölsäure spielen. 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 komplexen Crosstalk zwischen Fettgewebe und glatten Muskelzellen in der Gefäßwand zu untersuchen.

Summary

Obesity is a hallmark of the metabolic syndrome and represents a major global health problem, which is frequently associated with the development of non-communicable diseases, including type 2 diabetes and cardiovascular disease. Interestingly, a complex inter-organ crosstalk scenario between liver, muscle, vasculature and fat underlies the progression of these diseases, with adipose tissue being on top of the crosstalk hierarchy. Today it is well known, that this is due to the huge diversity of proteins and bioactive peptides, the so-called adipokines, secreted from adipose tissue, which is now considered as one of the major endocrine organs. Moreover, several studies revealed that beside the increased secretion of adipokines also the enhanced release of free fatty acids in obesity is associated with cardiovascular risk and the development of atherosclerosis. In this regard, considerable research in recent years has focused on the impact of single adipokines with or without free fatty acids on VSMC proliferation, migration and signaling, trying to provide insight into the complex cellular mechanisms linking obesity and cardiovascular disease. Although one study investigated the effects of conditioned medium from mouse cell lines and rat adipose tissue explants on human vascular smooth muscle cells, the impact of the whole human adipocyte secretory output on the crosstalk with human vascular smooth muscle cells is only incompletely unraveled. Therefore, the main topic of this work was to address the atherogenic impact of adipokines and free fatty acids at the level of human vascular smooth muscle cells. Here, conditioned media from *in vitro* differentiated human adipocytes (which does not contain free fatty acids) induced proliferation and migration of vascular smooth muscle cells. Furthermore, conditioned media enhanced the expression of the adhesion molecule ICAM-1 and activated proliferative and pro-inflammatory signaling pathways (p38 MAPK, NF-κB, and mTOR). The combination of conditioned media with oleic acid synergistically induced proliferation and enhanced the activation of the above mentioned proliferative and pro-inflammatory signaling pathways in comparison to both factors alone. Moreover, only combined treatment also increased iNOS expression, NO production, inducing a marked release of VEGF of human vascular smooth muscle cells.

Interestingly, the proliferative impact of various conditioned media correlated highly significantly with their content of VEGF. Human recombinant VEGF alone enhanced proliferation of smooth muscle cells, and the combination of VEGF and oleic acid induced proliferation in an additive way. Blocking VEGF with a specific antibody significantly reduced the proliferation under all conditions. Furthermore, the incubation of smooth muscle cells with oleic acid alone or in combination with conditioned media enhanced the triglyceride concentration of smooth muscle cells significantly. Here, we could also

observe that conditioned media induced the expression of the fatty acid transporter CD36. Silencing CD36 with specific siRNA also significantly reduced smooth muscle cell proliferation after treatment with oleic acid, conditioned media and VEGF alone or in combination. Interestingly, CD36 silencing abrogates VEGF-induced ERK signaling without affecting VEGF receptor expression. In addition, perivascular adipose tissue explants from diabetic patients release significantly higher amounts of VEGF and induce stronger proliferation of smooth muscle cells as compared to subcutaneous fat obtained from the same subject and both fat depots of non-diabetics, in parallel to marked induction of CD36 and VEGF receptors.

The combination of conditioned media and oleic acid synergistically induced NF-κB activation. Investigations of distinct NF-κB target genes revealed different results after smooth muscle cell treatment with the monounsaturated fatty acid, oleic acid, and the saturated fatty acid, palmitic acid, in combination with conditioned media. Whereas the combination of conditioned media with oleic acid significantly decreased the mRNA expression levels of cytosolic superoxide dismutase 1, matrix-metalloproteinase 1 and activin A, the combination of palmitic acid and conditioned media enhanced IL-6 mRNA expression and secretion, ROS production as well as matrix-metalloproteinase 1 activity in comparison to both factors alone, respectively. Furthermore, the combination of conditioned media with oleic acid as well as palmitic acid significantly reduced mRNA expression of angiopoietin-1 and the lipid droplet coating protein CIDEA in human vascular smooth muscle cells. These findings indicate that the combination of protein factors and lipid mediators augments the detrimental effects compared to either conditioned media or fatty acids alone.

In conclusion, the atherogenic impact of the whole secretory output of human adipocytes alone or in combination with fatty acids on vascular smooth muscle cell function was investigated. Here, we identified VEGF and CD36 as essential factors for the synergistic effect of adipokines and oleic acid-induced smooth muscle cell proliferation. Taken together, the presented work illustrates several novel aspects of the complex crosstalk between adipose tissue and smooth muscle cells in the vessel wall.

Table of Contents

List of Abbreviations

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
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
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	lipid chromatography
LMW	low molecular weight
LPS	lipopolysaccharide

MAPK	mitogen-activated protein kinase
MCP	monocyte chemotactic protein
MIF	macrophage migration inhibitory factor
MMP	matrix metalloproteinase
MMW	middle molecular weight
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

CHAPTER 1

General Introduction

1.1 Obesity

Obesity and overweight are defined as abnormal or excessive fat accumulation that may impair health. In the last century the incidence of obesity and obesity-related comorbidities has risen dramatically. Worldwide obesity has more than doubled since 1980 and is a growing health problem in developed as well as developing countries. In 2008, 1.5 billion adults over the age of 20 were overweight with a body mass index (BMI) greater than or equal to 25. Of these over 200 million man and nearly 300 million women were obese with a BMI greater than or equal to 30. Each year at least 2.8 million people die globally as a result of being overweight or obese, whereby overweight and obesity become the fifth leading risk for global death. Moreover, the incidence of obesity is associated with non-communicable diseases such as cardiovascular disease, type 2 diabetes, hypertension, respiratory diseases, dyslipidemia, fatty liver, Alzheimer`s disease, osteoarthritis and even some cancers[1,2].

The fundamental causes of obesity and overweight are lifestyle changes, such as caloric excess and reduced physical activity, in combination with genetic factors likely modifying individual predisposition to environmental factors. Obesity develops when energy intake persistently exceeds energy expenditure. It is well known that the increase of fat mass depends on multiple factors such as diet, gender, and the localization of the adipose tissue depot. Interestingly, women have a greater extent of body fat compared to men with the same BMI[3]. Also the distribution of adipose tissue diversifies between women and men. A way to measure fat distribution is the circumference of the waist, which

divides people into two categories: individuals with an android fat distribution (often called “apple” shape), meaning that most of their body fat is intra-abdominal and distributed around their stomach and chest and puts them at a greater risk of developing obesity-related diseases. Individuals with a gynoid fat distribution (often called “pear” shape), meaning that most of their body fat is distributed around their hips, thighs and bottom are at greater risk of mechanical problems. Obese men are more likely to be “apples“, while women are more likely to be “pears”[4]. This difference is mainly due to the local fat cell number between the genders. Interestingly, due to this fact men are more susceptible to enhanced triglycerides, fasting glucose, and insulin levels than adiposity-matched women. In addition, women with abdominal obesity showed a male risk profile, indicating that the abdominal fat depots may be of special importance for metabolic alterations, which is due to their unique position and relationship to the portal vein[3]. Another study showed that liposuction of subcutaneous adipose tissue in women did not significantly improve obesity-related metabolic disorders such as insulin resistance[5]. Furthermore, comparison of diabetic patients to age- and BMI-matched non-diabetic patients revealed that diabetic patients had a substantial higher amount of abdominal fat, also referred to visceral fat[6]. All these studies suggest the location of adipose tissue is more responsible for metabolic disturbances, rather than its total amount.

Adipose tissue has two key roles: 1. to ensure sufficient energy status by storing triglycerides after food intake, and releasing triglycerides during the fasting state; 2. to insulate and cushion the body. Beside these functions, adipose tissue secretes a considerable quantity of proteins and bioactive peptides, also known as adipokines. Therefore, our view of the role of adipose tissue has changed, from being solely a passive storage organ, to an important endocrine organ, that modulates metabolism, immunity, and satiety. Until now the factors and mechanisms regulating adipose tissue mass are not fully understood. In obesity lipid accumulation promotes both an increase in fat cell number (hyperplasia) as well as enhanced fat cell volume (hypertrophy), with storage occurring mainly in pre-existing adipocytes. Hypertrophy is well documented and is thought to be the most important mechanism whereby fat depots increase in adults[7,8]. The number of adipocytes is set during childhood and adolescence, and even after weight loss in adulthood and reduced adipocyte volume, the fat cell number remains the same, suggesting that the number of adipocytes represents an important determinant for the fat mass. Studies showed that there is a remarkable turnover within this population, indicating that adipocyte number is tightly controlled but not influenced by energy balance[8]. Analysis of adipocyte turnover has recently shown that adipocytes are a dynamic and highly regulated population of cells. New adipocytes are constantly formed to replace lost adipocytes, such that approximately every eight years 50 % of adipocytes

in human subcutaneous fat mass are replaced[8]. During this continuous turnover adipocytes progenitor cells (pre-adipocytes) originating from the stroma-vascular fraction of adipose tissue are known to proliferate and differentiate into mature cells *in vitro*. Recently, white adipose tissue progenitor cells were identified *in vivo*[9,10] and shown to be capable of reconstituting the adipocyte mass in lipodystrophic mice[11,12,13]. Furthermore, necrotic as well as apoptotic adipocytes are also found in adult human adipose tissue[14], even though no decrease in adipocyte number is seen. These data suggest that pre-adipocytes are recruited to become lipid-filled mature adiopcytes at the same rate that adipocytes die, and that in this way the fat mass is in constant flux, and adipocyte number is kept constant[15]. In obesity the number of pre-adipocytes in the stroma-vascular fraction of abdominal subcutaneous adipose tissue is reduced[16,17], indicating that possible mechanisms could be an enhanced apoptosis of pre-adipocytes or a decreased pre-adipocyte differentiation. In addition, the number of pre-adipocytes able to differentiate in mature adipocytes was negatively correlated with both BMI and adipocyte cell size[18]. Recent studies suggest that tumor necrosis factor (TNF)α in human adipose tissue serves as an important regulator of fat cell size and number in healthy subjects[19]. In the context of obesity, chronically elevated TNFα levels led to a complete trans- or dedifferentiation of pre-adipocytes to another phenotype, including inflammatory cells.

Besides the intake of nutrients, hormones are also able to regulate the growth of adipocytes, including aldesterone[20], thyroid hormones[21], and glucocorticoids[22], which promote adipocyte differentiation, or growth hormone (GH), which is known to inhibit differentiation[23]. Moreover, numerous transcription factors have been shown to play an important role in the adipogenic process, notably 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[24]. Besides these, the peroxisome proliferator-activated receptor (PPAR)γ is the glucose metabolism and is a molecular target for thiazolidinediones (TZD), e.g. troglitazone or rosiglitazone[25].

Adipocyte turnover is not only important for regulating the total fat mass but also for the development of hyperplasia and hypertrophy. Decreased adipogenesis and relative adipocyte death rate were demonstrated in hypertrophic compared to hyperplastic individuals[26]. Furthermore, subjects with hypertrophy have a significantly lower number of adipocytes than those with hyperplasia, independent of the total fat mass[26]. In addition, two independent studies showed that adipose hypertrophy is an independent risk factor for type 2 diabetes[27,28]. Larger adipocytes have a greater rate of triglycerol synthesis, lipolysis and therefore greater rates of transmembrane fatty acid flux. Furthermore, they display a more detrimental profile of cytokine secretion[29], leading

to higher amounts of pro-inflammatory cytokines, e.g. TNFα, and reduced abundance of anti-inflammatory cytokines, such as adiponectin[30].

1.1.1 Adipose tissue dysfunction

During the progression from the lean to the obese state, adipose tissue undergoes hyperplasia as well as hypertrophy in an attempt to cope with the increased demand for triglyceride storage. Beyond a critical threshold, adipocytes begin to exhibit several dysfunctions, including apoptotic signalling, endoplasmatic reticulum (ER) stress, mitochondrial dysfunction, production of reactive oxygen species (ROS), enhanced fatty acid release, and altered adipokine secretion, leading to a chronic low-grade inflammation[31,32]. Over the past years it has become well accepted that changes in inflammatory signalling by adipocytes and infiltration of adipose tissue by distinct immune cells are key features of obesity-associated metabolic diseases in animal models and humans[33]. Despite the correlation between increased adipose tissue inflammation and metabolic dysfunction, the presence of immune cells in adipose tissue is not uniformly detrimental[33]. Macrophages may play a role in the extensive tissue remodelling that occurs during adipose tissue growth[34,35]. Recent studies showed the importance of the specific activation state of immune cells in determining functional consequences[33]. The classically activated M1 macrophages are stimulated by interferon-γ and lipopolysaccharide to secrete pro-inflammatory factors such as TNFα and IL-6 and to produce ROS[36], whereas the alternatively activated M2 state plays a role in wound healing, tissue repair, lipid metabolism, and resolution of acute inflammation via anti-inflammatory signalling[37,38]. Besides macrophages, recent studies indicate a role for other immune cells in adipose tissue inflammation, such as natural killer (NK) cells[39], B cells, mast cells, regulatory T cells, T-helper cell type 1 (T_H1), and T-helper cell type 2 (T_H2)[40,41,42]. 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[38]. The pro-inflammatory T_H1 subset has been suggested to be involved in macrophage recruitment and activation of the M1 phenotype[42]. Conversely, the T_H2 T-cell populations, like adipocytes, secrete IL-4 and IL-13 and are expected to favour the M2 profile in macrophages[43].

In the context of obesity, both adipocytes and macrophages secrete a number of cytokines including TNFα, IL-6, IL-1β, and migration inhibitory factor (MIF)[38], activating inflammatory signalling pathways via c-Jun N-terminal kinase (JNK), and inhibitor of kappa β kinase (IKKβ) in both immune and neighbouring nonimmune cells[44,45]. This results in

increased inflammation, as well as possible alterations in other metabolic targets, that in combination contribute to overall metabolic dysfunction.

Increased exposure to free fatty acids (FFA), whether because of increased fat content or aberrant lipolysis in adipocytes, has been proposed as one of the key activators of metabolic and immune signalling in obesity[38]. In addition, FFA and intracellular lipids have been shown to elevate diacylglycerol levels and induce classical and novel protein kinase (PK)C isoforms[46,47]. One mechanism by which FFA have been proposed to directly influence immune cell signalling is through recognition by pathogen-sensing molecules, including toll-like receptors (TLR) and subsequent JNK activation and engagement of inflammatory signalling cascades[48,49]. Interestingly, not all FFA-mediated signalling is detrimental in the context of metabolism and inflammation. FFA signalling through the PPAR family of nuclear receptors has been shown to have beneficial effects on metabolic parameters[50].

1.1.2 Adipose tissue-derived factors

The adipokine production and signalling of adipocytes is another important aspect of adipose tissue that is altered in obesity. Most of the adipokines are significantly enhanced in obesity and are good predictors of the development of type 2 diabetes[51], and are correlated to metabolic and cardiovascular complications observed in the obese state. To gain insights accounting the important regulatory impact of adipocytes, in figure 1.1 most of the adipose tissue-derived factors were clustered according to their postulated functions, including adipokines involved in inflammation, metabolism, oxidative stress, contributing to the extracellular matrix, or the control of angiogenesis and blood pressure.

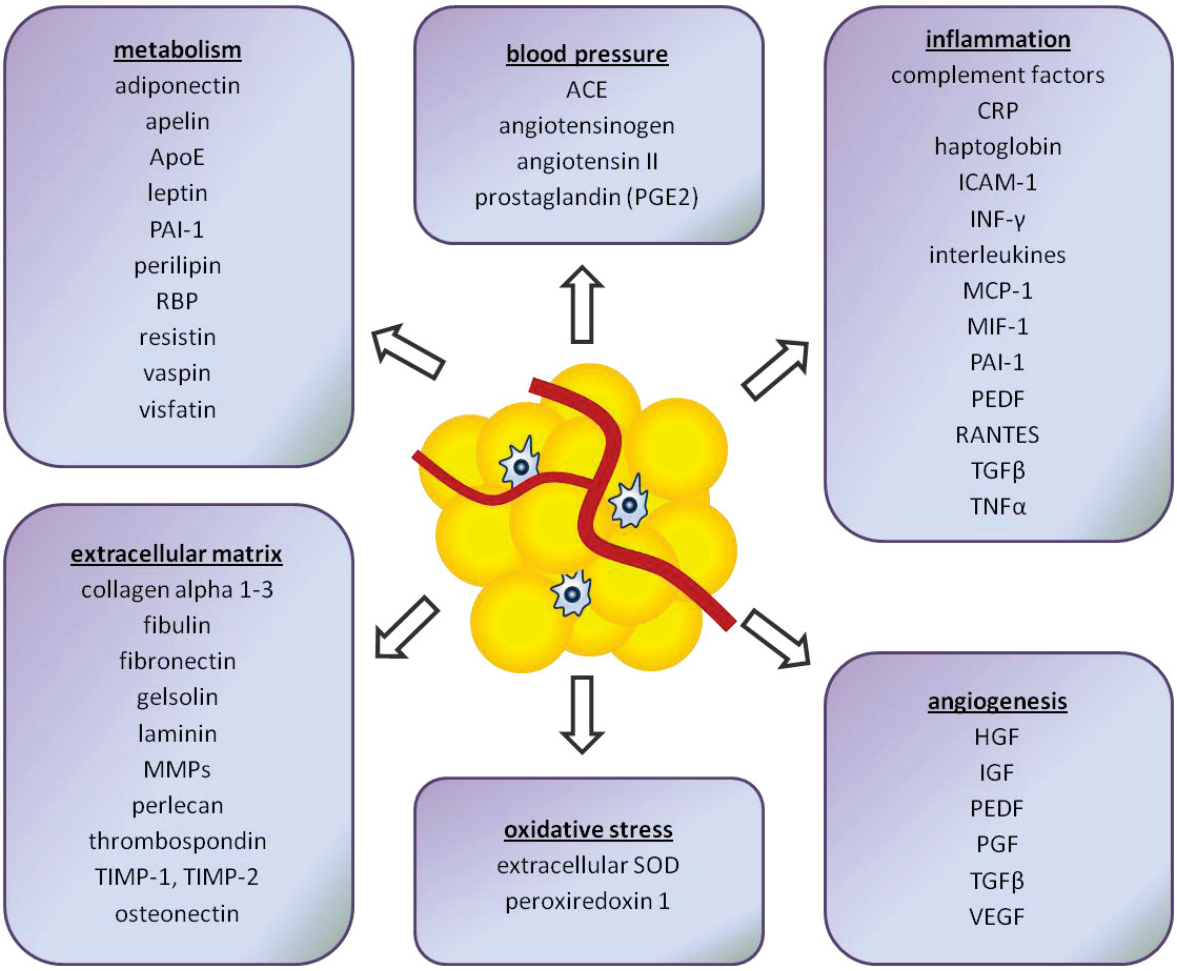

Figure 1.2 Adipose tissue-derived factors. MMPs, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinases; ACE, angiotensin converting enzyme; TNF, tumor necrosis factor; IFN, interferon; TGF, transforming growth factor; IL, interleukin; MCP, monocyte chemotactic protein; ICAM, intercellular 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 *(according to dissertation of Daniela Lamers)*

A very well analyzed adipokine is TNFα, which was the first adipose-tissue derived factor suggested to represent a link between obesity, inflammation and diabetes[52]. Produced by macrophages within adipose tissue and by adipocytes themselves[53], and stimulated by ER stress and unfolded protein response (UPR)[31], TNFα inhibits lipoprotein lipase activity and increases lipolysis[54]. In several animal models of obesity TNFα is upregulated[55,56], but its role in human physiology is still unclear[57]. Among humans losing

weight, macrophage expression of TNFα decreases and is inversely proportional to lipoprotein lipase activity[58]. Due to the fact that a major activity of lipoprotein lipase is breakdown of circulating triglycerides and VLDL, decreased lipase activity due to increased adipose tissue TNFα concentrations may partly account for hypertriglyceridemia of obesity[58]. In patients with type 2 diabetes TNFα concentrations are higher and correlate with fasting glucose and insulin in obese individuals[59]. Moreover, TNFα inhibits insulin action in adipocytes, possibly through inhibition of IRS-1 by JNK[60], leading to adipocyte insulin resistance[61]. TNFα is also likely involved in adiposity-related vascular dysfunction, as in animal models of metabolic syndrome, endothelial dysfunction is associated with an increase of TNFα expression; blockade of TNFα restored endothelial vasodilatation[62]. This effect of TNFα on vascular function may relate to its importance as a stimulant of ROS and inhibitor of nitric oxide release[63].

Adipose tissue secretes also other peptides that have peripherally and centrally effects. The most investigated of these is leptin, a satiety factor which was first characterised in a rodent model of monogenic obesity, the ob/ob mice[64]. Leptin is secreted by adipocytes and modulates food intake by suppressing orexigenic peptides, such as neuropeptide Y, and upregulates anorexigenic peptides, e.g. corticotrophin-releasing hormone, in the brain[65]. In humans, congenital leptin deficiency causes severe obesity, impaired thermogenesis, and insulin resistance[66]. Furthermore, leptin stimulates fatty acid oxidation and prevents lipid accumulation in adipose tissue[67,68].This forms a negative feedback mechanism, where enhanced fat mass produces more leptin, which reduces food intake, inhibiting further adipose tissue expansion and limiting leptin expression. It was initially thought that this feedback loop could be used to inhibit food intake in the obese, but clinical trials of leptin analogues had little success, because endogenous leptin has since been found to be increased in the obese, who often exhibit leptin resistance[69]. Therefore, leptin treatment appears to be only a valuable option in rare diseases states in which leptin levels are low[70].

Another small polypeptide hormone produced by adipocytes as well as immunocompetent cells, is resistin[71,72]. In 2001 Steppan et al. suggested that resistin represented a hormone that links obesity and diabetes, inducing insulin resistance[73]. Later studies revealed that the source of resistin is different between rodents (adipocytes) and humans (macrophages)[71,74]. In animals, increased resistin levels are associated with obesity and it seems to be implicated in inducing insulin resistance in liver, skeletal muscle, and adipose tissue[75]. In contrast, the role of resistin in humans is less clear[74,76,75]. Resistin expression correlates with the release of markers such as TNFα and IL-6[77,78]. In human adiocytes and macrophages resistin also induces the secretion of TNFα, further pointing to the pro-inflammatory nature of this adipokine[79,80].

Monocyte chemotatic protein (MCP)-1 is a member of the family of inducible cytokines, which is expressed by adipocytes and other cell types, including smooth muscle cells and endothelial cells, when exposed to pro-inflammatory stimuli[81]. In the context of obesity, MCP-1 is overexpressed in rodents[82,83], and it also attains significantly higher plasma levels in obese human individuals[84,85]. MCP-1 plays a role in the recruitment of monocytes and T lymphocytes to the site of injury and infection[81] and its expression is higher in visceral adipose tissue compared to subcutaneous adipose tissue, which correlated closely to the number of residing macrophages[86]. *In vivo*[87,88] as well as *in vitro*[82,89] studies revealed good evidence for a relationship between MCP-1 and the development of insulin resistance. Furthermore, MCP-1 was found to be elevated in patients with CAD, possibly leading to atherosclerosis[90].

Another multifunctional pro-inflammatory adipokine is IL-6, which is tightly regulated and expressed at low levels in healthy individuals. Elevated IL-6 expression is associated with a variety of diseases, including inflammatory conditions such as CVD, obesity and type 2 diabetes. IL-6 serum levels are increased in obesity and correlate with the development of type 2 diabetes[70] and cardiovascular mortality[91]. Interestingly, the detailed function of IL-6 in various diseases is not completely clear. *In vivo* studies showed that recombinant IL-6 administration resulted in hyperglycemia and compensatory hyperinsulinaemia in rodent models and humans[92,93]. Similarly, also *in vitro* data pointed to a correlation between IL-6 and insulin resistance in skeletal muscle[94] and 3T3-L1 adipocytes[95]. In contrast, IL-6 secretion from muscle is increased after exercise and leads to higher energy expenditure[96]. This acute stimulation of IL-6 results in increased glucose uptake and ß-oxidation via activation of AMPK[97]. Another study showed that injection of IL-6 increased whole body insulin sensitivity in rat skeletal muscle cells[98]. Moreover, IL-6 induces the induction of CRP production, which is now known to be an independent risk factor for cardiovascular complications[99]. Some studies have suggested that IL-6 is an indirect marker of vascular dysfunction, while others have suggested a more active role in vascular dysfunction[100]. Furthermore, in various animal models a positive as well as negative role of IL-6 in the development of atherosclerosis was reported[101]. Therefore, IL-6 may have a biphasic effect on metabolic disorders as a reflection of its pro-inflammatory or anti-inflammatory nature activities[102,103,104].

One very well examined and unique adipokine is adiponectin, which is synthesised almost exclusively by adipocytes, but also produced by skeletal muscle cells, endothelial cells and cardiomyocytes[105,106]. Furthermore, 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, 12-18mers) multimers. Adiponectin exerts anti-inflammatory[107] and anti-atherogenic[108,109]

properties, and in contrast to most other adipokines, circulating levels of adiponectin are diminished in obesity and in a number of obesity-related co-morbidities such as type 2 diabetes[110,111], the metabolic syndrome[112,113], non-alcoholic steatohepatitis[114] and CAD[109,115]. It has also been found that adiponectin is anti-diabetic, increasing insulin sensitivity, glucose uptake and fat oxidation, as well as suppressing hepatic glucose output[116,117,118]. Adiponectin is normally present in plasma at high concentrations, but in obese patients adiponectin levels were found to be decreased and correlate with visceral adiposity[119]. In patients with type 2 diabetes and coronary heart disease, circulating levels of HMW adiponectin are selectively reduced due to an impaired secretion of this oligomer from adipocytes[120].

1.2 Obesity-related disorders

1.2.1 Obesity and Type 2 Diabetes

Diabetes mellitus is defined as a metabolic disorder of several etiologies characterized by chronic hyperglycemia resulting from a defective insulin secretion and insulin action. The prevalence and incidence of diabetes is dramatically increasing worldwide, whereas 2007 246 million adults were diagnosed with diabetes[121], it is suggested that 2030 the number of people with diabetes will nearly double[122]. Two main forms of diabetes mellitus exist: type 1 and type 2 diabetes[123]. Type 1 diabetes, accounting for 5-10 % of all diabetic patients, is an autoimmune disorder usually diagnosed in childhood or adolescence, leading to destruction of insulin-producing pancreatic β-cells, which in turn results in an absolute insulin deficiency. Therefore, patients with type 1 diabetes need a lifelong insulin treatment[124]. With 90-95 % of patients with diabetes, the much more prevalent form is type 2 diabetes, which is caused by a combination of resistance to insulin action and an inadequate compensatory insulin secretory response. Today it is well known, that a long time before diabetes is diagnosed, the degree of hyperglycemia is sufficient to cause pathological and functional changes in various tissues, but without clinical syndromes. Factors that increase the risk of type 2 diabetes as well as cardiovascular disease are impaired fasting glucose (IFG) and /or impaired glucose tolerance (IGT).

Type 2 diabetes is a heterogeneous metabolic disorder, causing microangiopathic, macroangiopathic, and neuropathic complications[125]. In this context, the advanced state of these complications appears in retina, kidney and nerves. An early event in the development of type 2 diabetes is insulin resistance of peripheral tissues and represents a main focus in the attempt to unravel the molecular mechanisms causing this disease[126]. Interestingly, insulin resistance occurring in skeletal muscle leads to persisting postprandial hyperglycemia, which can occur 20 years before manifestation of clinical symptoms[127,128]. In adipocytes, insulin resistance leads to increased circulating FFA levels, in turn decreasing insulin sensitivity in nonadipose tissue such as liver, pancreas, and skeletal muscle[129], due to an increase in ectopically stored lipid products[47].

In the last years, a rising problem is the increasing number of obese children and adolescents who are diagnosed with type 2 diabetes. Currently, a few thousands children and adolescents are diagnosed with type 2 diabetes, but the number is supposed to increase since the prevalence of overweight and obesity is also rising[130,131].

1.2.2 Obesity and Dyslipidemia

Dyslipidemia are disorders of lipoprotein metabolism, including lipoprotein overproduction and deficiency which is associated with obesity. They may manifest as one or more of the following: elevated total cholesterol, low-density lipoprotein cholesterol (LDL), and triglycerides levels or as decreased high-density lipoprotein cholesterol (HDL)[132,133,134,135,136,137,138,139]. It is widely accepted that dyslipidemia is a risk factor for coronary heart disease. Several studies showed that increased triglyceride levels and decreased HDL levels correlate with the development of atherosclerosis and CVD[140]. Furthermore, hypertriglyceridemia is associated with insulin resistance in type 2 diabetes. There is good evidence that insulin resistance correlates with plasma triglyceride concentrations, as triglycerides may influence an early step in insulin action pathway; alternatively, insulin resistance may cause hypertriglyceridemia[141]. With higher glucose in the blood, more LDL is glycated, so that the affinity of LDL for modified LDL receptors on macrophages is enhanced, promoting foam cell formation, endothelial cell toxicity and smooth muscle cell proliferation[142]. Hypertriglyceridemia is also associated with abnormalities of clotting, the fibrinolytic system, and raised levels of CRP, fibrinogen, plasminogen activator inhibitor (PAI), and IL-6, all of which play an important role in the pathogenesis of CAD. Furthermore, another important factor that play a role in dyslipidemia is PPAR-α, a major regulator of intra- and extracellular lipid metabolism. In addition, PPARs regulate the interaction between HDL cholesterol and apolipoprotein B containing lipoproteins[143]. With the growing prevalence of obesity, insulin resistance, and type 2 diabetes worldwide, prevention and management of this dyslipidemic state is critically important for the prevention of coronary artery and macrovascular disease[144].

1.2.3 Obesity and Hypertension

Obesity is also considered as one of the causes of high blood pressure, also known as hypertension. It has been indicated that almost two-thirds of the people suffering from obesity are at a risk of hypertension. The exact mechanism how obesity is a cause for hypertension is still unknown. Obesity is associated with increased blood flow, vasodilatation, cardiac output, and hypertension. Although cardiac index (cardiac output divided by body weight) does not enhance, cardiac output and glomerular filtration rate do. However, renal sodium retention also increases, leading to hypertension[145,146,147]. The factors generally considered responsible for obesity-related alterations in the pressure-

natriuresis (a compensatory mechanism to maintain blood pressure within the normal range) curve include enhanced sympathetic tone, activation of the renin-angiotensin system, hyperinsulinemia, structural changes in the kidney, and the involvement of adipokines such as leptin. Sympathetic blockade (combined alpha and beta blockade) prevents obesity-related hypertension in animal models as well as humans[145,146,147,148]. Similarly, leptin can cause hypertension, which in turn can also be prevented by combined sympathetic blockade, indicating that leptin contributes to obesity-induced hypertension primarily through sympathic activation. Interestingly, renal denervation prevents the development of hypertension in some animal models of obesity-related hypertension[149,145,146,147,150,151,152,148], indicating that sympathetic activation seems to be related to activation of renal nerve traffic and subsequent alteration of pressure-natriuresis relationship. Moreover, there is also activation of the renin-angiotensin system in hypertension with elevations of circulating renin, angiotensinogen, and angiotensinogen II, despite the fact that renal sodium retention is augmented[153,146,147,150,151]. The reasons for the activation of the renin-angiotensin system are not completely understood. The elevation of renin activity observed in obesity could be a result of sympathetic activity. In any case, elevations of angiotensin II directly increase renal tubular reabsorbation of sodium and stimulate synthesis of the sodium-retaining hormone aldosterone. Similarly, obesity is associated with hyperinsulinemia[149,151]. The roles for the renin-angiotensin system and hyperinsulinemia in this process are indirectly supported by the beneficial effects on blood pressure of angiotensin-converting enzyme inhibitors and PPAR-γ agonists[154,155,156]. Recently the so-called obesity-paradox has been described, meaning that, while obese individuals are more likely to develop cardiovascular diseases, they may have better survival than lean individuals with hypertension. Although controversial, this idea is intriguing, as is the notion that obese individuals with hypertension may have an increased risk of renal insufficiency[145,155,146,147,148].

1.2.4 Obesity and Metabolic Syndrome

The metabolic syndrome, also known as syndrome X or insulin resistance syndrome, describes a cluster of several metabolic conditions that occur together and increase the risk for cardiovascular disease and type 2 diabetes[157,158]. At the moment it is not sure whether the metabolic syndrome is due to one single cause, but all of the risk factors for this syndrome are related to obesity. In Europe metabolic syndrome affects

nearly 30 % of the population[159] and more than 40 % in the USA[160]. The incidence of this syndrome has been estimated to increase with age for individuals over 50 years. People with metabolic syndrome are estimated to have twice the risk to developing CVD compared to healthy individuals and a five-fold increased risk of type 2 diabetes[161,158]. However, the underlying pathophysiological processes leading to the development of metabolic syndrome are unclear and there is also confusion over its conceptual definitions and criteria. The risk factors seen in metabolic syndrome include abdominal obesity, high triglyceride levels, low high-density lipoprotein cholesterol, high blood pressure, and fasting blood glucose (Table 1.1). According to NCEP-ATP III there must be at least three of the before mentioned five metabolic risk factors for an individual to be diagnosed with metabolic syndrome. Other risk factors are age, genetic disposition, hormone changes, and daily lifestyle (e.g. low physical activity and excess caloric intake). The cause of metabolic syndrome is still unknown and its pathophysiology is extremely complex and has been only partially elucidated. Moreover, there is a debate regarding whether obesity or insulin resistance is the cause of the metabolic syndrome or if obesity and insulin resistance are consequences of more far-reaching metabolic dysfunction.

Table 1.1 *Clinical identification of the metabolic syndrome (according to ATP III[132])*

Risk Factor	**Defining level**
Abdominal obesity (waist circumference) Men Women	 > 102 cm > 88 cm
Triglycerides	≥ 150 mg/dL
HDL cholestersol Men Women	 > 40 mg/dL > 50 mg/dL
Blood pressure	≥ 130/≥ 85 mm Hg
Fasting glucose	≥ 110 mg/dL

1.3 Atherosclerosis in the context of obesity

1.3.1 Pathophysiology of Atherosclerosis

Atherosclerosis is a complex pathological process in the walls of blood vessels that develops over many years resulting in coronary heart disease (heart attack) and cerebrovascular disease (stroke). It is responsible for a large proportion of cardiovascular diseases (CVD), which is with over 17 million deaths per year, the leading cause of death worldwide[162]. In 2008, out of the 17.3 million cardiovascular deaths, heart attacks were responsible for about 7 million deaths and strokes were responsible for about 6 million deaths[162]. Atherosclerosis is characterized by a thickening and hardening of artery walls, affecting large and medium-sized arteries. Important hallmarks of atherosclerosis are vascular inflammation, endothelial dysfunction and the buildup of low-density lipoprotein cholesterol, lipids, calcium, and cellular debris within the intima of the vessel wall. These detrimental alterations result in vascular remodelling, plaque formation, acute and chronic luminal obstruction, abnormalities in blood flow, and diminished oxygen supply to target organs. Until now the complex interaction between the cellular elements of atherosclerotic lesion is not completely understood. These cellular elements are endothelial cells, smooth muscle cells, platelets, and leukocytes. Vasomotor function, the thrombogenicity of the blood vessel wall, the state of activation of the coagulation cascade, the fibrinolytic system, smooth muscle cell proliferation and migration, and cellular inflammation are complex and interrelated biological processes, contributing to atherogenesis and the clinical manifestations of atherosclerosis.

Although several factors are known for their influence on the development of atherosclerosis, all seem to originate from a general inflammatory process of the vascular endothelium. Until now, the underlying cause of the inflammation remains unclear, but an important and broadly supported theory is the so-called “response-to-injury” theory[163]. This hypothesis assumes that vessel wall damage with ensuing dysfunction of the endothelium is the first step of atherosclerosis. Endothelial damage precedes smooth muscle cell proliferation and migration, deposition of intracellular and extracellular lipids, and accumulation of extracellular matrix[164]. Factors identified as damaging to the endothelium include hypertension, diabetes mellitus, elevated low-density cholesterol levels, free radicals, genetic abnormalities, various infections or a combination of these factors[165,166,167,168]. The endothelium is a dynamic monolayer lining the blood vessel wall

and is responsible for maintaining the vascular tone through the production of vasodilators, e.g. NO, and vasoconstrictors, e.g. endothelin-1. In addition, the endothelium releases other factors, such as von Willebrand factor, plasminogen inhibitors, and prostacyclin, which regulate thrombosis by inhibiting platelet aggregation[169]. The earliest changes that precede the formation of lesions in atherosclerosis include increased permeability to lipoproteins and other plasma continuants, which is mediated by NO, prostacyclin, platelet-derived growth factor (PDGF), angiotensin II (AngII), and endothelin-1. Specific arterial sites, such as branches, bifurcations, and curvatures, cause characteristic alterations in the blood flow, including decreased shear stress and increased turbulence. Rolling and adhesions of monocytes and T cells occur at these sites as a result of the upregulation of adhesion molecules on both the endothelium (e.g. E-selelctin, P-selectin, intracellular adhesion molecule (ICAM)-1, and vascular adhesion molecule (VCAM)-1) and leukocytes (such as L-selectin, and integrins)[164]. Another important factor contributing to the increased recruitment of monocytes and leukocytes to the vessel wall is oxidized low-density lipoprotein (oxLDL), which induces endothelial cell and smooth muscle cell dysfunction, secretion of inflammatory mediators, and expression of adhesion molecules, culminating in monocyte and leukocyte accumulation in the subendothelial space[170]. Once located in the arterial intima, monocytes acquire the morphological characteristics of macrophages, undergoing a series of changes that lead to foam cells (lipid-laden macrophages). The monocytes elevate the expression of scavenger receptors of modified lipoproteins, such as the scavenger receptor A and CD36, and then internalize modified lipoproteins[171]. These lipid-laden macrophages characterize the early atherosclerotic lesion and this is the first step in fatty streak formation. Besides foam cells, fatty streaks initially consist of T lymphocytes. Later they are joined by various numbers of smooth muscle cells, which migrate from the media into the intima. As fatty streaks progress to intermediate and advanced lesions, they tend to form a fibrous cap, as a result of progressive lipid accumulation and the ongoing migration and proliferation of smooth muscle cells. The smooth muscle cells are responsible for the deposition of extracellular connective tissue matrix and form a fibrous cap that overlies a core of lipid-laden foam cells, extracellular lipid, and necrotic cellular debris[164]. In unstable or vulnerable atherosclerotic plaques, denudation of the overlying endothelium, rupture of the protective fibrous cap or ulceration of the fibrous plaque can rapidly lead to thrombosis and usually occurs at sites of thinning of the fibrous cap that covers the advanced lesion. Thinning of the fibrous cap is apparently due to the continuing influx and activation of macrophages, releasing metalloproteinases and other proteolytic enzymes. 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 and occlusion of the artery[164].

1.3.2 Impact of adipose tissue-derived factors on atherosclerosis

Dysfunctional adipose tissue with low-grade, chronic and systemic inflammation links metabolic and vascular pathogenesis including dyslipidemia, low-grade inflammation and insulin resistance and is a hallmark of disorders such as type 2 diabetes and cardiovascular disease. Today, it is generally accepted that inflammation induced by obesity accelerates atherosclerosis. Therefore, obesity is recognized as an important determinant of cardiovascular morbidity and mortality, including stroke, congestive heart failure, myocardial infarction, and cardiovascular death[172,173]. Many studies in humans and various animal models have shown that obesity is strongly related to the development of atherosclerosis[174,175]. Adipose tissue plays a pivotal role in the development of a systemic low-grade inflammation state that contributes to obesity-associated vascular dysfunction and cardiovascular risk. Adipocytes produce large numbers of hormones, peptides and other molecules that affect cardiovascular function, not only in an endocrine manner, but also by autocrine and paracrine mechanisms[66]. In this context, the local secretion of adipokines and FFA by perivascular adipose tissue may provide a direct link between obesity and cardiovascular dysfunction[176].

One of the most extensively examined pro-inflammatory cytokines is TNF-α, which plays an important role in atherosclerosis as well as in other inflammatory and metabolic disorders, which are known risk factors for cardiovascular diseases. Upregulation of TNF-α in the vascular wall of carotid and coronary arteries promotes endothelial apoptosis thus leading to impairment of endothelial function[177,178]. In TNF-α/ApoE double knockout mice most pro-atherosclerotic factors such as IL-1β, MCP-1, and NF-κB are downregulated[179]. Furthermore, TNFα plasma concentrations are positively correlated with carotid intima-media thickness (IMT)[180] and increased in patients with premature CAD[181], thus further emphasizing the important role of TNF-α for the development of cardiovascular diseases.

The results of clinical studies investigating the contributions of leptin to the pathophysiology of cardiovascular complications are controversial, leaving the precise role of leptin unclear[182]. Some studies have reported elevated leptin levels in patients with acute coronary syndrome (ACS)[183,184] and described an association between circulating leptin levels and risk of coronary artery disease (CAD)[185,186]. However, the

findings of other studies have indicated no clinically relevant association with risk of CAD[187,188,189]. The effects of leptin on endothelial cells and vascular SMC are better investigated and comprise increased NO production via activation of endothelial NO synthase (eNOS)[190], and increased expression and activity of inducible NO synthase (iNOS), respectively[191]. However, leptin also increases the expression of PAI-1[192] and CRP[193] in human vascular endothelial cells. Furthermore, leptin-deficient mice, which are extremely obese, are protected from atherosclerosis despite other metabolic risk factors, indicating that this adipokine contributes directly to cardiovascular diseases[194].

In opposite to the adipokines described above, adiponectin levels are diminished in human obese subjects[195]. It has been shown to positively influence energy consumption and fatty acid oxidation in muscle and liver, thereby reducing the triglyceride content[196]. Transgenic mice overexpressing adiponectin exert an improved lipid profile[197,198]. Adiponectin has been suggested to be an important factor modulating the cardiovascular system due to its anti-atherogenic and anti-inflammatory effects. In macrophages and endothelial cells, it acts via suppression of TNFα[199] and pro-inflammatory cytokines such as IL-6[200], and directly ameliorates endothelial dysfunction by increasing nitric oxide (NO) production[201,202]. The role of adiponectin as a cardioprotective adipokine is further supported by results from clinical studies. While increased adiponectin levels are associated with a decreased risk of myocardial infarction[203], hypoadiponectinemia is observed in patients with coronary atherosclerosis and ACS[204,205]. In a recent study, it has been shown that serum adiponectin is associated with biomarkers of insulin resistance, inflammation, and endothelial dysfunction, which are independent risk factors for cardiovascular diseases[206]. In addition, results from animal studies have revealed that adiponectin exerts beneficial effects at mostly all stages of the atherosclerotic process [207].

1.3.3 Impact of adipose-tissue derived factors on smooth muscle cells

Under physiological conditions VSMC play an essential role in providing structural integrity of the vessel wall and in controlling vascular tone and blood pressure[208,209]. 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[210,211] and leading to the modulation of VSMC relaxation[212]. Furthermore, the complex system, which regulates VSMC responses and modulates the contractile process, involves the expression of receptors for catecholamines,

acetylcholine, serotonin, histamine, purinergic mediators, AngII, bradykinin, neuropetide Y, vasopressin, leukotrienes and growth factors, such as PDGF, epidermal growth factor (EGF), TGF-β, fibroblast growth factor (FGF), insulin, and insulin-like growth factor (IGF)-1. The signal transduction system following membrane activation mostly consists of guanine nucleotide regulatory proteins, phosphoinositide metabolism, cyclic nucleotides (cAMP and cGMP), and calcium[213,214]. Different agonists modulate VSMC responses by activating tyrosine kinases through receptor and non-receptor mechanism. In particular, IGF-1 through its specific receptor leads to direct activation of extracellular signal regulated kinases (ERK1/2), whereas AngII and other mediators activate tyrosine kinase pathway by indirect mechanism such as increased hydrogen peroxide (H_2O_2) production mediated by NADPH oxidase[213,214].

Following repeated or chronic arterial wall injury, such as in arterial hypertension and exposure to other cardiovascular risk factors, VSMC respond by migration into the intima, secretion of cytokines, as well as by increased proliferation[215,216,164]. VSMC migration from the media to the intima depends on mechanisms regulated by soluble growth factors and chemoattractants, as well as by interactions with extracellular matrix[215,216,164]. In atherosclerosis VSMC phenotypic modulation plays a key role and is classically defined as a switch from the “contractile” state to a “synthetic” phenotype, whereby genes that define the contractile type are suppressed and proliferative and migratory mechanisms are induced, contributing to plaque growth. Furthermore, there is also evidence that VSMC may take on a “pro-inflammatory” phenotype, whereby VSMC secrete cytokines, e.g. IL-8 and IL-6, and express cell adhesion molecules, such as VCAM, which functionally regulate monocyte and macrophage adhesion and other processes during atherosclerosis[217].

Several indications suggest that adipokines play a pivotal role in inducing the synthetic phenotype of VSMC and consequently affect VSMC function. As far as VSMC are concerned, pro-inflammatory mediators and growth factors, such as IL-2, AngII and VEGF induce the synthesis and release of IL-6 in VSMC, in turn augmenting proliferation and migration of VSMC[218,219,220,221]. Additionally, IL-6 stimulates VSMC proliferation via PDGF-dependent and –independent mechanisms[222,223], and is involved in VSMC migration, by interplaying with VEGF and TNF-α[223,224,225]. Interestingly, VSMC are both source as well as target of TNF-α[226], stimulating together with other cytokines IL-6 production[227]. Through NF-κB signaling pathway, TNF-α enhances synthesis and the release of MMP from VSMC[228], indicating that TNF-α is supremely involved in plaque inflammation and instabilization. At the present, the effects of leptin on the arterial wall have not been fully elucidated. Human *in vitro* studies showed that leptin induced VSMC proliferation, migration and the expression of MMP-2, by inducing ERK 1/2, PKC, and NF-κB signaling

pathways[229]. Furthermore, leptin enhances the cardiovascular risk by stimulating osteogenic differentiation and consequently vascular calcification[230]. Also, indirect mechanisms responsible for other leptin effects on VSMC are referred to oxidative stress which can cause VSMC damage and stimulation of low-grade vascular inflammation[231]. Interestingly, the adipokine visfatin influences VSMC phenotype switch from a proliferative synthetic to a non-proliferative contractile one[232], and promotes proliferation of VSMC in perivascular adipose tissue by a paracrine mechanism[233]. Resistin is also known to induce proliferation of human VSMC through both ERK 1/2 and AKT signaling pathways[234]. Furthermore, hypoxia enhances resistin expression in rat VSMC[234]. In contrast, adiponectin suppresses proliferation and migration of VSMC by directly binding to several growth factors, particularly PDGF BB, FGF, and heparin-binding epidermal growth factor-like growth factor (HB-EGF)[235,115]. Moreover, adiponectin may favor plaque stabilization decreasing MMP activity by modulating the expression of the tissue inhibitor of metalloproteinase-1 (TIMP-1) through increased IL-10 secretion[115].

1.3.4 Inflammation, a link between Obesity and Cardiovascular disease

Atherosclerosis and obesity are distinct diseases, but there are similarities in the development of their pathophysiological concepts. In the past both diseases were traditionally known as lipid-storage disease, principally involving triglycerides in adipose tissue and cholesteryl ester in plaques[236]. Classical risk factors, such as smoking, hypertension, dyslipidemia, gender, age, family history, and diabetes mellitus, cannot adequately explain the high incidence of CVD[237]. Interestingly, treatment of typical atherosclerosis risk factors, e.g. plasma lipid control, blood pressure lowering, and diabetes treatment, reduced but did not eliminate CVD risk[238]. Therefore, it is supposed that obesity beside the traditional risk factors might be a new therapeutic target. The relation between obesity and CVD is indeed complicated. Some investigators suggest that the connection is indirect and depends on the enhanced prevalence of diabetes, hypertension and dyslipidemia, whereas others have demonstrated a direct association between obesity, especially abdominal obesity, and CVD risk[239,240]. A study showed that obesity in young men is associated with accelerated coronary atherosclerosis, indicating that the relationship between obesity and CVD appears to develop at a relatively young age[241]. When individuals become obese, adipose tissue undergoes cellular as well as

molecular alterations that subsequently impair systemic metabolism. Macrophages accumulate within adipose tissue, leading to local inflammation, which is believed to result in numerous metabolic dysfunctions that accompany obesity, including systemic inflammation and atherosclerosis. Moreover, clinical and experimental data support a link between systemic inflammation and endothelial dysfunction, a potential early marker of the development of atherosclerosis[242]. Elevated C-reactive protein (CRP) levels were identified as an independent risk factor for endothelial dysfunction. This might provide an important clue for linking a systemic marker of inflammation to the progression of atherosclerotic disease. Furthermore, blood vessels express receptors for most of the adipocyte-derived factors, suggesting that adipose tissue plays a key role in cardiovascular physiology via the existence of a network of local and systemic signals[242]. In the context of obesity the development of atherosclerosis stems from a constellation of interrelated pro-atherogenic mechanisms. Today it is well known that higher BMI is associated with subclinical inflammation, as reflected by increased CRP levels[243], and enhanced systemic oxidative stress that is independent of blood glucose and diabetes[244]. In addition, the orchestrated effects of hyperinsulinemia, hyperglycemia, nonesterified fatty acids (NEFA), and pro-inflammatory mediators all promote vascular wall oxidative stress, inflammation and endothelial dysfunction, contributing to the development of atherosclerosis.

1.4 Objectives

In light of the dramatically increased number of overweight and obese patients worldwide and considering that obesity is an important risk factor for CVD, the global leading cause of death, it is of particular importance to understand the responsible mechanisms and to find ways of how to combat competently both diseases. As mentioned in the previous sections, profound evidence established a negative crosstalk between increased adipose tissue mass in obesity and peripheral tissues and organs such as the vessel wall. In this context, adipose tissue is an endocrine organ secreting numerous adipokines, which are associated with inflammation, insulin resistance and atherosclerosis. Although several studies analyzed the impact of single adipokines on VSMC proliferation, migration and signaling, the complete secretory output of human adipocytes has not been investigated until now. In this context, the effects of free fatty acids in combination with the whole mixture of adipokines on VSMC remain incompletely characterized. In that perspective, the identification of factors and mechanisms contributing to VSMC dysfunction in obesity could lead to the discovery of new pharmacological targets for the treatment of atherosclerosis and vascular diseases, or of novel biomarkers indicating a person´s individual risk of developing atherosclerosis or vascular diseases later in life. Therefore, the starting point of the presented work is the characterization of the impact of *in vitro* differentiated human adipocytes on human VSMC.

- It is apparent that expanded fat mass in obesity, especially by its secretory output, is a strong risk factor for the development of CVD. The crosstalk of adipose tissue with cells of the arterial wall, especially smooth muscle cells, is not completely understood. Therefore, the first objective of this study was to provide new insights into the complex cellular mechanisms linking obesity and atherosclerosis by assessing the role of lipid mediators and protein factors in the crosstalk between human VSMC and adipose tissue. In this regard, the impact of adipocyte-CM in combination with fatty acids on smooth muscle cell proliferation, migration and pro-inflammatory signaling was investigated.

- In obesity, FFA coincide with an altered adipokine profile, enabling synergistic influences. However, so far only few studies have analyzed the combined impact of fatty acids and adipokines in VSMC. In addition, until now the factors and mechanisms, which are responsible for aberrant VSMC proliferation induced by lipid mediators and adipokines, are not fully understood. Based on the observations of the first study the second objective of this thesis was to investigate factors and mechanisms which are responsible for the synergistic effect of adipokines and oleic acid on VSMC proliferation.

- It is well established, that the transcription factor NF-κB plays a central role as a signal integrator controlling the process of vascular inflammation. Beside its function in triggering differentiation, survival, and proliferation of cells, NF-κB also controls the expression of various genes encoding inflammatory cytokines (MCP-1), adhesion molecules (VCAM-1, ICAM-1), and multiple enzymes (e.g. COX-2, iNOS), which are known to be involved in the further formation of the atherosclerotic plaque. Additionally, we reported that the combination of CM and OA leads to a synergistic activation of the NF-κB pathway, which was found to be essential for VSMC proliferation. In this context, the third aim of this thesis was to analyze selected NF-κB target genes, which are regulated by adipocyte-CM and/or fatty acids OA and PA, possibly involved in SMC proliferation and inflammation.

CHAPTER 2

Study 1

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 mm^2 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. Treating hVSMC with CM led to a significant 3-fold increase in the migration of hVSMC compared to untreated cells (Fig. 1E).

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). We could demonstrate that hVSMC migrated into the scratch already after incubation with CM for 6 h (Fig. 1D).

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 hVSMC had no effect on 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.

Acknowledgments

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The authors confirm that there are no conflicts of interest.

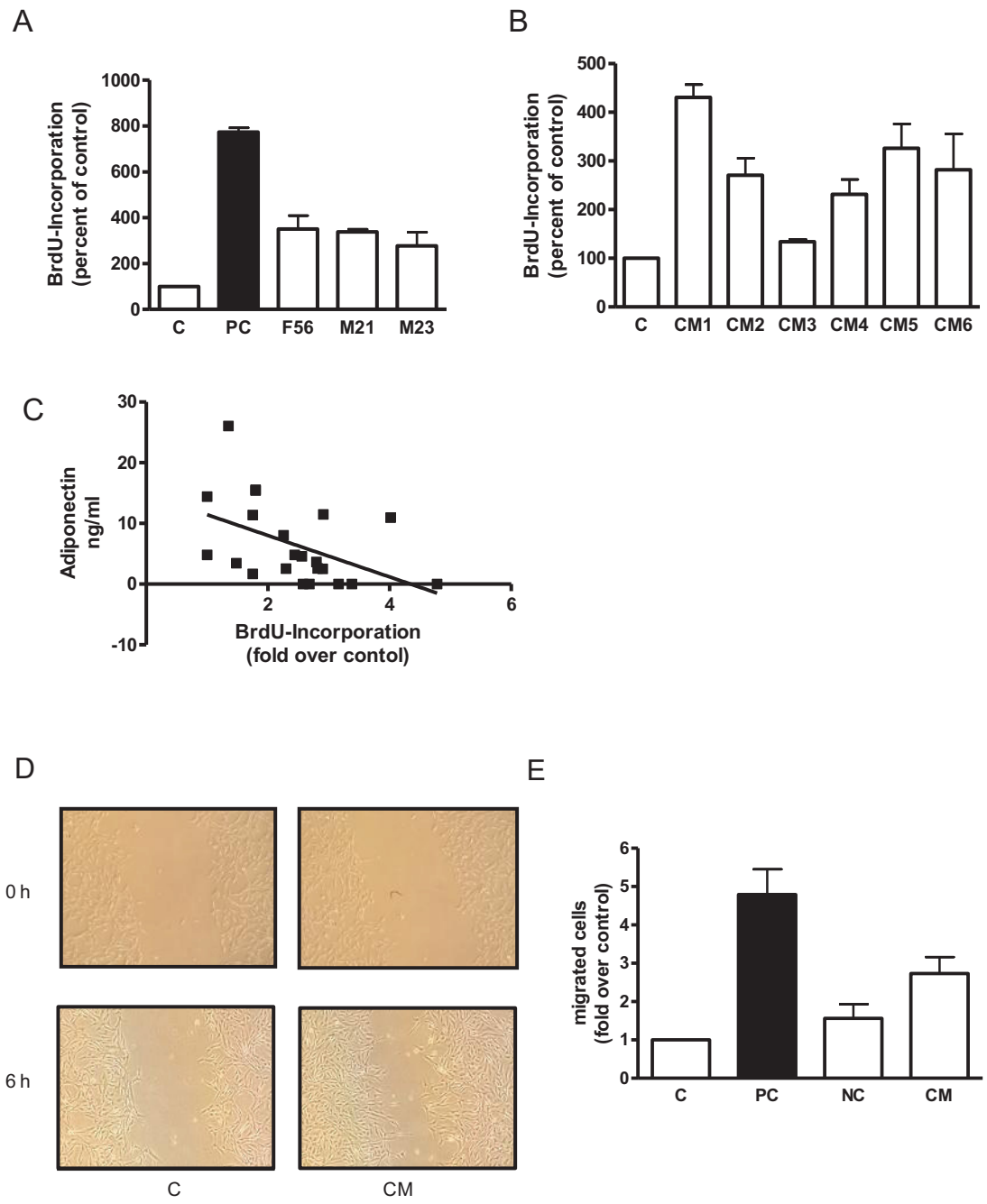

Figure 2.1 Effect of CM on proliferation (A, B, C) and migration (D, E) of hVSMC. The proliferation was determined by measuring the incorporation of BrdU into DNA. Data are expressed relative to the basal control value, taken as 100 %. FCS was used as a positive control (PC). (A) Effect of CM from a 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.

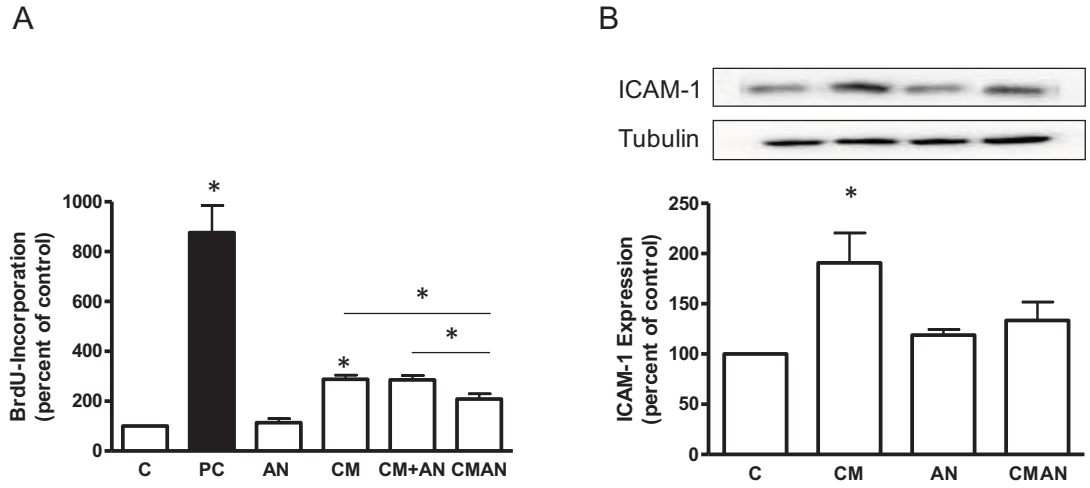

Figure 2.2 Analysis of CM generated in the absence or presence of AN. CM were generated for 48 h with or without 10 nM full length AN (CMAN). Further, AN was added to CM just before the incubation with hVSMC (CM+AN). (A) Effect of AN, CMAN and CM+AN on BrdU incorporation into DNA in hVSMC. Data are expressed 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.

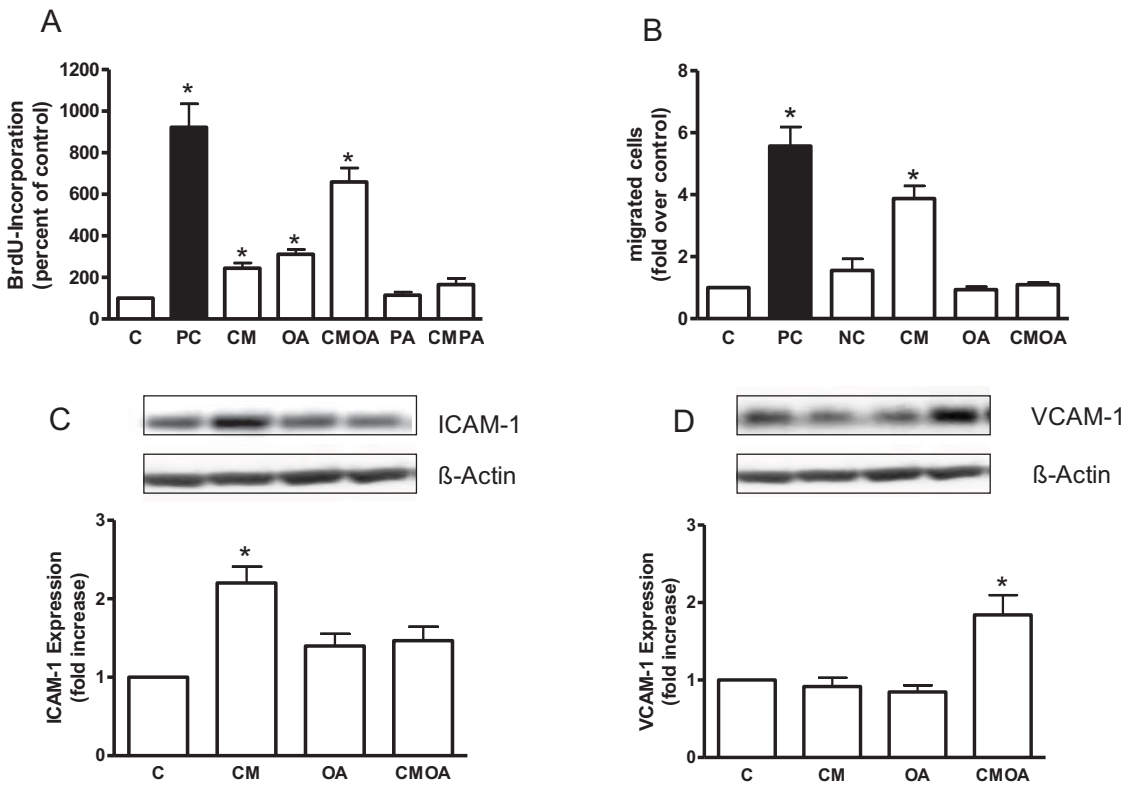

Figure 2.3 Effect of OA and PA (100 µmol/l) on hVSMC proliferation (A), migration (B) and the expression of adhesion molecules ICAM-1 (C) and VCAM-1 (D). (A) For the proliferation assay, hVSMC were serum starved for 24 h and subsequently incubated with BrdU in the absence or presence of CM, OA, PA or 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.

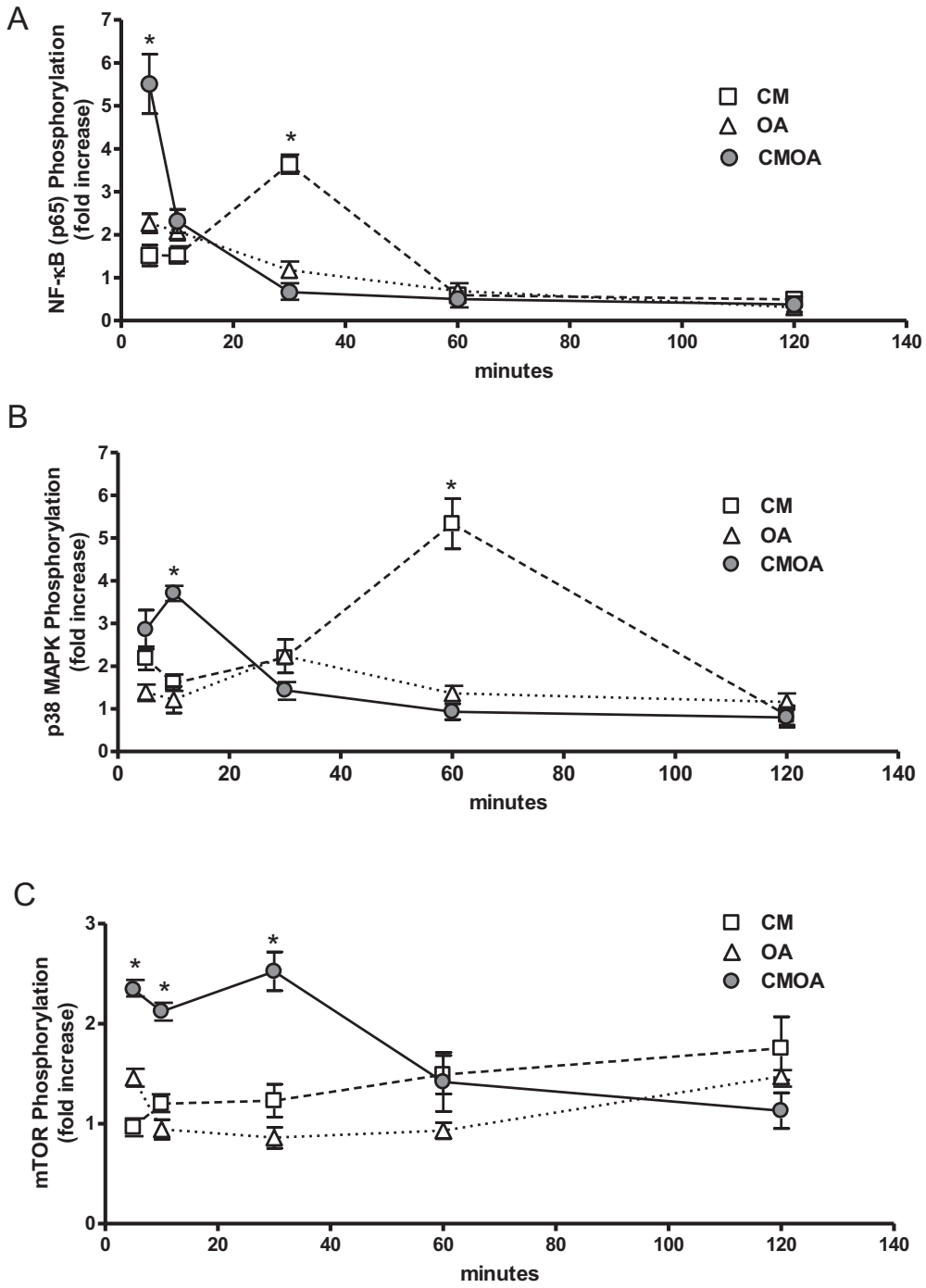

Figure 2.4 CM, OA and the combination of both acutely activate multiple intracellular signaling pathways. hVSMC were serum starved for 24 h and then exposed to CM, 100 µmol/l OA and the combination CMOA for the indicated times. Total cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies 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)

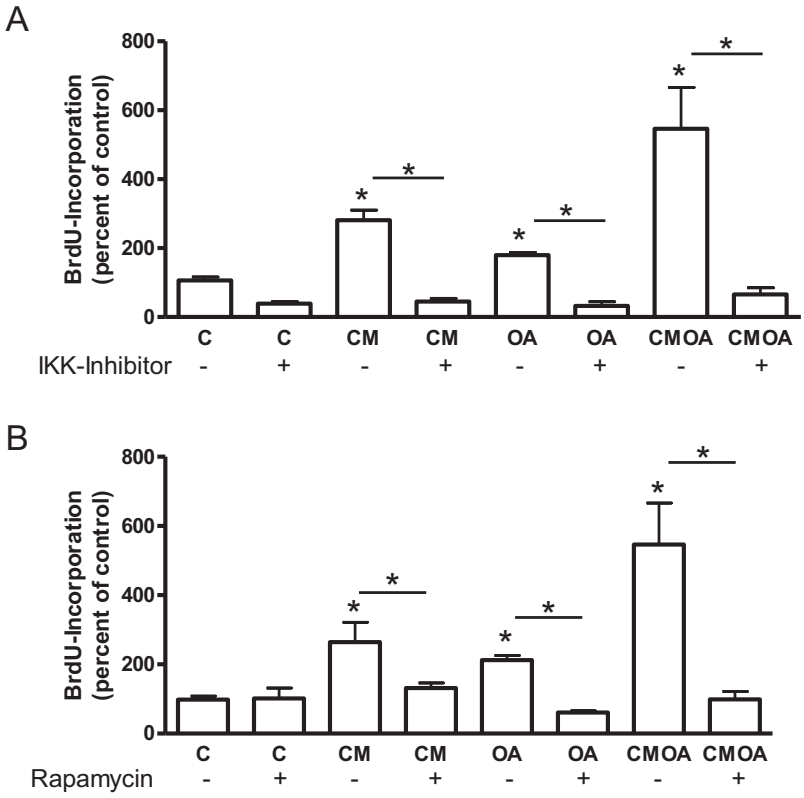

Figure 2.5 Impact of rapamycin and IKK-Inhibitor on the proliferative effect of CM, OA and CMOA in hVSMC. Cells were treated with CM, OA and CMOA as described in the legend to Fig. 3, without or with 10 nmol/l rapamycin (A) or 10 µmol/l IKK-Inhibitor (B) for 24 h. Proliferation was measured by the incorporation of BrdU into DNA . Data are expressed relative to the basal control value. *$p<0.05$ ($n = 3$-4).

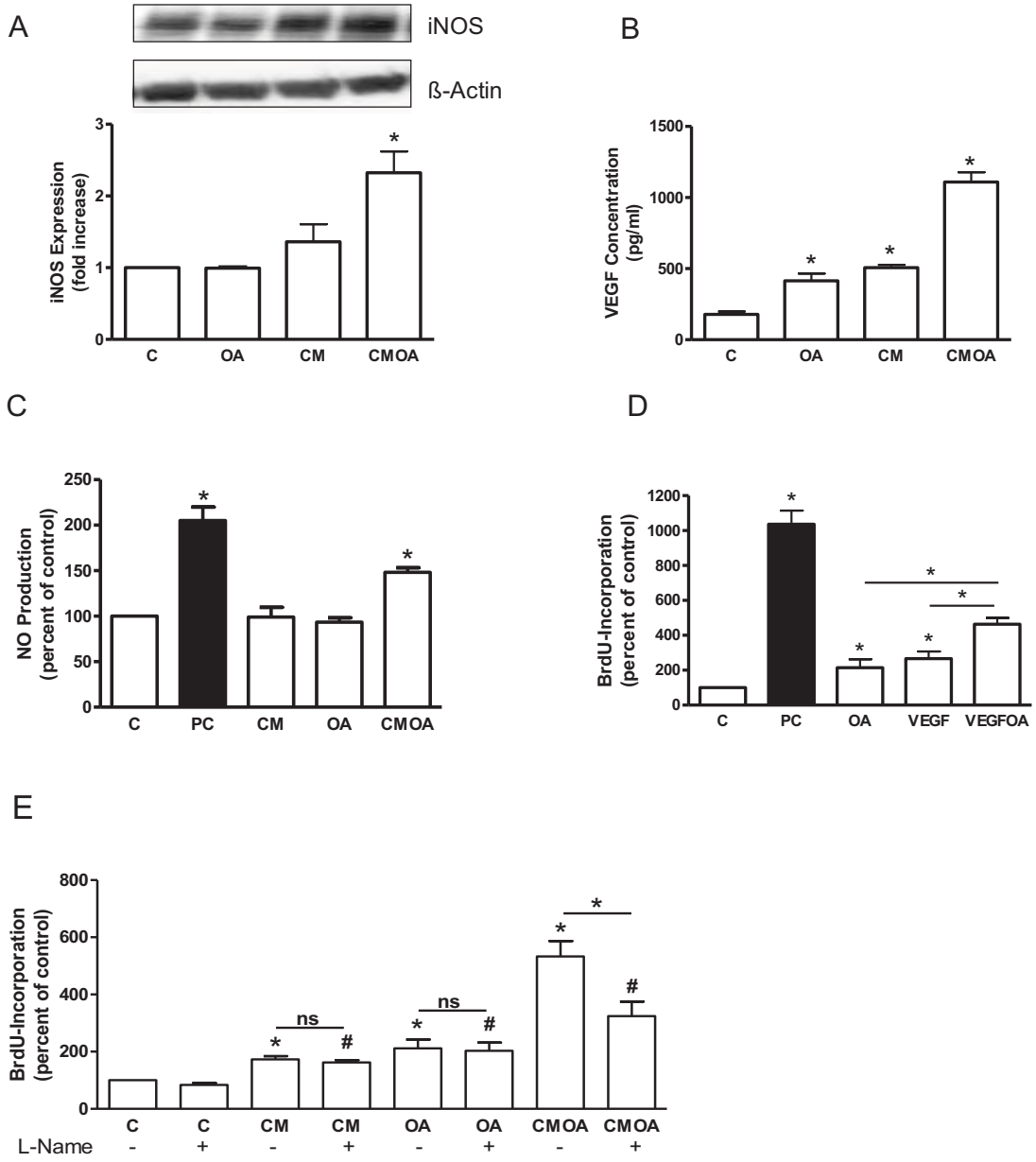

Figure 2.6 Effects of OA, CM and the combination of both on iNOS expression, VEGF concentration and NO production and impact of VEGF and NOS inhibitor L-NAME on proliferation. hVSMC were treated as described in the legend to Fig. 3. (A) Total cell lysates were resolved by SDS-PAGE and immunoblotted with 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. 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).

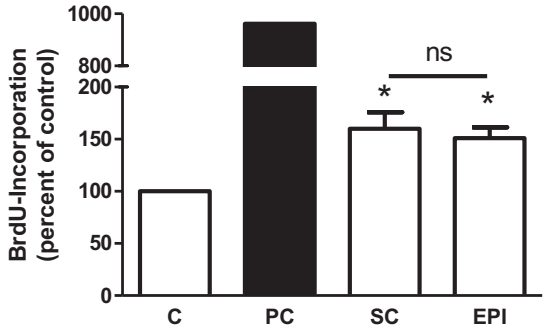

Figure 2.7 Effect of CM from subcutaneous (sc) and epicardial (epi) fat explants on the proliferation of hVSMC. The proliferation was determined by measuring the incorporation of BrdU into DNA. Data are expressed relative to the basal control value, which was set as 100 %. Data are presented as mean ± 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.

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CHAPTER 3

Study 2

Adipokine-induced increase of CD36 and VEGF are mediators of adipokine- and oleic acid-induced proliferation of smooth muscle cells†

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Abbreviations: 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; VSMC, 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, adipose tissue secrets various adipokines and free fatty acids, which may contribute to obesity-associated vascular dysfunction and cardiovascular risk. The combination of adipokines and oleic acid increases proliferation of human vascular smooth muscle cells (VSMC) in a synergistic way. In this study we investigated which factors and mechanisms are responsible for the synergistic effect of adipokine and oleic acid-induced proliferation of VSMC. *Methods and Results:* Adipocyte-conditioned media (CM) generated from human adipocytes induces proliferation of VSMC in correlation to its VEGF content. CM-treatment also increases CD36 and VEGF-R expression and VEGF secretion of VSMC, while oleic acid stimulates VEGF secretion from VSMC and lipid accumulation. CM and oleic acid in combination induce the strongest VEGF secretion from VSMC. Perivascular adipose tissue (PAT) explants from diabetic patients release significantly higher amounts of VEGF and induce stronger proliferation of VSMC as compared to subcutaneous fat (SAT) and SAT/PAT of non-diabetics, in parallel to marked induction of CD36 and VEGF-R. VEGF neutralization and CD36 silencing abrogate CM- and OA-induced proliferation and considerably reduce proliferation induced by the combination of CM and OA. CD36 silencing abrogates VEGF-induced ERK signaling without affecting VEGF-R expression. *Conclusion:* Our results identified VEGF as a critical growth factor and adipokine-mediated induction of CD36 as a mechanism in the adipokine and oleic acid-induced proliferation of VSMC. The combination of CM and oleic acid synergistically induced proliferation of VSMC via increased CD36 and VEGF-R expression as well as VEGF secretion of VSMC, with CD36 being necessary for an enhanced VEGF signaling. Combined elevated circulating free fatty acids and increased adipokine secretion might be a link between adipose tissue inflammation and VSMC dysfunction.

Keywords: smooth muscle cells, proliferation, adipokines, oleic acid, VEGF, CD36

Introduction

Obesity has become a major worldwide health problem, especially in industrial countries, which is associated with a number of metabolic diseases, including insulin resistance, type 2 diabetes, atherosclerosis and cardiovascular disease [1]. It is well established, that adipose tissue is an endocrine organ releasing lipid mediators and a variety of cytokines, the so-called adipokines [2,3,4]. Increasing evidence indicates that obesity is causally linked to a chronic low-grade systemic inflammatory state [5,6] that contributes to obesity-associated vascular dysfunction and cardiovascular risk [7]. Obesity is strongly related to the development of atherosclerosis in human beings as well as in

various animal models [8,9]. In the pathophysiology of vascular diseases, perivascular adipose tissue might play an important role because almost all blood vessel are surrounded by this fat depot. Due to the fact that perivascular adipocytes are not separated from the blood vessel wall by an anatomic barrier, the secretion of adipokines by this fat depot may provide a new link between obesity and vascular complications [10]. However, till now the mechanism how perivascular fat enhances the risk of metabolic and cardiovascular disease is not completely unraveled. Besides endothelial cells, vascular smooth muscle cells (VSMC) represent one of the major cell types of the vascular wall retaining homeostasis of the vessel wall. Arterial wall thickening is mediated by migration of VSMC from the media to intima and their concomitant proliferation. Recently, an *in vivo* study could demonstrate that lipids induced phenotype switching of VSMC [11]. Other studies showed that adipokines such as leptin and resistin affected VSMC proliferation and function [12,13]. The secretory output of expanded adipose tissue is a strong risk factor for the development of cardiovascular diseases [14]. We previously showed that adipocyte-conditioned medium (CM) induced VSMC proliferation and the combination of CM with oleic acid (OA) increased the proliferation in a synergistic way via induction of iNOS expression, NO-production and pro-inflammatory signaling [15]. However, until now the factors and mechanism, which are responsible for aberrant VSMC proliferation induced by lipid mediators and adipokines is not fully understood. Therefore, the main objective of this study was to provide insight into the complex cellular mechanisms of crosstalk between adipose tissue and VSMC potentially linking obesity and atherosclerosis. Our study demonstrates that CM-treatment increases CD36 and VEGF receptor-1 and 2 (VEGF-R1 and 2) expression and VEGF secretion of VSMC while OA-treatment induces VEGF secretion and triglyceride accumulation of VSMC. The prominent proliferative effect of the combination of CM and OA is mediated by increased VEGF release, CD36 and VEGF-R expression of VSMC with CD36 being necessary for an enhanced VEGF signaling.

Materials and Methods

Materials

Reagents for SDS-PAGE were supplied by Amersham Pharmacia Biotech (Braunschweig, Germany) and by Sigma (München, Germany). Polyclonal antibodies anti VEGF-R 1 and 2, 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 came from Abcam (Cambridge, Great Britain), anti-tubulin from Calbiochem (Merck Biosciences, Schwalbach, Germany) and anti-fatty acid

transport protein-4 (FATP-4) from Abnova (Heidelberg, Germany). Vascular endothelial growth factor (VEGF) neutralization was achieved by pretreatment with 1 µg/ml VEGF-neutralizing antibody from R&D Systems (Wiesbaden-Nordenstadt, Germany) for 1 hour. Anti-CD36 antibody was a kind gift from J.F. Glatz (Maastricht, Netherlands). 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 and BSA (fraction V, fatty acid free, low endotoxin) were obtained from Sigma (München, Germany). Human recombinant VEGF was purchased from Millipore GmbH (Schwalbach, Germany). The Cell Proliferation ELISA (BrdU, chemiluminescent) and protease inhibitor cocktail tablets were from Roche (Mannheim, Germany). VEGF ELISA kits were purchased from BioVendor GmbH (Heidelberg, Germany). FCS was supplied by Gibco (Invitrogen, Carlsbad, USA). HiPerFect transfection reagent was obtained from Qiagen (Hilden, Germany) and non-silencing control small interfering RNA (siRNA) and Silencer® select pre-designed siRNA specific for human CD36 were products from Ambion (Applied Biosystems, Darmstadt, Germany). Sodium salt of OA (Sigma, München, Germany) was dissolved in water as a 6 mM stock solution, and was further diluted in sterile serum-free VSMC medium containing 4 % (wt/v) BSA. OA was applied to VSMC at a final concentration of 100 µmol/l for 18 h. All controls of experiments involving fatty acids were treated with BSA alone. All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma.

Culture of adipocytes and fat explants

Subcutaneous adipose tissue was obtained from healthy 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 [16]. Seeded preadipocytes were induced to differentiation into adipocytes over 15 days as previously described by us [15]. The degree of differentiation was determined by oil red staining and induction of adiponectin. Differentiated adipocytes were used for the generation of adipocyte-CM, as recently described by us [17]. Briefly, CM was generated by culturing adipocytes for 48 h in VSMC basal medium (PromoCell) with addition of 50 ng/ml amphotericin b and 50 µg/ml gentamycin. Each CM was tested for its proliferative effect and the content of adiponectin (negatively correlated to proliferation). A more-detailed characterization of CM was described previously by us [17,18]. Human perivascular and subcutaneous fat biopsies were obtained from patients with (n=5, body mass index 28.8 ± 6.9, and aged 67.8 ± 9.0 years) or

without type 2 diabetes (n=9, body mass index 27.6 ± 3.7, and aged 68.7 ± 11.6 years) undergoing coronary artery bypass surgery. Adipose tissue was collected and used to generate CM as described [19]. Briefly, fat explants were cultured in DMEM F12 containing 10% fetal calf serum, 33 µmol/l biotin, 17 µmol/l panthothenate and antibiotic-antimycotic (Invitrogen, Carlsbad, USA) overnight in a humidified atmosphere (95% air and 5% CO_2). To generate CM, 100 mg of adipose tissue explants were cultured in 1 ml fresh 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 (VSMC)

Primary human coronary artery VSMC were obtained from PromoCell (Heidelberg, Germany). VSMC from four different donors (Caucasian, male, 23, 31, 40 years old; female, 56 years old) were used as subconfluent cells of passage 3. Cells were characterized as VSMC by morphologic criteria and by immunostaining with smooth muscle α-actin.

Silencing of CD36 in VSMC

The siRNA was used at 20 nmol/l for CD36 with HiPerFect as transfection reagent according to the manufacturer´s protocol. The applied siRNA was either non-silencing RNA as negative control or siRNA specifically silencing CD36. Lysates for immunoblotting were prepared 48 h after transfection. To analyze the effect of CD36 silencing on growth-promoting activity, VSMC were seeded on 96 well plates and allowed to attach for 24 h. Transfection with non-silencing and CD36 siRNA was preformed as described above. After 24 h, the medium was changed and the cells were stimulated with CM, VEGF and OA for 18 h, in the presence of BrdU, as detailed below.

In vitro analysis of VSMC proliferation

To monitor DNA synthesis VSMC 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 18 h as outlined above in the presence of BrdU (10 µM). 10.000 VSMC 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).

Immunoblotting

VSMC 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 [20]. 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 VersaDoc work station (Bio-Rad Laboratories, München, Germany).

Triglyceride Assay and Nile-Red staining

Biovision´s Triglyceride Quantification Kit (BioCat, Heidelberg, Germany) was used to assay triglyceride content. VSMC were treated as described above and lysed in a 5 % Triton-X100 solution. Lipids were dissolved by heating the lysates to 95°C for 5 minutes followed by slowly cooling down to room temperature. This was preformed twice before the lysates were cleared by centrifugation (13000 rpm, 5 min). The supernatants were used for the triglyceride assay according to the manufacturer´s instruction. For the Nile-Red staining, VSMC were fixed and stained for 20 min with 100 µg/ml Nile Red (Biomol, Hamburg, Germany) dissolved in DMSO. VSMC were viewed with a 20x plan objective lense using a Zeiss Axiovert 200M microscope and Zeiss LSM 5 PASCAL software (Zeiss, Jena, Germany). Simultaneous excitation with wavelengths of 488 nm and 543 nm was applied to visualize red as well as green/gold fluorescence.

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, USA) considering a P value of less than 0.05 as statistically significant. Corresponding significance levels are indicated in the figures.

Results

Adipocyte-conditioned medium and VEGF induce VSMC proliferation

CM from *in vitro* differentiated human adipocytes induces proliferation of human coronary artery VSMC which is donor dependent (Figure 1A). The large majority of the CMs tested induce a prominent proliferation of more than 2-fold compared to control that strongly correlates with the VEGF-content of the respective CM (Figure 1B), indicating that VEGF might play a pivotal role for the CM-induced proliferation. We previously reported that the proliferative effect of CM negatively correlates with its adiponectin concentrations but no correlation was found with IL-6 in CM [15]. While CM and OA induce similarly strong proliferation of VSMC, their combination augmented the proliferation in a synergistic way (Figure 1C), as reported previously [15]. Replacing CM by VEGF at the average concentration found in the tested CM (250 pg/ml human recombinant VEGF), the proliferative effect of CM could be mimicked (Figure 1D). In combination with OA, an additive proliferative effect of VEGF was observed, implying that other adipokines in addition to VEGF might play a role in CMOA-induced proliferation of VSMC. Treatment with CM, OA or the combination of CMOA induced VEGF secretion of VSMC (Figure 1E). The combination of CMOA induced a significantly increased VEGF secretion by VSMC compared to CM or OA alone, suggesting that VSMC significantly contribute to proliferation by releasing VEGF for an autocrine/paracrine stimulation (Figure 1E). CM and the combination of CMOA also increased the expression of VEGF-R1, whereas OA had no effect on its expression (Figure 1F). In addition, VEGF-R2 is regulated in the same way (data in supplement).

CM induces CD36 and augments triglyceride accumulation in VSMC

Incubation of VSMC with OA significantly enhanced triglyceride accumulation in VSMC that was additionally increased by the combined treatment of CM and OA (Figure 1G). CM alone had no effect on the triglyceride content of VSMC. CM substantially induced the expression of the fatty acid transporter CD36 (Figure 1H), while OA had no effect on CD36 expression and could not further increase CD36 induction by CM (data not shown). Furthermore, also VEGF induced the expression of CD36 (Figure 1H). Our data suggests, that the up-regulation of CD36 might enhance the uptake of OA, followed by an increased triglyceride concentration in VSMC after the incubation with the combination of CM and OA.

Perivascular fat of diabetic patients releases increased amounts of VEGF and induces significant proliferation of VSMC

In order to validate our findings in a more pathophysiological setting, we collected paired samples of subcutaneous (SAT) and perivascular (PAT) adipose tissue from patients with and without type 2 diabetes. Explants of SAT and PAT were used to generate CM for the measurement of VEGF release and the test of their proliferative capacity. The release of VEGF was comparable from SAT and PAT of the non-diabetic subjects and from SAT of patients with type 2 diabetes (Figure 2A) while its release by PAT of the diabetic patients was significantly increased. Accordingly, CM from PAT of diabetic patients induced the strongest proliferative effect on VSMC (Figure 2B). In both groups of patients, CM from PAT induced a stronger proliferation as compared to CM from SAT. Furthermore, CM from PAT of patients with type 2 diabetes induced a 4.6-fold increase of VEGF-R1 and 2 expression in VSMC (Figure 2C, data on VEGF-R2 in supplement). In contrast, PAT of non-diabetics exerted a comparable effect to the respective SAT. Expression of CD36 was also regulated by CM of SAT and PAT in a different way with PAT inducing CD36 expression above the level of SAT in both groups (Figure 2D). The strongest induction of CD36 was observed in VSMC treated with CM from PAT of type 2 diabetes patients. Our findings suggest that VEGF is an important adipokine produced in PAT especially from patients with type 2 diabetes that might induce expression of VEGF-R1 in VSMC. Furthermore, yet unknown adipokines specifically released by PAT might contribute to CM-induced proliferation.

VEGF is an important factor for CM- and OA-induced VSMC proliferation

VEGF was neutralized with a specific antibody and the dose-dependent proliferative effect of VEGF at concentrations ranging from 250 pg/ml to 1 µg/ml, according to the VEGF release by VSMC in the different conditions, were effectively blocked (Figure 3A). The induction of VEGF-R1 by CM and the combination of CM and OA was also completely prevented by VEGF-blocking (Figure 3B), indicating that the autorelease of VEGF by VSMC and VEGF in CM could be responsible for the regulation of this VEGF-R. Furthermore, neutralizing VEGF prevented the CM-, VEGF- and OA-induced proliferation completely (Figure 3C and D), underlining our hypothesis that CM and OA increase VSMC proliferation via VEGF. VEGF-blocking significantly reduced the proliferative effect of the combination CMOA, which is still substantially elevated compared to untreated control (Figure 3C), illustrating that under this condition VEGF alone is not the only proliferative mechanism.

CD36 is crucial for triglyceride accumulation, but not for VEGF secretion or VEGF-R expression of VSMC

CD36 content of VSMC was manipulated by silencing CD36 with specific siRNA, resulting in a reduction of CD36 protein expression by more than 80% after 48 h (Figure 4A). All further experiments were done after 48 h silencing. As a proof for CD36 being essential for the increase of triglyceride concentration induced by OA and the combination of CM and OA, reducing CD36 content prevented triglyceride accumulation in both situations (Figure 4B). In contrast, CD36 silencing had no effect on VEGF secretion of VSMC (Figure 4C) and expression of VEGF-R1 (Figure 4D).

CD36 is essential for proliferation of VSMC and VEGF-induced ERK phosphorylation

Proliferation induced by CM and the combination of CM and OA could be significantly reduced by CD36 silencing while the effect of OA could be completely abrogated (Figure 5A). In addition, proliferation induced by VEGF and VEGF combined with OA could be significantly diminished by CD36 silencing (Figure 5B). Activation of ERK1/2 by VEGF could be significantly blocked by CD36 silencing (Figure 5C) despite an unchanged regulation of VEGF-R1 (Figure 4D). As both neutralization of VEGF by a specific antibody and siRNA-mediated reduction of CD36 partially prevent the synergistic proliferative effect of the combination of CM and OA, both strategies were applied simultaneously. As shown in Figure 5D, concomitant neutralization of VEGF and silencing of CD36 depress most of the CMOA-induced proliferation, suggesting that both VEGF and CD36 are required to induce VSMC proliferation. However, we hypothesize that CM containing various adipokines other than VEGF could induce VSMC proliferation by other mechanisms still unknown.

Discussion

Obesity is strongly related to the development of atherosclerosis [21,22]. This study was designed to elucidate mechanisms how the secretory output from adipose tissue is related to VSMC proliferation as a crucial event in the genesis of atherosclerotic lesions [23]. We could show in a previous study that CM of *in vitro* differentiated adipocytes induces proliferation of VSMC in negative correlation to the adiponectin content of CM [15]. Searching for an active component of CM being responsible for VSMC proliferation, we found VEGF in CM to be significantly correlated with proliferation. VEGF is traditionally known as an endothelial cell-specific growth factor which modulates vascular disease by inducing endothelial proliferation mainly through the VEGF-R2 [24]. However, an increase of VEGF and VEGF-Rs could be observed in other injured arterial wall cells like monocytes and VSMC [25]. Vascular inflammation and the proliferation of endothelial cells as well as VSMC are enhanced through angiotensin II-induced VEGF release and expression of VEGF-R [26,27,28,29]. Here, we report that CM and OA as well as their combination induce release of VEGF by VSMC which in turn might auto-stimulate proliferation (for schematic summary of results see Figure 6). This effect is similar to hypoxia-induced proliferation of VSMC, where an autocrine mechanism of VEGF has been described [30]. In addition, it has been described that pro-inflammatory stimulation including JNK activation or stimulation with angiotensin II and IL-1beta induce VEGF release by VSMC [31,32]. It might be speculated that proinflammatory adipokines present in CM are therefore responsible for the induction of VEGF. As we observed previously OA stimulates VEGF release by VSMC [15]. In addition to stimulating the release of VEGF from VSMC, CM induced VEGF-R1 and 2 expression which mediate proliferation and migration of VSMC [33]. We propose that both VEGF-R are regulated by VEGF itself, as neutralizing VEGF prevented CM-induced augmentation of these receptors in parallel to proliferation. VEGF is a pro-inflammatory factor [34,35] and we previously demonstrated that CM activates NF-κB signaling which might be related to VEGF but also to other pro-inflammatory adipokines [15]. Blocking VEGF with a specific neutralizing VEGF-antibody reduced the CM, OA and VEGF-induced proliferation of VSMC completely. In contrast, the strong proliferative effect of the combination of CM and OA was not totally abrogated, illustrating that VEGF is not the only important factor for the synergism of CMOA. As CM contains various adipokines such as IL-6, IL-8 or MCP-1 [17], it is possible that some of these factors induce proliferation in addition to VEGF VEGF and that these factors might be causal for the synergistic effects of CM and OA by yet undescribed mechanisms.

The synergistic effect of CM and OA on VSMC proliferation is accompanied by a strong increase in triglyceride accumulation in VSMC, which is significantly higher compared to the treatment with OA alone. Analysis of CD36 expression revealed a strong upregulation of this fatty acid translocase and scavenger receptor in CM-treated

VSMC. Upregulation of CD36 has also been observed after treatment of VSCM with oxidized LDL and high glucose [36], which might be related to oxidative stress that is also induced by CM [18]. CD36 has been intensively studied for its role in facilitating fatty acid uptake and oxidation and is implicated in the pathophysiology of metabolic diseases including cardiovascular alterations [37,38,39,40]. OA-induced triglyceride accumulation is CD36-dependent in our human cell model, which confirms recent data obtained in rodent cells [41]. CM-induced expression of CD36 enhances OA-induced triglyceride accumulation but this effect cannot be attributed to VEGF as CD36 is not regulated by VEGF. Silencing CD36 prevented triglyceride accumulation without affecting VEGF release or expression of VEGF-R1. One of the major positive regulators of smooth muscle cell proliferation is the ERK signaling pathway [42] and activation of ERK is involved in the PDGF-induced VEGF expression in human VSMC [43]. Interestingly, VEGF signaling after CD36 silencing as assessed by ERK activation was significantly impaired. Previous studies could show that a link between VEGF-R and CD36 exists, which might explain why CD36 silencing impairs VEGF signaling. CD36 associates with VEGF-R2 in endothelial cells integrating angiogenic signals from thrombospondin and VEGF [44]. Recently data indicates that CD36 associates with VEGF-R2 and that VEGF-A, VEGF-R2 and CD36 are necessary for an angiogenic switch in endothelial cells [45]. Our results point to a potential interaction of CD36 and VEGF-R in a way that the presence of both receptors is necessary for normal VEGF signaling in human VSMC. Neither VEGF-blocking nor CD36 silencing could completely decrease CMOA-induced proliferation of VSMC, but combining both approaches depresses most of the CMOA-induced effect, suggesting that both VEGF and CD36 are required for CMOA-induced VSMC proliferation. Further studies will be needed to identify the factors in CM that induce CD36 and which might be additionally responsible for the synergism of CMOA.

Recently, it has been proposed that PAT might be involved in the development of atherosclerosis by a paracrine crosstalk with cells of the vessel wall where inflammation is induced [46]. PAT of obese rodents is characterized by higher macrophage accumulation, which also produce VEGF, and inflammation associated with abdominal aortic aneurysms [47]. Here, we demonstrate that PAT from patients with type 2 diabetes is characterized by a significantly higher VEGF release as compared to SAT and also compared to PAT from non-diabetics. Higher VEGF release is paralleled by a stronger induction of VEGF-R1 and 2. In addition, CM from PAT also significantly increases CD36 expression in comparison to SAT, both from healthy controls and type 2 diabetic patients. CD36 induction reflects the stronger proliferative effect of CM from PAT. Our results imply that VEGF and CD36 might be essential factors produced and induced by PAT from patients with type 2 diabetes, respectively. The use of paired biopsies from human SAT and PAT further strengthens the notion of VEGF release from PAT for a potential paracrine crosstalk. Anyway, the VEGF content of CM from, SAT and PAT from

non-diabetics as well as SAT from diabetics was equal, but had not the same effect on VSMC proliferation, indicating that VEGF is probably not the unique parameter for the observed effects. The induction of CD36 possibly leading to enhanced lipid accumulation in VSMC might be involved in a negative crosstalk between PAT and cells of the vascular wall. The restricted use of these biopsies only for key experiments is a technical limitation of our study. Further work will be needed to elucidate the differences in adipokine expression and secretion between PAT and other fat depots in health and disease in order to identify other putative factors responsible for PAT-induced vascular dysfunction.

In conclusion, our results provide new insights into the pro-atherogenic mechanisms of a negative crosstalk between human adipose tissue and VSMC identifying 1) VEGF as a critical growth factor being released by SAT as well as PAT and 2) adipokine-mediated induction of CD36 as a mechanism in the synergistically induced proliferation of VSMC after treatment with CM and OA (for schematic summary of results see Figure 6). We suggest that the combined elevated circulating free fatty acids and increased adipokine secretion by adipose tissue in obesity and type 2 diabetes might be a link between adipose tissue inflammation, vascular dysfunction and the development of atherosclerosis.

Acknowledgments

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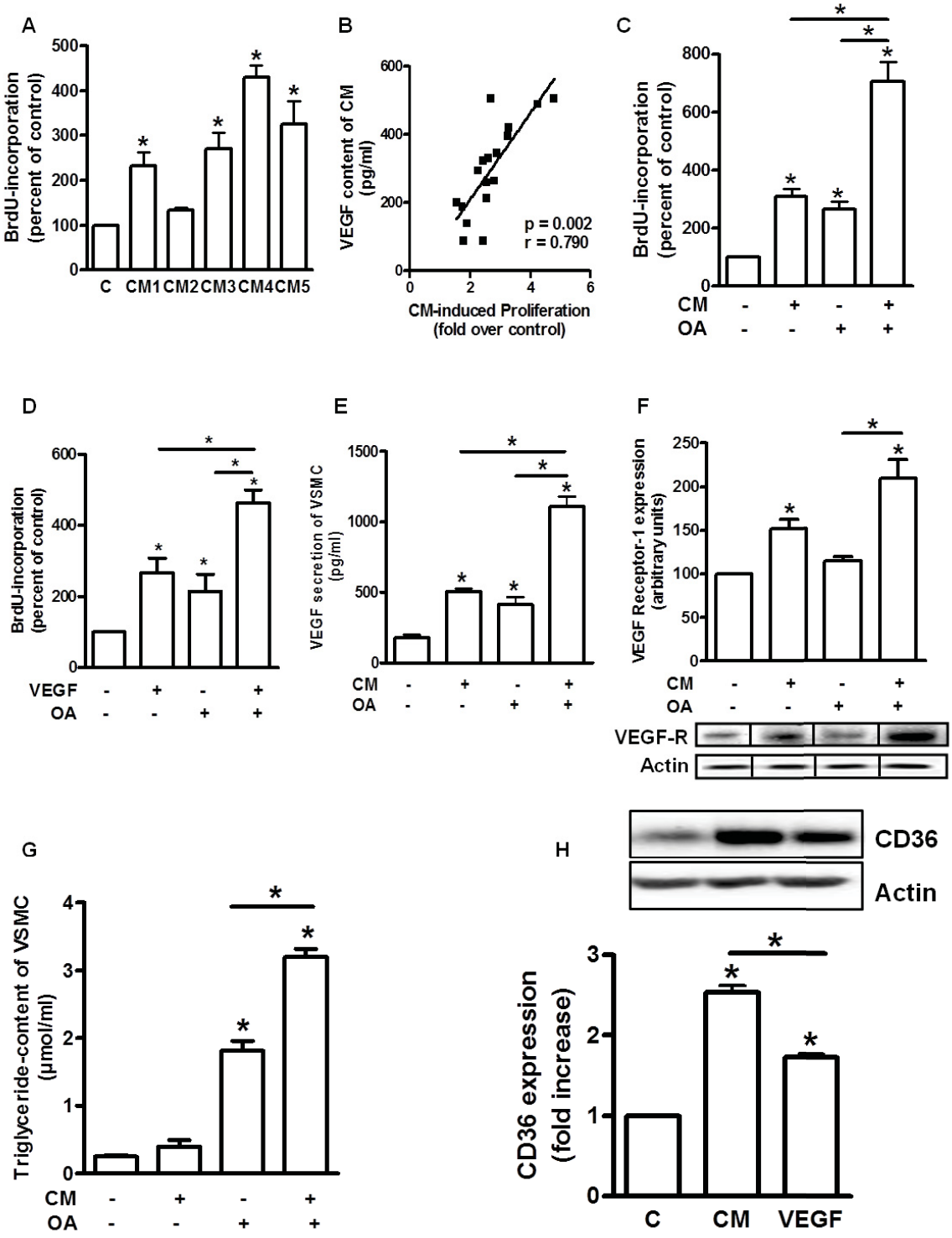


Figure 3.1 CM-induced proliferation is mediated by VEGF. (A) Proliferative effects of CM from five different adipose tissue donors determined on VSMC. VSMC medium was used as control (C). (B) The proliferative effect of CM correlates highly significantly with its content of VEGF measured by ELISA (n=17). (C and D) VSMC were serum starved for 24 h and subsequently incubated with CM, 250 pg/ml VEGF, 100 µmol/l OA or the combination of CM or VEGF and OA in the abscence or presence of BrdU for 18 h.

Incorporation of BrdU into DNA was measured. Data are expressed relative to control, taken as 100 %. Data are mean values ± SEM of three independent experiments. *p<0.05 compared to control or designated treatment. (E and F) VSMC were treated with CM, OA and their combination for 24 h and supernatant were collected for VEGF measurement in duplicates by ELISA. Total cell lysates were resolved by SDS-PAGE and immunoblotted with a specific VEGF-R1 antibody. Data are mean values ± SEM of three independent experiments. Lanes were excised from a single western blot and displayed in the present order. All data were normalized to the level of actin expression and are expressed relative to the control. CM induces CD36 expression and increases OA-induced triglyceride accumulation. (G) The triglyceride concentration was measured by a Triglyceride Quantification Kit. (H) VSMC were treated with CM from three different donors and VEGF for 24 h. Total cell lysates were resolved by SDS-PAGE and immunoblotted with a specific CD36 antibody. All data were normalized to the level of actin expression and expressed relative to the control. Data are mean values ± SEM of three independent experiments. *p<0.05 compared to control or designated data.

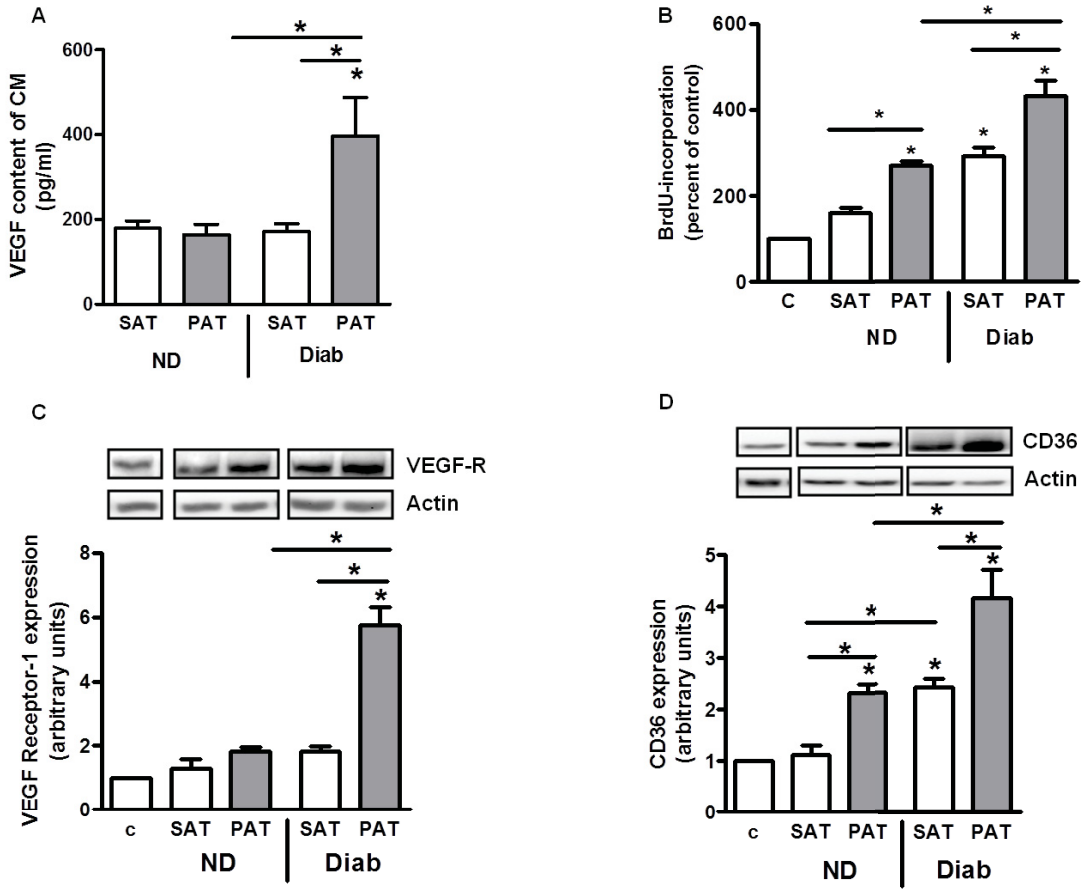


Figure 3.2 VEGF-concentration is higher in perivascular adipose tissue (PAT) from diabetics (Diab) correlating with VSMC proliferation, VEGF-R1 and CD36 expression. (A) VEGF content of the various fat depots was measured in duplicates by ELISA. (B) VSMC were treated with subcutaneous adipose tissue (SAT) and PAT CM from Diab or non-diabetics (ND) for 24 h. 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 PAT and SAT were obtained from the same patient. (C and D) Total cell lysates were resolved by SDS-PAGE and immunoblotted with a specific CD36 or VEGF-R1 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. *$p<0.05$ compared to control or designated data.

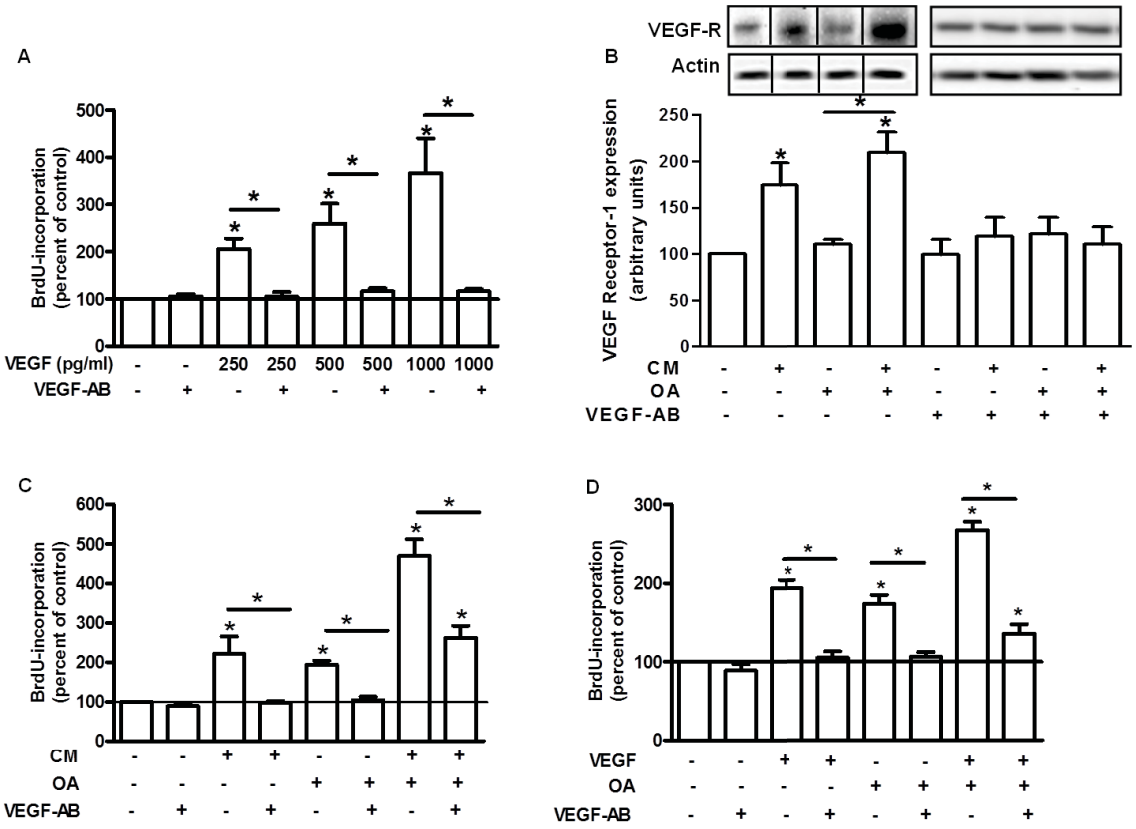


Figure 3.3 VEGF-neutralization prevents CM- and OA-induced proliferation. Cells were treated with 250, 500 and 1000 pg/ml VEGF (A), CM, OA and CMOA (B and C) and accordingly VEGF, OA and VEGFOA (D) as described in the legend to Figure 1 in the presence or absence of specific neutralizing VEGF antibody for 24 h. (A,C and D) BrdU incorporation into DNA was determined as described in the legend to Figure 1. Data are expressed relative to control. (B) Total cell lysates were resolved by SDS-PAGE and immunoblotted with a specific VEGF-R1 antibody. Lanes were excised from a single western blot and displayed in the present order. 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 or designated data.

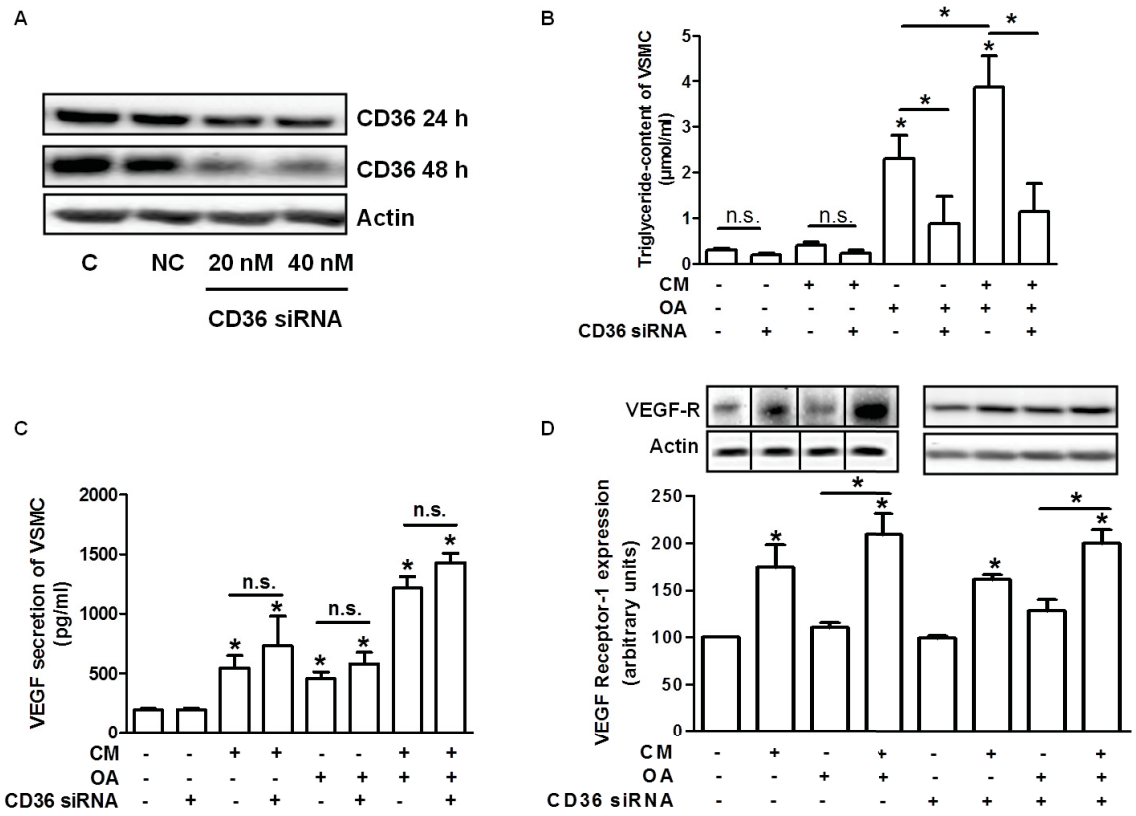


Figure 3.4 CD36 silencing prevents triglyceride accumulation and has no effect on VEGF concentration and VEGF-R1 expression. (A) VSMC were transfected with small interfering (si) RNA negative control (NC) or CD36 for 24 h or 48h and then treated with CM, 100 µmol/l OA and the combination of CM and OA (B-D). (A and D) Total cell lysates were resolved by SDS-PAGE and immunoblotted with a specific CD36 or VEGF1 antibody. Lanes were excised from a single western blot and displayed in the present order. 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) The triglyceride concentration was measured by Triglyceride Quantification Kit. (C) VEGF secretion of VSMC was measured in duplicates by ELISA.

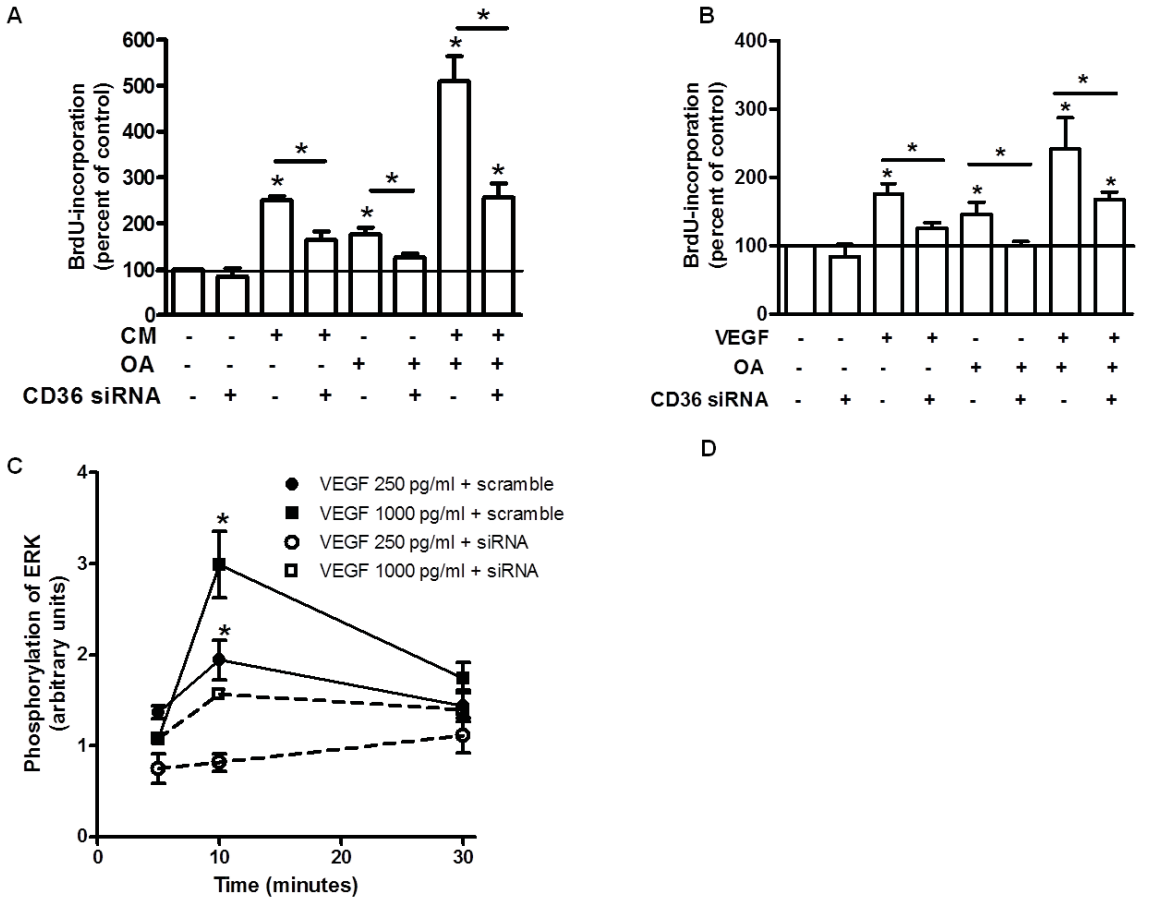


Figure 3.5 CD36 silencing prevents CM- and OA-induced proliferation and VEGF-induced ERK phosphorylation. VSMC were transfected with small interfering (si) RNA negative control (NC) or CD36 for 48h and then treated with CM or 250 or 1000 pg/ml VEGF, 100 µmol/l OA and the combination of CM or 250 pg/ml VEGF and OA. (A and B) BrdU incorporation into DNA was determined as described in the legend to Figure 1. Data are expressed relative to the basal control value. (C) Total cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies to phosphorylated and unphosphorylated ERK. 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. (D) Combination of VEGF neutralization and CD36 silencing decreases the CMOA-induced proliferation. VSMC were serum starved for 24 h and subsequently incubated with CM, 100 µmol/l OA or the combination of CM and OA with or without CD36 siRNA and specific neutralizing VEGF antibody. BrdU incorporation into DNA was determined as described in the legend to Figure 1. Data are expressed relative to control, taken as 100 %. Data are mean values ± SEM of three independent experiments. *p<0.05 compared to control or designated treatment.

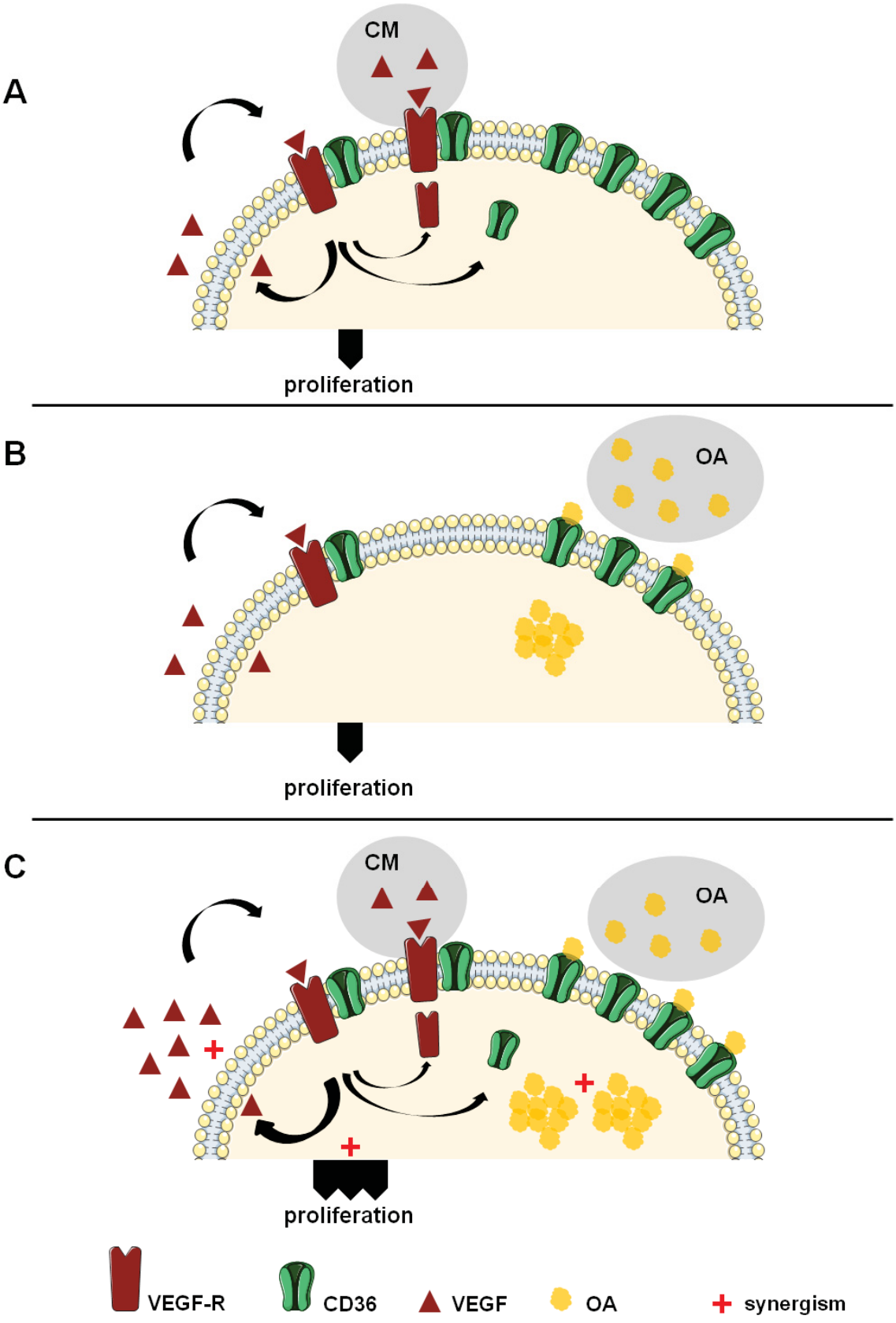
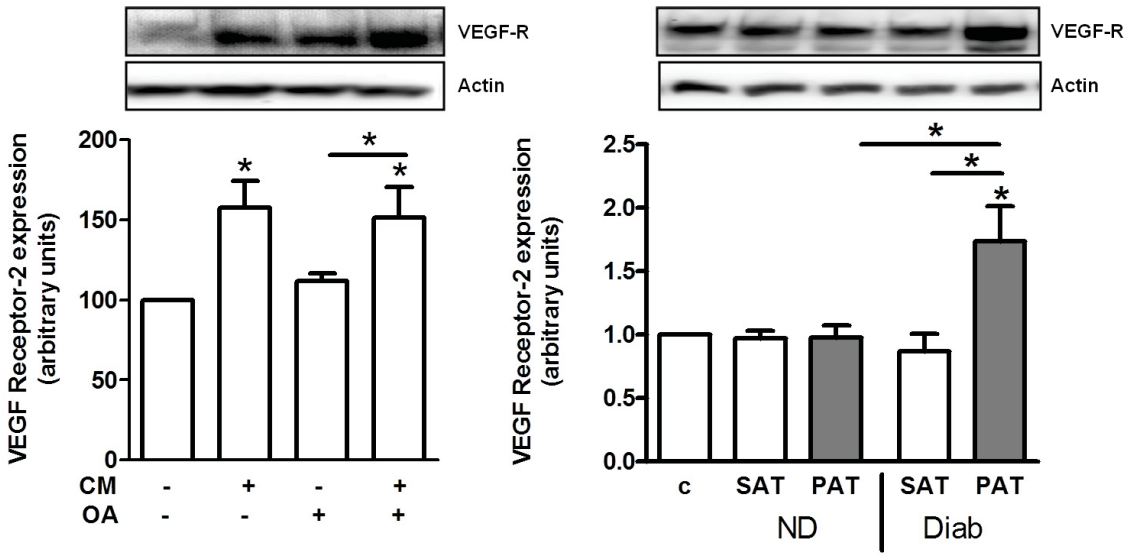

A
CM
proliferation
B
OA
proliferation
C
CM
OA
proliferation
VEGF-R
CD36
VEGF
OA
synergism

Figure 3.6 Schematic representation of the biological effects of (A) CM, (B) OA and (C) the combination of CM and OA in VSMC. (A) CM increased CD36 and VEGF-R expression and VEGF secretion of VSMC, (B) OA induced also VEGF secretion and triglyceride accumulation of VSMC and (C) the combination of CM and OA synergistically induced triglyceride accumulation and proliferation of VSMC via increased CD36 and VEGF-R expression and VEGF secretion of VSMC.



Supplement Figure 3.7 Expression of VEGF-R2. VSMC were serum starved for 24 h and then exposed to CM, 100 µmol/l OA and the combination of CM and OA or with CM from SAT and PAT from non-diabetic or diabetic patients. Total cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies to VEGF-R2. 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 or designated data.

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CHAPTER 4

Study 3

Differential impact of oleate, palmitate, and adipokines on expression of NF-κB target genes in human vascular smooth muscle cells†

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Abbreviations: CM adipocyte-conditioned medium; OA oleic acid, PA palmitic acid; SMC smooth muscle cells; ang-1 angiopoietin-1; MMP matrix-metalloproteinase; BrdU bromdesoxyuridin; FFA free fatty acids; CIDEA cell death-inducing DNA fragmentation factor α-like effector A; ang pre angiotensinogen precursor; ROS reactive oxygen species; SOD superoxide dismutase

Abstract

It is widely accepted that obesity is a major risk factor for the development of atherosclerosis. In this context, adipose tissue produces a variety of adipokines and releases free fatty acids, contributing to a chronic-low grade inflammation state implicated in vascular complications. In this study we investigated the role of adipokines, oleic acid (OA), palmitic acid (PA), and the combinations on activation of NF-κB target genes in human vascular smooth muscle cells (SMC). Adipocyte-conditioned medium (CM), generated from human adipocytes, in combination with low concentrations of OA, but not PA, induces SMC proliferation and activation of the transcription factor NF-κB in a synergistic way. Combined treatment of CM and OA further regulates a set of downstream NF-κB target genes including angiopoietin-1, activin A, and MMP-1, all critically involved in SMC dysfunction. This suggests that the lipotoxic potential of fatty acids is substantially enhanced by the presence of adipocyte-derived factors.

Keywords: Vascular smooth muscle cells; oleic acid; palmitic acid; adipocyte-conditioned medium; NF-κB; inflammation; vascular dysfunction

Introduction

Obesity is considered as a major determinant of cardiovascular morbidity and mortality[1,2], and several studies in humans and various animal models could demonstrate that obesity strongly correlates with the development of atherosclerosis[3,4]. Furthermore, it is now widely appreciated that adipocyte-secreted proteins, also known as adipokines, and free fatty acids (FFA) are involved in the development of a chronic low-grade inflammatory state that contributes to vascular dysfunction thereby providing a direct link between obesity and vascular complications[5,6]. A major cell type in the vessel wall is represented by smooth muscle cells (SMC), which migrate from the media to the intima in the early phase of the development of atherosclerosis. The subsequent proliferation of SMC in the intima and the concomitant phenotypic change from the contractile to the synthetic state are further features of SMC in the progression of atherosclerosis. Recently, we could show that conditioned media (CM) derived from in vitro differentiated human adipocytes induce a prominent migration and proliferation of human vascular smooth muscle cells[7]. Furthermore, oleate (OA) but not palmitate (PA) induces proliferation of SMC. CM, which itself does not contain fatty acids, in

combination with oleic acid markedly increased proliferation in a synergistic way. Additionally, we reported that the combination of CM and OA leads to a synergistic activation of the nuclear factor κB (NF-κB) pathway, which was found to be essential for SMC proliferation. A body of work has illuminated the central role for the transcription factor NF-κB as a signal integrator controlling the process of vascular inflammation[8]. Beside its function in triggering differentiation, survival, and proliferation of cells, NF-κB also controls the expression of various genes encoding inflammatory cytokines (MCP-1), adhesion molecules (VCAM-1, ICAM-1), and multiple enzymes (e.g. COX-2, iNOS), which are known to be involved in the recruitment of immune cells and/or the further formation of the atherosclerotic plaque[9,10]. Therefore, the aim of this study was to further provide insight into the complex cellular mechanisms linking obesity and atherosclerosis by analyzing selected NF-κB target genes, which are regulated by adipocyte-CM and/or fatty acids OA and PA, possibly involved in SMC proliferation and inflammation.

Materials and Methods

Materials

Reagents for SDS-PAGE were supplied by Amersham Pharmacia Biotech (Braunschweig, Germany) and by Sigma (Munich, Germany). Polyclonal antibodies anti-phospho-NF-κB (P65) (Ser536) and anti-NF-κB (P65) were supplied by Cell Signalling Technology (Frankfurt, Germany). The anti-actin antibody came from Abcam (Cambridge, GB). HRP-conjugated goat anti-rabbit and goat anti-mouse IgG antibodies came from Promega (Mannheim, Germany). Reagents for RT-PCR were obtained from QIAGEN (Hilden, Germany) and Applied Biosystems (Darmstadt, Germany). Collagenase NB4 was obtained from Serva (Heidelberg, Germany). TNFα, BSA (fraction V, fatty acid free, low endotoxin), sodium palmitate and sodium oleate were obtained from Sigma (Munich, Germany). Protease inhibitor cocktail tablets were from Roche (Mannheim, 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). 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. 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=9, body mass index 28.9 ± 1.5, and aged 47.7 ± 2.9 years) undergoing plastic surgery. The procedure was approved by the ethical committee of the Heinrich-Heine-University (Düsseldorf, Germany), and all subjects gave their written consent. 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 (Hauner et al., 1995). 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 % CO2. 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. Differentiated adipocytes were used for the generation of CM, as recently described by us (Dietze-Schroeder et al., 2005). 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 (Dietze-Schroeder et al., 2005). Using the Fatty Acid Assay Kit from Biovision (Biocat, Heidelberg, Germany) and HPLC (Sell et al., 2008) revealed that there were no measurable concentrations of FFA in CM.

Culture of human vascular smooth muscle cells (hVSMC)

Primary human coronary artery SMC were obtained from PromoCell (Heidelberg, Germany). SMC from four different donors (Caucasian, male, 31, 55, 58 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 SMC 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 human vascular SMC 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 18 h with CM, OA, PA or the combinations of CM with each fatty acid in the presence of BrdU (10 µM). 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).

RNA isolation and RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN) according to the manufacturer`s instructions. RNA concentration was measured with NanoDrop 2000 (Thermo Scientific, Schwerte, Germany). RNA was converted into cDNA using Omniscript RT Kit (QIAGEN). Primer Assays for IL-6, CIDEA, MMP-1, SOD-1, activin A, ang-1, ang pre, and RANTES were obtained from QIAGEN. Samples were run in triplicate in 25 µl or 10 µl reaction volumes using StepOne plus (Applied Biosystems). Amplification was done using SYBR Green technology (Applied Biosystems).

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 (Wichelhaus et al., 1994). 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 antibody. After washing, filters were incubated with a secondary HRP-coupled antibody and processed for enhanced

chemiluminescence detection using Immobilon HRP substrate (Millipore, Billerica, USA). Signals were visualized and evaluated on a VersaDoc 4000 MP (BioRad) workstation.

Determination of reactive oxygen species (ROS)

Human VSMC were pretreated as indicated for 18 h. Then, cells were washed in PBS without Ca/Mg and subsequently incubated with 10 µM 2',7'-dichlorodihydrofluorescein diacetate (DCF, Molecular Probes, Karlsruhe, Germany) solved in SMC basal medium (PromoCell) for 30 min at 37°. As a positive control, cells were treated with 0.3 % H2O2 for 30 min in parallel to DCF incubation. Subsequently, cells were lysed in PBS containing 0.1% (v/v) Triton X-100 and 0.5 mmol/l EDTA. Fluorescence was measured with excitation/emission wavelengths of 493/538 nm using an InfiniteM200 plate reader (Tecan, Maennedorf, Switzerland).

ELISA and Activity Assay

IL-6 secretion by hVSMC was determined using ELISA kits purchased from Hoelzel (Cologne, Germany). Human active MMP-1 was measured using Fluorokine® E Enzyme Activity Assay from R&D Systems (Wiesbaden-Nordenstadt, Germnay). 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 (Cell Biolabs, Inc., San Diego, USA). hVSMC were grown to confluence and serum-starved for 24 h. 300 µl cell suspension containing 3 x 104 detached cells was added to the upper compartment. Serum-free medium, CM, FCS or OA were placed in the lower compartment (500 µl/well). 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.

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 in combination with OA induces synergistic proliferation and NF-kappaB activation in hVSMC

In a previous study we could show that conditioned medium derived from in vitro differentiated human adipocytes induced migration and proliferation of human VSMC[7]. We could confirm the synergistic stimulation of SMC proliferation by the combination CMOA (10-fold) in comparison to CM (3-fold) and OA (2.7-fold) alone (fig. 1A). Moreover, treating cells with PA had no effect on SMC growth (1.2-fold), whereas the combination of PA and CM induced no further proliferation compared to the effect of CM alone (3-fold) (fig. 1A). Furthermore we could also observe that CM alone induced migration of human VSMC (fig. 1B).

As proliferation of SMC is regulated by different pathways including NF-κB, we further analyzed the activation of this transcription factor after treatment with CM, OA, PA and the combinations CMOA and CMPA. As shown in fig. 1C, the combination of adipocyte-CM and OA led to a substantial, 5-6 fold synergistic activation of NF-κB after 5 min, which was much more prominent than with CM (1.5-fold, not significant) or OA (2.2-fold) alone[7]. Furthermore, treatment with CM alone increased phospho-NF-κB significantly after 30 min, whereas at later time points NF-κB activation was no longer detectable. In the present study we could additionally observe that PA alone as well as the combination of CMPA did not induce NF-κB phosphorylation within 120 min. However, long-term activation of NF-kappaB by CMOA (1.5fold) as well as CMPA (1.6 fold) was observed after 18 h (fig. 1D).

The combination of CM and PA induces IL-6 expression and release

Additionally, we could observe that CMPA synergistically induces the expression of IL-6 (8-fold) after 4 h, in comparison to CM (2-fold) and PA (4-fold) alone (fig. 2A). Measuring IL-6 release after 24 h by ELISA revealed a significant IL-6 secretion after treatment with PA (2-fold) and with the combination CMPA (2.4-fold) (fig. 2B). Due to the fact that IL-6 is one of the best established NF-κB dependent genes in SMC, we analyzed the IL-6 release after treatment with a specific IKK-inhibitor. After the incubation with the IKK-inhibitor the PA-, CMOA- and CMPA-induced release of IL-6 was significantly reduced (fig. 2B).

Effects of CM and fatty acids on expression of NF-κB target genes

To further elucidate the downstream effects of the enhanced NF-κB activation, we analyzed several NF-κB target genes involved in SMC physiology and relevant for the development and progression of atherosclerosis. Figure 3 shows that OA as well as PA induced mRNA expression of RANTES (fig. 3A), a chemotactic cytokine having a role in leukocyte recruitment to inflammatory sites. In comparison to OA and PA alone, CM as well as the combination of CM with each fatty acid had no impact on RANTES expression. Furthermore, OA, and PA led to a significant induction of angiotenstin II (fig. 3B). Interestingly, positive control TNFα exerts no effect on angiotensin mRNA levels, suggesting that that the TNFα-mediated activation of the TNF-receptor-NF-κB pathway cannot mimic the effects of FFA in this case. However, after treatment with the combination of both CM and fatty acids, angiotensin expression does not change. In addition we could show that only the combinations CMOA and CMPA lead to a significant decrease in the expression of angiopoietin-1 (ang-1; fig. 3C) and lipid-droplet coating protein cell death-inducing DNA fragmentation factor-α like effector A (CIDEA; fig. 3D).

The combination of CM and PA leads to synergistic ROS production in comparison to CM and PA alone

Because reactive oxygen species (ROS) production is known to promote SMC proliferation, we further elucidated whether an enhanced ROS production is responsible for our observed proliferative effects. Surprisingly, only CM and the combination of CM and PA induced ROS significantly, indicating that ROS are not responsible for the synergistic effect of CMOA on proliferation (fig. 4A). One should notice that the assay is able to detect several types of ROS (hydrogen peroxides, hydroxyl radicals, peroxyl radicals, peroxynitrite anions). To investigate the role of superoxide anions, we additionally analyzed the effects of CM and fatty acids on the NF-κB target superoxide dismutase (SOD)-1, which protects the cell against high levels of superoxide anions. Here we could demonstrate that only the combination of CMOA reduced SOD-1 expression significantly (fig. 4B), possibly indicating that the production of superoxide anions is increased in this situation.

The combination of CM and OA leads to a significant decrease in MMP-1 and activin A mRNA expression

As matrix-metalloproteinases (MMP) were shown to play a profound role in intimal thickening, we investigated the expression of the collagenase MMP-1. As shown

in figure 5A, only the combination of CM and OA reduces mRNA expression of MMP-1 (about 40 %), whereas treatment with PA with or without CM exerts no effects. Interestingly, MMP-1 activity was substantially increased only after CMPA treatment (fig. 5B). Furthermore, we investigated the expression of activin A, which is involved in proliferation and differentiation of several cell types. OA and the combination CMOA reduce mRNA expression of activin A (fig. 5C), whereas the downregulation by CMOA is much more prominent (50 % vs. 20 %).

Discussion

It has been speculated that perivascular fat tissue by its secretory activity might directly contribute to the pathogenesis of atherosclerosis[11]. Consistently, the effect of single adipokines on inducing smooth muscle cell proliferation and migration has been demonstrated[12,13]. In our previous study we reported that the secretory output of human in vitro differentiated adipocytes in the form of CM induces proliferation and NF-κB activation in human vascular SMC[7], an effect synergistically increased by oleate but not palmitate. Here we assessed the expression of several NF-κB target genes involved in SMC macrophage recruitment/ inflammation (RANTES, activin A, selectin-P), production of reactive oxygen species (SOD-1), plaque rupture (MMP-1), proliferation/migration (angiopoietin-1, angiotensinogen precursor), and lipid storage capacity (CIDEA) to finally get insight into the molecular mechanisms triggered by adipokines and fatty acids in human vascular SMC. Our data show a complex, differential regulation of these NF-κB target genes, most likely involving additional pathways like ROS production.

Recently it was demonstrated that palmitate activates inflammatory NF-κB signaling in several cell types including vascular endothelial cells[14], as well as human and rat SMC[14,15]. However, we could not observe an increased activity of NF-κB after treatment with relatively low concentrations of palmitate (100 µmol/l) within 120 min. However, it could be possible that activation of NF-κB occurs at later time points, as its activation could be shown in endothelial cells after 3-6 h of treatment with palmitate[16]. Interestingly OA, especially in combination with CM, exerts a much stronger effect on NF-κB signaling within 120 min than PA with or without combination with CM, which should be further investigated. Moreover, the combinations of CMOA and CMPA activated NF-κB substantially after 18 h, whereas the fatty acids alone exerted no effect.

Furthermore we could report that PA increases IL-6 mRNA expression (3.5-fold) in VSMC after 4 h, which is in accordance to a study showing that PA induces IL-6 mRNA in human endothelial and smooth muscle cells[17]. In addition, IL-6 secretion was also significantly induced after stimulation with PA.Interestingly, the combination of CMPA increases IL-6 mRNA levels in a synergistic way (8.5-fold) in comparison to CM and PA alone. With regard to IL-6 secretion, the combination of CMPA induced a significant

induction of IL-6 in comparison to all other conditions. In addition, the specific IKK-inhibitor reduced the PA and CMPA-induced IL-6 secretion significantly, providing direct evidence for the key role of NF-kappaB in mediating the fatty acid and CM effects

The NF-κB target gene RANTES, otherwise known as CCL5, exerts a multitude of generally proinflammatory effects like T cell, monocyte chemoattraction or T cell proliferation. RANTES can be stimulated in response to cytokines such as IL-6 or TNFα and is also known to be secreted by vascular SMC[18,19]. In accordance to the literature we could show in the present study that TNFα led to a significant 25-fold increase of RANTES mRNA level. However, the combination of CM and fatty acids did not modify RANTES expression, suggesting that this chemokine is not involved in augmenting SMC inflammation under our experimental conditions. Similar results were observed for angiotensinogen precursor (ang pre), which is necessary for the synthesis of angiotensin II. Angiotensin II exerts profound effects on SMC by inducing inflammation, thrombosis and cell proliferation through stimulation of the production of cytokines and growth factors[20].

Interestingly, only the combination of CM with each fatty acid significantly reduced the expression of angiopoietin 1 (ang-1). Ang-1 exerts a vessel-sealing effect[21], acts as an anti-inflammatory agent[22,23] and protects against cardiac allograft atherosclerosis[24]. Obviously, this effect is not NF-κB-dependent, since TNFα did not modify the ang-1 mRNA level. These data further support our notion of the deleterious impact of the combination of fatty acids and adipokines. In line with this we could show that the combined treatment with CMOA and CMPA significantly decreased the expression of CIDEA mRNA in comparison to CM, OA, and PA alone. CIDEA is a lipid droplet coating protein, which colocalizes with perilipin, a regulator of lipolysis[25]. 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[26]. In this regard, 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[25]. To our knowledge, CIDEA expression in SMC has never been investigated and its functional role in this cell type is unknown. It may play a role in lipid handling, but future studies will be needed to address this issue.

It is well accepted that the excessive generation of ROS, leading to oxidative stress by outstripping the antioxidant system, contributes to the development of CVD including atherosclerosis and diabetes mellitus. Measuring ROS in the current study showed that CM induces a significant 1.4-fold increase in ROS compared to control, indicating that CM also induces oxidative stress in SMC, which was previously observed in skeletal muscle cells by our group[27]. OA, PA, and the combination CMOA had no effect on ROS production, whereas the combination of CM and PA could induce ROS (2-fold) in

comparison to CM and PA alone. Recent studies conducted in 3T3 adipocytes demonstrated that palmitate, but not oleate (250 µmol/l) induced ROS[28] and in L6 skeletal muscle cells also palmitate, but not oleate induced ROS production via de novo synthesis of ceramide[29]. Mattern and Hardin showed that the low oleate oxidation in SMC was not due to a limitation in fatty acid transport or to storage of oleate as triglyceride[30]. However, palmitate most likely causes oxidative stress because the metabolism of palmitate, unlike oleate, results in the formation of ceramide[31] and ROS[32]. In our setting PA alone did not induce ROS, which is probably due to the lower concentration (100 µmol/l) used, but the combination with CM increased ROS, possibly due to an enhanced formation of ceramide This finding suggests that CMOA-induced proliferation is not generally dependent on ROS production. On the other hand, only this combination induced a profound reduction of the NF-κB target gene SOD-1, suggesting that superoxide anions are involved in SMC proliferation in response to CMOA. However, future work will be necessary to analyze superoxide anion production by CMOA and to investigate other ROS scavenging enzymes like glutathion-peroxidase, catalase, or SOD-2, probably involved in this complex scenario.

Only the combination of CMOA led to a reduction in matrix-metalloproteinase (MMP)-1 mRNA levels after 4 h, whereas CM and OA alone exert no effects. This reduction could possibly lead to a higher production of extracellular collagen, resulting in an accelerated intimal thickening by CMOA. In contrast the combination of CMPA increased the MMP-1 activity significantly after 24 h, indicating that higher active MMP-1 levels enhance the risk of plaque rupture. Additionally, OA as well as CMOA significantly decreased activin A mRNA levels, by 20 % and 50 % respectively. It has been postulated that activin A modulates the proliferation and differentiation of several cell types involved in atherogenesis, notably endothelial cells (EC), macrophages and SMC. In this context it inhibits the propagation of human EC[33] and it enhances the differentiation of monocytic cells into macrophages[34] and inhibits foam-cell formation[35]. The role of activin A in inducing proliferation of SMC is controversially discussed at the moment. Whereas in some studies activin has induced DNA synthesis in rat SMC[36,37], other reported that activin did not affect rat SMC growth[38]. Further it could be shown that activin A mediates the differentiation of proliferating human SMC towards a contractile phenotype[39], thereby inducing a redifferentiation, suggesting a rather negative impact of CMOA on plaque stability. Activin A was also reported as a novel component of conditioned medium obtained from vasoactive G-protein-coupled receptor (GPCR) agonists-stimulated hVSMC[40]. Furthermore activin A was demonstrated to be increased after vascular injury. Thus, this molecule seems to play an important role in the control of cellular processes including proliferation and migration associated with the progression of vascular lesions.

In conclusion, we show here that the combination of adipocyte-derived factors and low concentrations of OA specifically and synergistically induce SMC proliferation, NF-κB activation, and a set of downstream targets critically involved in atherosclerosis progression. This includes the anti-inflammatory agent ang-1 and additionally MMP-1 and activin A, both involved in vascular remodeling. These data support our notion that the lipotoxic potential of fatty acids is substantially enhanced by the presence of adipocyte-derived factors.

Acknowledgements

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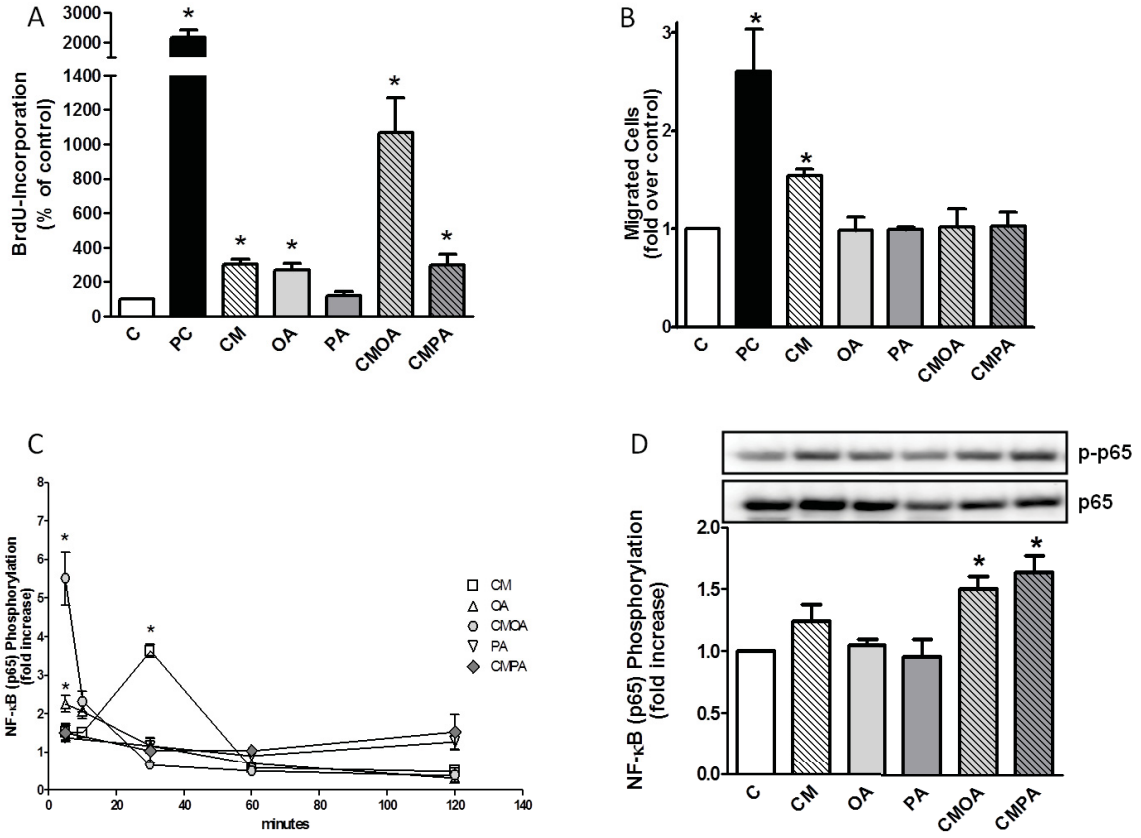

Figure 4.1 Effect of CM, OA, PA and the combinations CMOA and CMPA on human vascular smooth muscle cell proliferation, migration and activation of NF-κB. hVSMC were serum starved for 24 h and subsequently incubated with BrdU in the absence or presence of CM, 100 µmol/l OA or PA and the combination of CM with each fatty acid for 18 h. (A) Proliferation was determined by measuring the incorporation of BrdU into DNA. Data are expressed relative to the basal control value, which was set at 100 %. FCS is used as positive control (PC). Data are means ± SEM of six independent experiments. (B) Quantitative analysis of hVSMC migration with a Transwell Cell migration assay . 5 % FCS was used as positive control (PC). Data are presented as mean ± SEM from three independent experiments. (C and D) Total cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies to phosphorylated forms of NF-κB. Data are mean values ± SEM of three to four 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 hSMC.

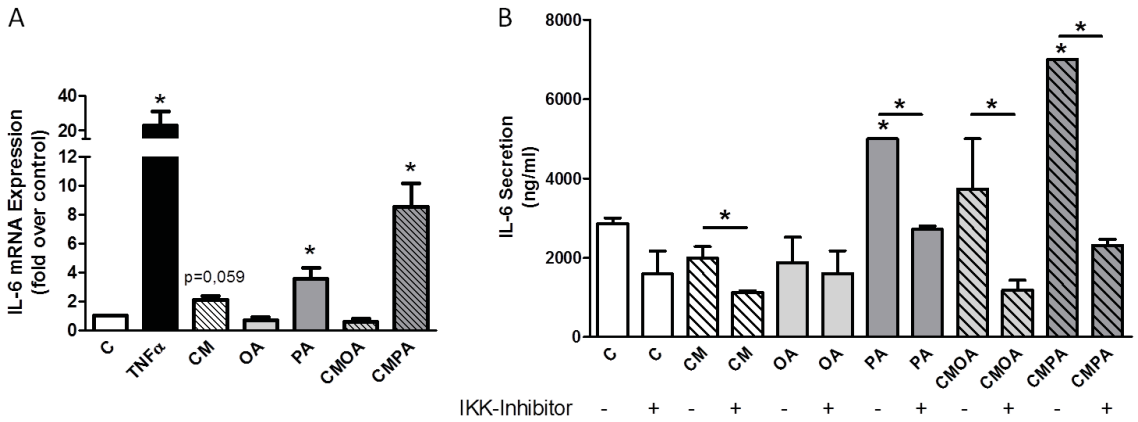

Figure 4.2 Expression of IL-6 mRNA as well as IL-6 secretion of hVSMC after incubation with CM and the combination with each fatty acid. Cells were serum starved for 24 h and subsequently treated with CM, OA, PA or the combination of CM with each fatty acid for 18 h with or without a specific IKK-inhibitor. mRNA level of IL-6 (A) were quantified by Real-time PCR and normalized to the level of actin. TNFα was used as positive control. Data are expressed relative to the basal control value and are means ± SEM of four to five independent experiments. (B) After 24 h supernatants were collected and IL-6 concentration was measured by ELISA assay. Data are means ± SEM of four to five independent experiments. *p<0.05 compared to untreated cells.

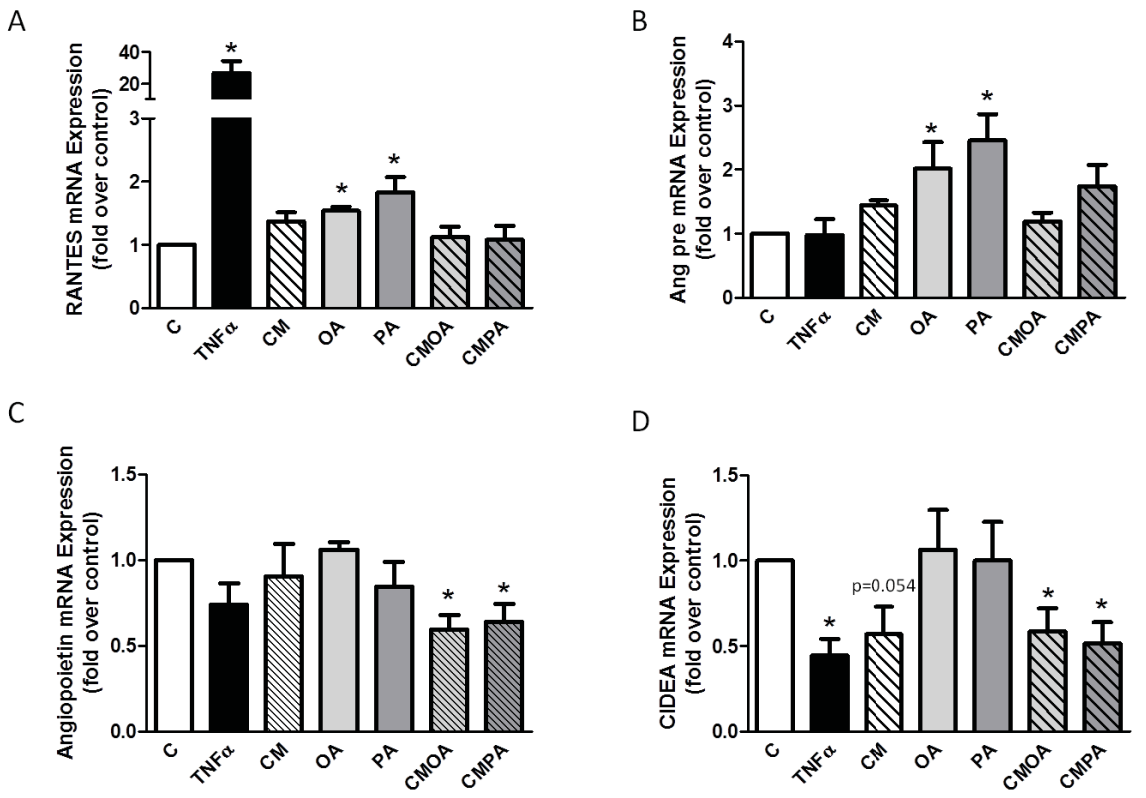

Figure 4.3 Impact of CM, OA, PA and the combinations CMOA and CMPA on NF-κB target gene expression in hVSMC. hVSMC were treated as described in legend to Fig. 2.Total RNA was isolated and converted into cDNA. Endogenous mRNA levels of RANTES (A), angiotensinogen precursor (B), angiopoietin 1 (C), and CIDEA (D) were quantified by Real-time PCR and normalized to the level of actin. TNFα was used as positive control. Data are expressed relative to the basal control value and are means ± SEM of four to five independent experiments. *$p<0.05$ compared to control.

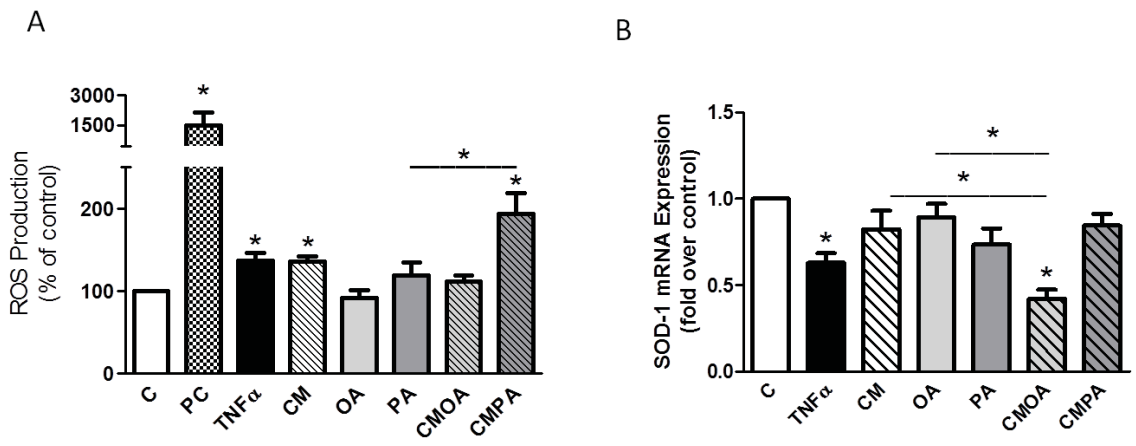

Figure 4.4 Effect of CM, OA, PA and the combinations CMOA and CMPA on ROS production and expression of SOD-1 mRNA. Cells were pretreated as indicated for 18 h. (A) For ROS measurement cells were incubated with DCF for 30 min at 37°. 0.3 % H_2O_2 was used as positive control (PC). Fluorescence was measured with excitation/emission wavelengths of 493/538 nm. Data are means ± SEM of four to five independent experiments and are expressed relative to the basal control value. (B) Cells were treated as described in legend to Fig. 2. mRNA level of SOD-1 was quantified by Real-time PCR and normalized to the level of actin. TNFα was used as positive control. Data are expressed relative to the basal control value and are means ± SEM of four to five independent experiments. *$p<0.05$ compared to untreated cells.

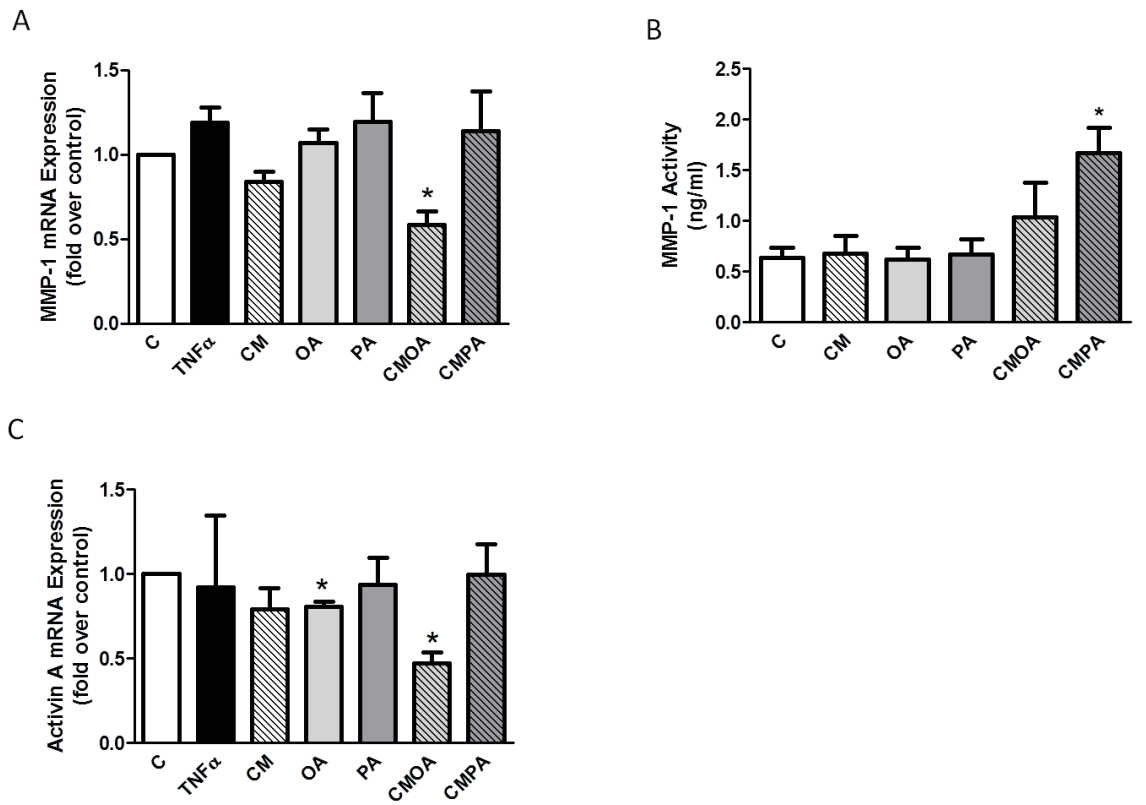

Figure 4.5 Analysis of MMP-1 and Activin A mRNA expression and MMP-1 activity after treatment with CM, and the combinations CMOA and CMPA. Cells were treated as described in legend to fig. 2. mRNA level of MMP-1 (A) and Activin A (C) were quantified by Real-time PCR and normalized to the level of actin. TNFα was used as positive control. Data are expressed relative to the basal control value and are means ± SEM of four to five independent experiments. (B) After 24 h supernatants were collected and active MMP-1 was measured by an Enzyme Activity Assay. Data are means ± SEM of three independent experiments. *$p<0.05$ compared to untreated cells or indicated data.

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CHAPTER 5

Study 4

Inflammation and Metabolic Dysfunction: Links to Cardiovascular Diseases†

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Summary

Abdominal obesity is a major risk factor for cardiovascular disease, and recent studies highlight a key role of adipose tissue dysfunction, inflammation and aberrant adipokine release in this process. An increased demand for lipid storage results in both hyperplasia and hypertrophy, finally leading to chronic inflammation, hypoxia, and a phenotypic change of the cellular components of adipose tissue, collectively leading to a substantially altered secretory output of adipose tissue. In this review we have assessed the adipo-vascular axis and an overview of adipokines associated with cardiovascular disease is provided. This resulted in a first list of more than 30 adipokines. A deeper analysis only considered adipokines that have been reported to impact on inflammation and NF-κB activation in the vasculature. Out of these, the most prominent link to cardiovascular disease was found for leptin, TNF-α, A-FABP, interleukins and several novel adipokines such as lipocalin-2 and PEDF. Future work will need to address the potential role of these molecules as biomarkers and/or drug targets.

Introduction

Obesity is a metabolic disorder of pandemic proportions and is associated with a variety of metabolic dysfunctions like hypertension, dyslipidemia, insulin resistance, and hyperglycemia [1]. Thus, it is considered a major risk factor for the development of chronic diseases such as type 2 diabetes [2] and cardiovascular diseases [3,4]. Adipose tissue enlargement ensues as a consequence of a persistent positive energy balance resulting from a sedentary lifestyle, conditioned by environmental and genetic factors. Formerly, the function of adipose tissue was thought to be restricted to insulate and cushion the body, store triglycerides during periods of excess energy, and provide the body with energy in the form of free fatty acids in states of energy shortage [5]. However, it has become increasingly evident that adipose tissue is also a secretory organ able to release various lipid mediators as well as a multitude of bioactive proteins and peptides, collectively referred to as adipokines [6].

Today, it is commonly accepted that adipokines have essential roles in energy homeostasis, glucose and lipid metabolism, cell viability, control of feeding, thermogenesis, neuroendocrine function, reproduction, immunity, and, importantly, cardiovascular function [7]. Accordingly, numerous studies in recent years have demonstrated the pivotal role of adipokines as molecular messengers in the crosstalk of adipose tissue with other organs and tissues as well as their contribution to the development of obesity-associated disorders. However, this picture has gained complexity since modern approaches applying highly sensitive analytical techniques

have revealed that the adipose tissue output is comprised of hundreds of different factors [8,9,10,11], with additional novel adipokines still being identified [12]. Furthermore, it has been described that the adipokine profile may vary between different adipose tissue depots and is altered in pathological conditions such as obesity. In this context, it has been shown that plasma levels of several pro-inflammatory cytokines as well as acute phase proteins such as C-reactive protein (CRP) are increased in obesity, contributing to a chronic state of low-grade inflammation [13,14,15]. This systemic inflammatory state has been suggested to be a causative link between obesity and related secondary complications such as cardiovascular diseases [16], by inducing inflammatory processes in the vessel wall. Such processes are considered to be critical determinants of pathological alterations of the vasculature such as thickening of vessel wall, fatty streak formation, or promotion of atherosclerotic plaques.

Previous studies have demonstrated local production of pro-inflammatory mediators by immune cells of atherosclerotic plaques (for reviews see [17,18]), however in this review we will elucidate the role of adipose tissue-derived factors in the induction of inflammatory processes in the vasculature, and focus especially on selected adipokines able to activate NF-kB signaling. Furthermore, we will discuss the special role of perivascular fat as a local adipose tissue depot and its contribution to the development of cardiovascular diseases. In this context, we propose a list of candidates, including well-known as well as novel adipokines, involved in the induction of inflammatory processes and possibly leading to atherosclerotic lesions as well as cardiovascular complications. With this, we provide new insights into the role of adipokines in the complex inter-organ crosstalk between adipose tissue and the vasculature. Understanding the molecular mechanisms linking inflammation, metabolic syndrome, and cardiovascular diseases is essential to identify possible biomarkers and potential drug targets. These are important steps to improve diagnosis and treatment of cardiovascular diseases.

Obesity-associated alterations of adipose tissue

Enlargement of adipose tissue ensues as a result of an imbalance between energy intake and expenditure. As a consequence, adipocytes undergo hyperplasia and hypertrophy to meet the increased demand for storage capacities [19,20]. However, a persistent state of energy excess represents an increased burden for the lipid storage and processing capacities of the expanding adipose tissue, resulting in various dysfunctions within the tissue like low-grade chronic inflammation and hypoxia [16,21]. These obesity-associated dysfunctions may lead to changes in the cellular composition of the tissue, including alterations in the number, phenotype, and localization of immune, vascular, and structural cells [1], resulting in an altered adipose tissue secretory

output. Increased expression of chemoattractant proteins like monocyte chemotactic protein-1 (MCP-1) induces recruitment and infiltration of additional macrophages. These contribute to the increased expression of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) [22], thereby further exacerbating the obesity-associated inflamed status of the adipose tissue [23,24,16]. Additionally, obesity-associated adipocyte hypertrophy has also been associated with a shift of the adipocyte secretome to a more pro-inflammatory composition [25]. In this context, a positive correlation has been described between adipocyte size and secretion of various pro-inflammatory factors such as TNF-α, IL-6, IL-8, MCP-1, leptin, and granulocyte colony stimulating factor [25]. While the majority of adipokines have been found to be increased in obesity, expression of adiponectin is decreased [26]. Unlike many other adipokines, adiponectin has been correlated to insulin-sensitizing, anti-inflammatory, and anti-proliferative properties [27,28,29]. Therefore, adiponectin has been attributed a cardioprotective role.

These observations demonstrate that the adipose tissue output dramatically changes in pathological conditions such as obesity. However, the adipokinome may already vary depending on the site of the adipose tissue depots [1]. Adipose tissue in the visceral and the subcutaneous compartment are the two most abundant depots and it has been shown that they produce unique profiles of adipokines [30,1]. In this context, visceral adipose tissue has received special attention, since various studies have found a positive correlation between the amount of visceral adipose tissue and cardiovascular diseases [31,32]. Recent studies have even proposed that visceral adiposity, measured as waist circumference, is a more precise risk indicator for type 2 diabetes and cardiovascular diseases than whole-body obesity [33,34,35,36]. On the one hand this may be attributed to its location, as it drains directly into the portal vein [37]. Special depots of visceral adipose tissue, perivascular and epicardial tissue, might also be located around blood vessels and the heart, respectively, where they specifically affect local tissues, as further discussed below. On the other hand, the visceral adipokinome contains many pro-inflammatory cardiovascular risk factors, such as IL-6 and PAI-1 [38,39], which contribute to the close association of visceral adipose tissue and cardiovascular disease.

The adipo-vascular axis

Obesity is often associated with and represents a major risk factor for the development of cardiovascular diseases. Cardiovascular diseases are responsible for one of the highest mortality rates worldwide, accounting for 16.7 million deaths each year [40,41], mostly due to the life-threatening complications of coronary artery and cerebrovascular disease [42]. While cardiovascular diseases may be characterized by alterations like coronary artery calcification, thickening of vessel wall, formation of fatty streak and atherosclerotic plaques, vessel stiffness, and/or hypertension, atherosclerosis

may be considered the principal contributor [43,44,45]. Originally atherosclerosis was believed to be a merely passive accumulation of cholesterol in the vessel wall, however since novel data indicate underlying inflammatory processes to play a major contributing role [27,46] this review aims to elucidate the role of pro-inflammatory adipokines in the pathogenesis of atherosclerosis.

In order to comprehend the influence of adipokines on the development of atherosclerosis, understanding the complex course of events taking place in the pathogenesis of atherosclerosis is important. It has been demonstrated that in the early stages of atherosclerosis, endothelial cells may be activated by various inflammatory stimuli, including a diet rich in saturated fat, hypercholesterolemia, obesity, hyperglycemia, insulin resistance, hypertension, and smoking, triggering the expression of adhesion molecules like vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and intracellular adhesion molecule-1 (ICAM-1) [47,43,27]. This increases the adherence of monocytes, which infiltrate the subendothelial space and accumulate within the intima [48,49]. In response to overexpression of macrophage colony-stimulating factor in the inflamed intima, which is induced by modified low-density lipoprotein, monocytes are converted to activated macrophages [27]. These may then convert to foam cells by receptor-mediated incorporation and accumulation of lipoprotein particles [50,27]. The formation of foam cells and their continued accumulation in the intima, accompanied by proliferation and migration of smooth muscle cells (SMC) from the media, leads to the first stage of the atherosclerotic lesion, the fatty streak [47]. Continued exposure to atherosclerotic factors promotes the progression to more complex atherosclerotic plaques, until destabilizing factors like thinning of the fibrous cap and high foam cell content elicit rupture of a plaque, triggering thrombus formation [47]. This in turn may either obstruct the lumen immediately or detach to form an embolus blocking blood flow distal to its origin. Consequently, such atherothrombosis may result in myocardial or brain infarction.

As mentioned above, a major risk factor for the development of atherosclerosis is obesity and the associated adipokines. In order to elucidate the molecular basis of this adipo-vascular-axis, numerous studies have attempted to assess the impact of various adipokines on the cells of the vessel wall. In this context it has been described that various processes may be affected by adipokines. As abnormal proliferation and migration of SMC located in the arterial intima has been suggested to be a central event in atherosclerosis [51,52], adipokines able to induce proliferation or migration have to be considered as potential players in this process. Similarly, activation of inflammatory signaling by adipokines like TNF-α, leptin, and PAI-1, has been suggested to contribute to the development of cardiovascular diseases [48,53], by stimulating the generation of endothelial adhesion molecules, proteases, and other mediators, which may enter the circulation in soluble form [27]. In this context the transcriptional regulator nuclear

transcription factor-κB (NF-κB) plays a central role, as it mediates the expression of a multitude of genes. Amongst many others, the expression of adhesion molecules ICAM-1, VCAM-1, and E-selectin has been described to be mediated via NF-κB activation [49,54]. Thus, adipokine-induced activation of NF-κB may promote adherence, diapedesis, and accumulation of immune cells such as monocytes or lymphocytes in the vessel wall, which play a central role in atherosclerotic plaque formation [27]. Additionally, NF-κB activation is involved in SMC proliferation [55] and mediates the expression of a variety of pro-inflammatory molecules by macrophages and SMC [56].

In recent years a number of studies have investigated the impact of selected adipokines on the different steps in the pathogenesis of atherosclerosis mentioned above. However, intensive research on the adipokinome in recent years have (1) demonstrated the very complex nature of the secretory output of adipocytes, (2) suggested novel roles for well-known adipokines, and (3) identified a number of novel adipokines associated with a vasoactive potential. As this multitude of studies conducted in various species and different models makes it difficult to identify promising candidates for drug target or biomarker validation, this review provides a novel summary of available data and evaluation of the vasoactive potential of currently known adipokines based on the evidence found in literature. As a general overview Table 1 provides a list of adipokines currently described to be associated with cardiovascular disease, including their impact on proliferation, inflammation and NF-κB activation. However, this list is likely to soon require updating as an increasing number of adipokines are rapidly identified and associated with a vasoactive potential.

The fact that this multitude of adipokines has been found to modulate vascular homeostasis underlines the pivotal role of the adipokinome in the adipose tissue-vessel crosstalk. Due to these findings we propose that obesity-induced inflammatory processes within the adipose tissue and the paracrine action of the associated pro-inflammatory adipokinome triggers endothelial dysfunction and vascular inflammation, which may ultimately lead to atherosclerosis, heart attack, or stroke (Figure 1).

Adipokines with a tight link to cardiovascular disease

To analyze in more detail the adipokines presented in Table 1, we selected those adipokines with reported effects on NF-κB activation. Since NF-κB is one of the major transcription factors that has been linked to both cardiovascular health and diseases, it is not suprising that NF-κB has been shown to influence numerous cardiovascular diseases including atherosclerosis [57]. The function of NF-κB is largely dictated by the genes that it targets for transcription and varies according to stimulus and cell type [57]. We are certainly aware that several adipokines induce cardiovascular diseases independent of NF-κB, however, this is not part of this review.

Here, we have distinguished two groups of adipokines. The first group represents adipokines with a tight link to cardiovascular diseases based on evidences from in vitro and clinical studies (Table 2) as well as from animal models (Table 3). The second group includes adipokines with less strong evidence for the development of cardiovascular diseases, since data from animal models are not available either because the animal model has not been generated up till now or the specific knockout is lethal, as for VEGF.

Adiponectin and Leptin - classical adipokines

Adiponectin, almost entirely produced by adipocytes, is one of the most comprehensively studied adipokines, and in human obese subjects its levels are diminished [58]. It has been shown to positively influence energy consumption and fatty acid oxidation in muscle and liver, thereby reducing the triglyceride content [59]. Transgenic mice overexpressing adiponectin exert an improved lipid profile [60,61]. Adiponectin has been suggested to be an important factor modulating the cardiovascular system due to its anti-atherogenic and anti-inflammatory effects. In macrophages and endothelial cells, it acts via suppression of TNF-α [48] and pro-inflammatory cytokines such as IL-6 [62], and directly ameliorates endothelial dysfunction by increasing nitric oxide (NO) production [63,64]. In addition, adiponectin reduces vascular SMC proliferation and migration [65]. The role of adiponectin as an cardioprotective adipokine is further supported by results from clinical studies. While increased adiponectin levels are associated with a decreased risk of myocardial infarction [66], hypoadiponectinemia is observed in patients with coronary atherosclerosis and acute coronary syndrome (ACS) [67,68]. In a recent study, it has been shown that serum adiponectin is associated with biomarkers of insulin resistance, inflammation, and endothelial dysfunction, which are independent risk factors for cardiovascular diseases [69]. In addition, results from animal studies have revealed that adiponectin exerts beneficial effects at mostly all stages of the atherosclerotic process [70].

Leptin, which directly influences food intake, was the first adipokine identified [71] and is primarily synthesized by white adipose tissue [72]. The results of clinical studies investigating the contributions of leptin to the pathophysiology of cardiovascular complications are controversial, leaving the precise role of leptin unclear [73]. Some studies have reported elevated leptin levels in patients with ACS [74,75] and described an association between circulating leptin levels and risk of coronary artery disease (CAD) [76,77]. However, the findings of other studies have indicated no clinically relevant association with risk of CAD [78,79,80]. The effects of leptin on endothelial cells and vascular SMC are better investigated and comprise increased NO production via activation of endothelial NO synthase (eNOS) [81], and increased expression and activity of inducible NO synthase (iNOS), respectively [82]. However, leptin also increases the expression of PAI-1 [83] and CRP [84] in human vascular endothelial cells.

Furthermore, leptin-deficient mice, which are extremely obese, are protected from atherosclerosis despite other metabolic risk factors, indicating that this adipokine contributes directly to cardiovascular diseases [85].

TNF-α and MIF – macrophage-associated cytokines

TNF-α is one of the most extensively examined pro-inflammatory cytokines that plays an important role in atherosclerosis as well as in other inflammatory and metabolic disorders, which are known risk factors for cardiovascular diseases. Upregulation of TNF-α in the vascular wall of carotid and coronary arteries promotes endothelial apoptosis thus leading to impairment of endothelial function [86,87]. Moreover, TNF-α induces phenotypic changes in vascular SMC [88] as well as their migration [89,90,88,91] proliferation [92] and apoptosis [93], which are all critical for the initiation and progression of vascular lesions. In TNF-α/ApoE double knockout mice most pro-atherosclerotic factors such as IL-1β, MCP-1, and NF-κB are downregulated [94]. Furthermore, TNF-α plasma concentrations are positively correlated with carotid intima-media thickness (IMT) [95] and increased in patients with premature CAD [91], thus further emphasizing the important role of TNF-α for the development of cardiovascular diseases.

MIF is expressed in various tissues such as adipose tissue, and regulates acute inflammatory as well as adaptive immune reactions [96]. MIF expression is induced by proatherogenic stimuli, such as oxidized low-density-lipoprotein [97], and it has been shown to become upregulated in macrophages, endothelial cells and SMC during the development of atherosclerotic lesions [98,97]. Its expression correlates with increased IMT and lipid deposition in the aorta of mice, and in advanced human carotid artery plaques [99,100]. A recent study suggested high MIF levels as an independent risk factor for future coronary events in CAD patients with IGT/T2DM [101]. MIF influences the proliferation and migration of macrophages and vascular cells [102,103], and MIF-deficient SMC display impaired proliferation. MIF-deficieny in LDL receptor-knockout mice prevented diet-induced atherogenesis, as shown by decreased IMT and lipid deposition in the aorta [102]. Taken together, these results suggest that MIF could be an important player in the pathogenesis of atherosclerosis and may represent a potential drug target for the treatment of inflammatory and cardiovascular diseases [104].

A-FABP and Lipocalin-2 – small lipid-binding proteins

A-FABP is one of the most abundant intracellular lipid transport proteins in adipocytes [105,106], regulating lipid metabolism by promoting diffusion, sequestration and transport of long chain fatty acids [107]. In addition, A-FABP is secreted and it has been shown that high levels are associated with a worse cardiometabolic risk profile [108]. Furthermore, A-FABP serum levels are positively associated with the Metabolic Syndrome [109], CAD [110], and carotid IMT [111], while inversely associated with endothelial

function [112]. In human endothelial cells, the expression of A-FABP can be induced by VEGF-A, bFGF [113] and lipids leading to reduced activity of eNOS and NO production [114]. In addition, knockdown of A-FABP reduced endothelial cell proliferation [113].

The expression of Lipocalin-2 in adipocytes was first described by Lin and colleagues [115], and is markedly induced during differentiation of pre-adipocytes to adipocytes [116]. Clinical as well as experimental studies indicate an important role of lipocalin-2 as an inflammatory adipokine in obesity and related complications [117,118,119]. In patients with CAD lipocalin-2 levels are increased and independently associated with systolic arterial blood pressure, insulin resistance and decreased HDL cholesterol levels [120]. In addition, high expression of lipocalin-2 has been shown in atheromatous human plaques which were associated with increased MMP-9 activity [121]. Serum lipocalin-2 levels are also elevated in various obese rodent models and human obesity [119,122,123], and positively correlated with adiposity, hypertriglyceridemia, hyperglycemia, insulin resistance and high-sensitive CRP [119]. In vascular SMC mRNA and protein expression of lipocalin-2 is increased upon IL-1β treatment in a NF-κB- dependent manner [124]. Furthermore, lipocalin-2 knockout mice are protected against diet-induced endothelial dysfunction [125]. However, lipocalin-2 may also have anti-inflammatory effects as it suppresses LPS-induced cytokine production in macrophages and antagonizes effects of TNF-α on adipocytes and macrophages [123].

A-FABP as well as lipocalin-2 are adipokines linking obesity with vascular diseases and are involved in the pathogenesis of atherosclerotic plaque. Interestingly, serum levels of A-FABP are positively associated with those of lipocalin-2 [126,127,128,108]. The increased release of these two adipokines in conditions of obesity may contribute to the pathogenesis of endothelial dysfunction and atherosclerosis.

Interleukins – family of immune system`s messengers

Pro-inflammatory cytokines of the interleukin family are considered to be key players in the chronic vascular inflammation that is typical for atherosclerosis and cardiovascular diseases [129]. IL-1ß is a prototypic pro-inflammatory cytokine with different biological functions, inducing the production of different cytokines and chemokines. In vitro studies demonstrated that IL-1ß increases the expression of cell adhesion molecules [130], MCP-1 [131] and lipocalin-2 [124] in vascular SMC. In addition, it stimulates the migration [132] and proliferation of these cells [133,134]. In the apoE/IL-1ß double knockout mouse model, IL-1ß deficiency decreases the severity of atherosclerosis [135,136], further supporting the important role of IL-1ß in vascular disorders.

IL-4 is a proinflammatory cytokine and plays a critical role in the progression of atherosclerosis. In endothelial cells IL-4 increases the expression of inflammatory mediators [137,138], the generation of ROS [139], as well as apoptosis [140], while it induces proliferation [141] and migration [142] in vascular SMC. A recent study described higher

circulating IL-4 levels in patients with CAD [143], while IL-4 mRNA is rarely [144] or not observed in atherosclerotic plaques [145]. Data obtained in animal models are controversial. While Davenport et al. showed that IL-4 deficiency reduced plaque area and atherosclerotic lesions [146], George et al. observed no protection from early atherosclerosis [147].

IL-8 is another cytokine that plays a role in the context of cardiovascular diseases [148]. It has been described as a modulator of monocyte–endothelial interaction [149] and its expression is induced by TNF-α [150] and homocysteine [151]. In addition, IL-8 promotes endothelial cell proliferation [152] and migration [153,154], the latter via activation of PI3K-Rac1/RhoA pathway [155]. In vascular SMC IL-8 has been shown to influence VCAM-1 expression [156,157], proliferation [141], and migration [158,159].

Moreover IL-18 act as a pro-atherogenic factor, as supported by data from IL-18-deficient mice which display reduced atherosclerosis [160]. A recent study demonstrated that IL-18 induced proliferation of human SMC and MMP induction [161].

In contrast, IL-10 is an anti-inflammatory cytokine, which is thought have an anti-atherogenic potential. IL-10 inhibits TNF-α-, IL-1-β- or LPS-induced expression of IL-6 and IL-8 in endothelial cell [137], and increases eNOS expression and NO production [162]. In vascular SMC IL-10 prevents migration and proliferation, partially mediated by NF-κB inactivation [163,164]. Furthermore, IL-10 transgenic mice showed reduced atherosclerosis while IL-10 deficient mice exhibited increased early atherosclerotic lesion formation [165], illustrating the anti-atherogenic potential of IL-10. However, results from clinical studies are not consistent as some report higher IL-10 levels in patients with ACS [166,167], while other report lower levels in patients with CAD [168] and unstable angina [169].

Thrombospondin-1 and PEDF – proteins associated with angiogenesis

TSP-1 is a member of a protein-family that mediates cell-matrix and cell-cell-interactions. Experimental data have shown that TSP-1 stimulates the aggregation and stability of platelet aggregates [170], induces the expression of cell adhesion molecules [171] in endothelial cells, and stimulates chemotaxis in vascular SMC [172,173]. In TSP-1 knockout mice more extensive postinfarction remodeling has been observed compared to wild-type mice [174]. Until now, no clinical data are available for TSP-1.

PEDF, first identified in retinal pigment epithelial cells, is a multifunctional, pleiotropic protein and is expressed in various tissues such as adipose tissue. The role of PEDF in cardiovascular diseases is not completely understood. PEDF has been shown to possess anti-angiogenic effects [175] and to inhibit VEGF-induced endothelial cell migration and proliferation [176] as well as PDGF-BB-induced proliferation and migration of vascular SMC [177]. However, our group recently reported an increased proliferation of vascular smooth muscle cells upon PEDF treatment as well as the activation of inflammatory signaling pathways [178]. In clinical studies higher PEDF levels in type 2 diabetic patients

[179,180] have been found, and it was shown that PEDF levels are strongly associated with the Metabolic Syndrome [180], vascular inflammation and carotid IMT [181].

In Fig. 2 we provide an additional schematic overview of the above described adipokines summarizing their impact on central features of cardiovascular diseases such as proliferation, migration, NO production, and induction of inflammatory cytokines.

Adipokines with less strong evidence for involvement in cardiovascular disease

The following adipokines are also important factors for the development of cardiovascular disease, but these adipokines have diminished evidence linking NF-κB inflammation and cardiovascular diseases, since KO or transgenic studies have not been reported.

Chemerin, Resistin, Visfatin, and Vaspin – novel adipokines

Chemerin is a newly described adipokine that is highly expressed in adipose tissue and liver and effects adipocyte metabolism [182]. In humans, its plasma levels have been shown to be associated with inflammation and various components of the Metabolic Syndrome such as BMI, triglycerides and hypertension [183,184], whereas no differences were observed between non-diabetic and type 2 diabetic subjects [185]. Studies in rodents that investigated the role of chemerin with regard to obesity and diabetes have revealed controversial data. In obese db/db mice expression in adipose tissue and serum levels of chemerin are reduced compared to lean controls [186], while its expression is higher adipose tissue of obese diabetic Psammomys obesus compared to lean normoglycemic P. obesus [185]. Experimental data have shown that chemerin promotes proliferation and migration of endothelial cells [187]. However, until till now the effects of chemerin on vascular SMC have not been investigated, and future studies have to determine the impact of chemerin on cells of the vessel wall in relation to cardiovascular diseases.

Resistin was identified as adipokine in 2001 and shown to be increased in diet-induced and genetic forms of obesity in rodents [188] as well as in morbidly obese subjects compared to lean controls [189]. In humans, resistin is expressed in and secreted by monocytes and macrophages in addition to adipocytes [190,191]. It promotes vascular SMC migration [192] and proliferation [193], induces monocyte-endothelial cell adhesion and increases the expression of VCAM-1, ICAM-1 and MCP-1 in endothelial cells [194]. Clinical studies have revealed that plasma resistin levels correlate with markers of inflammation and are predictive of coronary atherosclerosis in humans [195]. Resistin may therefore represent a potential link between obesity and cardiovasculare diseases.

Visfatin is an adipokine which was suggested to act pro-inflammatory since it induces cytokine production [196]. In clinical studies it has been shown that visfatin

expression was increased in plaques from patients with unstable carotid and coronary atherosclerosis [197]. In addition, adipose tissue levels of visfatin were significantly higher in CAD patients relative to control subjects [198]. Visfatin induces endothelial cell proliferation and migration, induces eNOS and iNOS, and has anti-apoptotic effects in both vascular cell types [199,200,201,202]. These data indicate that visfatin may be implicated in the pathogenesis of atherosclerosis and cardiovascular disease [203]. Further studies will be needed to clearly define the impact of visfatin in this context.

Vaspin, a serine protease inhibitor, was originally identified as an adipokine predominantly secreted from visceral adipose tissue in Otsuka Long-Evans Tokushima fatty, an animal model of obesity and type 2 diabetes [204]. In humans, vaspin mRNA expression in adipose tissue is regulated in a fat depot-specific manner and is associated with parameters of obesity, insulin resistance, and glucose metabolism [205]. Plasma levels of vaspin are associated with age, gender, BMI and insulin resistance [206,207,208]. Studies investigating an association between circulating vaspin and cardiovascular diseases reported controversial results. One study have reported an association between plasma level and artherosclerosis in women [206] while another study have found no association between vaspin concentration and parameters of atherosclerosis severity [209]. However, the expression of vaspin have been described in vascular smooth muscle cells and foam cells in atherosclerotic lesions [210]. In addition, anti-apopotic effects of vaspin in endothelial cells have been reported [211]. In vascular SMC, vaspin have been shown to inhibit TNF-α induced ROS generation, NF-κB activation, and expression of ICAM-1 pointing towards a protective role of vaspin [212]. Clearly, further investigations have to be conducted to clarify the impact of vaspin for cardiovascular disease.

Angiotensin II and VEGF – proliferative proteins

Angiotensin II, the major effector of the renin-angiotensin-system, has many functions including vasoconstriction, cell growth, generation of oxidative stress and inflammation. Angiotensin II has pro-inflammatory effects in the vascular wall by inducing gene expression of inflammatory cytokines and cell adhesion molecules [213]. In vitro studies have revealed that angiotensin II induces proliferation and migration in human vascular SMC [214] and endothelial cells [215]. These experimental findings point to the important cardiovascular actions of angiotensin II. However, the precise mechanisms of angiotensin II in the context of cardiovascular diseases need further investigations.

VEGF is a well-characterized growth factor, which is involved in the regulation and differentiation of the vascular system. However, the results of studies investigating the role of VEGF in the context of cardiovascular diseases are controversial until now. On the one hand, studies predict a beneficial role VEGF for cardiovascular health by

enhancing protective vascular functions [216]. But on the other hand data obtained in some studies in mouse models of atherosclerosis seem to promote a pro-atherogenic role of angiogenesis [217,216]. Although VEGF has an important role in various physiological processes, the very same qualities cause it to play a part in the origin and maintenance of various pathological processes, including atherosclerosis [218]. To date our understanding of the mechanisms and the precise role of VEGF in the context of cardiovascular disease in humans remains unclear and is an important question to be addressed in future studies.

Perivascular adipose tissue and cardiovascular diseases

Perivascular adipose tissue (PVAT) is defined as adipose tissue around blood vessels which occurs in a way that no fascial layer separates this fat depot from the vascular wall. In addition to this barrier-free connection between PVAT an infiltration of adipocytes into the outer region of the adventitia has been observed [219]. In obesity, perivascular adipose tissue is increased in humans and rodents [220,221]. The amount of PVAT was highly associated with visceral obesity and moderately correlated with subcutaneous adipose tissue and body mass index [222]. Adipocytes in PVAT have been compared to subcutaneous and visceral adipocytes in humans and rodents in various studies and there is still controversy as for the classification of PVAT as a depot of white adipose tissue or brown adipose tissue. It appears that PVAT surrounding abdominal and thoracic aortas might be multifaceted as for its adipocyte phenotype [219,223].

In the obese state, adipose tissue is characterized by infiltration of various immune cells including macrophages and T-lymphocytes [23] and low-grade chronic inflammation. Adipocytes of obese patients are characterized by increased release of various pro-inflammatory adipokines such as IL-6 and MCP-1. Most of the studies analyzing differences in adipokine expression and release of adipocytes in the obese state work with subcutaneous or visceral adipocytes. In contrast, little is known on adipokine expression and release in PVAT compared to other fat depots and in pathological states. In vitro differentiated human PVAT adipocytes are characterized by lower adiponectin release and higher secretion of MCP-1 [219]. In rodent, PVAT expression of adiponectin and FABP4 (A-FABP) was lower, and expression of leptin and MCP-1was higher in high-fat diet fed mice as compared to controls [219,223]. Data on the presence of immune cells in PVAT are controversial. A very recent publication analyzed macrophage content in thoracic PVAT in comparison to white and brown adipose tissue and found PVAT to be resistant to HF-diet induced immune cell infiltration similar to brown adipose tissue [224]. In contrast, adipose tissue inflammation in PVAT and macrophage infiltration could be described to be responsible for a loss of anti-contractile function of this fat depot in the mesenteric bed [225]. Furthermore, adipokines released from PVAT strongly

induced the chemotaxis of peripheral blood leukocytes to the interface between PVAT and the adventitia in human atherosclerotic arteries [226]. These chemotactic effects have been ascribed to IL-8, and MCP-1, and have been proposed to underlie the accumulation of macrophages and T-cells in atherosclerosis.

Vascular relaxation factors, pro-atherogenic and pro-inflammatory adipokines, and growth factors secreted from PVAT were found to directly regulate vascular function through paracrine and endocrine effects on the vascular wall. Adipokines that are increased in the obese state such as leptin have been described to affect vascular function on the level of endothelial dysfunction [227]. Endothelial dysfunction preceding atherosclerosis is characterized by deregulation of vasoreactivity, increased inflammatory and oxidative stress, and impaired barrier function [228]. Oxidative stress has been shown to be increased in PVAT in obesity [229]. Vasorelaxing effects of PVAT are lost in human obesity which is associated with expansion of PVAT [230]. Addition of TNF-α and inhibition of adiponectin inhibit the vasodilator activity of PVAT around healthy blood vessels while blocking of TNF-α by specific antibodies could reverse obesity-induced defects in vasodilatation. In addition to TNF-α and adiponectin, leptin and resistin could be described to be mediators of endothelial dysfunction [231].

Migration of vascular SMC from the media to the intima and their proliferation in the synthetic state are crucial steps in arterial wall thickening in atherosclerosis. In vitro, PVAT explants induce proliferation of vascular SMC [232,55]. Secretory products of PVAT from diet-induced obese rats significantly induced human SMC proliferation compared to lean controls [232]. Increased neointima formation diet-induced obese mice was accompanied by a decreased expression of adiponectin and induction of inflammatory markers such as MCP-1, TNF-α, IL-6, and PAI-1 in PVAT [233]. Importantly, adiponectin-deficient mice display increased neo-intima formation when compared with wild-type mice, and this effect could be reversed by local administration of adiponectin to the periadventitial area [234]. In line with this, adiponectin has been found to abrogate adipokine-induced SMC proliferation [55]. It is currently unknown which factors secreted from PVAT contribute to vascular SMC migration and proliferation. Potential candidates include leptin, resistin, and visfatin, which have been found to directly affect SMC [193,235,236].

For the comprehensiveness of this review, a short paragraph should be devoted to epicardial adipose tissue that is a major fat depot associated with obesity and cardiovascular diseases. As epicardial adipose tissue is directly lying on the surface of the myocardium and also in direct contact with coronary vessels, this depot can also be seen as a special perivascular adipose tissue. While an association between epicardial adipose tissue thickness and the prevalence of cardiovascular diseases and the metabolic syndrome is widely accepted (recently reviewed in [237]), few studies analyze a crosstalk between epicardial adipose tissue and cardiomyocytes. Secretory products from

epicardial adipose tissue induces insulin resistance and defects in contractility and calcium influx in cardiomyocytes with the responsible adipokines still to be identified [238]. Cardioprotective effects have been assigned to adiponectin [239], while resistin has been characterized as cardiodepressant [240].

Conclusion

Enlarged adipose tissue releases a host of adipokines that play a key role in the inter-organ crosstalk between adipose tissue and the vasculature. Here we present a novel strategy to narrow down the huge number of potential biomarkers and drug targets by analyzing molecules that activate NF-κB pro-inflammatory signaling pathways. Comprehensive literature analysis resulted in a final list of known and novel adipokines that we suggest as major candidates for future work. This should address the role of these molecules in linking obesity, chronic inflammation and cardiovascular disease, and their use as potential targets for diagnosis and treatment.

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Table 5.1 Overview of adipokines associated with cardiovascular disease

adipokine	proliferation	inflammation	NFkB-activation	depot
adiponectin	↓[55,28,241]	↓[54]	↓[54]	↑v[242,243]; ↑sc[244,245]
adipsin	-	↑[246,247]	-	↑v[38]
A-FABP	↑[113]	↑[29]	↑[29]	↑v[5]
Angiotensin II	↑[248,241]	↑[249,250]	↑[251]	-
ANGPTL2	-	↑[1,165]	-	↑sc[252]
apelin	-	↑[246]	-	-
chemerin	↑[187]	↑[253] ↓[254]	↑[255]	↔[256]

CXCL5	-	↑[1]	-	-
DPP-4	↑[12]	-	-	↑v[12]
G-CSF	↑[257]	↑[258]	-	-
IL-1β	↑[241,247]	↑[246,247]	↑[250,259]	↔[260]
IL-4	↑[261] ↓[262]	↑[246]	↔[259]	-
IL-6	↑[241,247]	↑[263,246,1]	-	↑v[264,265]
IL-8	↑[241]	↑[246]	↑[266]	↔[260]; ↑v[267]
Il-10	↓[241]	↓[268]	↓[163,268]	(-)
IL-18	↑[269]	↑[246,1]	↑[269]	(-)
Leptin	↑[270,241,271]	↑[263,1,247]	↑[271]	↑sc[272,38]
lipocalin 2	-	↑[1] ↓[123]	↓[118]	-
MCP-1	↑[241,247]	↑[246,1]	-	↑v[273]
MIF	↑[241] ↔[103]	↑[246,103]	↑[274]	↑v[275]; ↔[276]
MIP-1a	↑[247,277]	↑[278]	-	-
Nesfatin-1	-	↑[254]	-	↑sc[279]
omentin	-	↓[280,254]	-	↑v[207]
PAI-1	↑[241,281,282] ↓[283]	↑[27] ↓[284]	-	↑v[53,264]; ↑sc[285]; ↔[286]
PDGF	↑[287]	-	-	-
PEDF	↑[178]	↑[178]	↑[178]	↑v[288]
RANTES	↔[289]	↑[290]	-	↑sc[291]
RBP4	-	↑[1,292]	-	↑v[118]; ↑sc[260]
resistin	↑[241]	↑[293,294,1]	↑[295,293]	↔[296]
Sfrp5	-	↓[263,1]	-	-
TGFβ	↑[297]	↑[247]	-	↔[286]

TNFα	↑[241,247]	↑[263,1]	↑[49,259]	↔[38,5]
TSP1	↑[298] ↓[299]	↓[298]	↓[300]	↑v[301]
vaspin	-	↓[254,212]	↓[212]	↑v[302]
VEGF	↑[187,303,304]	↑[305]	↑[306]	↑v[264]
visfatin	↑[241]	↑[201,1]	↑[201]	↔[307]

ANGPTL2, angiopoietin-like 2; A-FABP, adipocyte fatty acid-binding protein; CXCL5, C-X-C motif chemokine 5; DPP-4, dipeptidyl peptidase-4; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; MCP-1, monocyte chemotactic protein-1; MIF, macrophage migration inhibitory factor; MIP-1α, macrophage inflammatory protein-1α; PAI-1, plasminogen activator inhibitor-1; PDGF, platelet-derived growth factor; PEDF, pigment epithelium-derived factor; RANTES, regulated upon activation, normal T-cell expressed, and secreted; RBP4, retinol binding protein 4; Sfrp5, secreted frizzled-related protein 5; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α; TSP1, thrombospondin 1; VEGF, vascular endothelial growth factor; ↑, parameter activated; ↓, parameter increased; ↔, parameter unchanged; ↑v, adipokine predominantly expressed in visceral adipose tissue; ↑sc, adipokine predominantly expressed in subcutaneous adipose tissue

Table 5.2 Overview of adipokines with a tight link to cardiovascular diseases based on data obtained in in vitro and in vivo studies

Adipokine	in vitro studies	clinical studies
Adiponectin	- suppression of inflammatory cytokines [48,62] - EC: ↑ NO production [308], ↓ NO inactivation [63], ↓ apoptosis [309,310,311] - VSMC: ↓ proliferation [28,55,65], ↓ migration [28,312]	- inversely asssociated with markers of insulin secretion, endothelial function and inflammation [69] - hypoadiponectinemia in patients with coronary atherosclerosis [67] and ACS [68] - increased levels associated with a decreased risk of myocardial infarction in healthy men [66]
A-FABP	- EC: expression induced by VEGF-A, bFGF [113] and lipids [114], associated with ↓ phosphorylated eNOS and ↓NO production [114], ↑proliferation [113]	- high levels associated with worse cardiometabolic risk profile [108] - serum levels positively associated with the Metabolic Syndrome [109], CAD [110], carotid IMT [111] - circulating levels inversely associated with endothelial function [112]
IL-1β	- EC: ↑ VCAM-1 ectodomain release [313] and ↑ expression of MCP-1 [314] - VSMC: ↑ expression of cell adhesion molecules [315], MCP-1 [131] and lipocalin-2 [124] - VSMC: ↑migration [132], ↑proliferation [133,134]	- high levels of IL-1β in patients with unstable angina [316] - ↑ levels of IL-1β mRNA in coronary arteries of patients with ischemic heart disease [317]
IL-4	- EC: ↑ expression of inflammatory mediators [137,138], ↑ ROS generation [139], ↑apoptosis [140] - VSMC: ↑proliferation and 12-lipoxygenase expression [141], ↑ migration [142]	- circulating levels higher in patients with CAD [168] - IL-4 mRNA rarely [144] or not observed in atherosclerotic plaques [145]

IL-8	- modulator of monocyte–endothelial interaction under flow conditions [149] - EC: expression induced by TNFα [150] and homocysteine [151], ↑ proliferation [152], ↑ migration [153,154] - VSMC: ↑ proliferation and 12-lipoxygenase expression [141], ↑ migration [158,159], regulation of VCAM-1 [156,157]	- concentrations significantly higher in CAD patients [318] - levels predicts cardiovascular events [319] - higher levels in patients with early coronary atherosclerosis [320] - expression increased in coronary atherectomy tissue [321] and atherosclerotic plaques [322]
IL-10	- macrophages: inhibition of inflammatory molecules, ↓ apoptosis [323] - EC: inhibition of TNFα-, IL-1β- or LPS-induced expression of IL-6 and IL-8 [137], ↑ eNOS expression, ↑NO production [162] - VSMC: inhibition of TNFα- and bFGF-stimulated proliferation and migration [163,164]	- elevated plasma levels predict long-term adverse outcomes in ACS [324] - some studies report higher levels in patients with ACS [166,167], while other studies report lower levels in patients with CAD [168] and unstable angina [169]
IL-18	- EC: secretion induced by CRP [325], ↑ apoptosis [269,326] - VSMC: ↑ proliferation [269,161], ↑ migration [269], ↑ expression of IL-6, IL-8 and MCP-1 [327]	- expressed in human carotid atherosclerotic plaques [328] - serum levels as a strong independent predictor of death from cardiovascular causes in patients with CAD [329] -serum levels associated with carotid IMT [330]
Leptin	- EC: ↑ PAI-1 [83] and CRP expression [84], ↑eNOS activation and NO production [81], - VSMC: ↑ iNOS expression and activity leading to ↑ NO production [82]	- elevated plasma levels in patients with ACS [74,75] - some studies report an association with risk of CAD [76,77], while other studies found no association with risk of CAD [78,79,80]

Lipocalin-2	- VSMC: ↑ expression after IL-1β treatment via NF-κB [124] - macrophages: supression of LPS-induced cytokine production [123]	- levels positively associated with e.g. adiposity, hypertriglyceridemia, high sensitivity CRP [119], and CAD [120] - high expression in atheromatous human plaques, associated with increased MMP-9 activity [121]
MIF	- EC and macrophages: induction by oxLDL [97] - VSMC: ↑ migration after short-term exposure [103] - MIF-deficient smooth muscle cells: impaired proliferation and lower proteolytic capacity [102]	- upregulated during progression of atherosclerosis toward inflammatory stages [97] - high levels as independent risk factor for future coronary events in CAD patients with IGT/T2DM [101]
PEDF	- EC: inhibition of VEGF-induced proliferation and migration [176] -VSMC: ↑ proliferation, activation of inflammatory signaling pathways [178] -VSMC: inhibition of PDGF-BB-induced proliferation and migration [177,331]	- higher levels in T2DM patients [179] - levels strongly associated with the Metabolic Syndrome [180], vascular inflammation and carotid IMT [181]
TNF-α	- EC: ↑ apoptosis [86] - VSMC: induction of phenotypic changes [88], ↑ migration [89,91], ↑ proliferation [92], ↑ apoptosis [93]	- increased plasma concentrations in patients with premature coronary artery disease [332] - levels positively correlated with carotid IMT [95]
TSP-1	- EC: ↑ expression of cell adhesion molecules [171] - VSMC: stimulation of chemotaxis [172,173] - ↑ aggregation and stability of platelet aggregates [170]	no data available

ACS, acute coronary syndrome; bFGF, basic fibroblast growth factor; CAD, coronary artery disease; CRP, C-reactive protein; EC, vascular endothelial cells; IMT, intima-media thickness; IGT, impaired glucose

tolerance; MMP, matrix metalloproteinase; oxLDL, oxidized low-density lipoprotein; ROS, reactive oxygen species; T2DM, type 2 diabetes mellitus; VSMC, vascular smooth muscle cells.

Table 5.3 Overview of adipokines with a tight link to cardiovascular diseases based on data obtained in animal model

Adipokine	Animal studies	
Adiponectin	KO model overexpression	- ↑ leukocyte-endothelial cell interactions, ↑ E-selectin and VCAM-1 expression, ↓ endothelial NO production [70] - ↓adiposity, altered expression of lipogenic enzymes, ↑ expression of UCPs [60] - ↓ fat storage, morbidity and mortality, oxidative DNA damage upon high fat diet [61]
A-FABP	KO model	- ↑ obesity, but no insulin resistance or diabetes, no TNF-α expression in adipose tissue [333] - improved peripheral insulin resistance, beneficial effect on pancreatic beta cell function and lipid metabolism [334]
IL-1β	KO model	- ↓ aortic sinus lesions and lesion area [135,136] - ↓ VCAM-1 and MCP-1 expression [136]
IL-4	KO model	- ↓ plaque area [146] - in contrast: no protection from early atherosclerosis, no differences in the presence and activity of 12/15-lipoxygenase in macrophages [147]
IL-8	KO model	- ↓ atherosclerotic lesions [335]
Il-10	KO model overexpression	- ↑ lesions [336], ↑ susceptibility to atherosclerosis, lipid accumulation and T-cell infiltration, ↓ collagen content [337] - ↓ lesions [336]
IL-18	KO model	- ↓ lesion size with a more stable phenotype, ↑ serum cholesterol [160]
Leptin	KO model	- ↑ plasma cholesterol and triglyceride levels, extensive atherosclerotic lesions throughout the aorta [85]

Lipocalin-2	KO model	- ↓endothelial dysfunction, ↑ basal and insulin-stimulated AKT/eNOS phosphorylation in the aorta [125]
MIF	KO model	- ↓abdominal aorta lipid deposition and IMT, marked retardation of atherosclerosis, ↓ cell proliferation [102]
PEDF	KO model	- ↑ microvascular density, excessive angiogenesis, epithelial cell hyperplasie [338]
TNF-α	KO model	- ↓ fatty-streak lesions, ↓expression of pro-atherosclerotic factors [94]
TSP-1	KO model	- ↑ postinfarction inflammatory response, infiltration of neighboring noninfarcted area with macrophages and myofibroblasts, ↑ left ventricular remodeling [174]

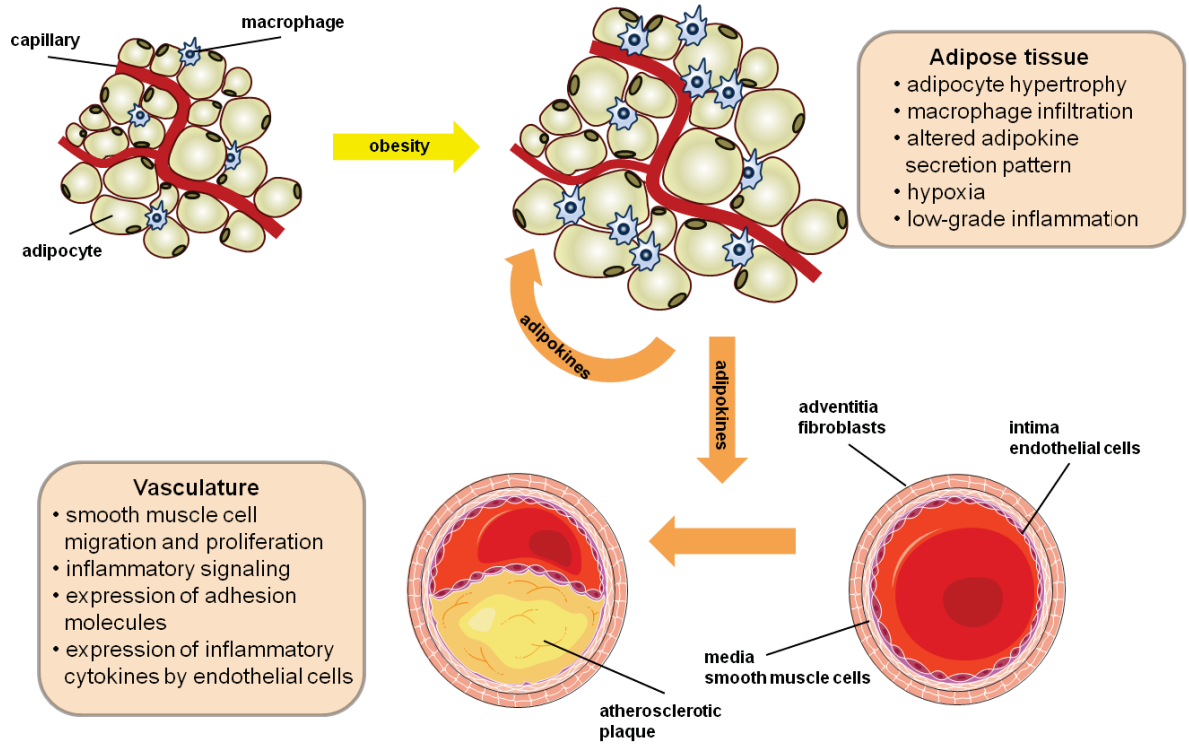

Figure 5.1 Obesity-induced adipose tissue inflammation triggers endothelial dysfunction and vascular inflammation

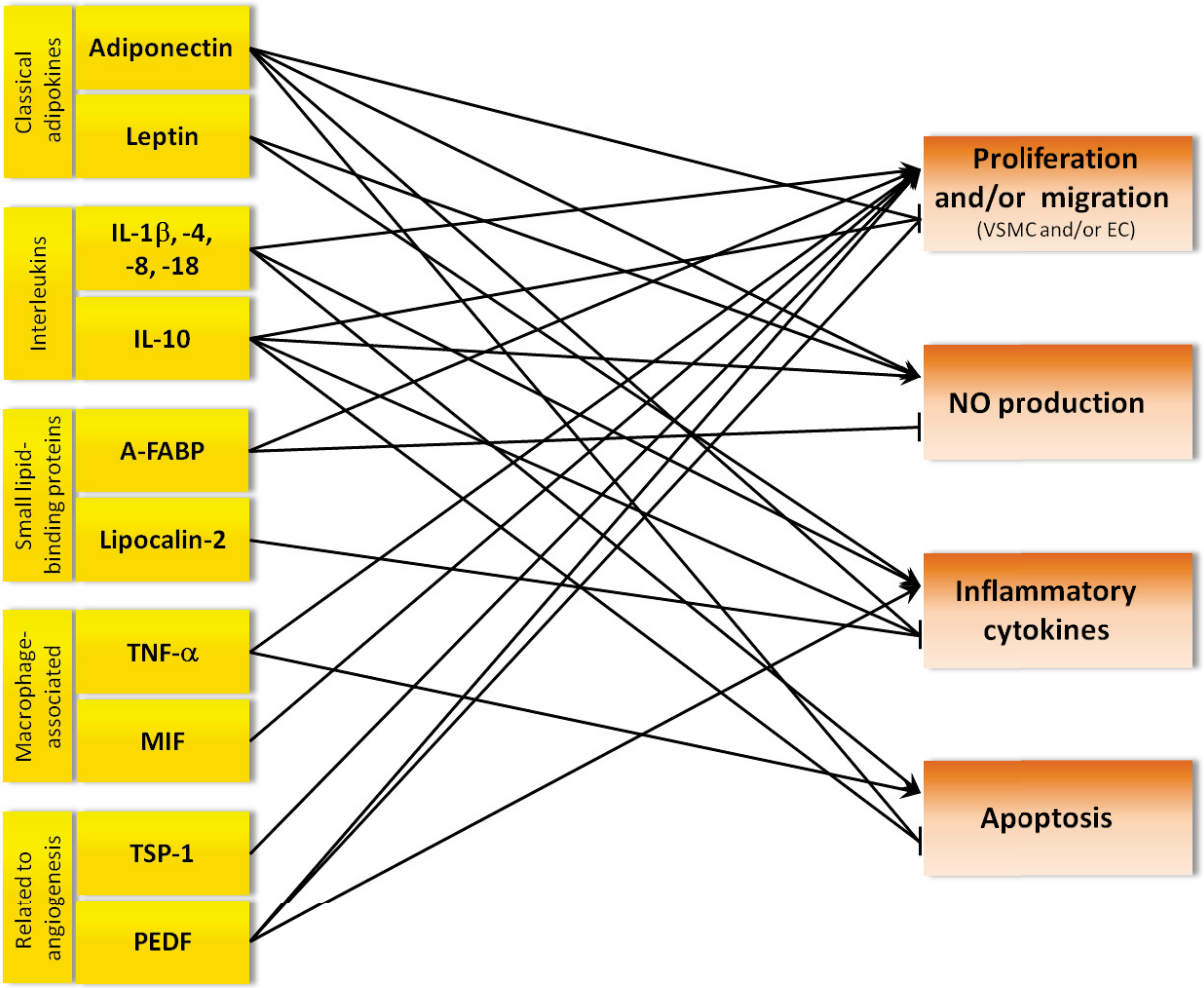

Figure 5.2 Effects of selected adipokines. Arrow indicates stimulation, line indicates inhibition of the respective process

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CHAPTER 6

General Discussion

6.1 Crosstalk between adipose tissue and vascular smooth muscle cells

As mentioned 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 non-communicable diseases, including type 2 diabetes and CVD[245]. The complex inter-organ crosstalk between liver, muscle, and fat obviously underlies the progression of these diseases, with adipose tissue on top of the crosstalk hierarchy[246]. The recognition that adipose tissue is also an endocrine organ affecting whole body energy homeostasis was a great breakthrough towards a better molecular understanding of obesity-related disorders[247].

Several studies in humans as well as animal models have demonstrated that expanded fat mass in obesity strongly correlates with the development of atherosclerosis[174,175]. Therefore obesity is considered as a major determinant of cardiovascular morbidity and mortality[172,173]. In the context of obesity, increased secretion of adipokines and FFA by enlarged adipose tissue plays a pivotal role in the development of a chronic low-grade inflammation state, associated with vascular

dysfunction and cardiovascular risk. However, the crosstalk of adipose tissue with cells of the arterial wall is not yet completely unraveled.

6.1.1 Role of adipokines

Concerning the multifunctional effects of adipokines, it is well known that specific adipocyte derived -factors such as leptin, IL-6, and resistin are involved in the regulation of vascular functions, including VSMC proliferation and migration[235,234,229]. While various studies have examined the effect of single adipokines on VSMC function, until now the impact of the whole secretory output of adipocytes has not been investigated. Disregarding of one study using adipocyte-conditioned medium (CM) from mouse cell lines and rat adipose tissue explants inducing proliferation in human VSMC[248], no further data on a direct interaction of adipocytes and VSMC exist. In the present study, we could demonstrate that CM from *in vitro* differentiated human adipocytes induces proliferation and migration of human VSMC. In this regard, CM of human adipocytes contains several 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 our observed effects. It should be noted that the release of FFA by *in vitro* differentiated adipocytes is very low and hardly detectable by high performance liquid chromatography, indicating that FFA do not have an important role in our setting. Interestingly, the majority of CM induced a 3-4-fold proliferation of human VSMC, whereas only a few lacked this effect. Measuring the amount of adiponectin in all used CM revealed that low adiponectin content is correlated with high proliferative impact of CM and vice versa. With regard to a previous study performed by our working group demonstrating that adiponectin exerts an autocrine effect on adipocytes decreasing the release of adipokines[249], we suggested that the adiponectin content in CM might be responsible for the differences in the proliferative potency of CM. Adiponectin has unlike the majority of adipokines a particular role in the cardiovascular system by exerting anti-inflammatory, anti-atherogenic and cardioprotective effects on multiple target organs and tissues like the heart and blood vessels[250]. Regarding the impact of adiponectin on the level of smooth muscle cells, it could be observed that adiponectin strongly suppressed PDGF-BB-induced proliferation and migration of VSMC through the direct association with this growth factor[235]. In this study we showed that adiponectin added to adipocytes during medium conditioning prevents the proliferative effect of CM. Interestingly, adiponectin added to CM after conditioning did not affect CM-induced proliferation, indicating that

this adipokine has to be in contact with adipocytes to exert its positive influence on adipokine release rather than binding adipokines and preventing their proliferative effects.

Due to the fact that proliferation of VSMC is regulated by distinct pathways, we conducted an initial screening with the Kinex^{TM} antibody microarray to elucidate which pathways are involved in CM-induced proliferation of VSMC. This microarray facilitates a screening of SMC lysates with 377 pan-specific and 273 phospho-site-specific antibodies. After 24 h incubation with CM, 87 proteins were upregulated (≥ +50 % vs. control) and 58 proteins were downregulated (≥ -50 % vs. control).

Therefore we focused on three of these proteins, including p38 MAPK, NF-κB, and mTOR. We observed that CM substantially increases the phosphorylation of all three signaling mediators after short time incubation. Moreover, the IKK inhibitor and rapamycin completely abrogated the CM-induced proliferation, indicating that there is a crosstalk between these two pathways. A recent study has shown that the downregulation of PTEN in hepatocytes is mediated by a signaling complex made of mTOR and NF-κB[251]. Thus, it is probably that both mTOR and NF-κB are responsible for the CM-induced proliferation of VSMC.

Furthermore, we demonstrated 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, VSMC can also express a number of cellular adhesion molecules such as ICAM-1, VCAM-1, selectins, or fractalkine (CX3CL1)[252]. Concerning the expression of ICAM-1, increased amounts of this adhesion molecule could be detected in smooth muscle cells in the intima of atherosclerotic lesions compared to normal smooth muscle cells in the adult aorta[253,254]. Furthermore, ICAM-1 expression was occasionally found in medial smooth muscle cells adjacent to atherosclerotic plaque[255]. VSMC interact with monocytes via adhesion molecules, so that monocytes concomitantly differentiate into macrophages[252,256]. The correlation between the extent of adhesion molecules in the atherosclerotic vessel wall and mononuclear cell infiltration suggests a potential role of VSMC in retaining monocytes and macrophages within the atherosclerotic lesion[257], representing a marker of the phenotypic switch from the contractile to the synthetic state. In addition, several studies, but not all, showed that single adipokines, e.g. TNFα, IL-1β, or IFNγ can induce ICAM-1 expression, depending on the different vascular regions smooth muscle cells were isolated from[252,258,259].

To validate our findings obtained with CM from subcutaneous adipose tissue, we also analyzed 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. In this study CM generated from subcutaneous and epicardial

adipose tissue induces a significant 1.5-fold increase of VSMC proliferation compared to control medium. Although the release of single adipokines may certainly differ between these two fat depots, these data show that the proliferative impact of subcutaneous adipose tissue 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 VSMC in the vessel wall due its considerable amount in the obese state. Since the amount of human perivascular adipose tissue from surgery is technically limited, it could only be used for key experiments. In addition, it should also be noted that CM from explants contains secretory products from all cell types represented 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 subcutaneous and perivascular adipose tissue.

6.1.2 Role of fatty acids

Besides the enhanced secretion of adipokines by enlarged adipose tissue, also increased levels of FFA play a pivotal role in the development of vascular dysfunction and cardiovascular risk. Adipose tissue and adipocytes are secretory active in a metabolic manner by releasing FFA into circulation. While the release of FFA is very low in our model system of *in vitro* differentiated human adipocytes, it plays a crucial role in human physiology. In the context of obesity, the regulation of fat storage and energy supply by adipose tissue is impaired so that plasma FFA levels become increased and excessive metabolism of FFA including triglyceride storage and high levels of FFA metabolites occurs in non-adipose tissue such as liver, muscle, pancreas, heart, and cells of blood vessels, e.g. smooth muscle cells[129,260]. Due to the fact that these organs are not capable of storing large amounts of lipids, ectopic fat stores exert lipotoxic effects on peripheral tissues. Moreover the accumulation of fat around the blood vessels can directly affect vascular function in a paracrine manner by the release of secretory factors, such as proatherogenic adipokines including smooth muscle cell growth factors[260]. In obesity, the high amounts of fat surrounding blood vessels could possibly contribute to the enhanced vascular stiffness in a mechanistic way. Interestingly, FFA can affect smooth muscle cells in the vasculature in a paracrine way, released by perivascular adipose tissue, as well as in an endocrine way by its circulating plasma levels mainly released by subcutaneous adipose tissue. As mentioned above, CM in our model of *in vitro* differentiated human adipocytes contains no detectable amounts of FFA, therefore we incubated VSMC with oleic acid (OA), a monounsaturated fatty acid (18:1n-9), or palmitic acid (PA), a saturated FA (16:0),

alone or in combination with CM, respectively. Plasma contains a mixture of many different FFA, but the most abundant unsaturated FA is OA and the most abundant saturated FA is PA, due to their occurrences in food[261]. In obese hypertensive subjects it was observed that plasma FFA were elevated, particularly OA[262], promoting the switch of VSMC from the contractile to the synthetic phenotype and contributing to the formation of organized atherosclerotic plaques[168]. It is also well known, that OA induces proliferation of rat VSMC as well as migration and plays a pivotal role in obesity and FA-induced atherosclerosis[263,264,265]. In this study we could reproduce the effect of OA on human VSMC proliferation, whereas PA had no effect. The differences between OA and PA are not due to apoptosis as both FA do not induce cell death at the concentrations used[266]. However, different effects on VSMC proliferation and migration could be explained by a differential activation of PGC-1α expression by OA and PA[267,268]. This study demonstrated that overexpression of PGC-1α blocked OA-induced proliferation, whereas suppression of PGC-1α via siRNA amplified these effects. In contrast, PA markedly induced PGC-1α expression[267].

Furthermore, this study is the first to test a combination of adipokines and FA for their effects on human VSMC, demonstrating a markedly increased proliferation of primary VSMC. Previous studies have shown that OA enhances the mitogenic activity of angiotensin II in rat VSMC in a synergistic way similar to the effect of OA in combination with CM in our study[110]. Moreover, the combination of CM and OA enhanced the expression of VCAM-1, which is essential for phenotypic modulation of cultured smooth muscle cells. Interactions of VCAM-1 and its ligand VLA_4 may influence the phenotype and synthetic capacity of smooth muscle cells[269]. In contrast, CM and OA had no effect on ICAM-1 expression. This different expression of VCAM-1 and ICAM-1 can be explained by the fact that the different regulation of these adhesion molecules by mTOR. In addition, mTOR downregulates thrombin-induced ICAM-1 expression[270], whereas another study observed that inhibition of mTOR decreases VCAM-1 expression[271].

As mentioned in the chapter before, we analyzed different pathways regulating VSMC proliferation, such as p38 MAPK, NF-κB, and mTOR. p38 MAPK can be activated by stress, inflammatory cytokines and growth factors[272]. OA alone did not activate p38 MAPK, confirming the observations from Lu and colleagues[273]. Moreover, the combination of CM and OA had prominent effects on the phosphorylation of p38 MAPK, NF-κB, and mTOR within 30 min, in comparison to the partly moderate effects of CM and OA alone. The inhibition of the IKK complex upstream of NF-κB with the IKK-inhibitor I229 and the inhibition of mTOR with rapamycin completely abrogated proliferation of VSMC induced by OA and the combination of CM and OA, respectively, revealing that NF-κB and mTOR are essential pathways for VSMC proliferation. Due to the fact that in hepatocytes

the downregualtion of PTEN triggered by OA is mediated by a signaling complex made of mTOR and NF-κB[251], we suggest that the proliferative potency of the combination of CM and OA could be partly explained by the stronger activation of mTOR and NF-κB.

Furthermore, it is well established, that the expression of iNOS can be induced by various pro-inflammatory cytokines, such as TNFα, IL-1β or IFN-γ[274,275] in a number of cell types including smooth muscle cells. A recent study showed that OA induces iNOS expression in human retinal pigment epithelium[276]. In this study, we observed a substantial increase of iNOS expression induced by the combination of CM and OA, which accompanies with NO production in VSMC. In correlation to this finding, the combination of CM and OA significantly enhanced the secretion of VEGF by VSMC, compared to the moderately induced VEGF secretion induced by CM and OA alone. Inhibition of iNOS by L-NAME partly inhibited the CMOA-induced VSMC proliferation, indicating that the synergistic proliferative effect 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 until now not completely understood. These results demonstrate that adipokine- and OA-mediated iNOS induction enhanced VEGF secretion. Moreover, the combination of human recombinant VEGF in combination with OA leads to an obvious augmentation of VSMC proliferation. Therefore, we assume 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 impact of the complex mixture of adipokines in CM.

In conclusion, we show here for the first time that lipid mediators and adipokines synergistically disturb VSMC function by inducing augmented proliferation and inflammatory signaling. Furthermore, enhanced iNOS expression, NO production and VEGF release by VSMC may be critically involved in this complex process. Therefore, we propose that the combined elevated release of adipokines and fatty acids by adipose tissue in obesity might be a link between, adipose dysfunction, VSMC dysfunction, vascular inflammation, and the development of atherosclerosis.

6.2 Critical mediators of adipokine and fatty acid induced proliferation

As outlined previously, obesity is strongly related to the development of atherosclerosis[277,278]. Almost all blood vessels are surrounded by perivascular adipose tissue, considering merely a structural support for the vasculature. Recent studies revealed that perivascular fat produces and releases various bioactive substances, playing a role in vascular function[279] and providing a direct link between obesity and vascular complications[280,176]. Furthermore, it was already demonstrated that chemotatic proteins secreted by perivascular adipose tissue modulate the function of infiltrating leukocytes at the interface between human perivascular adipose tissue and the adventitia of atherosclerotic aortas[281].

This study was designed to elucidate mechanisms how the secretory output from adipose tissue is related to VSMC proliferation as a crucial event in the genesis of atherosclerotic lesions[282].

6.2.1 Impact of VEGF

In a previous study, we observed that CM of *in vitro* differentiated adipocytes induces proliferation of VSMC in negative correlation to the adiponectin content of CM[283]. Searching for an active component of CM being responsible for VSMC proliferation, we identified VEGF in CM to be significantly correlated with proliferation. VEGF is a well-characterized growth factor, which is involved in the regulation and differentiation of the vascular system. Traditionally VEGF is known as an endothelial cell-specific growth factor which modulates vascular disease by inducing endothelial proliferation mainly through the VEGF-R2[284]. However, an increase of VEGF and its receptors could be observed in other injured arterial wall cells such as monocytes and VSMC[285]. Furthermore, several studies revealed that vascular inflammation and the proliferation of endothelial cells as well as VSMC are enhanced through angiotensin II-induced VEGF release and expression of VEGF-R[286,287,288,289]. However, the results of studies investigating the role of VEGF in the context of cardiovascular diseases are controversial until now. On the one hand, studies predict that VEGF would be beneficial for cardiovascular health by enhancing protective

vascular functions[290]. But on the other hand some studies of VEGF in mouse models of atherosclerosis seem to promote a pro-atherogenic role of angiogenesis[291,290]. Although VEGF has an important role in various physiological processes, the very same qualities cause it to play a part in the origin and maintenance of various pathological processes, including atherosclerosis[292]. To date our understanding of the mechanisms and the precise role of VEGF in the context of cardiovascular disease in humans remains unclear. Here, we report that CM and OA as well as their combination induce release of VEGF by VSMC which in turn might auto-stimulate proliferation. This effect is similar to hypoxia-induced proliferation of VSMC, where an autocrine mechanism of VEGF has been described[293]. In addition, existing data described that pro-inflammatory stimulation including JNK activation or stimulation with angiotensin II and IL-1beta induce VEGF release by VSMC[294,295]. In this context, it might be speculated that pro-inflammatory adipokines, which are present in adipocyte-CM, are responsible for the induction of VEGF release by VSMC. In addition to stimulating the release of VEGF from VSMC, CM induced VEGF receptor 1 and 2 expression, which are known to mediate proliferation and migration of VSMC[296]. Moreover, we propose that both VEGF receptors are regulated by VEGF itself, as neutralizing VEGF prevented CM-induced augmentation of these receptors in parallel to proliferation. VEGF is a pro-inflammatory factor[297,298] and we previously demonstrated that CM activates NF-κB signaling which might be related to VEGF but also to other pro-inflammatory adipokines[299]. Blocking VEGF with a specific neutralizing VEGF-antibody reduced the CM, OA and VEGF-induced proliferation of VSMC completely. In contrast, the strong proliferative effect of the combination of CM and OA was not totally abrogated, illustrating that VEGF is not the only important factor for the synergism of CM combined with OA. Due to the fact, that CM contains various adipokines such as IL-6, IL-8 or MCP-1[249], it is possible that some of these factors induce proliferation in addition to VEGF and that these factors might be causal for the synergistic effects of CM and OA by yet undescribed mechanisms.

Adipose tissue in the visceral and the subcutaneous compartment are the two most abundant depots and it has been shown that they produce unique profiles of adipokines[300,301]. In this context, visceral adipose tissue has received special attention, since various studies have found a positive correlation between the amount of visceral adipose tissue and cardiovascular diseases[302,303]. Recent studies have even proposed that visceral adiposity, measured as waist circumference, is a more precise risk indicator for type 2 diabetes and cardiovascular diseases than whole-body obesity[304,305,306,307]. On the one hand this may be attributed to its location, as it drains directly into the portal vein[308]. On the other hand, the visceral adipokinome contains many pro-inflammatory

cardiovascular risk factors, such as IL-6 and PAI-1[309,310], which contribute to the close association of visceral adipose tissue and cardiovascular disease.

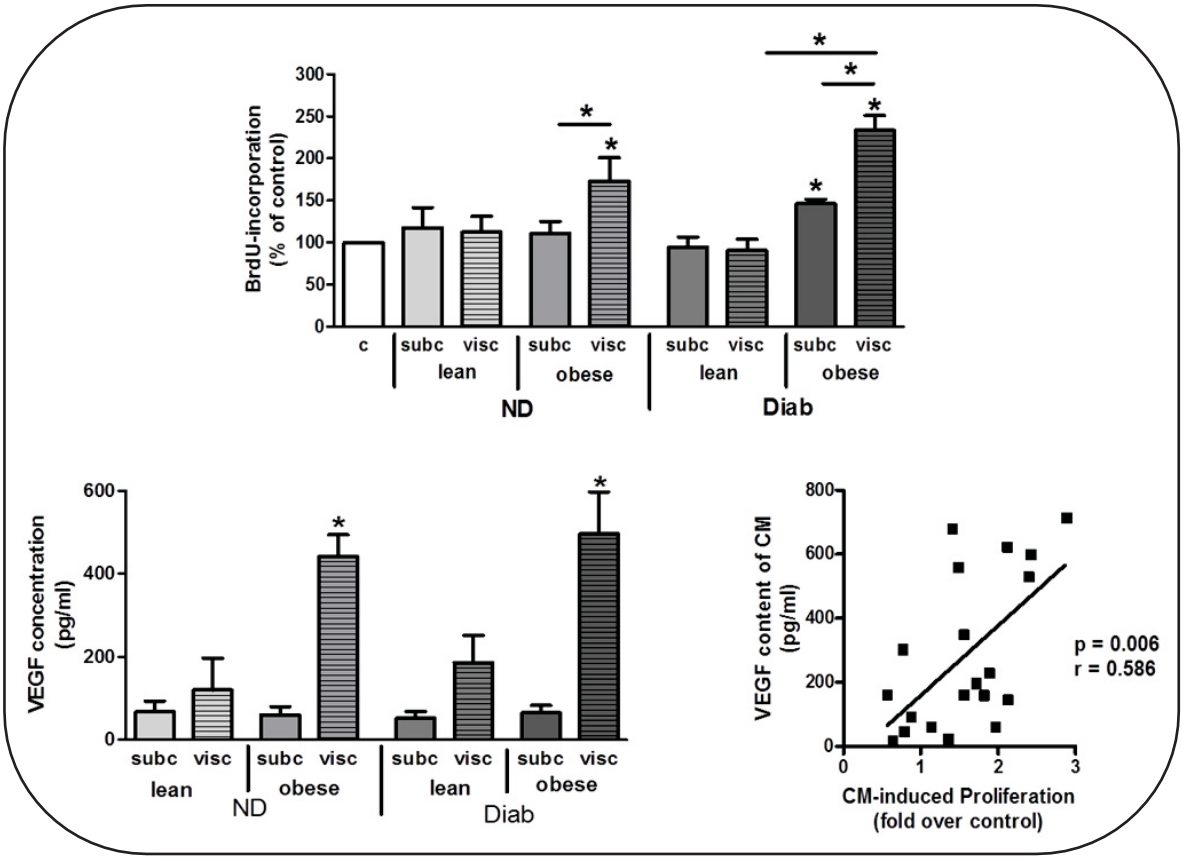

Figure 6.1 Effect of adipocyte-conditioned media of subcutaneous (subc) and visceral (visc) adipose tissue from lean and obese diabetic (Diab) and non-diabetic (ND) patients on vascular smooth muscle cell (VSMC) proliferation. VSMC were serum starved for 24 h and then exposed to subc or visc CM of lean and obese diabetic and non-diabetic patients in the presence or absence of BrdU for 18 h. Incorporation of BrdU into the DNA was measured according to the manufacturer´s instructions (ROCHE). Data are expressed relative to VSMC medium control (c), taken as 100 %. VEGF concentration of CM was measured in duplicates via ELISA. The proliferative impact of CM significantly correlates with its content of VEGF (n=20). Data are mean values ± SEM of three independent experiments. *p≤0.05 compared to control or indicated data.

Therefore, we analyzed the proliferative impact of CM from paired subcutaneous and visceral adipose tissue explants from lean and obese non-diabetic and diabetic patients on human VSMC (figure 6.1). Here, preliminary data demonstrated that visceral-CM of obese subjects significantly enhanced VSMC proliferation in comparison to the subcutaneous fat depots with no differences between diabetic or non-diabetic subjects.

Furthermore, only the VEGF concentration in CM of visceral adipose tissue from obese subjects was substantially enhanced. Interestingly, the VEGF content of visceral-CM correlated with their proliferative impact, indicating that VEGF is also in visceral adipose tissue from obese subjects an important factor for adipokine-induced VSMC proliferation. Future work will be needed to elucidate the precise role of VEGF in the context of VSMC function.

6.2.2 Impact of CD36

The membrane glycoprotein CD36 is present on various cells including platelets, mononuclear phagocytes, adipocytes, hepatocytes, myocytes, and some epithelia[311], reflecting the multiple cellular functions of CD36. On endothelial cells, CD36 is a receptor for thrombospondin-1[312,313]. In this context, it has been shown to play a role as an endogenous negative regulator of angiogensis[314], and therefore involved in inflammation, tumor growth, wound healing, and other pathological processes requiring neovascularization. Furthermore, CD36 also recognizes oxLDL[315,316], indicating that CD36 plays a role in foam cell formation, fatty streak development, and hence atherosclerotic diseases. In its function as fatty acid transporter, CD36 has been suggested to contribute to intracellular lipid accumulation[317,318], thereby promoting lipotoxicity and possibly contributing to the pathogenesis of metabolic disorders, such as type 2 diabetes and obesity. Here, the synergistic effect of CM and OA on VSMC proliferation is accompanied by a strong increase in triglyceride accumulation in VSMC, which is significantly higher compared to the treatment with OA alone. Analysis of CD36 expression revealed a strong upregulation in CM-treated VSMC. In addition, upregulation of CD36 has also been observed after treatment of VSMC with oxidized LDL and high glucose[319], which might be related to oxidative stress that is also induced by CM[320]. CD36 has been intensively studied for its role in facilitating fatty acid uptake and oxidation and is implicated in the pathophysiology of metabolic diseases including cardiovascular alterations[321,322,323,324]. Furthermore, OA-induced triglyceride accumulation is CD36-dependent in our human cell model, which confirms recent data obtained in rodent cells[325]. CM-induced expression of CD36 enhances OA-induced triglyceride accumulation but this effect cannot be attributed to VEGF as CD36 is not regulated by VEGF. Silencing CD36 prevented triglyceride accumulation without affecting VEGF release or expression of VEGF receptor 1.

One of the major positive regulators of smooth muscle cell proliferation is the ERK signaling pathway[326] and activation of ERK is involved in the PDGF-induced VEGF

expression in human VSMC[327]. Interestingly, VEGF signaling after CD36 silencing as assessed by ERK activation was significantly impaired. Previous studies could show that a link between VEGF receptors and CD36 exists, which might explain why CD36 silencing impairs VEGF signaling. CD36 associates with VEGF receptor 2 in endothelial cells integrating angiogenic signals from thrombospondin and VEGF[328]. Recently data indicates that CD36 associates with VEGF receptor 2 and that VEGF-A, VEGF receptor 2 and CD36 are necessary for an angiogenic switch in endothelial cells[329]. Our results point to a potential interaction of CD36 and VEGF receptors in a way that the presence of both receptors is necessary for normal VEGF signaling in human VSMC. Neither VEGF-blocking nor CD36 silencing could completely decrease CMOA-induced proliferation of VSMC, but combining both approaches depresses most of the CMOA-induced effect, suggesting that both VEGF and CD36 are required for CMOA-induced VSMC proliferation. Further studies will be needed to identify the factors in CM that induce CD36 and which might be additionally responsible for the synergism of CMOA.

Recently, it has been proposed that PVAT might be involved in the development of atherosclerosis by a paracrine crosstalk with cells of the vessel wall where inflammation is induced[330]. PVAT of obese rodents is characterized by higher macrophage accumulation, which also produces VEGF, and inflammation associated with abdominal aortic aneurysms[331]. Here, we demonstrate that PVAT from patients with type 2 diabetes is characterized by a significantly higher VEGF release as compared to SAT and also compared to PVAT from non-diabetics. Higher VEGF release is paralleled by a stronger induction of VEGF receptor 1 and 2. In addition, CM from PVAT also significantly increases CD36 expression in comparison to SAT, both from healthy controls and type 2 diabetic patients. CD36 induction reflects the stronger proliferative effect of CM from PVAT. Our results imply that VEGF and CD36 might be essential factors produced and induced by PVAT from patients with type 2 diabetes, respectively. The use of paired biopsies from human SAT and PVAT further strengthens the notion of VEGF release from PVAT for a potential paracrine crosstalk. Anyway, the VEGF content of CM from, SAT and PVAT from non-diabetics as well as SAT from diabetics was equal, but had not the same effect on VSMC proliferation, indicating that VEGF is probably not the unique parameter for the observed effects. The induction of CD36 possibly leading to enhanced lipid accumulation in VSMC might be involved in a negative crosstalk between PVAT and cells of the vascular wall. The restricted use of these biopsies only for key experiments is a technical limitation of our study. Further work will be needed to elucidate the differences in adipokine expression and secretion between PVAT and other fat depots in health and disease in order to identify other putative factors responsible for PAT-induced vascular dysfunction.

In conclusion, our results provide new insight into the pro-atherogenic mechanisms of a negative crosstalk between human adipose tissue and VSMC identifying VEGF as a critical growth factor being released by SAT as well as PVAT and adipokine-mediated induction of CD36 as a mechanism in the synergistically induced proliferation of VSMC after treatment with CM and OA. Therefore, we suggest that the combined elevated circulating free fatty acids and increased adipokine secretion by adipose tissue in obesity and type 2 diabetes might be a link between adipose tissue inflammation, vascular dysfunction and the development of atherosclerosis.

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6.3 Effect of adipokines and fatty acids on NF-κB target genes

Atherosclerosis can be viewed as a multistep, chronic inflammatory disease that involves the interplay between various soluble mediators, endothelial cells, monocytes and smooth muscle cells. It has been demonstrated that NF-κB is constitutively active in VSMC *in vitro*, and its inhibition leads to apoptosis in low-density but not high-density cultures[332]. In this context, NF-κB appears to be essential for VSMC proliferation induced by serum, thrombin, or TNFα[333,334]. High levels of NF-κB are present in VSMC of atherosclerotic lesions[335]. Furthermore, VEGF generates signals that lead to NF-κB activation and NF-κB-dependent expression of antiapoptotic genes, protecting cells from apoptosis during (aberrant) proliferation[336]. Thus, there are multiple ways in which NF-κB can contribute to the initiation and progression of atherosclerosis. In our previous study we reported that the secretory output of human *in vitro* differentiated adipocytes in the form of CM induces NF-κB activation in human VSMC[283], an effect synergistically increased by oleic acid but not palmitic acid. Here we assessed the expression of several NF-κB target genes involved in SMC macrophage recruitment/ inflammation (RANTES, activin A, selectin-P), production of reactive oxygen species (SOD-1), plaque rupture (MMP-1), proliferation/migration (angiopoietin-1, angiotensinogen precursor), and lipid storage capacity (CIDEA) to finally get insight into the molecular mechanisms triggered by adipokines and fatty acids in human VSMC. Our data show a complex, differential regulation of these NF-κB target genes, most likely involving additional pathways like ROS production.

Recently it was demonstrated that palmitic acid activates inflammatory NF-κB signaling in several cell types including vascular endothelial cells[337], as well as human and rat SMC [337,338]. However, we could not observe an increased activity of NF-κB after treatment with relatively low concentrations of palmitate (100 µmol/l) within 120 min. However, it could be possible that activation of NF-κB occurs at later time points, as its activation could be shown in endothelial cells after 3-6 h of treatment with palmitate[339]. Interestingly OA, especially in combination with CM, exerts a much stronger effect on NF-κB signaling than PA with or without combination with CM, which should be further investigated. Moreover, the combinations of CMOA and CMPA activated NF-κB substantially after 18 h, whereas the fatty acids alone exerted no effect.

Furthermore we could report that PA increases IL-6 mRNA expression in VSMC after 4 h, which is in accordance to a study showing that PA induces IL-6 mRNA in human endothelial and smooth muscle cells[340]. In addition, IL-6 secretion was also significantly induced after stimulation with PA. Interestingly, the combination of CMPA increases IL-6 mRNA levels in a synergistic way in comparison to CM and PA alone. With regard to IL-6 secretion, the combination of CMPA induced a significant induction of IL-6 in comparison to all other conditions. In addition, the specific IKK-inhibitor reduced the PA and CMPA-induced IL-6 secretion significantly, providing direct evidence for the key role of NF-κB in mediating the fatty acid and CM effects.

The NF-κB target gene RANTES, otherwise known as CCL5, exerts a multitude of generally pro-inflammatory effects like T cell and monocyte chemoattraction or T cell proliferation. RANTES can be stimulated in response to cytokines such as IL-6 or TNFα and is also known to be secreted by VSMC[341,342]. However, the combination of CM and fatty acids did not modify RANTES expression, suggesting that this chemokine is not involved in augmenting VSMC inflammation under our experimental conditions. Similar results were observed for angiotensinogen precursor (ang pre), which is necessary for the synthesis of angiotensin II. Angiotensin II exerts profound effects on SMC by inducing inflammation, thrombosis and cell proliferation through stimulation of the production of cytokines and growth factors[343].

Interestingly, only the combination of CM with each fatty acid significantly reduced the expression of angiopoietin 1 (ang-1). Ang-1 exerts a vessel-sealing effect[344], acts as an anti-inflammatory agent[345,346] and protects against cardiac allograft atherosclerosis[347]. Obviously, this effect is not NF-κB-dependent, since TNFα did not modify the ang-1 mRNA level. These data further support our notion of the deleterious impact of the combination of fatty acids and adipokines. In line with this we could show that the combined treatment with CMOA and CMPA significantly decreased the expression of CIDEA mRNA in comparison to CM, OA, and PA alone. CIDEA is a lipid droplet coating protein, which colocalizes with perilipin, a regulator of lipolysis[348]. 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 [349]. In this regard, 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[348]. To our knowledge, CIDEA expression in VSMC has never been investigated and its functional role in this cell type is unknown. It may play a role in lipid handling, but future studies will be needed to address this issue.

It is well accepted that the excessive generation of ROS, leading to oxidative stress by outstripping the antioxidant system, contributes to the development of CVD including

atherosclerosis and diabetes mellitus. Measuring ROS in the current study showed that CM induces a significant increase in ROS compared to control, indicating that CM also induces oxidative stress in SMC, which was previously observed in skeletal muscle cells by our group[320]. OA, PA, and the combination CMOA had no effect on ROS production, whereas the combination of CM and PA could induce ROS in comparison to CM and PA alone. Recent studies conducted in 3T3 adipocytes demonstrated that palmitate, but not oleate (250 µmol/l) induced ROS[350] and in L6 skeletal muscle cells also palmitate, but not oleate induced ROS production via de novo synthesis of ceramide[351]. Mattern and Hardin showed that the low oleate oxidation in SMC was not due to a limitation in fatty acid transport or to storage of oleate as triglyceride[352]. However, palmitate most likely causes oxidative stress because the metabolism of palmitate, unlike oleate, results in the formation of ceramide[353] and ROS[354]. In our setting PA alone did not induce ROS, which is probably due to the lower concentration (100 µmol/l) used, but the combination with CM increased ROS, possibly due to an enhanced formation of ceramide This finding suggests that CMOA-induced proliferation is not generally dependent on ROS production. On the other hand, only this combination induced a profound reduction of the NF-κB target gene SOD-1, suggesting that superoxide anions are involved in SMC proliferation in response to CMOA. However, future work will be necessary to analyze superoxide anion production by CMOA and to investigate other ROS scavenging enzymes like glutathion-peroxidase, catalase, or SOD-2, probably involved in this complex scenario.

Only the combination of CMOA led to a reduction in matrix-metalloproteinase (MMP)-1 mRNA levels after 4 h, whereas CM and OA alone exert no effects. This reduction could possibly lead to a higher production of extracellular collagen, resulting in an accelerated intimal thickening by CMOA. In contrast the combination of CMPA increased the MMP-1 activity significantly after 24 h, indicating that higher active MMP-1 levels enhance the risk of plaque rupture. Additionally, OA as well as CMOA significantly decreased activin A mRNA levels, by 20 % and 50 % respectively. It has been postulated that activin A modulates the proliferation and differentiation of several cell types involved in atherogenesis, notably endothelial cells, macrophages and VSMC. In this context it inhibits the propagation of human endothelial cells[355] and it enhances the differentiation of monocytic cells into macrophages[356] and inhibits foam-cell formation [357]. The role of activin A in inducing proliferation of SMC is controversially discussed at the moment. Whereas in some studies activin A has induced DNA synthesis in rat smooth muscle cells [358,359], other reported that activin did not affect rat smooth muscle cell growth[360]. Further, it could be shown that activin A mediates the differentiation of proliferating human VSMC towards a contractile phenotype[361], thereby inducing a redifferentiation, suggesting a rather negative impact of CMOA on plaque stability. Activin A was also reported as a

novel component of conditioned medium obtained from vasoactive G-protein-coupled receptor (GPCR) agonists-stimulated VSMC[362]. Furthermore activin A was demonstrated to be increased after vascular injury. Thus, this molecule seems to play an important role in the control of cellular processes including proliferation and migration associated with the progression of vascular lesions.

In conclusion, we show here that the combination of adipocyte-derived factors and low concentrations of OA specifically and synergistically induce SMC proliferation, NF-κB activation, and a set of downstream targets critically involved in atherosclerosis progression. This includes the anti-inflammatory agent ang-1 and additionally MMP-1 and activin A, both involved in vascular remodeling. These data support our notion that the lipotoxic potential of fatty acids is substantially enhanced by the presence of adipocyte-derived factors.

6.4 Perivascular adipose tissue and cardiovascular disease

Perivascular adipose tissue (PVAT) is defined as adipose tissue around blood vessels which occurs in a way that no fascial layer separates this fat depot from the vascular wall. In addition to this barrier-free connection between PVAT an infiltration of adipocytes into the outer region of the adventitia has been observed[363]. In obesity, perivascular adipose tissue is increased in humans and rodents[364,365]. Furthermore, the amount of PVAT is highly associated with visceral obesity and moderately correlated with subcutaneous adipose tissue and BMI[366]. In various human and animal studies adipocytes of PVAT have been compared to subcutaneous and visceral and there is still controversy as for the classification of PVAT as a depot of white adipose tissue or brown adipose tissue. It appears that PVAT surrounding abdominal and thoracic aortas might be multifaceted as for its adipocyte phenotype[363,367].

In the obese state, adipose tissue is characterized by infiltration of various immune cells including macrophages and T-lymphocytes[368] and low-grade chronic inflammation. Adipocytes of obese patients are characterized by increased release of various pro-inflammatory adipokines such as IL-6 and MCP-1. Most of the studies analyzing differences in adipokine expression and release of adipocytes in the obese state work with subcutaneous or visceral adipocytes. In contrast, little is known on adipokine expression and release in PVAT compared to other fat depots and in pathological states. *In vitro* differentiated human PVAT adipocytes are characterized by lower adiponectin release and higher secretion of MCP-1[363]. In rodent, PVAT expression of adiponectin and FABP4 (A-FABP) was lower, and expression of leptin and MCP-1 was higher in high-fat diet fed mice as compared to controls [363,367]. Furthermore, data on the presence of immune cells in PVAT are controversial. A very recent publication analyzed macrophage content in thoracic PVAT in comparison to white and brown adipose tissue and found PVAT to be resistant to high-fat-diet induced immune cell infiltration similar to brown adipose tissue[369]. In contrast, another study described that adipose tissue inflammation in PVAT and macrophage infiltration is responsible for a loss of anti-contractile function of this fat depot in the mesenteric bed[370]. Moreover, adipokines released from PVAT strongly enhanced the chemotaxis of peripheral blood leukocytes to the interface between PVAT and the adventitia in human atherosclerotic arteries[371]. These chemotactic effects have been ascribed to IL-8, and MCP-1, and have been proposed to underlie the accumulation of macrophages and T-cells in atherosclerosis.

Vascular relaxation factors, pro-atherogenic and pro-inflammatory adipokines, and growth factors secreted from PVAT were found to directly regulate vascular function through paracrine and endocrine effects on the vascular wall. Adipokines that are increased in the obese state such as leptin have been described to affect vascular function at the level of endothelial dysfunction[372]. Endothelial dysfunction preceding atherosclerosis is characterized by deregulation of vasoreactivity, increased inflammatory and oxidative stress, and impaired barrier function[373]. Oxidative stress has been shown to be increased in PVAT in obesity[374]. Vasorelaxing effects of PVAT are lost in human obesity which is associated with expansion of PVAT[375]. Addition of TNFα and inhibition of adiponectin inhibit the vasodilator activity of PVAT around healthy blood vessels while blocking of TNFα by specific antibodies could reverse obesity-induced defects in vasodilatation. In addition to TNFα and adiponectin, leptin and resistin could be described to be mediators of endothelial dysfunction[376].

Migration of vascular SMC from the media to the intima and their proliferation in the synthetic state are crucial steps in arterial wall thickening in atherosclerosis. In vitro, PVAT explants induce proliferation of vascular SMC[248,283]. Secretory products of PVAT from diet-induced obese rats significantly induced human SMC proliferation compared to lean controls[377]. Increased neointima formation in diet-induced obese mice was accompanied by a decreased expression of adiponectin and induction of inflammatory markers such as MCP-1, TNFα, IL-6, and PAI-1 in PVAT[378]. Importantly, adiponectin-deficient mice display increased neo-intima formation when compared with wild-type mice, and this effect could be reversed by local administration of adiponectin to the periadventitial area[379]. In line with this, adiponectin has been found to abrogate adipokine-induced VSMC proliferation[283]. It is currently unknown which factors secreted from PVAT contribute to vascular VSMC migration and proliferation. Potential candidates include leptin, resistin, and visfatin, which have been found to directly affect SMC[380,381,382].

It is well established, that epicardial adipose tissue is a major fat depot associated with obesity and CVD. As epicardial adipose tissue is directly lying on the surface of the myocardium and also in direct contact with coronary vessels, this depot can also be seen as a special perivascular adipose tissue. While an association between epicardial adipose tissue thickness and the prevalence of cardiovascular diseases and the metabolic syndrome is widely accepted (recently reviewed in[383]), only a few studies analyze a crosstalk between epicardial adipose tissue and cardiomyocytes. Secretory products from epicardial adipose tissue induces insulin resistance and defects in contractility and calcium influx in cardiomyocytes with the responsible adipokines still to be identified[384].

In addition, cardioprotective effects have been assigned to adiponectin [385], while resistin has been characterized as cardiodepressant [386].

In conclusion, enlarged adipose tissue releases a host of adipokines that play a key role in the inter-organ crosstalk between adipose tissue and the vasculature. Further studies should address the role of adipose tissue-derived factors in linking obesity, chronic inflammation and CVD, and their use as potential targets for diagnosis and treatment.

To summarize the presented work, combined treatment of human VSMC with the whole secretory output of adipocytes in terms of CM resulted in enhanced proliferation and migration. Furthermore, CM activates proliferative and pro-inflammatory signaling mediators such as p38 MAPK, NF-κB, and mTOR and enhances the expression of the adhesion molecule ICAM-1. The combined treatment of CM and OA augments the proliferation and NF-κB activation in a synergistic way via iNOS expression, NO production, and VEGF release. Further investigations identified VEGF as an essential factor and CD36 as a mechanism in the adipokine and OA-induced VSMC proliferation (figure 6.2).

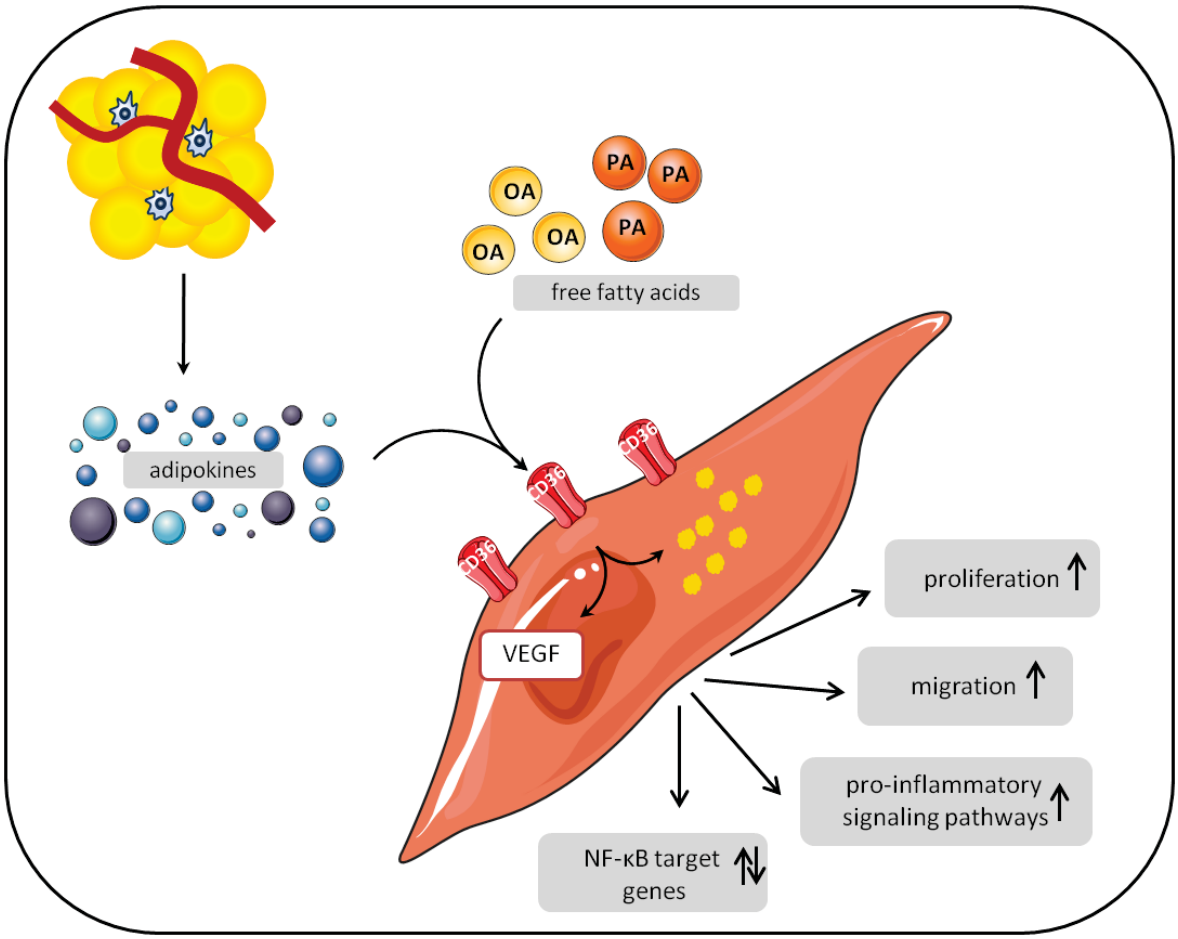

Figure 6.2 Schematic summary of presented work. Combined treatment of vascular smooth muscle cells (VSMC) with adipokines and fatty acids increases CD36 expression, VEGF release and accumulation of intracellular lipid droplets. As a result, proliferation of VSMC is synergistically increased, as well as pro-inflammatory signaling.

The combination of CM and OA synergistically induced proliferation via increased CD36 and VEGF receptor expression as well as VEGF secretion of VSMC, with CD36 being necessary for an enhanced VEGF signaling. Moreover, RT-PCR profiling of distinct NF-κB target genes revealed that CMOA significantly reduced SOD-1, CIDEA, MMP-1, and activin a mRNA levels in comparison to CM and OA alone, whereas the combination of CMPA enhances IL-6 secretion, ROS production as well as MMP-1 activity.

6.5 Perspectives

The dissertation presented here met several objectives proposed in the introduction. In the first study of this work, it was shown that adipocyte-CM, representing the whole secretory output of human adipocytes, induced proliferation and migration of human VSMC. Furthermore, we could also observe that CM increases ICAM-1 expression and pro-inflammatory signaling in VSMC. Several studies showed that beside the increased secretion of adipokines also the enhanced release of FFA in obesity is associated with cardiovascular risk. In this context, the absence of FFA in adipocyte-CM in our model system provides a good tool to investigate the effects of adipokines and lipid mediators separately or in combination. The combined treatment of VSMC with CM and OA synergistically increased VSMC proliferation and pro-inflammatory signaling, in comparison to CM and OA alone. The underlying mechanisms responsible for these observed effects are not yet elucidated. The identification of the responsible candidates and mechanisms revealed that the growth factor VEGF and the fatty acid transporter CD36 are essential factors for CM and OA-induced VSMC proliferation. Our results point to a potential interaction of CD36 and VEGF-R in a way that the presence of both receptors is necessary for normal VEGF signaling in human VSMC. In this context, the potential interaction of CD36 and VEGF-R in VSMC represents a novel and promising aspect that warrants further investigations. Additionally, future studies will be needed to identify the factors in CM that induce CD36 and VEGF release and which might be additionally responsible for the synergism of the combination of CM and OA.

In addition, preliminary data demonstrate that PVAT from patients with type 2 diabetes is characterized by a significantly higher VEGF release, increased CD36 expression, and a stronger effect of CM on human VSMC proliferation. These results imply that VEGF and CD36 might be essential factors produced and induced by PVAT from patients with type 2 diabetes, respectively. The use of paired biopsies from human SAT and PVAT further strengthens the notion of VEGF release from PVAT for a potential paracrine crosstalk. Further work will be needed to elucidate the differences in adipokine expression and secretion between PVAT and other fat depots in health and disease in order to identify other putative factors responsible for PVAT-induced vascular dysfunction.

Furthermore, the combination of CM and OA synergistically induced the activation of NF-κB in VSMC compared to CM or OA alone. Future studies have to unravel the underlying mechanisms, which are responsible for this observed effect. The investigation of distinct NF-κB target genes revealed that the expression of the anti-inflammatory agent

ang-1 and additionally MMP-1 and activin A, both involved in vascular remodeling, were significantly decreased after treatment with CM and OA. These data support our notion that the lipotoxic potential of fatty acids is substantially enhanced by the presence of adipocyte-derived factors. Moreover, the combination of CM and OA also reduced the expression of cytosolic SOD-1 mRNA levels, probably leading to the enhanced production of ROS, which could explain the synergistic effect of CMOA on VSMC proliferation.

Taken together, this model system to analyze the crosstalk between adipose tissue and smooth muscle cells, applied in this thesis, provides a promising tool to investigate the effects of several other FFA and to further study the influence of a combination of specific fatty acids on human VSMC, which have not been analyzed so far.

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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 contributions to each publication are outlined.

Chapter 2

- Effect of adipocyte-conditioned medium in combination with fatty acids on proliferation, migration and expression of adhesion molecules of human vascular smooth muscle cells
- Impact of adipocyte-conditioned medium and oleic acid on inflammatory and proliferative signaling pathways (NF-κB, p38 MAPK, mTOR)
- Impact of rapamycin and IKK-inhibitor on the proliferative effect of adipocyte-conditioned medium and oleic acid
- Effects of adipocyte-conditioned medium and oleic acid on iNOS expression, VEGF concentration and NO production and impact of L-NAME on proliferation
- Analysis of proliferative effect of conditioned medium from epicardial and subcutaneous fat explants on human vascular smooth muscle cells together with Dr. Daniela Lamers
- Preparation of the manuscript together with Dr. Daniela Lamers, Dr. Henrike Sell and Prof. Jürgen Eckel

Chapter 3

- Impact of VEGF and specific VEGF-blocking antibody on the proliferation of human smooth muscle cells
- Effect of CD36 siRNA on adipocyte-conditioned medium-, VEGF- and oleic acid-induced proliferation
- Analysis of protein abundance of VEGF-receptors, CD36 and FATP-4
- Performance of triglyceride quantification

- Analysis of conditioned medium from perivascular and subcutaneous fat explants from diabetic and non-diabetic patients on proliferation, VEGF secretion and expression of CD36 and VEGF receptor of human vascular smooth muscle cells
- Preparation of the manuscript together with Dr. Daniela Lamers, Dr. Henrike Sell and Prof. Jürgen Eckel

Chapter 4

- Preparation of the manuscript together with Dr. Daniela Lamers, Dr. Henrike Sell and Prof. Jürgen Eckel
- Effect of CM and fatty acids on proliferation, migration and NF-κB signaling
- Analysis of IL-6 secretion of vascular smooth muscle cells and impact of IKK-inhibitor on IL-6 secretion
- Effect of conditioned medium and fatty acids on MMP-1 activity

Chapter 5

- Preparation of the manuscript together with Dr. Annika Taube, Dr. Henrike Sell, Dr. Kristin Eckardt and Prof. Jürgen Eckel
- Literature research on various topics including adipokines, cardiovascular disease, atherosclerosis, smooth muscle cell, endothelial cells
- Preparation of schematic figure with Dr. Kristin Eckardt

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

Raphaela Schlich

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