HEINRICH HEINE
UNIVERSITÄT DÜSSELDORF

Involvement of adipokines in the induction of smooth muscle cell dysfunction and the development of arteriosclerosis

INAUGURAL-DISSERTATION

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von
Nina Wronkowitz
aus Castrop-Rauxel
2013

Diese Arbeit wurde angefertigt am

Deutschen Diabetes-Zentrum
Paul-Langerhans-Gruppe, Integrative Physiologie

an der Heinrich-Heine-Universität Düsseldorf
Leibniz-Zentrum für Diabetes-Forschung

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Referent: Prof. Dr. Jürgen Eckel
Korreferent: Prof. Dr. Rolf Wagner

Tag der mündlichen Prüfung: 27.01.2014

„Am Ende wird alles gut“ „

Zusammenfassung

Das Fettgewebe wurde lange Zeit nur als ein Depot zur Speicherung von Lipiden angesehen. Mittlerweile ist jedoch hinreichend bekannt, dass es sich hierbei ebenso um ein endokrines Organ handelt, welches eine große Anzahl von Proteinen und bioaktiven Peptiden, auch Adipokine genannt, sezerniert. Diese Adipokine beeinflussen Prozesse wie Metabolismus, Inflammation und Koagulation. Im Krankheitsbild der Adipositas wird die physiologische Funktion des Fettgewebes durch die Hypertrophie der Adipozyten und die chronische Inflammation innerhalb des Fettgewebes gestört. Die Fehlfunktion des Fettgewebes führt zu einer veränderten Produktion und Sekretion der Adipokine, welche wiederum zahlreiche periphere Gewebe wie Leber, Skelettmuskel und Blutgefäße sowohl endo- als auch parakrin beeinflussen. Im Kontext der Adipositas wurde dem Fettgewebe eine Schlüsselrolle in der Pathogenese des Type 2 Diabetes und kardiovaskulärer Erkrankungen zugeschrieben. Bis heute ist jedoch das Sekretom der Adipozyten noch nicht vollständig aufgeklärt, so dass fortlaufend neue Adipokine entdeckt und validiert werden. In einer vorrausgegangenen Proteom-Analyse des Sekretoms humaner Adipozyten konnte unsere Arbeitsgruppe lösliches DPP4 (sDPP4) als neues Adipokin identifizieren. DPP4 ist ein ubiquitär expremiertes Oberflächenprotein mit Protease-Aktivität, welches vor allem durch die Degradierung der Inkretinhormone als wichtiger Regulator des Blutzuckerspiegels bekannt ist. Durch Abspaltung von der Zellmembran wird eine lösliche Form dieses Proteins im Plasma und in anderen Körperflüssigkeiten freigesetzt. Demzufolge war das erste Ziel dieser Dissertation, sDPP4 als neues Adipokine zu charakterisieren und zu validieren. Hierbei konnten wir zeigen, dass während der Adipozytendifferenzierung die Freisetzung von sDPP4 deutlich ansteigt und dass Adipozyten hauptsächlich für die Freisetzung von sDPP4 aus dem Fettgewebe in die Blutzirkulation verantwortlich sind. Die Untersuchung verschiedener Fettdepots zeigte, dass DPP4 am höchsten im viszeralen Fettgewebe adipöser Patienten exprimiert wird. Dementsprechend wies das Serum adipöser Patienten erhöhte Level von sDPP4 auf, welche zudem mit Parametern des Metabolischen Syndroms korrelierten. Endo- und parakrine Effekte dieses Proteins konnten in Form einer sDPP4-induzierten Verminderung der Insulin Signalweiterleitung in Adipozyten und Skeletmuskelzellen aufgezeigt werden.

Es ist hinreichend bekannt, dass die mit Adipositas einhergehende Expansion des Fettgewebes negativen Einfluss auf die Zellen der Gefäßwand, besonders auf glatte Muskelzellen, ausübt. Daher befasste sich das zweite Ziel dieser Arbeit mit der Untersuchung direkter Effekte sDPP4s auf humane vaskuläre glatte Muskelzellen. Hierbei charakterisierten wir einen neuen sDPP4-induzierten und Rezeptor-vermittelten Signalweg. Unter Verwendung physiologischer DPP4 Konzentrationen zeigte sich sowohl eine kurzzeitige als auch eine längerfristige Aktivierung von ERK1/2. Zusätzlich wurde eine DPP4-vermittelte Phosphorylierung der NF-κB Untereinheit p65 beobachtet. Als Folge der ERK und NF-κB Aktivierung induziert sDPP4 sowohl die Expression von iNOS als auch die Freisetzung von pro-inflammatorischen Zytokinen wie IL-6, IL-8 und MCP-1. In Übereinstimmung mit dem sDPP4-induzierten Stress-Signal, führte sDPP4 zu einer erhöhten Proliferation und zur Verminderung der Insulin-Signalweiterleitung in vaskulären glatten Muskelzellen. Alle direkten Effekte von sDPP4 auf die verschiedenen Signalwege, Proliferation und Inflammation konnten vollständig durch die enzymatische Inhibition von DPP4 verhindert werden. In Anbetracht der DPP4-induzierten Signalwege und durch bioinformatische Analyse stellte sich sDPP4 als möglicher Agonist des Protease-aktivierten Rezeptors 2 heraus. Durch die Hemmung der Genexpression und Einsatz eines spezifischen Antagonisten dieses Rezeptors konnten die sDPP4-induzierten Effekte auf die Proliferation und Inflammation vollständig inhibiert werden.

Ein weiteres Thema dieser Arbeit befasste sich mit dem atherogenen Einfluss des gesamten Adipozytensekretoms in Form von konditioniertem Medium (CM) auf differenzierte vaskuläre glatte Muskelzellen. CM steigerte die Expression des für glatte Muskeln specifischen α-Aktins, des miRNA-143/145 Clusters und verminderte deutlich die Insulin-vermittelte Phosphorylierung von Akt und seinem Substrat eNOS. Zudem führte das CM zu einer vermehrten Phosphorylierung von SMAD2 und p38, welche beide in Verbindung mit der Induktion des miRNA-134/145 Clusters stehen. Demzufolge konnte sowohl die durch CM bedingte Induktion des miRNA-143/145 Clusters als auch die Inhibition der Insulin-vermittelten Akt und eNOS Phosphorylierung durch pharmakologische Inhibition von Alk-4/5/7 und p38 verhindert werden. Die Transfektion von vaskulären glatten Muskelzellen mit Precursor miRNA-143, jedoch nicht mit Precursor miRNA-145, resultierte in einer verminderten Insulin-vermittelten Akt und eNOS Phosphorylierung. Die Inhibition des Insulins Signalweges sowohl durch CM als auch miRNA-143 war mit einer reduzierten

Expression von oxysterol-binding protein-related protein 8 (ORP8) assoziiert. Letztendlich führte die Inhibition der ORP8 Expression ebenfalls zu einer verminderten, Insulin-vermittelten Akt Phosphorylierung in vaskulären glatten Muskelzellen.

Zusammenfassend lässt sich sagen, dass sDPP4 als neues Adipokin charakterisiert und validiert wurde. Ebenfalls wurde das atherogene Potential sowohl dieses neuen Adipokins als auch des gesamten Adipozytensekretoms auf Ebene der vaskulären glatten Muskelzellen untersucht. Hierbei stellte sich heraus, dass sDPP4 möglicherweise eine Rolle als molekularer Link zwischen Adipositas und dem Metabolischen Syndrom sowie kardiovaskulären Erkrankungen spielt. Zudem konnte mit der Untersuchung der miRNAs ein neuer Aspekt in dem komplexen Crosstalk zwischen Adipozyten und glatten Muskelzellen der Gefäßwand aufgezeigt werden.

Summary

Adipose tissue has long been seen as a passive lipid storage depot but it is now considered as an endocrine organ releasing a large number of proteins and bioactive peptides, the so-called adipokines. These adipokines affect several processes, such as metabolism, inflammation and coagulation. In obesity, adipocyte hypertrophy and the chronic low-grade inflammation within adipose tissue affects its normal physiological function. Adipose tissue dysfunction results in an altered production and secretion of adipokines, which in turn affects several peripheral tissues in an endo- and paracrine manner such as liver, skeletal muscle and the vasculature. Thus, in the context of obesity, adipose tissue has gained considerable interest as a key player in the pathogenesis of several metabolic diseases, such as type 2 diabetes and cardiovascular disease. However, the secretome of adipocytes remains still incompletely characterized and the discovery and validation of new adipocyte-derived factors is still in progress. With comprehensive proteomic profiling of the human adipocyte secretome our working group previously identified soluble DPP4 (sDPP4) as novel adipokine. DPP4 is a ubiquitously expressed cell-surface protease, which is widely known for its role in glycaemic control through catabolism of incretin hormones. Due to shedding from the membrane, a soluble form of DPP4 lacking the cytoplasmatic tail and the transmembrane region can be found in plasma and other body fluids. Therefore, the first aim of this thesis was to further characterize and validate sDPP4 as novel adipokine. Here, we demonstrated that sDPP4 release is considerably increased during adipocyte differentiation. Comparison with preadipocytes and adipose tissue macrophages showed that adipocytes most likely represent the major source of sDPP4, which is released from the intact organ into the circulation. Comparing different fat depots, DPP4 shows the highest expression in visceral adipose tissue of obese patients. Accordingly, circulating levels of sDPP4 were increased in obesity and correlate with parameters of the metabolic syndrome. Furthermore, sDPP4 exerts autocrine and paracrine effects as shown by impaired insulin signaling in adipocytes and skeletal muscle cells.

It is well accepted that expanded fat mass in obesity negatively affects cells of the vascular wall, especially smooth muscle cells. Therefore, the second objective of this thesis was to study direct effects of sDPP4 on human vascular smooth muscle. We characterized a novel sDPP4-induced and receptor-mediated signaling cascade. Using physiological

concentrations of sDPP4, we observed a short- as well as a long-term activation of ERK1/2. Additionally, sDPP4 treatment induced phosphorylation of the NF-κB subunit p65. Downstream of ERK and NF-κB, sDPP4 leads to iNOS induction and increased expression and release of pro-inflammatory cytokines, such as interleukin-6, interleukin-8 and monocyte chemoattractant protein-1. In accordance with sDPP4-induced stress and inflammatory signaling, sDPP4 stimulates proliferation and impairs insulin signaling in vascular smooth muscle cells. All direct effects of sDPP4 on signaling, proliferation and inflammation were completely prevented by enzymatic inhibition of sDPP4. Bioinformatic analysis and signaling signature induced by sDPP4 suggest that sDPP4 might be an agonist for protease-activated receptor 2. After silencing of this receptor and using a receptor antagonist, sDPP4-induced proliferation and inflammation was totally abolished.

A further topic of this work was to address the atherogenic impact of the whole secretory output of human adipocytes in terms of conditioned medium (CM) on differentiated human vascular smooth muscle cells. CM increased the expression of smooth muscle α-actin, and the miRNA-143/145 cluster, but markedly impaired the insulin-mediated phosphorylation of Akt and its substrate endothelial nitric oxide synthase (eNOS). Furthermore, CM promoted the phosphorylation of SMAD2 and p38, which have both been linked to miRNA-143/145 induction. Accordingly, the induction of miRNA-143/145 as well as the inhibition of insulin-mediated Akt- and eNOS-phosphorylation was prevented when vascular smooth muscle cells were treated with pharmacological inhibitors for Alk-4/5/7 and p38 before the addition of CM. Transfection of vascular smooth muscle cells with precursor miRNA-143, but not with precursor miRNA-145, resulted in impaired insulin-mediated phosphorylation of Akt and eNOS. This inhibition of insulin signaling by CM and miRNA-143 is associated with a reduction in the expression of the oxysterol-binding protein-related protein 8 (ORP8). Finally, knock-down of ORP8 resulted in impaired insulin-mediated phosphorylation of Akt in hVSMC.

In conclusion, sDPP4 was characterized and validated as novel adipokine. Furthermore, the atherogenic impact of this novel adipokine and of the whole secretory output of adipocytes was investigated on the level of vascular smooth muscle cells. In this context, we identified sDPP4 as a promising candidate linking obesity to the metabolic syndrome and cardiovascular disease. The presented work also illustrated miRNAs as novel players in the complex crosstalk between adipocytes and vascular smooth muscle cells.

Table of Contents

List of Abbrevations

ADA	adenosine deaminase
Alk	activin receptor-like linase
aPKC	atypical protein kinase C
AP	activating peptide
APS	accessible pedestrian signals
AT	adipose tissue
BMI	body mass index
BMPR2	bone morphogenic protein receptor II
BNP	brain natriuretic peptide
Cbl	casitas B-lineage lymphoma
CM	adipocyte conditioned medium
CVD	cardiovascular disease
DPP4	dipeptidyl peptidase 4
ECM	extracellular matrix
EDHF	endothelium-derived hyperpolarizing factor
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cells
Erk	extracellular signal regulated kinase
ET-1	endothelin-1
FDA	Food and Drug Administration
FFA	free fatty acids
Gab1	GRB2-associated-binding protein 1
GIP	glucose-dependent insulinotropic peptide
GLP-1	glucagon-like-peptide 1
GLUT	glucose transporter
Grb	growth factor receptor bound protein
GSK	glycogen synthase kinase
HbA1c	glycated hemoglobin
HDL	high density lipoprotein
HUVEC	human umbilical vein endothelial cells
IGF	insulin-like growth factor
IL	interleukin
iNOS	inducible nitric oxide synthase
IRAK1	interleukin-1 receptor-associated kinase 1
IRR	insulin receptor-related receptor
IRS	insulin receptor substrate
JNK	c-Jun NH2-terminal kinase

LDLR	low-density lipoprotein receptor
LPS	lipopolysaccharide
M6P/IGF2R	mannose-6-phosphate receptor/IGF-2 receptor
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
miRNA	micro RNA
MMP	matrix metalloproteinases
NF-κB	nuclear factor kappa B
NO	nitric oxide
NPR-A	natriuretic peptide receptor type A
NPY	neuropeptide Y
ORP8	oxysterol-binding-protein-related protein
oxLDL	oxidized LDL
PAI-1	plasminogen activator inhibitor-1
PAR	protease-activated receptor
PDGF	platelet derived growth factor
PI3K	phosphatidylinositol 3-kinase
PIP3	trisphosphorylated inositol
PKA	protein kinase A
PKB	protein kinase B
PKC-θ	protein kinase C-θ
PMN	polymorphonuclear neutrophi
Pre-miRNA	precursor micro RNA
Pri-mirRNA	primary micro RNA
PTP1B	protein tyrosine phosphatase 1B
PVAT	perivascular adipose tissue
RAS	rat sarcoma protein
RBP4	retinol-binding protein-4
RER	rough endoplasmic reticulum
ROS	reactive oxygen species
SAT	subcutaneous adipose tissue
SDF	stromal derived factor
sDPP4	soluble dipeptidyl peptidase 4
SHP2	phosphotyrosine phosphatase 2
SIRP	signal regulatory protein
SOCS	suppressor of cytokine signaling
SU	sulfonylureas
T2D	type 2 diabetes mellitus
TAG	triacylglycerides
TGFβ	transforming growth factor-β
TGFβR2	transforming growth factor β receptor II
TL	tethered ligand

TLR	toll-like receptor
TNFα	tumor necrosis factor α
Tollip	toll interacting protein
TZD	thiazolidinediones
VAT	visceral adipose tissue
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VLDL	very low-density lipoprotein
VMSC	vascular smooth muscle cells
WHO	World Health Organization
αSMA	α-smooth muscle actin

List of Figures

CHAPTER 1: General Introduction

1.1. Obesity

Overweight and obesity are characterized by abnormal or excessive fat accumulation that may impair health (1). Overweight in adults has been defined as a body mass index ([BMI]; the weight in kilograms divided by square of height in meters) of 25.0-29.9 kg/m^2 and obesity as a BMI of 30 kg/m^2 or higher. Overweight and obesity have reached epidemic proportions globally along with an adoption of a westernized lifestyle characterized by a combination of excessive food intake and inadequate physical activity. According to the World Health Organization (WHO) worldwide obesity has nearly doubled since 1980 and is now so common within the world's population that it is replacing undernutrition and infectious diseases as the most significant contributor to ill health. In 2008, more than 10% of the world´s adult population was obese. But also the prevalence of overweight and obesity in children is dramatically increasing in the last three decades. In 2011, more than 40 million children under the age of 5 were overweight. Once considered a high-income country problem, overweight and obesity are now increasing in low- and middle-income countries, particularly in urban settings. Thus, it is not surprising that more than 30 million overweight children are living in developing countries and 10 million in developed countries. Overweight and obesity in adolescence are strong determinants for obesity and related morbidity and mortality in later life, with 50-80% of obese adolescents becoming obese as adults (2;3). Since at least 2.8 million adults die each year as a result of being overweight or obese, obesity became the fifth leading risk for global deaths. Obesity causes or exacerbates many health problems, both independently and in association with other diseases including type 2 diabetes mellitus (T2D), coronary heart disease, certain forms of cancer, and sleep-breathing disorders. Thus, 44% of the diabetes burden, 23% of the ischaemic heart disease and between 7% and 41% of certain cancers are attributable to overweight and obesity. Some of these pathophysiologic sequelae, especially T2D and cardiovascular disease (CVD), can be linked to obesity-associated insulin resistance (4). Although there is a strong relationship between obesity and insulin resistance (5-8), not all obese patients are insulin resistant (9;10). Data from the European Group for the Study of Insulin Resistance suggest that nearly 25% of obese individuals [BMI>35 kg/m^2] are insulin sensitive (8;10).

Nevertheless, under normal conditions, the pancreatic islet β-cells increase insulin release sufficiently to overcome the reduced efficiency of insulin action, thereby maintaining normal glucose tolerance (11-13). Thus, most obese, insulin-resistant individuals do not develop hyperglycaemia. Since obesity and its related comorbidities are costly to treat, obesity clearly has a considerable economic impact and has emerged as one of the most serious public health concerns in the 21st century. Conservative estimates of the economic costs of obesity in developed countries are between 2 and 7% of the total health costs, which represents a significant expenditure of national health-care budgets (14). Obesity is a complex disease with multifactorial etiology, including behavioral, environmental and genetic factors. From a series of family, twin and adoption studies the genetic contribution to obesity in humans has been established and the estimated heritability of BMI seems to range between 50 and 90% (15). Not surprisingly, many obesity gene variants appear to be involved in pathways affecting energy homeostasis. Although the susceptibility to obesity is determined largely by genetic factors it is hypothesized that the environment determines phenotypic expression (16). The environmental influences act most likely through the physiological mediators of energy intake and expenditure. This means that globally there has been an increased intake of energy-dense foods that are high in fat; and an increase in physical inactivity due to the increasingly sedentary nature of many forms of work, changing modes of transportation, and increasing urbanization. Thus, obesity treatment and sustained weight loss is most successful with a combination of decreased food intake and physical activity (17).

1.1.1. Adipose Tissue

Adipose tissue (AT) is the primary storage organ for excess energy. The classical functions of AT are to insulate and cushion the body, to store free fatty acids (FFA) after food intake, and to release FFAs during periods of fasting to ensure sufficient energy availability (18). While the role of AT as a central source of energy has been recognized for centuries, in the past decade it has become increasingly clear that AT also displays characteristics of an endocrine organ releasing a number of AT-specific factors, known as adipokines. For that reason AT is actively involved in physiological processes such as angiogenesis, adipogenesis,

extracellular matrix dissolution and reformation, steroid metabolism, immune response and energy homeostasis (19).

Typically, only one third of the AT is constituted by adipocytes and the remaining part is represented by the stroma-vascular fraction of fibroblasts, endothelial cells, macrophages and preadipocytes (20). During positive caloric balance lipid storage capacity is buffered by AT growth resulting in an increase in adipocyte number (hyperplasia) as well as enhanced adipocyte volume (hypertrophy). In growth stages, AT is increased mainly through hyperplasia. In the adult life, adipocytes initially undergo hypertrophy, which normally triggers AT paracrine signaling for the purpose of adding functional fat cells and towards maintaining AT physiologic functions during increased energy storage (21-23). Nevertheless, with aging the capacity of preadipocytes to become fully functional mature adipocytes declines (24). Thereby the inability of an individual to increase cell numbers by this process contributes to the development of metabolic diseases (25). However, the pathogenesis of metabolic diseases is significantly influenced not only by the increased storage of lipids (adipocyte hypertrophy versus hyperplasia), but also where the lipids are stored (26). The adipose organ includes two major discrete anatomical depots, namely subcutaneous and visceral AT. Subcutaneous adipose tissue (SAT) is the depot below the skin in the abdominal region and stores >80% of total body fat in the body. A layer of connective tissue (Scarpa's fascia) separates deep from superficial SAT. These subcutaneous layers are functionally distinct and independently correlate with metabolic complications of obesity (27). Visceral adipose tissue (VAT), which represents approximately 20% of total body fat is associated with digestive organs, and include the omental (covered by the peritoneum), the mesenteric (associated with the intestine), and epiploic (along the colon) AT. Visceral AT accumulation is the fat depot most characterized as being associated with an increased risk of metabolic disease (28). Location is one of the more important reasons why different fat depots have different pathogenic potential. Only VAT has a direct connection with the liver and secretes various adipocyte factors (such as FFA and adipokines) into the portal vein. In addition, mobilization of FFAs is more rapid from visceral than from subcutaneous fat cells because of the higher lipolytic activity in visceral adipocytes. Thus, VAT probably contributes significantly to the FFA levels in the systemic circulation. Therefore patient populations described as metabolically healthy (no metabolic disease), but obese, often have less visceral AT distribution than obese patients with metabolic disease (29;30). Conversely, patients who

are metabolically obese (those with metabolic disease), but normal weight, often have more VAT than individuals of similar weight without metabolic diseases (30).

1.1.2. Adipose tissue-derived factors

It is now well established that AT plays a critical role in the maintenance of energy homeostasis through secretion of a large number of proteins, including cytokines such as adiponectin, resistin, leptin, visfatin, IL-6, TNFα, Retinol-Binding Protein-4 (RBP4) and components of the renin–angiotensin system. These adipokines are able to act at both the local (autocrine and/or paracrine) and systemic (endocrine) level. Thus, adipokines enable an interaction of the AT with central as well as peripheral organs such as the brain, liver, pancreas, vasculature and skeletal muscle to control diverse processes, including food intake, energy expenditure, carbohydrate and lipid metabolism, blood pressure, blood coagulation, and inflammation (31-33) (Figure 1). Although the expression and secretion of many adipokines is high in adipocytes, expression of only a few is consistently restricted to adipocytes. Of these, adiponectin shows the most restricted expression pattern, with few reports of its expression in cells other than adipocytes, except under conditions of extreme hepatic steatosis, during which time the entire adipogenic cascade is induced in hepatocytes (34). Other AT-derived factors, such as leptin, exhibit expression patterns that are less adipocyte-specific (35;36), and several adipokines, such as resistin and visfatin, are produced by several other tissue-types as well (37-39). Much research in this area has focused on leptin and adiponectin, two prototypical adipokines that show beneficial effects on several physiological processes.

Leptin was first identified in 1994 as the product of the *ob* gene (40) and is mainly produced by adipocytes and in low levels by the gastric fundic epithelium, intestine, skeletal muscle, mammary epithelium, placenta, and brain (41). Adipocytes secrete leptin in direct proportion to AT mass as well as nutritional status, and this secretion is greater from subcutaneous relative to visceral AT (42;43). Leptin expression and secretion are also regulated by a variety of other factors, including insulin, glucocorticoids, tumor necrosis factor α (TNFα), FFA and peroxisome proliferator-activated receptor-γ agonists. Leptin regulates energy metabolism by increasing energy expenditure and decreasing energy consumption thereby acting as a metabolic signal for satiety. In addition to its central

effects, leptin also acts peripherally to directly increase hepatic lipid oxidation and lipolysis in skeletal muscle and adipocytes (44;45). The leptin receptor is a member of the interleukin (IL) 6 receptor family of class I cytokine receptors (46-48) and is expressed in both the CNS and periphery (49). Leptin also has significant effects on human type I proinflammatory immune responses. Accordingly, leptin deficient mice are resistant to a wide range of autoimmune diseases and display features of immune deficiency (50).

Adiponectin was independently characterized in 1995 and 1996 by four groups using different methods (51). Adiponectin is known to enhance insulin sensitivity, to decrease influx of non-esterified FAs, to increase FA oxidation in liver and muscle, and to decrease the expression of adhesion molecules within the vascular wall, resulting in a decrease in atherogenic risk (51). These diverse molecular actions of adiponectin are mediated by its receptors, AdipoR1 and AdipoR2. AdipoR1 is ubiquitously expressed, whereas AdipoR2 is only present in mouse liver. Adiponectin circulates in serum as a range of multimers from low molecular weight trimers to high-molecular-weight dodecamers (52-54). It is a highly abundant plasma protein with serum concentrations varying from 2 to 10 μg/ml (55) even though it has a rapid plasma turnover (53).

Beside those beneficial adipokines, there are also adipokines like resistin and RBP4, both linked to the development of insulin resistance. Resistin is expressed within adipocytes in rodents (56) and by stromal-vascular macrophages and preadipocytes in humans (57). A causal role for resistin in glucose homeostasis is mainly based on studies in experimental animals with altered serum resistin levels. For example, administration of mouse resistin results in severe hepatic but not peripheral insulin resistance, suggesting that acute resistin treatment blunts hepatic insulin action (58). By contrast, reducing circulating resistin protects against obesity-induced hyperglycemia, primarily by restoring hepatic insulin responsiveness (56;59;60). Together, these studies suggest that, in rodents, resistin may counterbalance the insulin sensitizing effects of adiponectin, but its role in humans and the underlying molecular mechanism of action are not completely understood.

RBP4 was first reported to be an adipokine in 2005 (61) and is mainly released by visceral AT (62), although the major source of circulating RBP4 is liver. RBP4 has been shown to produce systemic insulin resistance by impairing insulin signaling in skeletal muscle (61) and in adipocytes (63), leading to increased glucose production in the liver (61).

However, adipocytes are not the only source of these inflammatory cytokines secreted from AT. AT cells from the stromal-vascular fraction, and in particular resident macrophages, are also responsible for the chronic inflammatory responses observed in obesity (64). Unlike the adipokines described above, TNFα and IL-6 are mainly produced by macrophages and exert potent paracrine effects on a variety of adipose tissue functions (65).

TNFα is an immunomodulatory and pro-inflammatory cytokine that undergoes cleavage by metalloproteinases and is released into the circulation as soluble, mature TNFα molecule (66). Although mature white adipocytes are capable of producing TNFα, macrophages from the stromal-vascular fraction of AT are the primary source of adipose tissue-derived TNFα. In obesity, elevated TNFα levels are due to an increased infiltration of AT by macrophages (64). TNFα inhibits lipogenesis, increases lipolysis, increases leptin secretion, decreases adiponectin secretion, decreases glucose transporter-4 expression, and impairs insulin signaling in adipocytes (67;68).

IL-6 is secreted by adipose tissue and enters the circulation (69;70). Thus, plasma IL-6 levels are increased in obesity and T2D and are positively correlated with plasma FFA levels and with body mass (71;72). Although approximately 30% of IL-6 detected in plasma is attributed to production from white AT (69), most of the adipose tissue-derived IL-6 is produced by cells of the stromal-vascular fraction. *In vitro*, IL-6 increases leptin secretion, reduces adiponectin secretion and increases lipolysis (73).

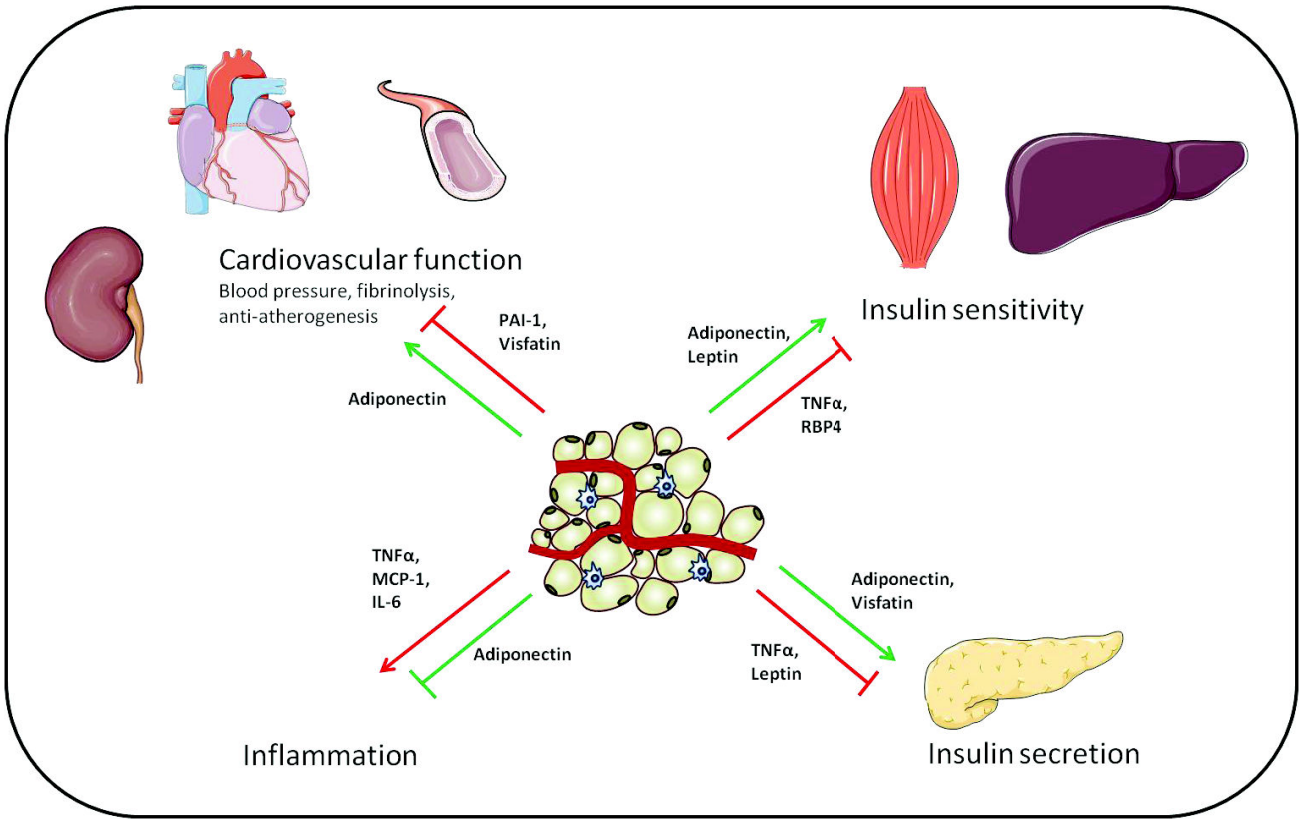


Figure 1: Adipose tissue as an endocrine organ.
Adipocytes secrete numerous proteins with endocrine functions designated as adipokines that regulate glucose metabolism in peripheral tissues, insulin secretion, inflammatory response and blood pressure, among others. Imbalanced secretion of some of these adipokines is associated with obesity. PAI-1, plasminogen activator inhibitor-1; TNFα, tumor necrosis factor α; IL, interleukin; MCP-1, monocyte chemotactic protein-1; RBP4, Retinol-Binding Protein-4 (modified from (74))

1.1.3. Adipose tissue dysfunction

Adipocytes have a particularly large capacity to synthesize and store triacylglycerides (TAG) during feeding, as well as to hydrolyse and release triglycerides as FFAs and glycerol during fasting (75;76). With the development of obesity, AT becomes increasingly dysfunctional due to an overload with TAG and a decreased buffering capacity for lipid, especially in the postprandial state (77). Consequently, circulating levels of FFA are elevated and non-ATs such as skeletal muscle, pancreatic islets and the liver tend to accumulate these lipid fuels in the form of TAG when the capacity to oxidize fatty acids is not sufficient (78). Numerous studies suggest that TAG accumulation in liver and skeletal muscle may play an important role in the development of insulin resistance and/or impaired insulin secretion in obese individuals. Furthermore, increased delivery of fatty acids to the liver leads to higher

glucose production and reduced insulin clearance by the liver resulting in conditions such as glucose intolerance, hyperlipidemia, and hyperinsulinemia, respectively (78).

In the progression of obesity, adipocytes undergo hypertrophy to meet the need for additional fat storage capacity. Adipocyte size can increase up to 140–180 μm in diameter during the development of obesity (79) but the capacity for adipocyte hypertrophy is limited. Since the diffusion distance for oxygen is at most 100 μm (80), enlarged adipocytes endure less than adequate oxygen supply and subsequently hypoxia occurs. Both hypoxia and adipocyte hypertrophy are associated with the dysregulation of its endocrine function (81-83). AT of obese insulin-resistant subjects is characterized by increased expression and/or secretion of pro-inflammatory molecules, including TNF-α (84-87), IL-6 (84;85;88;89), plasminogen activator inhibitor-1 (PAI-1) (90;91) and leptin (84;92-94). Conversely, the insulin-sensitizing and anti-inflammatory factor adiponectin is downregulated in obese and insulin resistant humans (95-97). A variety of adipokines that may link obesity to insulin resistance are upregulated in enlarged human and rodent adipocytes (98-100). Furthermore, *in vitro* it has been shown that large adipocytes seem to differentially express pro-inflammatory and anti-inflammatory factors compared to smaller adipocytes, with a shift toward dominance of pro-inflammatory adipokines (101). Especially, monocyte chemoattractant protein-1 (MCP-1) has been shown to play an important role in macrophage infiltration in AT. MCP-1 is a chemokine and member of the small inducible cytokine family, which plays a crucial role in monocyte and lymphocyte recruitment to sites of injury and infection (102). MCP-1 is produced by macrophages, endothelial cells and adipocytes (102;103), and its expression is closely related to the number of residing macrophages (104). Therefore, adipocytes are contributing to the infiltration of AT by inflammatory cells resulting in the obesity associated chronic low-grade inflammation (105;106). AT of lean subjects usually consists of approximately 5–10% macrophages, whereas in obese patients, macrophage content in AT can be as high as 50% of the total number of cells (107). Furthermore, macrophage-secreted factors like TNFα and IL-6 impair human fat cell differentiation (108) and induce inflammatory events in 3T3-L1 adipocytes by activating the nuclear factor kappa B (NF-κB) pathway (109). Therefore, macrophage infiltration in AT in obesity could be integral to the inflammatory response in this tissue and to the development of insulin resistance.

1.2. Obesity-related disorders

The burden of obesity on health extends across multiple organ systems and diseases with an increased risk of insulin resistance, T2D, fatty liver disease, atherosclerosis, degenerative disorders including dementia, airway disease and some cancers (110). Over the past decade, the search for a potential unifying mechanism behind the pathogenesis of obesity-associated diseases has revealed a close relationship between the state of excess AT and chronic low-grade inflammation. The metabolic syndrome describes a cluster of several obesity-related metabolic conditions that increase the risk for CVD and T2D (111;112). According to the International Diabetes Federation, the metabolic syndrome is defined as elevated waist circumference in combination with at least two further features such as high fasting triglycerides levels, low high density lipoprotein (HDL) cholesterol levels, high blood pressure and elevated fasting blood glucose levels. Subjects with metabolic syndrome have a three times higher risk of suffering a heart attack or stroke, twice of dying from such an event, and fivefold greater risk of developing T2D when compared to people without the metabolic syndrome (113).

1.2.1. Type 2 diabetes

Diabetes mellitus is defined as a metabolic disorder characterized by chronic hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. This in turn leads to disturbances of carbohydrate, fat and protein metabolism. The current diagnostic criteria for diabetes mellitus are (1) a fasting plasma glucose value ≥ 126 mg/dl, (2) a random plasma glucose value ≥ 200 mg/dl, (3) or if the plasma glucose value 2 hours after a 75 g oral load of glucose is over 200 mg/dl. According to the International Diabetes Federation, 371 million people lived with diabetes in 2012 worldwide and this is expected to rise to 552 million by 2030 (114). Most people with diabetes live in low- and middle-income countries, and these countries will also see the greatest increase over the next 19 years (114). Equally alarming and less well known is the fact, that only around one half of these people are known to have the condition. In 2012 4.8 million people died of diabetes and 471 billion US dollar were spent in the health care system due to diabetes. Diabetes mellitus can

be categorized into two major types, type 1 and T2D. Type 1 diabetes mellitus, also known as insulin-dependent diabetes mellitus, accounts for 5-10 % of all diabetic patients and results from an auto-immune destruction of the pancreatic insulin-producing β-cells. Since type 1 diabetic patients have only little or no endogenous insulin secretory capacity, they require a lifelong insulin therapy. T2D, formerly noninsulin-dependent diabetes mellitus**,** is the commonest form of diabetes and is characterized by chronic hyperglycemia due to insulin resistance, following increasing and then declining insulin production, and eventual pancreatic β-cell failure (115;116). In the initial stages of the disease, the pancreatic β-cell compensates the peripheral insulin resistance by secreting an increased amount of insulin. During this compensatory hyperinsulinaemic phase, progressive β-cell dysfunction occurs (117). As a consequence, the insulin secretory reserves cannot compensate for the defects in peripheral insulin action anymore and overt diabetes results. Thereafter, insulin resistance results in decreased glucose uptake by the muscle and AT, altered lipogenesis, and increased glucose output by the liver. Elevated circulating levels of lipids and glucose might exacerbate the peripheral insulin resistance and damage the insulin-producing β-cells ending up in a vicious cycle (Figure 2). In the advanced state of the disease, hyperglycemia can cause microangiopathies (diabetic nephro-, retino-, and neuropathy) and macroangiopathies (atherosclerosis, stroke) (118). T2D is generally attributed to a combination of genetic and environmental factors. Genome-wide association scans and candidate gene approaches now have identified 64 genetic variants associated with T2D (119) that are mainly related to β-cell dysfunction, with fewer involved in pathways related to insulin resistance independent of obesity (120;121). Although numerous diabetes-associated genes have been identified, the known genes are estimated to predict only 15% of T2D (122). Thus, environmental factors are largely responsible for the modern day epidemic of T2D. It is well accepted that obesity and physical inactivity are the major risk factors for the development of T2D (123). Therefore in many cases weight reduction, an increase in daily energy expenditure and anti-hyperglycaemic agents are sufficient to achieve adequate glycaemic control.

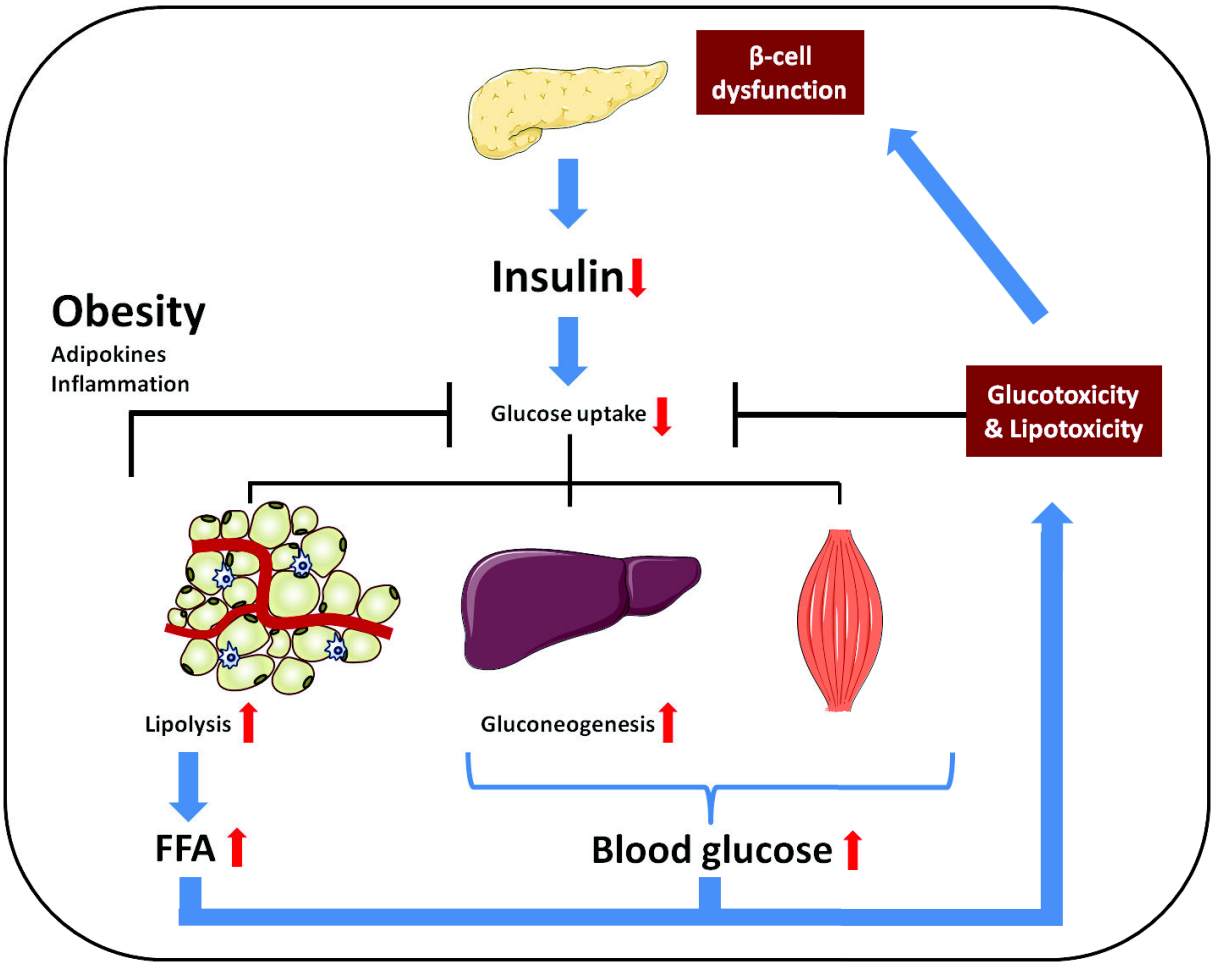


***Figure 2**: **Association between obesity and type 2 diabetes**.*
The chronic low-grade inflammation in adipose tissue and the increased release of pro-inflammatory adipokines in obesity lead to insulin resistance in peripheral organs. Subsequently, hepatic gluconeogenesis is increased while glucose uptake by muscle is decreased, both resulting in hyperglycemia. Insulin resistance in AT is associated with elevated FFA levels due to increased lipolysis. The increased glucose levels together with the elevated FFA levels can synergize to further adversely affect β-cell health and insulin action ending up in a vicious cycle that leads to the development of T2D. FFA, Free Fatty Acids (modified from (124))

1.2.1.1. Insulin signaling

Despite periods of feeding and fasting, plasma glucose remains in a narrow range between 4 and 7 mM in metabolically normal individuals (125). This tight control is governed by the balance between glucose absorption from the intestine, production by the liver and uptake and metabolism by peripheral tissues. Insulin increases glucose uptake in muscle and AT, and inhibits hepatic gluconeogenesis. Thus, insulin acts as the primary regulator of blood glucose concentration. Insulin also stimulates cell growth and differentiation, and promotes

the storage of substrates in AT, liver and muscle by stimulating lipogenesis, glycogen and protein synthesis, and inhibiting lipolysis, glycogenolysis and protein breakdown (125) (Figure 3). But also in not classical insulin-sensitive tissues like vasculature, insulin plays an important role in vasorelaxation and endothelial cell function.

Insulin is first synthesized as a single polypeptide called pre-proinsulin in the β cells of the pancreatic islets of Langherhans. Pre-proinsulin is formed by sequential synthesis of a signal peptide, the B-chain, the connecting C-peptide and the A-chain comprising a single chain of 100 amino acids. The signal peptide directs the nascent polypeptide chain to the rough endoplasmic reticulum (RER). After translocation into lumen of the RER, the signal peptide is cleaved and proinsulin is formed (126). Once in the RER, proinsulin undergoes appropriate folding so that the disulfide linkage between the A- and B- chains of insulin are correctly aligned (127). After its assembly in the endoplasmic reticulum, proinsulin is transported to the trans-Golgi network where proinsulin enters immature secretary vesicles and is cleaved to yield insulin and C-peptide. Insulin forms zinc-containing hexamers which are insoluble and represent the storage form of insulin (128). When mature granules are secreted into the circulation by exocytosis, insulin, and an equimolar ratio of C-peptide are released. Once the hexamers are secreted from the β-cell and diffuse into the blood down their concentration gradient, a combination of electrostatic repulsion and decreased concentration of insulin favors the dissociation of insulin into its monomeric form. The monomer consists of 51 amino acids with a molecular weight of 5.8 kDa and is the active form of insulin.

Insulin mediates its actions through binding to the insulin receptor. The insulin receptor belongs to a subfamily of receptor tyrosine kinases that includes the insulin-like growth factor (IGF)-I receptor and the insulin receptor-related receptor (IRR) (129). The insulin receptor is a tetrameric protein consisting of two α- and two β- glycoprotein subunits linked by disulfide bonds and is located on the cell membrane (130). Insulin binds to the extracellular α-subunit leading to derepression of the kinase activity in the β-subunit followed by transphosphorylation of the β-subunits and a conformational change that further increases kinase activity (129). This enables tyrosine phosphorylation of intracellular substrate proteins, including members of the insulin receptor substrate family (IRS1/2/3/4) (131), the Shc adapter protein isoforms, signal regulatory protein (SIRP) family members, GRB2-associated-binding protein 1 (Gab-1), Cbl (casitas B-lineage lymphoma), and accessible

pedestrian signals (APS) (132). The tyrosine phosphorylation of IRS family members generates docking sites for several SH2-containing proteins (131) including important enzymes such as phosphatidylinositol 3-kinase (PI3K) and phosphotyrosine phosphatase 2 (SHP2), and other proteins that lack enzymatic activity but which link IRS-1 and other intracellular signaling systems, e.g. the adaptor protein growth factor receptor-bound protein 2 (Grb2) which connects with the rat sarcoma protein (RAS) pathway.

PI3K consists of a p110 catalytic subunit and a p85 regulatory subunit that possesses two SH2 domains that interact with tyrosine-phosphorylated motifs in IRS proteins (133). By rapid increase in trisphosphorylated inositol (PIP3) concentration, PI3K activates serine and threonine kinases such as Akt/protein kinase B (PKB) and the atypical protein kinase C (aPKC) (130). Akt has received much attention due to its ability to phosphorylate proteins that regulate lipid synthesis, glycogen synthesis, cell survival, and protein synthesis (130). The Akt kinase exists as three different isoforms, all of which are activated by phosphorylation on Threonin 308 and Serin 473 (130). Upon insulin stimulation, Akt localizes near the plasma membrane, where it becomes phosphorylated. The activated enzyme has the ability to induce translocation of the glucose transporter GLUT4 from intracellular stores to the plasma membrane. v-SNAREs and t-SNAREs have been identified to facilitate GLUT4 vesicle translocation. VAMP-2 is the main v-SNARE found in GLUT4 vesicles. The main t-SNAREs found in the plasma membrane of insulin-sensitive tissues are syntaxin 4 and SNAP-23 (134).

RAS operates as a molecular switch, stimulating a serine kinase cascade through the stepwise activation of Raf, MEK and extracellular signal regulated kinase (ERK). Activated ERK is translocated into the nucleus, where it catalyses the phosphorylation of transcription factors such as $p62^{TCF}$, initiating a transcriptional program that leads to cellular proliferation or differentiation (135). Thus broadly, PI3K mediates metabolic effects of insulin, e.g. cellular glucose uptake, while RAS mediates mitogenic effects of insulin (130).

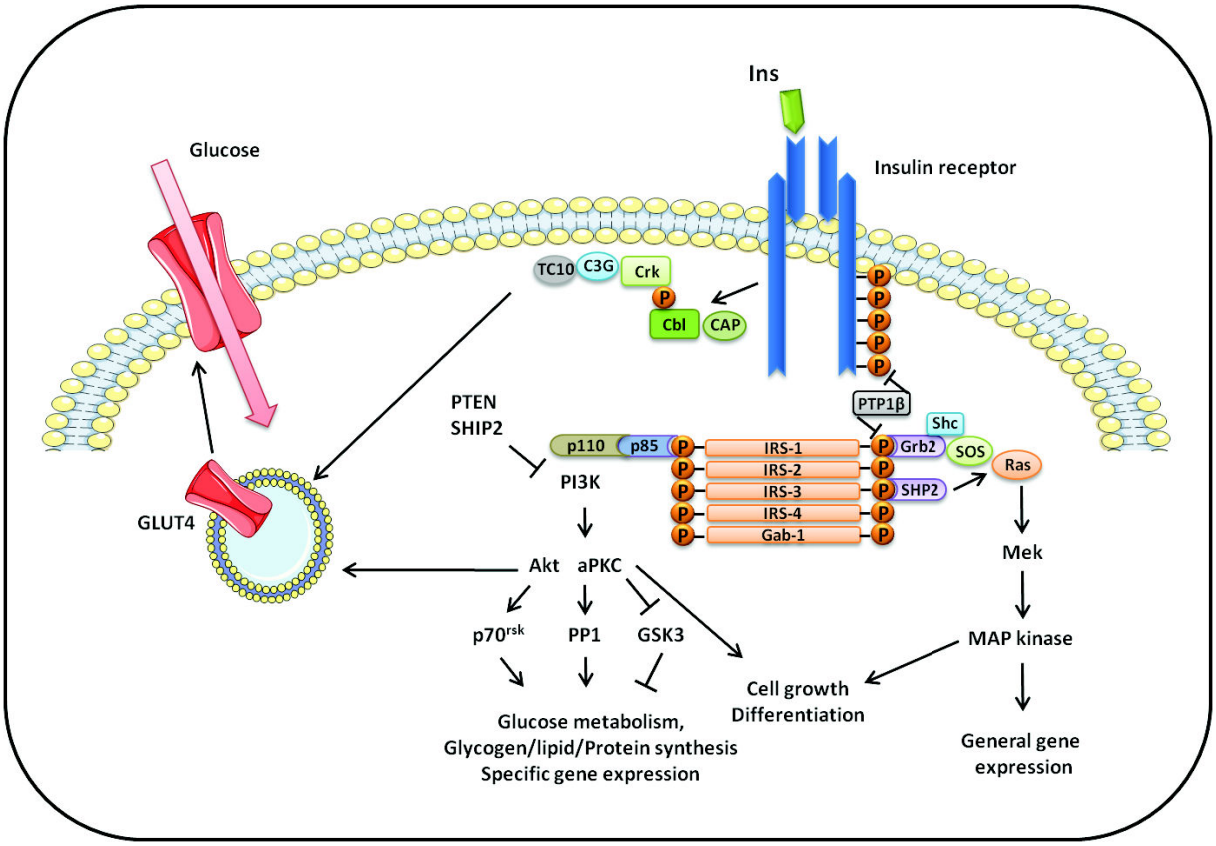


Figure 3: Principal components of the insulin signaling pathway.
Activation of the insulin receptor by insulin induces autophosphorylation of the receptor, creating docking sites for downstream interacting proteins, such as IRS 1-4. These are themselves phosphorylated and provide further docking sites for SH2 domain-containing proteins. This results in the activation of three major pathways, the PI3K, CAP/Cbl/Tc10 and the MAPK pathway, allowing the transmission of the signals that result ultimately in the diverse biological effects of insulin (modified from (125)) .

1.2.1.2. Impact of adipose tissue-derived factors on Insulin resistance

Insulin resistance is a condition characterized by the impaired action of insulin in insulin-sensitive target tissues such as the liver, muscle and AT. The phenotypes of insulin resistance include, reduced glucose uptake in muscle and AT, increased lipolysis in AT, and increased glucose production and output by the liver (136). In most cases insulin resistance is believed to be manifest at the cellular level via post-receptor defects in insulin signaling. These defects can occur at many levels, with decreases in receptor concentration and kinase activity, the concentration and phosphorylation of IRS-1 and -2, PI3K activity, glucose transporter translocation, and the activity of intracellular enzymes (132). Furthermore, there

are inhibitory molecules for insulin signaling such as the protein tyrosine phosphatase 1B (PTP1B), the suppressor of cytokine signaling (SOCS) and the growth factor receptor bound protein 10 (Grb10) that suppress insulin signaling by inducing insulin receptor dephosphorylation, physical blocking of substrate phosphorylation, and degradation of the insulin receptor and/or IRS (137). Nevertheless, activation of the MAP kinase pathway by insulin is not reduced in the insulin resistant state.

Obesity has been shown to increase insulin resistance and the risk of T2D (136). However, the loss of AT also leads to metabolic disorders that result in severe states of insulin resistance and potential diabetes (138). These findings indicate that AT may not only be a target of insulin, but that it may also influence whole-body insulin sensitivity. In fact, adipokines secreted by AT signal to other organs, including the brain, liver, skeletal muscle and the cardiovascular system, to modulate their insulin sensitivity (139). Some adipokines, such as adiponectin (140), apelin (141) and vaspin (142) can improve insulin sensitivity, whereas others have a role in producing insulin resistance, such as RBP4 (61), TNFα (86) and resistin (56). Adiponectin may be the most biologically active adipokine in improving insulin sensitivity (140). Stimulation of adenosine monophosphate kinase and inhibition of acetyl coenzyme A carboxylase may be key factors in adiponectin signaling resulting in decreased hepatic glucose production, increased fatty acid oxidation and glucose uptake in muscle (143). These metabolic effects might be the primary mechanisms by which adiponectin enhances insulin sensitivity. In humans, there is an inverse relationship between *in vivo* insulin sensitivity and plasma levels or adipocyte release of adiponectin (144-146). In addition, low level of adiponectin displays an independent risk factor for development of T2D (147) and appears before T2D is manifested (148).

Conversely, pro-inflammatory adipokines like TNF-α, IL-6 and IL-1β induce SOCS1/3, thereby enhancing the degradation of IRS1/2 through E3 ubiquitin ligase activation (149). Another mechanism to induce insulin resistance is the IRS phosphorylation on serine or threonin residues that negatively affects the binding of IRS to the insulin und IGF-1 receptors (150). Thus, pro-inflammatory adipokines are able to inactivate IRS through the activation of serine/threonine kinases like IKKβ kinase, c-Jun NH_2-terminal kinase (JNK), protein kinase C-θ (PKC-θ) and ERK. IKK and JNK are the major cascades activated in response to inflammatory signals and desensitize the insulin signaling cascade. In response to TNFα, JNK1 promotes the phosphorylation of Ser307 of IRS-1 which interferes with the interaction of IR and IRS-1, thus

preventing insulin-induced Tyr phosphorylation of IRS-1 (151). Indeed, JNK1-deficient mice show decreased adiposity and significantly improved insulin sensitivity (152). IKKβ is part of the IKK complex that phosphorylates the inhibitor of NF-κB, IκB. This results in degradation of IκB, allowing the activation of NF-κB (153). Heterozygous deletion of IKKβ (IKKβ$^{+/-}$) protects against the development of insulin resistance during high-fat diet (HFD) and in obese Lep$^{ob/ob}$ mice (150;154;155). Both pathways are activated in obesity, not only in response to adipokines, but also by increased FFA concentrations and oxidative stress (156). The list of IRS kinases implicated in the development of insulin resistance is growing rapidly, concomitant with the list of potential Serin/Threonin phosphorylation sites in IRS proteins (157).

1.2.1.3. Anti-hyperglycaemic agents in the treatment of type 2 diabetes

Maintaining glycaemic levels as close to the non-diabetic range as possible has been demonstrated to have a substantial impact on diabetes-related complications, including retinopathy, nephropathy, and neuropathy (158-160). When lifestyle changes fail to reduce glucose levels to the desirable range, the conventional approach is to begin therapy with an oral anti-hyperglycaemic agent. Glycated hemoglobin (HbA1c) levels reflect the average plasma glucose concentration over prolonged periods of time and are therefore a suitable indicator for blood glucose control. The most recent glycaemic goal recommended by the American Diabetes Association is, in general, an HbA1C level of less than 7% (161;162). More stringent HbA1C levels of 6.5% or less have been proposed by earlier guidelines (163); however, studies have found these lower glycaemic targets to be associated with either excess CVD mortality (at HbA1C <6%) or to have no benefit on primary CVD outcomes (164;165). Treatment options have greatly expanded in the past two decades. Thus, the development of new classes of glucose-lowering medications to supplement older drugs (insulin, sulfonylureas, metformin) has certainly broadened the palette of available treatments and possible combinations. Available agents reduce glucose levels often through a variety of mechanisms and can be divided into two major categories: 1. Agents that improve insulin sensitivity (Biguanides and Thiazolidinediones (TZDs)) and 2. Agents that

increase circulating insulin levels (Sulfonylureas (SU), glucagon-like-peptide 1 (GLP-1) receptor agonists and dipeptidyl peptidase 4 (DPP4) –Inhibitors).

Agents that improve insulin sensitivity

The only biguanide available in most parts of the world and a cornerstone of T2D treatment is metformin. Metformin has been shown to increase insulin sensitivity and enhance glucose uptake (166) thereby consistently lowering HbA1C by 1–2% (167). Although it is known that metformin reduces hepatic glucose production by stimulating AMP-activated protein kinase, an enzyme that plays a role in the expression of hepatic gluconeogenic genes (168), the exact mechanism remains unknown. Other nonglycemic benefits have been reported, including improvements in fibrinolysis (169), inflammatory markers, and endothelial function (170). Notably, metformin as monotherapy remains the only agent associated with the potential for weight loss (171-173). Although lactic acidosis is rarely reported, metformin should be used with caution in elderly diabetic individuals with renal impairment. Gastrointestinal side effects may occur, especially if therapy is initiated at higher doses.

TZDs (pioglitazone, rosiglitazone, troglitazone, ciglitazone) are peroxisome proliferator–activated receptor γ activators that enhance insulin sensitivity in peripheral tissues and reduce hepatic glucose production (168). The HbA1C lowering effect of the TZDs is equivalent and typically in the same range as that achieved by the SU or metformin (174;175). The most common adverse effects associated with TZDs include weight gain by increasing subcutaneous adiposity and fluid retention. The latter complication usually manifests as peripheral edema, although new or worsened heart failure may occur. Pioglitazone is the only remaining TZD for prescription after the restricted use of rosiglitazone recommended by Food and Drug Administration (FDA) due to increased cardiovascular events reported with this compound (176).

Agents that increase circulating insulin levels

SU are generally well tolerated and lower glucose levels via enhancing insulin secretion by the pancreatic β cell. Treatment with SU agents generally yields a mean absolute HbA1C reduction of 1–2% (177-179). The major adverse effect associated with sulfonylureas is

hypoglycemia, with severe episodes (accompanied by coma or seizures) being infrequent and more common in elderly patients (180).

Incretin hormones, the major ones being glucose-dependent insulinotropic peptide (GIP) and GLP-1, are involved in the regulation of blood glucose and insulin and glucagon secretion (181). Both GLP-1 and GIP are glucose-dependent hormones that are released from endocrine cells in the small intestine when glucose levels rise above fasting levels and that indirectly stimulate insulin secretion by activating G protein–coupled receptors on pancreatic β-cells (180). GLP-1 also reduces the secretion of glucagon, a hormone produced by the pancreas that stimulates the liver to convert glycogen to glucose. Additionally, GLP-1 is known to have central effects, including a reduction in gastric emptying and appetite, and an increased sensation of satiety. Due to rapid inactivation by the proteolytic enzyme dipeptidyl peptidase-4, the actions of GLP-1 and GIP in vivo are short-lived. Incretin mimetics and inhibitors of the protease DPP4 are new classes of anti-diabetic agents first introduced in the years 2005 (exenatide) and 2007 (sitagliptin), respectively. Because these agents stimulate insulin secretion in a glucose-dependent manner, the risk of hypoglycemia appears to be very low (182). In addition, emerging evidence suggests incretin-based therapies may have a positive impact on inflammation, cardiovascular and hepatic health, sleep, and the central nervous system (183). GLP-1–receptor agonists (exenatide and liraglutide) are injectable agents that are structurally similar to endogenous GLP-1 (but with a longer half-life and slower elimination) and activate GLP-1 receptors in many tissues. These agents, which are used as an adjunct to other treatments (eg, SU, metformin, TZDs), lower HbA1C levels by 0.5% to 1%, primarily by decreasing postprandial blood glucose levels (180). Other effects include delayed gastric emptying and appetite suppression, typically resulting in a weight loss of approximately 2 to 4 kg over 6 months (168). Exenatide is known to cause a relatively high frequency (30%-45%) of gastrointestinal disturbances (eg, nausea, vomiting, diarrhea), which may subside over time (184). The Food and Drug Administration (FDA) issued a warning in 2007 following reports of pancreatitis in some patients taking exenatide. However, definitive causal relationship between GLP-1 agonists and pancreatitis has not been established, a possible association remains to be further studied (185). Liraglutide, approved more recently, is a longer-acting GLP-1 analogue that generally displayed better efficacy and fewer gastrointestinal side effects. However, the FDA also issued a warning regarding the ability of liraglutide to cause dose-dependent and treatment-duration–

dependent thyroid C-cell tumors in rats and mice. Since the potential of liraglutide to cause thyroid C-cell tumors in humans is unknown, the FDA recommends that patients with thyroid nodules be referred to an endocrinologist for further evaluation (180).

DPP4 inhibitors, also called incretin enhancers, exert glucose regulatory actions by prolonging the effects of GLP-1 and GIP, ultimately increasing glucose-mediated insulin secretion and suppressing glucagon secretion (186). Five DPP4 inhibitors (sitagliptin, linagliptin, saxagliptin, vildagliptin, alogliptin) are currently approved in the United States and Europe for the treatment of T2D, and a number of other DPP4 inhibitors are in late-stage development (185). These agents are considered small molecules that are rapidly absorbed following oral dosing, resulting in over 80% inhibition of DPP4 and a 2- to 3-fold increase in peripheral plasma concentrations of GLP-1 and GIP. In clinical studies, DPP4 inhibitors have been shown to lower A1C levels by 0.6% to 0.9%, and have shown neutral effects on weight as well as the potential for the preservation or enhancement of β-cell function (180;185;187). The most compelling indication for the use of DPP-4 inhibitors appears to be in combination with metformin in patients with early T2D who require their first combination therapy. The complementary pharmacology of DPP4 inhibition and biguanide action may lead to increased glucose-dependent insulin secretion, suppression of hepatic gluconeogenesis, and improvement in insulin sensitivity. Based on clinical evidence suggesting that a substantial proportion of patients receiving a combination of saxagliptin and metformin achieved statistically significant improvements in glycaemic control (compared with either treatment alone), a fixed-dose metformin-saxagliptin product was recently approved for T2D (180;188). Because DPP4 is expressed in many tissues, including immune cells, DPP4-inhibitors have the potential to influence immune function, as evidenced by an increased incidence of infection (180;189).

Both DPP4 inhibitors and GLP-1 analogues have been demonstrated to exert beneficial effects on classic cardiac risk factors by reducing blood pressure, weight, triglycerides, and low-density lipoprotein cholesterol, and increasing high-density lipoprotein cholesterol (180;185). Several large cardiovascular outcome trials are currently under way to determine the impact of incretin-based therapies on macrovascular risk.

1.2.2. Cardiovascular disease

Cardiovascular diseases (CVD) are still the leading cause of death worldwide. CVD are usually a result of atherosclerosis, thrombosis or high blood pressure, which compromise organ function and myocardial infarction or stroke occurs. According to the WHO an estimated 17.3 million people died from CVD in 2008, representing 30% of all global deaths. Of these deaths, an estimated 7.3 million were due to coronary heart disease and 6.2 million were due to stroke. Over 80% of CVD deaths take place in low- and middle-income countries and occur almost equally in men and women (1). Since behavioral risk factors are responsible for about 80% of coronary heart disease and cerebrovascular disease, most CVDs can be prevented by addressing risk factors such as tobacco use, unhealthy diet and obesity and physical inactivity.

1.2.2.1. Pathogenesis of atherosclerosis

Atherosclerosis is the dominant cause of CVD and usually develops many years before any clinical symptoms are manifest. Atherosclerosis is mainly located in the intima of many middle sized and large arteries, especially where the vessels divide. The major complications of atherosclerosis are associated with occlusion or inadequate blood flow to organs perfused by the affected artery. Nevertheless, atherosclerosis typically does not produce symptoms until the vessel's luminal diameter has been decreased by 70 to 80%. Acute events, such as a heart attack, stroke, or sudden death, often are caused by the rupture of atherosclerotic plaques resulting in the formation of a blood thrombus on its surface. The underlying pathogenesis of atherosclerosis involves an imbalanced lipid metabolism and a maladaptive immune response entailing a chronic low-grade inflammation of the arterial wall. Endothelial cells, leukocytes, and intimal smooth muscle cells are the major players in the development of this disease.

The atherosclerotic process is characterized, in its earliest stages, by perturbations in endothelial function. Hypercholesterolemia and hypertension, two major risk factors for atherosclerosis, causes focal endothelial activation. When plasma levels of the cholesterol-rich very low-density lipoprotein (VLDL) and LDL rise, the lipoproteins infiltrate the artery wall to an extent that exceeds the capacity for elimination and are retained in the

extracellular matrix (190;191). LDL is modified, through enzymatic attack or non-enzymatic oxidation in the intima, and becomes cytotoxic, pro-inflammatory, chemotactic, and pro-atherogenic. Oxidized LDL (oxLDL) activates endothelial cells resulting in an overexpression of adhesion molecules and the release of pro-inflammatory cytokines. As the vascular cell adhesion molecule-1 (VCAM-1) is typically upregulated in response to such stimuli, monocytes and lymphocytes carrying counterreceptors for VCAM-1 (e.g. α4-integrin) preferentially adhere to these sites (191). On the other hand, endothelial dysfunction, aside from denoting a specific state of "endothelial activation," also comprises impaired endothelium-dependent vasodilation. This includes a reduction of the bioavailability of vasodilators, in particular, nitric oxide (NO), whereas endothelium-derived contracting factors are increased (192). Increased cellular adhesion and reduced NO bioavailability then "sets the stage" for the recruitment of inflammatory cells, release of cytokines and recruitment of lipid into the atherosclerotic plaque.

Activated endothelium expressing adhesion molecules allows mononuclear leukocytes, such as monocytes and T-cells, to attach to the endothelium and penetrate into the intima. Within the intima, monocytes differentiate into macrophages and internalize the atherogenic lipoproteins (e.g. oxLDL) via so called scavenger receptors, of which SR-A and CD36 have been demonstrated to play significant roles in experimental atherosclerosis. The rapid uptake of highly oxidized (and otherwise modified) LDL particles by macrophages, leads to foam-cell formation, which is a hallmark of both early and late atherosclerotic lesions. With continuing supply of atherogenic lipoproteins, the macrophages take up lipoproteins until they die. The death of macrophages by apoptosis and necrosis contributes to the formation of a soft and destabilizing lipid-rich core within the atherosclerotic plaque.

In disease progression, the immune-inflammatory response is joined by a fibro-proliferative response mediated by intimal smooth muscle cells. Macrophages produce cytokines and growth factors, which in turn cause further recruitment of macrophages and induce phenotype change of vascular smooth muscle cells (VSMC) from the quiescent "contractile" state to the active "synthetic" state. Under physiological conditions, the predominant phenotype is the quiescent or noted as contractile or differentiated VSMC, which has its major function in the regulation of the blood flow (193). The synthetic, migratory and proliferative phenotype is present in the response to injury. Consequently,

activated VSMC proliferate and migrate from the media to the intima and synthesize collagen, elastin, proteoglycans, growth factors and cytokines.

Although the cellular characteristics of atherosclerotic lesions are complex and therefore difficult to classify, three major categories of lesions are commonly considered. These categories are fatty streaks, fibro-lipid lesions and complicated lesions. The earliest significant lesion is called a fatty streak. It is a yellow linear elevation of the intimal lining and is composed of masses of lipid-laden macrophages. These fatty streaks have no clinical significance. They may disappear from the arterial intima, but in patients at risk they progress to atherosclerotic plaques. The next step in the evolution of the atherosclerotic lesion is the fibro-lipid, or raised lesion. This kind of lesion is distinguished by two morphological components; one being a fibrous cap of smooth muscle cells and abundant extracellular matrix and the other a lipid-rich core region consisting of cellular debris. In addition to alterations in the intima, a thinning of the media can be observed. The fibro-lipid lesion develops without narrowing the lumen and is considered as a stable plaque due to a robust fibrous cap. It may progress to form a complicated lesion which is characterized by diverse events including necrosis, calcification and mural thrombosis. These unstable plaques are rich in macrophages and foam cells and the fibrous cap separating the lesion from the arterial lumen is usually weak and prone to rupture. Ruptures of the fibrous cap expose thrombogenic material, such as collagen to the circulation and eventually induce thrombus formation in the lumen. Upon formation, intraluminal thrombi can occlude arteries completely, but more often they detach, move into the circulation and eventually occluding smaller downstream branches causing thromboembolism.

1.2.2.2. Impact of adipose tissue-derived factors on atherosclerosis

The American Heart Association has reclassified obesity as a ‘major, modifiable risk factor’ for coronary heart disease (194). In obesity, the increased size of adipocytes and the chronic low-grade inflammation within AT alter its normal physiological function. AT dysfunction results in an altered production and secretion of adipokines, which in turn affect several tissues in a para- or endocrine manner like liver, skeletal muscle and vasculature. Numerous circulating pro-inflammatory mediators participating in the development of CVD

are released directly from adipocytes. However, AT also surrounds almost every vessel in the human body and this depot is referred to as perivascular adipose tissue (PVAT). This fat depot is not separated from vascular adventitia by fascia, thus allowing easy access for factors secreted by both organs. Adipokines have contrasting actions on hypertension, endothelial function, cardiomyocyte actions, cardiac pathology, atherosclerosis and inflammation thereby linking obesity to an increased cardiovascular risk.

Inflamed AT contributes to vascular dysfunction via multiple mechanisms. Firstly, local hypoxia and inflammation abolishes the protective anti-contractile properties of PVAT and shifts the paracrine effects of PVAT to vasoconstriction, thereby leading to heightened vascular tone, a propensity to vasospasm and impaired hemodynamic (195-197). These detrimental effects of inflamed PVAT can be attributable to impaired secretion of anti-contractile factors including adiponectin and angiotensin 1–7 paralleled by elevated production of vasoconstriction molecules, including superoxide, angiotensin II and aldosterone (195;198;199). Secondly, many pro-inflammatory AT-derived factors, such as TNF-α, resistin and adipocyte fatty acid-binding protein, act in an endocrine and/or paracrine manner on the endothelium to impair endothelial NO production and to induce apoptosis, thus leading to endothelial damage (195;200). Thirdly, various chemokines released from PVAT, such as MCP-1, IL8, RANTES induce the recruitment and infiltration of monocytes, lymphocytes and neutrophils into the blood vessel wall to instigate the local vascular inflammation (201). Fourthly, the secreted factors from inflamed AT, such as leptin and visfatin, can stimulate VSMC proliferation and migration, and promotes neointimal formation (195;202;203).

1.3. Objectives

The previous sections highlight that in the context of obesity, AT has gained considerable interest as a key player in the pathogenesis of several metabolic diseases like T2D and CVD. AT is an endocrine organ, which affects peripheral organs in an endo- and/or paracrine manner by the secretion of numerous adipokines. It is well accepted that the adipokine profile is altered in obesity and that plasma levels of pro-inflammatory and pro-atherogenic

adipokines are increased, contributing to a chronic state of low-grade inflammation and thereby potentially linking obesity to CVD. However, the secretome of human adipocytes remains incompletely characterized. Considering the dramatically increased prevalence of overweight and obesity worldwide and the high mortality of CVD, it is of particular importance to understand the crosstalk between AT and the vasculature for the development of novel therapeutic strategies to competently combat both diseases. Emerging clinical and experimental studies propose that VSMC may be functionally impaired in obesity and diabetes and thus contribute to the increased incidence of macrovascular complications. Although this idea has general support, the underlying molecular mechanisms are currently unknown and hence are the subject of intense research. Therefore, the starting point of the present work is to characterize the impact of the secretory output of *in vitro* differentiated adipocytes on human VSMC.

- With comprehensive proteomic profiling of the human adipocyte secretome soluble dipeptidyl peptidase 4 (DPP4) was identified by our group as a novel adipokine. Based on this observation the first objective of this thesis was to investigate the expression and regulation of DPP4 in human adipocytes and to assess the impact of DPP4 on adipocytes, skeletal muscle cells and VSMC. In this regard, the expression and secretion of DPP4 in three independent clinical studies performed on lean and obese subjects was analyzed.

- Soluble DPP4 turned out to be a promising candidate potentially linking obesity to CVD. Although DPP4-inhibitors, a class of oral anti-hyperglycaemic agents, hold interesting promise for cardiovascular protection, the underlying mechanism of these beneficial effects are not known. Therefore, the second objective of this thesis was to further characterize the direct effects of soluble DPP4 on VSMC and to identify the underlying mechanism.

- It is well accepted that expanded fat mass in obesity negatively affect cells of the vascular wall, especially VSMC. Although the detrimental effects of the whole secretory output of human adipocytes on VSMC has been investigated, the

underlying mechanism of these effects are poorly understood. Therefore the third objective of this thesis was to provide novel insight into the complex cellular mechanism linking obesity to atherosclerosis by assessing the role of miRNAs in the crosstalk between AT and VSMC. In this context, the impact of adipocyte conditioned medium (CM) on miRNA expression and the influence on the insulin signaling pathway was investigated.

CHAPTER 2

Study 1

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

Daniela Lamers[1]*, Susanne Famulla[1]*, Nina Wronkowitz[1]*, Sonja Hartwig[2], Stefan Lehr[2], D. Margriet Ouwens[2], Kristin Eckardt[1], Jean M. Kaufman[3], Mikael Ryden[4], Stefan Müller[5], Franz-Georg Hanisch[5], Johannes Ruige[3], Peter Arner[4], Henrike Sell[1] and Juergen Eckel[1]

[1]*Paul-Langerhans-Group and* [2]*Institute of Clinical Biochemistry and Pathobiochemistry, German Diabetes Center, Duesseldorf, Germany*
[3]*Department of Endocrinology, Ghent University Hospital, Ghent, Belgium*
[4]*Department of Medicine, Karolinska Institute at Karolinska Hospital, Stockholm, Sweden*
[5]*Institute of Biochemistry II, Medical Faculty, University of Cologne, Cologne, Germany*

*These authors contributed equally to the work

published in *Diabetes* 60(7): 1917-1925, July 2011

Abstract

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

Keywords

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

Introduction

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

DPP4 is an ubiquitously expressed transmembrane glycoprotein, which cleaves N-terminal dipeptides from a variety of substrates including growth factors and hormones, neuropeptides and chemokines (6). Two substrates of DPP4, glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) are released from the intestinal mucosa and are responsible for about 60% of post-prandial insulin secretion, the so-called incretin effect (7). Since GLP-1 remains active under hyperglycemic conditions in type 2 diabetes, DPP4 has gained considerable interest as a therapeutic target, and a variety of DPP4-inhibitors that prolong the insulinotrophic effect of GLP1 are now in clinical use as anti-diabetic drugs (8). Substantial DPP4 activity is also found in plasma and other body fluids due to a soluble form of DPP4 lacking the cytoplasmic tail and the transmembrane region of this protein (9). Both, the membrane abundance and the circulating activity of DPP4 have been found to be altered in a variety of neurological and inflammatory diseases (6). However, although a fraction of soluble DPP4 most likely originates from cells of the immune system (10), the major source of circulating DPP4 and its regulation remain unknown.

Furthermore, essentially no data are currently available regarding the potential effects of soluble DPP4 on insulin target tissues including muscle and fat. In the present

investigation we combined *in vitro* experiments with two independent clinical studies, aiming to validate DPP4 as a novel adipokine and to characterize the association of DPP4 to different parameters of the metabolic syndrome. Here we show that i) DPP4 is a novel adipokine released from differentiated human adipocytes and that it may exert auto- and paracrine effects leading to insulin resistance, ii) DPP4 expression is substantially elevated in visceral fat of obese subjects and that serum DPP4 correlates with adipocyte size and all parameters of the metabolic syndrome, and that iii) adipose tissue explants from obese subjects release substantially more DPP4 with a prominent decrease after weight reduction. In light of the well-accepted interference of DPP4 with the incretin system, we now suggest that DPP4 may play a role in linking obesity to insulin resistance and the metabolic syndrome.

Materials and Methods

Materials

Reagents for SDS-PAGE were supplied by GE Healthcare (Freiburg, Germany) and by Sigma (München, Germany). Polyclonal antibodies for adiponectin and actin were supplied by Abcam (Cambridge, UK). HRP-conjugated goat anti-rabbit and goat anti-mouse IgG antibodies came from Promega (Mannheim, Germany). Collagenase NB4 was obtained from Serva (Heidelberg, Germany). Troglitazone, TNF-α, BSA (fraction V, fatty acid free, low endotoxin) were obtained from Sigma (München, Germany). Adiponectin was purchased from Biovendor (Heidelberg, Germany). Complete protease inhibitor cocktail and PhosStop phosphatase inhibitor cocktail were from Roche (Mannheim, Germany). FCS was supplied by Gibco (Invitrogen, Carlsbad, CA, USA). All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma. Human recombinant DPP4 was purchased from R&D Systems and a polyclonal antibody from Abnova (Heidelberg, Germany). The specific DPP4 inhibitor K579 was purchased from Biozol (Eching, Germany).

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

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

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

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

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

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

Adipocyte isolation and culture

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

Skeletal muscle cell culture

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

The cells were then differentiated and fused by culture in α-modified Eagles medium for 4 days and used for experiments.

Smooth muscle cell culture and proliferation

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

Immunoblotting

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

ELISA

DPP4 and HO-1 secretion by human primary adipocytes and macrophages was determined using ELISA kits purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany) and Stressgene (Lörrach, Germany). The assays were performed in duplicates according to the manufacturer's instructions.

Presentation of data and statistics

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

Results

DPP4 is a novel adipokine exhibiting regulated release from human adipocytes

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

To validate this novel adipokine, we used in vitro differentiated human adipocytes and macrophages isolated from adipose tissue. DPP4 expression in human adipocytes is significantly increased during differentiation with a maximum reached at day 7 (4-fold over undifferentiated control) (Fig. 1A). DPP4 expression is paralleled by a marked release of this adipokine (Fig. 1B), which was significantly elevated as compared to the undifferentiated control starting at day 4 and increasing up to day 9 (1.1 ng/ml released over 24h by 3.5 x 10^5 cells). DPP4 in the supernatant of adipocytes was quantified by ELISA and additionally confirmed by Western blotting (Fig. 1B). We further analyzed the release of DPP4 with prominent regulators of adipocyte secretory activity such as troglitazone, TNF-α, insulin and adiponectin (15; 20; 21). As shown in Fig. 1C, DPP4 release is significantly upregulated by TNF-α and insulin. In addition to adipocytes, adipose tissue-derived macrophages release

measurable amounts of DPP4 (Fig. 1D). This, however, is only 1/3 compared to adipocytes, pointing to a major contribution of adipocytes to DPP4 output from adipose tissue.

Soluble DPP4 exerts direct effects on fat and muscle cells

The soluble form of DPP4 may bind to the extracellular matrix (22) and affect a variety of cells, yet this has not been investigated so far. To assess potential direct effects of soluble DPP4 on peripheral cells, we studied insulin signaling in adipocytes and skeletal muscle cells. DPP4 treatment of human adipocytes results in a dose-dependent decrease in insulin-stimulated Akt phosphorylation which reached significance using a dose of 200 ng/ml (Fig. 2A). This demonstrates an autocrine effect of DPP4 on adipocytes. It should be noted that circulating DPP4 concentrations were found in the range of 200 to 600 ng/ml in healthy patients. The effect of DPP4 on insulin-stimulated Akt phosphorylation can be completely blocked by a specific DPP4 inhibitor (Fig. 2B). Validation experiments using this compound prove inhibition of DPP4 *in vitro* which remained unaltered for a period of at least 8 h (data not shown). Similar to adipocytes, DPP4 also induces insulin resistance in skeletal muscle cells at the level of Akt phosphorylation in a dose-dependent way but less prominent compared to adipocytes (Fig. 2C-D). To prove if DPP4 has a functional impact not only on insulin signaling, we determined DPP4-stimulated proliferation and insulin signaling in primary human smooth muscle cells. In addition to the induction of insulin resistance at the level of Akt in this cell type, DPP4 induced a 1.6 fold increase in cell proliferation which can be completely blocked by the DPP4 inhibitor (Fig. 3A-C).

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

Measuring DPP4 in serum from age-matched lean and morbidly obese man (patient characteristics in supplementary table 1) revealed that obese subjects are characterized by significantly increased DPP4 concentrations (Fig. 4A). DPP4 expression in adipose tissue biopsies from the same patients revealed that DPP4 protein expression is regulated by both the fatness of the individual and the adipose tissue depot (Fig. 4B). While there is only a

trend for higher DPP4 expression in visceral fat of lean subjects, obese patients are characterized by significantly higher DPP4 in visceral adipose tissue as compared to subcutaneous adipose tissue. Furthermore, expression of DPP4 in both depots is significantly higher in obese subjects as compared to lean ones. DPP4 levels positively correlate with BMI, the size of subcutaneous and visceral adipocytes, insulin and leptin, while a negative correlation with age and adiponectin could be found (Fig. 5A-G). Adjusting DPP4 for age has no impact on these correlations. However, when adjusted for BMI, DPP4 serum concentrations only significantly correlate with the size of subcutaneous adipocytes ($p=0.04$, $r=0.32$) pointing to a very close relation between the size of adipocytes and the release of this adipokine.

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

We investigated lean and obese after weight reduction and we analyzed the release of DPP4 from whole adipose tissue. Adipocytes from lean subjects are significantly smaller than those from obese patients (Fig. 6A). Surgery induced weight loss reduced the average size of adipocytes below the size from lean subjects. DPP4 release is significantly increased from adipose tissue of obese subjects as compared to lean ones (Fig. 6B) while weight reduction by bariatric surgery normalized the DPP4 release to the lean level. This was paralleled by a significant reduction in the circulating DPP4 level, supporting the notion that adipose tissue is an important source of serum DPP4 (Fig. 6C). In the group of lean and obese subjects, DPP4 release from adipose tissue significantly correlates with BMI, waist circumference, percent body fat, triglycerides, HOMA, adipocyte volume and leptin while the correlation is negative with HDL-cholesterol (Fig. 6D-K). All these factors are denominators of the metabolic syndrome. Notably, leptin shows similar correlations with the above mentioned parameters except for triglycerides where no correlation could be found. Importantly, the release of DPP4 from adipose tissue correlates with many parameters that correlate with circulating DPP4 concentrations. There is also a strong correlation between adipose secretion of leptin and DPP4 (Fig. 6K).

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

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

Discussion

Our proteomics approach identified DPP4 as a novel adipokine released by fully differentiated human adipocytes. This was confirmed by Western blot, ELISA and determination of enzymatic activity. DPP4 release increased substantially upon fat cell differentiation, and comparison to preadipocytes and adipose tissue macrophages showed, that adipocytes most likely represent the major source of DPP4 released from the intact organ to the circulation. DPP4 is a multifunctional, type II integral membrane glycoprotein exhibiting ubiquitous expression including adipose tissue (23), being highly abundant in the kidney, on T lymphocytes and endothelial cells (22). DPP4 is certainly different from many other adipokines, in that i) the protein is not secreted but released from the plasma membrane as soluble DPP4 subsequent to proteolytic cleavage (24), ii) DPP4 exerts dual functions both as a regulatory protease and a binding protein, and iii) this protein is already an established target for treatment of type 2 diabetes (8), supporting our notion that DPP4 may potentially link adipose tissue to type 2 diabetes and the metabolic syndrome. Regulators of DPP4 release are presently unknown, but we show here that both insulin and TNF augment the shedding of soluble DPP4 by about 50% despite an unaltered expression.

Thus, factors related to insulin resistance and adipose tissue inflammation enhance the release of this novel adipokine from the fat cell. In addition to the endocrine effects of DPP4 released to the circulation, both cell surface resident and soluble DPP4 may have multiple auto-/paracrine functional implications for adipose tissue physiology. First, DPP4 recruits adenosine deaminase to the cell surface (25), which may modulate the well-established antilipolytic effects of adenosine. Second, DPP4 is a very strong inhibitor of the anti-lipolytic activity of neuropeptide Y (NPY) (23), which is one of the best peptide substrates of the enzyme (26). Therefore, enhanced abundance of both resident and soluble DPP4 within adipose tissue of obese subjects may substantially augment the lipolytic activity of enlarged adipocytes. Finally, DPP4 inactivates or alters the specificity of many chemokines including RANTES, eotaxin, MDC, SDF-I and many others (22), making it likely that DPP4 plays a yet undefined functional role in the intra-organ crosstalk between macrophages, adipocytes and other components of the stroma-vascular fraction.

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

Serum DPP4 is altered in many pathophysiological conditions such as different types of cancer, allergic asthma or hepatitis C (for review (10)). To the best of our knowledge, this is the first study to analyze circulating DPP4 in the context of obesity and adipose tissue. Morbidly obese men are characterized by elevated DPP4 levels as compared to lean controls. DPP4 serum concentrations are significantly correlated with BMI, size of adipocytes in subcutaneous and visceral fat as well as with the adipocyte hormones adiponectin (negatively) and leptin, showing that DPP4 is not only related to increased body weight but also to other important parameters of adipose tissue in particular. Interestingly, DPP4 is negatively associated with age but all of the above mentioned parameters are still significantly correlated with DPP4 even after adjustment for age. Differently, BMI-adjustment causes the disappearance of most of these correlations except for the size of subcutaneous adipocytes. In addition to circulating DPP4, the protein expression of this adipokine is significantly different not only between lean and obese subject but also between their fat depots. Former studies report contradicting data as both decreased and increased mRNA expression of DPP4 in adipose tissue of obese men was described (23; 27). At the protein level, we now clearly demonstrate that obesity leads to a prominent induction of DPP4 abundance in both subcutaneous and visceral adipose tissue, and that in obese subjects the visceral fat exhibits the highest DPP4 level. We therefore conclude that enlargement of visceral adipocytes in obesity may substantially contribute to the augmented level of circulating DPP4 in obese patients. It is noteworthy that we measured DPP4 serum concentration and not its activity. However, in additional experiments, other samples from the same patients were used to determine DPP4 activity that is significantly correlated with circulating DPP4 levels (data not shown). Thus, DPP4 activity is also significantly increased in obese as compared to lean subjects.

DPP4 expression in adipose tissue is increased in obese as compared to lean individuals, a fact that is reflected by an increased release of DPP4 from adipose tissue explants of obese patients as compared to lean controls. Similar to circulating DPP4, its release from adipose tissue correlates with various classical markers for the metabolic syndrome, namely BMI, waist circumference and plasma triglycerides, HOMA as an index of insulin resistance, as well as with fat cell volume and the adipokine leptin. In addition, DPP4 release can be reversed to normal levels by surgery-induced weight loss which is also reflected by DPP4 being significantly reduced in serum of these patients. Except for one

study reporting on DPP4 levels in obese children before and after weight loss (28), this is the first description of significantly decreased DPP4 levels after weight loss in adults induced by obesity surgery. Thus in obesity, both circulating levels of DPP4 and DPP4 release by adipose tissue are increased but can be reduced to control levels by substantial weight loss.

Both circulating DPP4 and DPP4 release by adipose tissue correlate strongly with the metabolic syndrome. Thus, DPP4 may be of relevance as a novel biomarker of the metabolic syndrome and for detection of obese subjects at high risk for obesity-associated complications. Future studies are needed to address this important issue and to define the molecular pathways that link adipose DPP4 to the metabolic syndrome and type 2 diabetes. An adipose-specific knockout of DPP4 would be required to prove a causal role of this protein and this mouse model is currently under development in our laboratory. However, several lines of evidence support our notion that the novel adipokine links obesity to the metabolic syndrome. First, DPP4 impairs the function of the incretin system which is of key importance for glucose homeostasis (29). Additionally, incretin-based therapies are known to preserve beta-cell function and to exert salutary effects on blood pressure and lipid profile (30). Second, DPP4 inhibitors are well known to improve glucose tolerance in animal models of obesity (31). More importantly, chronic DPP4 inhibition in ZDF rats was shown to delay the onset of type 2 diabetes (31). Finally, preclinical data suggest that GLP-1 is cardioprotective (32) and DPP4 inhibition was shown to improve cardiovascular outcomes in rodents (33). Our data strongly support the current view (20) that adipocytes and specifically adipose tissue play a major, most likely causative role in the pathogenesis of metabolic diseases.

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

Author Contributions

D.L. researched data. S.F. researched data. N.W. researched data. S.H. researched data. S.L. contributed to discussion and reviewed/edited manuscript. D.M.O. contributed to discussion and reviewed/edited manuscript. K.E. reviewed/edited manuscript. J.M.K researched data. M.R. researched data. S.M. researched data. F.H. researched data. J.R. researched data and reviewed/edited manuscript. P.A. researched data and contributed to discussion. H.S. researched data and wrote the manuscript. J.E. wrote the manuscript.

Acknowledgements

This work was supported by the Bundesministerium für Gesundheit, the Deutsche Forschungsgemeinschaft (SE 1922/2-1), the Commission of the European Communities (Collaborative Project ADAPT, contract number HEALTH-F2-2008-201100; Integrated Project HEPADIP, contract number Contract LSHM-CT-2005-018734), and EU COST Action BM0602, Swedish Research Council (K2008-54X-01034-42-4 and 2007-2489), Swedish Diabetes Association, Swedish Heart and Lung Association, Diabetes programme at Karolinska Institutet and the Novo Nordisk Foundation. We wish to thank Prof. Liebau and her team, Dept. of Plastic Surgery, Florence-Nightingale-Hospital Düsseldorf, for support in obtaining adipose tissue samples. The technical assistance of Andrea Cramer, Angelika Horrighs, Birgit Knobloch and Kerstin Wåhlén and the secretarial assistance of Birgit Hurow (Paul-Langerhans-Group, German Diabetes Center) are gratefully acknowledged.

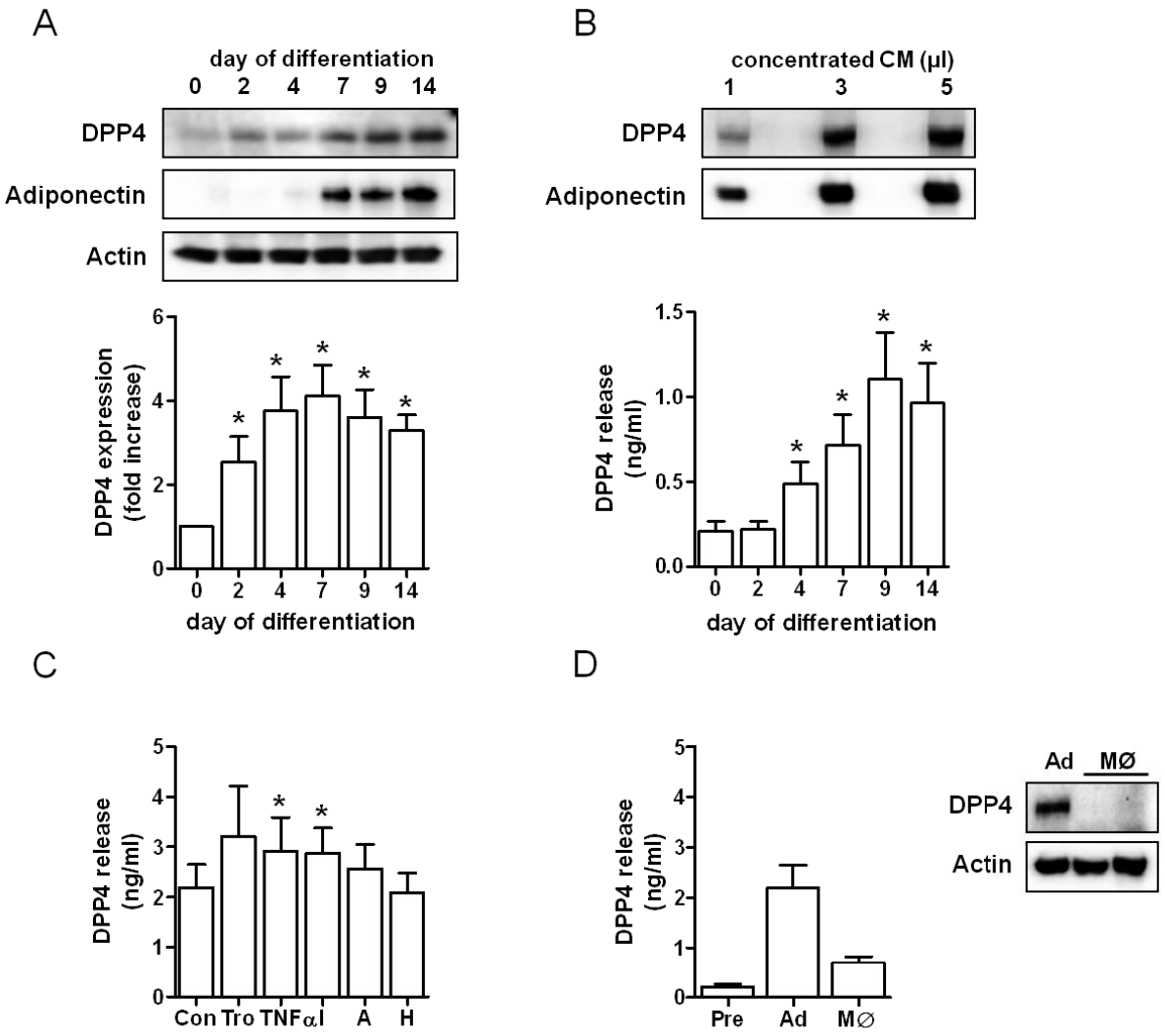


Figure 1 DPP4 protein level and release during adipocyte differentiation and after stimulation with different regulatory factors. **(A)** Human primary adipocytes were differentiated as described in materials and methods, and DPP4 protein level during differentiation was analyzed by SDS-PAGE and Western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

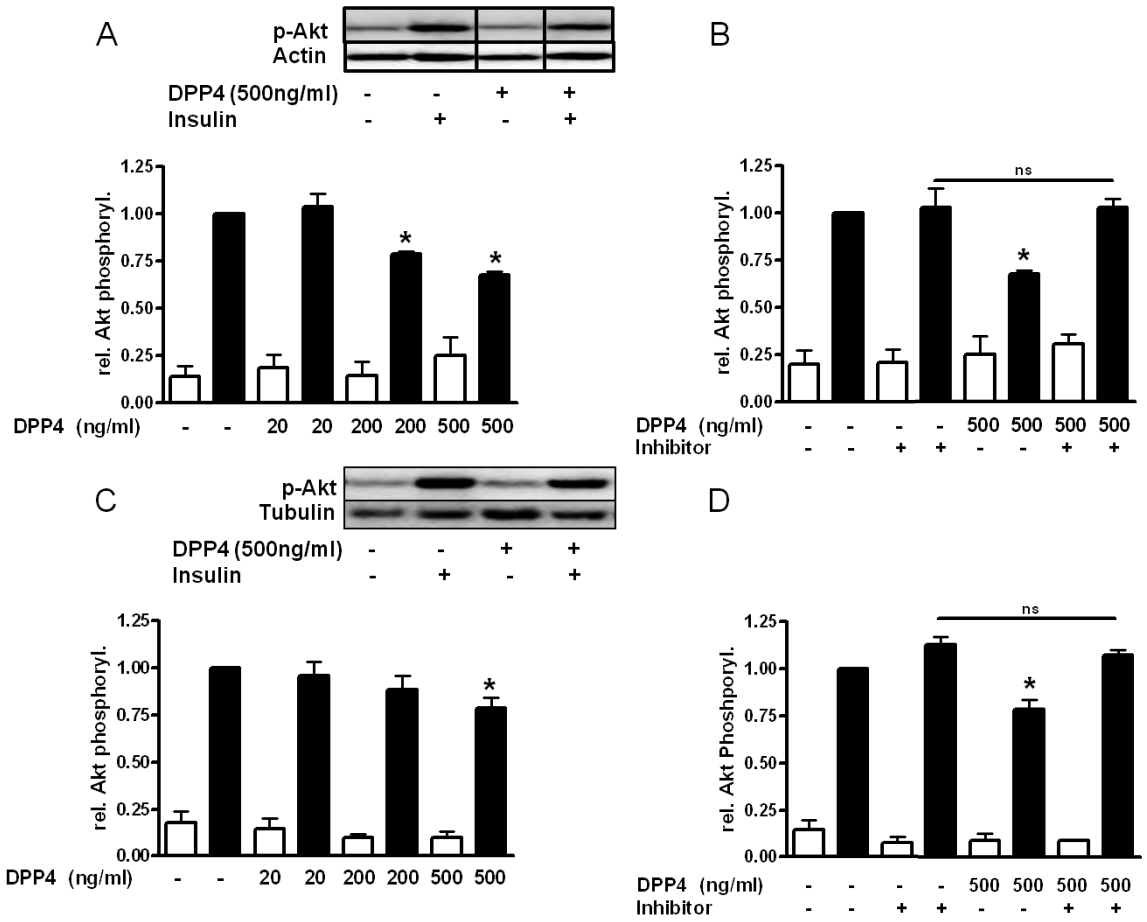


Figure 2 Effect of DPP4 on insulin-stimulated Akt phosphorylation in adipocytes and skeletal muscle cells. Differentiated human adipocytes **(A-B)** and skeletal muscle cells **(C-D)** were treated with the indicated amounts of DPP4 without and with concomitant administration of a specific DPP4 inhibitor for 24 h. After stimulation with insulin (100 nM, 10 min) the cells were lysed and 5-10 µg of total lysates were resolved by SDS-PAGE and blotted to PVDF membranes. Membranes were blocked with 5% milk in TBS containing 0.1% Tween-20 and incubated overnight with p-Akt antibody. After incubation with the appropriate HRP-coupled secondary antibody, the signal was detected by ECL. Signals were analyzed on a LUMI Imager Work Station. Data are actin normalized mean values ± SEM (n = 3-8). Representative Western Blots are presented. For **(A)**, lanes were excised from a single Western Blot and displayed in the presented order. White bars – basal, black bars – insulin-stimulated. ns – not significant. * significantly different from insulin-stimulated control or indicated situation.

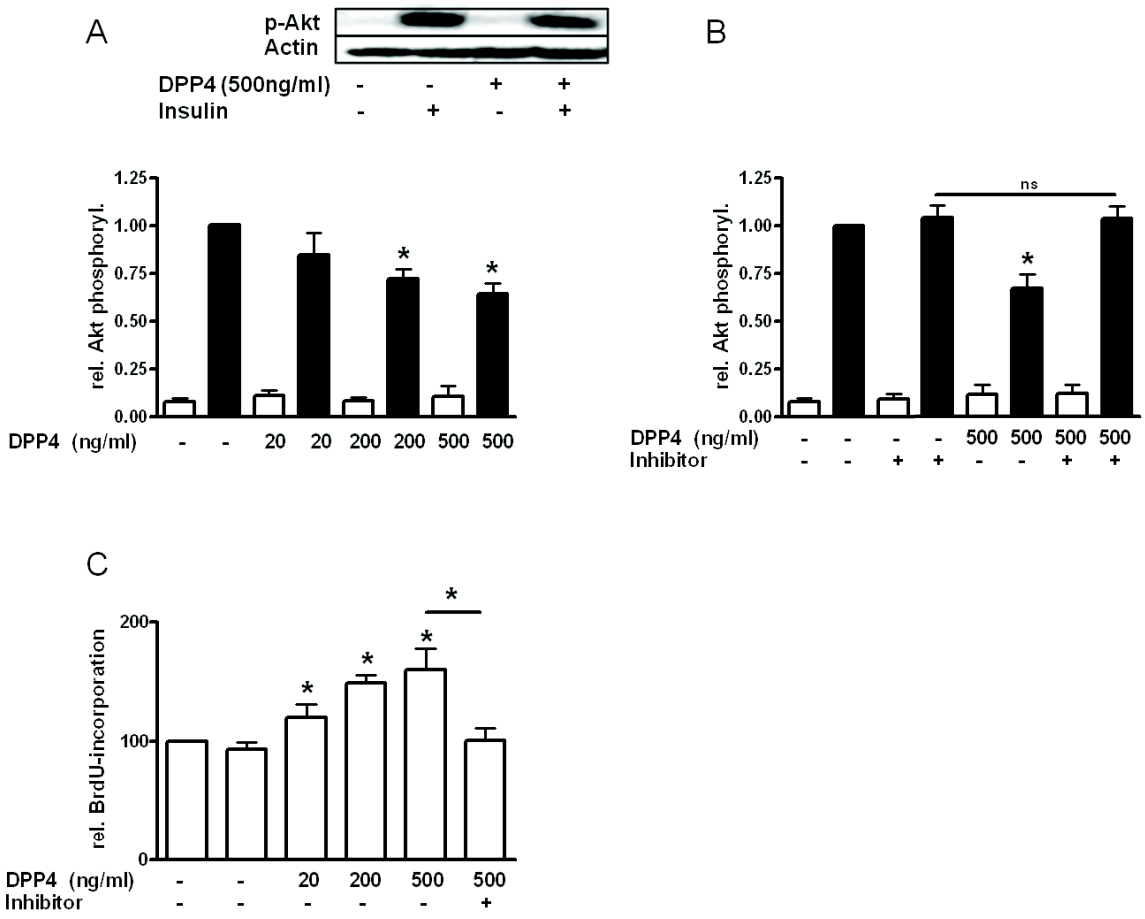


Figure 3 Effect of DPP4 on insulin-stimulated Akt phosphorylation and proliferation in smooth muscle cells. **(A-B)** Smooth muscle cells were treated with the indicated amounts of DPP4 without and with concomitant administration of a specific DPP4 inhibitor for 24 h. After stimulation with insulin (100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

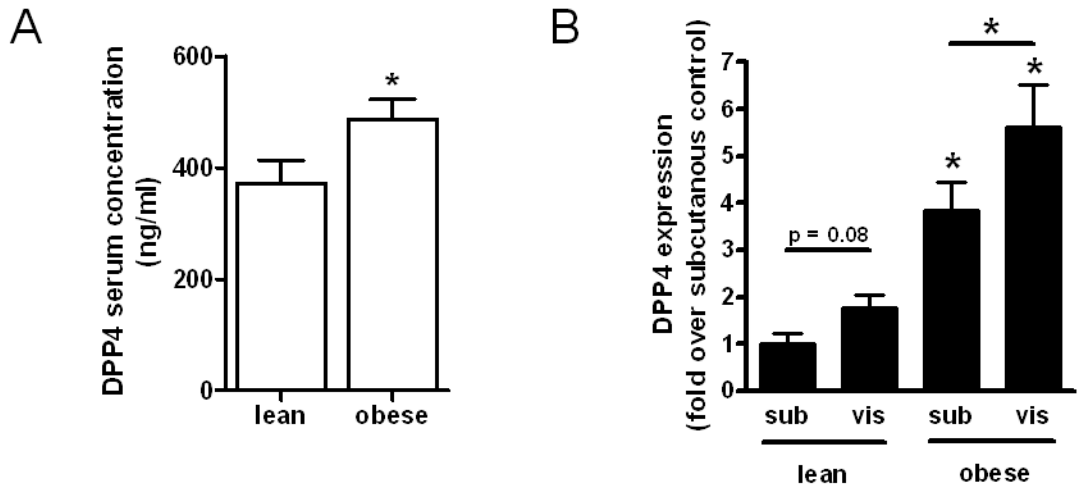


Figure 4 DPP4 serum concentration and expression in adipose tissue from lean compared to obese patients (Clinical study 1). **(A)** Sera from lean (n=20) and morbidly obese men (n=20) were analyzed for their DPP4 concentration by ELISA. Data are mean values ± SEM, *p<0.05 vs. lean group. **(B)** DPP4 protein level in adipose tissue biopsies was analyzed by SDS-PAGE and Western Blot. Data were normalized to the protein level of actin and are expressed relative to subcutaneous adipose tissue from lean subjects. Data are mean values ± SEM, n=8 for lean and n=14 for obese patients, *p<0.05 respective subcutaneous or designated group.

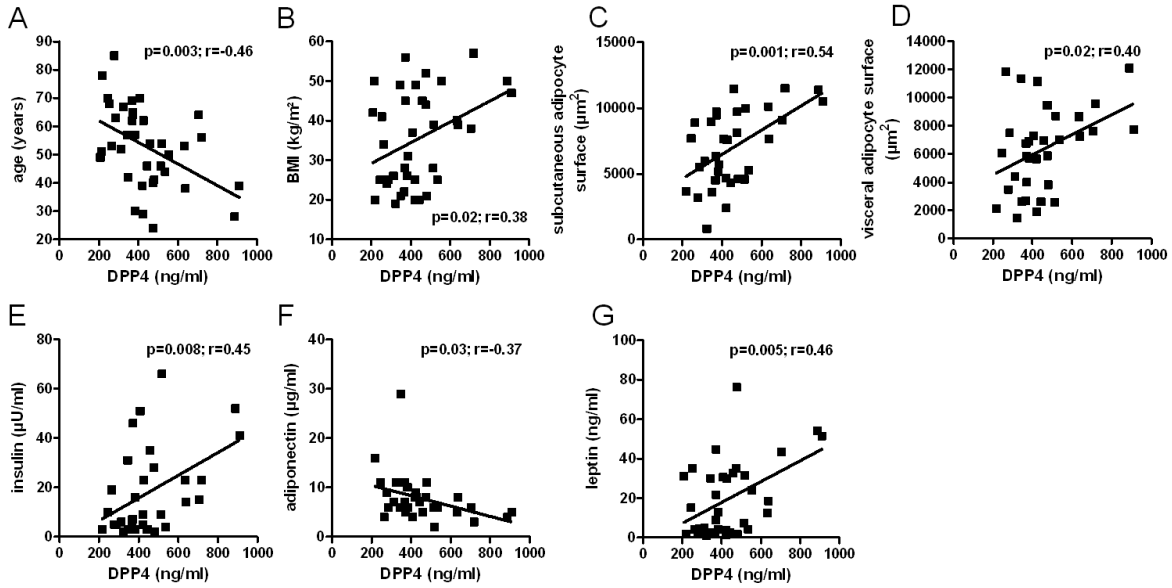


Figure 5 DPP4 serum concentrations correlate with various clinical and biochemical parameters (Clinical study 1). Sera from lean (n=20) and morbidly obese men (n=20) were analyzed for their DPP4 concentration by ELISA. Linear regression analysis of DPP4 serum concentration and patient characteristics like age (A), BMI (B), size of subcutanous (sc) (C) and visceral (vis) adipocytes (D), insulin concentration (E), adiponectin concentration (F) and leptin concentration (G). Statistical evaluation is indicated in each graph.

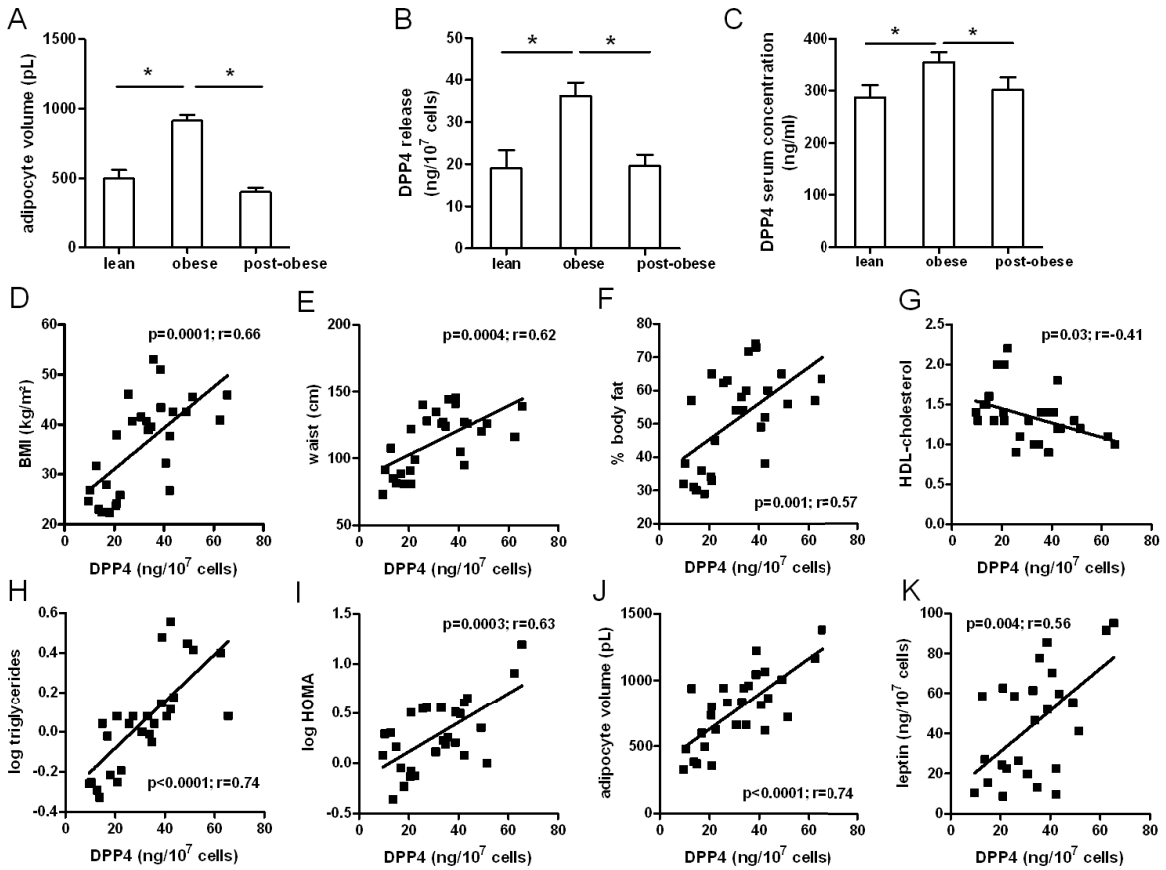


Figure 6 DPP4 release of explants obtained from adipose tissue of lean controls and obese patients before and after bariatric surgery as well as linear correlation with various clinical and biochemical parameters (Clinical study 2). **(A-B)** Samples of adipose tissue were obtained from lean controls (n=10) and obese patients before (n=19) and after (n=16) bariatric surgery, and used to generate explants as described in material and methods. The size of adipocytes for each subject was measured **(A)**. DPP4 release was analyzed by ELISA and related to the quantity of adipocytes **(B)**. **(C)** DPP4 serum concentration was measured in lean and in obese patients before and after bariatric surgery. **(D-K)** Linear regression analysis of DPP4 release per 10^7 cells and patient characteristics like BMI **(D)**, waist circumference **(E)**, percent of body fat **(F)**, HDL-cholesterol concentration **(G)**, triglycerides concentration **(H)**, HOMA **(I)**, adipocyte volume **(J)** and leptin **(K)**. **(A-C)** Data are mean values ± SEM . *p<0.05 between respective groups.

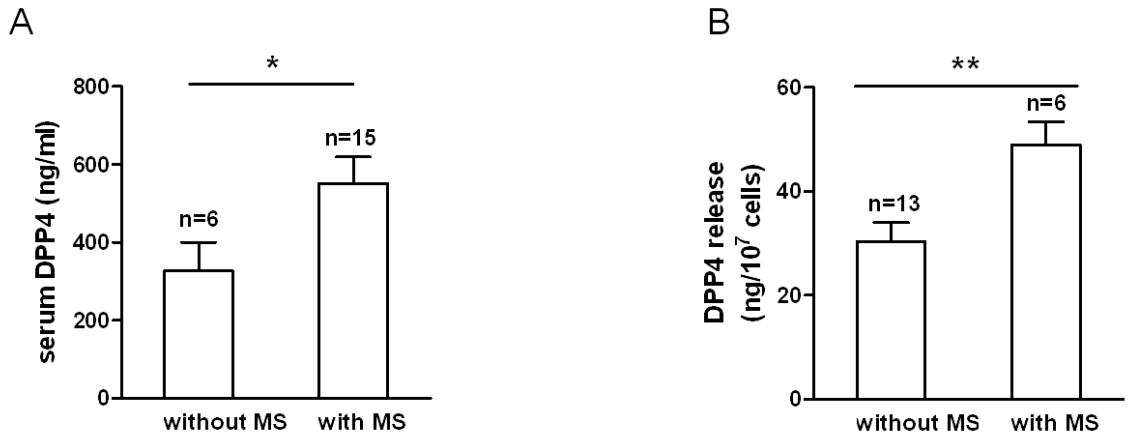


Figure 7 DPP4 in serum and release from adipose tissue explants in relation to a risk score for the metabolic syndrome. A risk score for the metabolic syndrome was calculated for all obese subjects from whom serum and adipose tissue explants were analyzed. Patients with a risk score ≥3 were qualified as "with metabolic syndrome (MS)". Patients with a score ≤2 were qualified as "without MS. Data was analyzed using t-test. Data are mean values ± SEM. *p<0.05.

References

1. Wellen KE, Hotamisligil GS: Inflammation, stress, and diabetes. *J Clin Invest* 115:1111-1119, 2005

2. Sell H, Dietze-Schroeder D, Eckel J: The adipocyte-myocyte axis in insulin resistance. *Trends Endocrinol Metab* 17:416-422, 2006

3. Arner P: The adipocyte in insulin resistance: key molecules and the impact of the thiazolidinediones. *Trends Endocrinol Metab* 14:137-145, 2003

4. Scherer PE: Adipose tissue: from lipid storage compartment to endocrine organ. *Diabetes* 55:1537-1545, 2006

5. Breitling R: Robust signaling networks of the adipose secretome. *Trends Endocrinol Metab* 20:1-7, 2009

6. Yazbeck R, Howarth GS, Abbott CA: Dipeptidyl peptidase inhibitors, an emerging drug class for inflammatory disease? *Trends Pharmacol Sci* 30:600-607, 2009

7. Drucker DJ, Nauck MA: The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet* 368:1696-1705, 2006

8. Ahren B: Dipeptidyl peptidase-4 inhibitors: clinical data and clinical implications. *Diabetes Care* 30:1344-1350, 2007

9. Lambeir AM, Durinx C, Scharpe S, De Meester I: Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. *Crit Rev Clin Lab Sci* 40:209-294, 2003

10. Cordero OJ, Salgado FJ, Nogueira M: On the origin of serum CD26 and its altered concentration in cancer patients. *Cancer Immunol Immunother* 58:1723-1747, 2009

11. Bonora E, Targher G, Alberiche M, Bonadonna RC, Saggiani F, Zenere MB, Monauni T, Muggeo M: Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. *Diabetes Care* 23:57-63, 2000

12. Kolaczynski JW, Morales LM, Moore JH, Jr., Considine RV, Pietrzkowski Z, Noto PF, Colberg J, Caro JF: A new technique for biopsy of human abdominal fat under local anaesthesia with Lidocaine. *Int J Obes Relat Metab Disord* 18:161-166, 1994

13. Lonnqvist F, Nordfors L, Jansson M, Thorne A, Schalling M, Arner P: Leptin secretion from adipose tissue in women. Relationship to plasma levels and gene expression. *J Clin Invest* 99:2398-2404, 1997

14. Lofgren P, Hoffstedt J, Naslund E, Wiren M, Arner P: Prospective and controlled studies of the actions of insulin and catecholamine in fat cells of obese women following weight reduction. *Diabetologia* 48:2334-2342, 2005

15. Dietze-Schroeder D, Sell H, Uhlig M, Koenen M, Eckel J: Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors. *Diabetes* 54:2003-2011, 2005

16. Curat CA, Miranville A, Sengenes C, Diehl M, Tonus C, Busse R, Bouloumie A: From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. *Diabetes* 53:1285-1292, 2004

17. Kim J, Choi YS, Lim S, Yea K, Yoon JH, Jun DJ, Ha SH, Kim JW, Kim JH, Suh PG, Ryu SH, Lee TG: Comparative analysis of the secretory proteome of human adipose stromal vascular fraction cells during adipogenesis. *Proteomics* 10:394-405

18. Rosenow A, Arrey TN, Bouwman FG, Noben JP, Wabitsch M, Mariman EC, Karas M, Renes J: Identification of Novel Human Adipocyte Secreted Proteins by Using SGBS Cells. *J Proteome Res*

19. Zhong J, Krawczyk SA, Chaerkady R, Huang H, Goel R, Bader JS, Wong GW, Corkey BE, Pandey A: Temporal Profiling of the Secretome during Adipogenesis in Humans. *J Proteome Res*

20. Deng Y, Scherer PE: Adipokines as novel biomarkers and regulators of the metabolic syndrome. *Ann N Y Acad Sci* 1212:E1-E19

21. Sell H, Dietze-Schroeder D, Eckardt K, Eckel J: Cytokine secretion by human adipocytes is differentially regulated by adiponectin, AICAR, and troglitazone. *Biochem Biophys Res Commun* 343:700-706, 2006

22. Gorrell MD: Dipeptidyl peptidase IV and related enzymes in cell biology and liver disorders. *Clin Sci (Lond)* 108:277-292, 2005

23. Kos K, Baker AR, Jernas M, Harte AL, Clapham JC, O'Hare JP, Carlsson L, Kumar S, McTernan PG: DPP-IV inhibition enhances the antilipolytic action of NPY in human adipose tissue. *Diabetes Obes Metab* 11:285-292, 2009

24. Iwaki-Egawa S, Watanabe Y, Kikuya Y, Fujimoto Y: Dipeptidyl peptidase IV from human serum: purification, characterization, and N-terminal amino acid sequence. *J Biochem* 124:428-433, 1998

25. Schrader WP, West CA, Miczek AD, Norton EK: Characterization of the adenosine deaminase-adenosine deaminase complexing protein binding reaction. *J Biol Chem* 265:19312-19318, 1990

26. Mentlein R: Dipeptidyl-peptidase IV (CD26)--role in the inactivation of regulatory peptides. *Regul Pept* 85:9-24, 1999

27. Bouchard L, Tchernof A, Deshaies Y, Marceau S, Lescelleur O, Biron S, Vohl MC: ZFP36: a promising candidate gene for obesity-related metabolic complications identified by converging genomics. *Obes Surg* 17:372-382, 2007

28. Reinehr T, Roth CL, Enriori PJ, Masur K: Changes of dipeptidyl peptidase IV (DPP-IV) in obese children with weight loss: relationships to peptide YY, pancreatic peptide, and insulin sensitivity. *J Pediatr Endocrinol Metab* 23:101-108

29. Drucker DJ: The biology of incretin hormones. *Cell Metab* 3:153-165, 2006

30. Rizzo M, Rizvi AA, Spinas GA, Rini GB, Berneis K: Glucose lowering and anti-atherogenic effects of incretin-based therapies: GLP-1 analogues and DPP-4-inhibitors. *Expert Opin Investig Drugs* 18:1495-1503, 2009

31. Sudre B, Broqua P, White RB, Ashworth D, Evans DM, Haigh R, Junien JL, Aubert ML: Chronic inhibition of circulating dipeptidyl peptidase IV by FE 999011 delays the occurrence of diabetes in male zucker diabetic fatty rats. *Diabetes* 51:1461-1469, 2002

32. Bose AK, Mocanu MM, Carr RD, Brand CL, Yellon DM: Glucagon-like peptide 1 can directly protect the heart against ischemia/reperfusion injury. *Diabetes* 54:146-151, 2005

33. Sauve M, Ban K, Momen MA, Zhou YQ, Henkelman RM, Husain M, Drucker DJ: Genetic deletion or pharmacological inhibition of dipeptidyl peptidase-4 improves cardiovascular outcomes after myocardial infarction in mice. *Diabetes* 59:1063-1073

Journal: *Diabetes*

Impact factor: 7.895

Contribution: Total: 37%

Conceived / designed experiments: 40%

Performed experiments: 40%

Analysed data: 40%

Contributed to discussion: 50%

Wrote the manuscript: 20%

Reviewed / edited manuscript: 30%

Author: 1st author (shared)

CHAPTER 3

Study 2

Soluble DPP4 Induces Inflammation and Proliferation of Human Smooth Muscle Cells via Protease-Activated Receptor 2†

Nina Wronkowitz[1], Sven W. Görgens[1], Tania Romacho[1], Laura A. Villalobos[2], Carlos F. Sánchez-Ferrer[2], Concepción Peiró[2], Henrike Sell*[1] and Jürgen Eckel*[1]

[1]*Paul-Langerhans-Group for Integrative Physiology, German Diabetes-Center, 40225 Düsseldorf, Germany*
[2]*Department of Pharmacology and Therapeutics, Universidad Autónoma de Madrid, 28049 Madrid, Spain*

**both authors contributed equally to this work*

† submitted to *Biochimica et Biophysica Acta - Molecular Basis of Disease*, October 2013

Abstract

DPP4 is an ubiquitously expressed cell-surface protease that is shedded to the circulation as soluble DPP4 (sDPP4). We recently identified sDPP4 as a novel adipokine potentially linking obesity to the metabolic syndrome. The aim of this study was to investigate direct effects of sDPP4 on human vascular smooth muscle cells (hVSMC) and to identify responsible signaling pathways. Using physiological concentrations of sDPP4, we could observe a concentration-dependent activation of ERK1/2 (3-fold) after 6h, which remained stable for up to 24h. Additionally, sDPP4 treatment induced a 1.5-fold phosphorylation of the NF-κB subunit p65. In accordance with sDPP4-induced stress and inflammatory signaling, sDPP4 also stimulates hVSMC proliferation. Furthermore we could observe an increased expression and secretion of pro-inflammatory cytokines like interleukin (IL)-6, IL-8 and MCP-1 (2.5-, 2.4- and 1.5-fold, respectively) by the sDPP4 treatment. All direct effects of sDPP4 on signaling, proliferation and inflammation could completely be prevented by DPP4 inhibition. Bioinformatic analysis and signaling signature induced by sDPP4 suggest that sDPP4 might be an agonist for PAR2. After silencing of PAR2, the sDPP4-induced ERK activation as well as the proliferation was totally abolished. Additionally, the sDPP4-induced upregulation of IL-6 and IL-8 could completely be prevented by the PAR2 silencing. In conclusion, we show for the first time that sDPP4 directly activates the MAPK and NF-κB signaling cascade in a PAR2-dependent manner resulting in the induction of inflammation and proliferation of hVSMC. Due to increased circulating levels of sDPP4 in obesity, sDPP4 may play a role in linking obesity to cardiovascular disease.

Introduction

Type 2 diabetes and obesity are frequently associated with the development of cardiovascular disease (CVD) [39]. CVD remains the major cause of death worldwide and represents a key global health problem due to the epidemic increase of obesity-induced insulin resistance and type 2 diabetes [18]. Adipose tissue was long seen as a passive lipid storage depot but it is now considered as an endo- and paracrine organ that produces a large number of mediators that affect metabolism, inflammation and coagulation. Expansion of adipose tissue in obesity is highly associated with an enhanced secretion of pro-

inflammatory and pro-atherogenic factors [32,34]. Thus, alterations in adipocyte derived factors, the so called adipokines, may link obesity to cardiovascular dysfunction.

Dipeptidyl peptidase 4 (DPP4) is an ubiquitously expressed cell-surface protease, which selectively cleaves N-terminal dipeptides from a variety of substrates including growth factors, hormones, neuropeptides and chemokines. Substantial DPP4 activity is also found in plasma and other body fluids because of a soluble form of DPP4 lacking the cytoplasmic tail and the transmembrane region [25]. In a previous study we could identify sDPP4 as a novel adipokine [26], which is widely known for its role in regulation of glycemia through catabolism of incretin peptides, and we could show that sDPP4 serum concentrations positively correlate with various parameters of the metabolic syndrome like BMI, adipocyte surface, leptin and insulin levels [26,33]. Interestingly, comparison of different fat depots showed the highest DPP4 expression in visceral adipose tissue of obese patients [33]. Furthermore, we were the first investigating direct effects of sDPP4 on human adipocytes, human skeletal muscle cells and hVSMC showing a sDPP4-induced insulin resistance and an increased hVSMC proliferation [26]. Therefore sDPP4 is a promising candidate linking obesity to CVD. Up to now DPP4 has gained most interest as a therapeutic target for type 2 diabetes treatment due to the degradation of the incretin hormone glucagon-like peptide (GLP)-1 by this enzyme. Thus, DPP4-inhibitors are able to prolong the insulinotropic effect of GLP-1 and are now in clinical use as anti-diabetic drugs [2]. Additionally, preclinical and preliminary clinical data suggest that DPP4-inhibitors hold interesting promise for cardiovascular protection. Since GLP-1 itself has favorable cardiovascular effects [3,31] most of these protective effects of DPP4 inhibition are ascribed to the increased bioavailability of GLP-1. Nevertheless, there are accumulating data suggesting that DPP4-inhibitors mediate vascular protection independent of GLP-1, involving endothelial repair [16], anti-inflammatory effects [12,16,38] and blunting of ischemic injury [41,42].

Vascular endothelial cells and smooth muscle cells represent the major cell types of the artery wall preserving vessel wall homeostasis. Migration of hVSMC from the media to intima and their concomitant proliferation occurring in the synthetic state are critical causes of arterial wall thickening and the development of arteriosclerosis [24]. Moreover, hVSMC are involved in the ongoing low-grade inflammation within arteriosclerotic lesions by

recruitment of immune cells [6]. However, the underlying mechanism of hVSMC dysfunction taking place in type 2 diabetes and obesity are not fully understood.

The aim of the present study was to further investigate direct effects of sDPP4 on hVSMC and to identify responsible signaling pathways. We report here a novel receptor-mediated pathway for sDPP4, involving PAR2-mediated activation of inflammation and proliferation, suggesting a major role of this adipokine in the pathophysiology of vascular complications.

Material and Methods

Cell culture of hVSMC

Primary human coronary artery SMCs were obtained from PromoCell (Heidelberg, Germany), tebu-bio (Offenbach, Germany) and Lonza (Basel, Switzerland). hVSMC from three different donors (Caucasian, male, 58 years old; two female, both 55 years old) were supplied as proliferating cells and kept in culture according to the manufacturer's protocol. For all experiments, subconfluent cells of passage 5 were used. Cells were characterized as hVSMC by morphologic criteria and by immunostaining with smooth muscle α-actin. Cells were seeded in smooth muscle cell growth medium (PromoCell, Heidelberg, Germany) with 5% Foetal calf serum (FCS) (Gibco Invitrogen, Carlsbad, CA, USA) and allowed to attach for 24 h. Afterwards cells were serum-starved for 24 h and then exposed to sDPP4 (R&D Systems, Wiesbaden-Nordenstadt, Germany) alone or in the combination with the specific DPP4-inhibitor K579 (Tocris Bioscience, Bristol, UK), the ERK1/2-inhibitor PD98059 (Calbiochem, Merck Biosciences, Schwalbach, Germany), the specific PAR2 antagonist GB83 or the highly specific IκB-Kinase (IKK)-inhibitor I229 [27]. The IKK-inhibitor was from Sanofi-Aventis (Frankfurt, Germany). It has submicromolar activity on the isolated IKK complex and is highly specific on IKK. The general structure of I229 is described in PCT/EP00/05340. The DPP4- and IKK-Inhibitor were dissolved in DMSO as a 100 mM stock solution, and were further diluted in sterile serum-free SMC medium up to 100 nM and 100 µM, respectively. The ERK1/2-inhibitor was already dissolved in DMSO as a 5 mg/ml stock solution, and was further diluted in sterile serum-free SMC medium up to 10 µM. Cells were 1 h preincubated with the ERK1/2- and the IKK-inhibitor and subsequently treated with sDPP4 with and without concomitant administration of the specific inhibitors. The specific PAR2 antagonist GB83 was

dissolved in DMSO as a 32,8 mM stock solution and was further diluted in sterile serum-free SMC medium up to 1 µM. All controls of experiments involving the DPP4-, the IKK-inhibitor or the PAR2 antagonist were treated with DMSO alone. Furthermore, hVSMCs were treated with the PAR2-activating peptide (AP) (Bachem, Bubendorf, Switzerland) alone or in combination with PAR2 antagonist GB83 for 10 min.

In vitro analysis of hVSMC proliferation

To monitor DNA synthesis, 10.000 hVSMC per 15 mm² well were seeded in 96 well culture dishes and allowed to attach for 24 h, followed by serum starvation for an additional 24 h period. hVSMC were exposed to sDPP4 alone or in combination with the ERK1/2-Inhibitor or the PAR2 antagonist in the presence of BrdU (10 µM) for 24 h. All controls of experiments involving ERK1/2-inhibitor or PAR2 antagonist were treated with DMSO alone. 5% FCS was used as positive control. The BrdU ELISA (chemiluminescent) (Roche, Mannheim, Germany) 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).

Transfection of hVSMC with PAR2 siRNA

To investigate the impact of PAR2 on the sDPP4-induced effects, hVSMCs were transfected with 20 nmol/l sramble siRNA (negative control) or PAR2 siRNA (Ambion, Life Technologies, Darmstadt, Germany) using Hiperfect (Qiagen, Venlo, Netherlands) as the transfection reagent. 24h after transfection cells were stimulated with sDPP4 and then analyzed for BrdU incorporation or lysed for protein or RNA isolation.

Analysis of protein expression and phosphorylation

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 hrs at 4°C on a rotation-shaker, the suspension was centrifuged at 10,000 g for 15 min. Thereafter, 5 µg protein of lysates were separated by SDS-PAGE using 10% horizontal gels and transferred to polyvenylidene fluoride filters in a semi-dry blotting apparatus [16]. Filters were blocked with Tris-buffered saline containing 0.1% Tween and 5% non-fat dry milk and subsequently incubated overnight with primary

antibodies for anti-phospho-NF-κB (P65) (Ser536) (Cell Signalling Technology, Frankfurt, Germany), anti-phospho-p44/42 MAPK (ERK1/2, Thr202/Tyr204) (Cell Signalling Technology, Frankfurt, Germany), anti-iNOS (BD Transduction Laboratories, Heidelberg, Germany), PAR2 (Santa Cruz Biotechnology, Heidelberg, Germany) and alpha-tubulin (Calbiochem Merck Biosciences, Schwalbach, Germany). After washing, filters were incubated with corresponding secondary HRP-coupled antibody (Promega, Mannheim, Germany) and processed for enhanced chemiluminescence detection using Immobilon HRP substrate (Millipore, Billerica, MA, USA). Signals were visualized and evaluated on a LUMI Imager work station.

Determination of cytokine secretion

To measure the secretion of pro-inflammatory cytokines, supernatants were collected after hVSMC were treated 24 h like indicated. IL-6, IL-8 and MCP-1 secretion was determined using ELISA kits purchased from Diaclone (Besancon Cedex, France). The assays were performed in duplicates according to the manufacturer's instructions.

RNA isolation, cDNA synthesis and qRT-PCR

To assess the effects on mRNA expression, hVSMC were harvested 24h after the addition of sDPP4 with and without the different inhibitors. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration and purity was measured with a NanoDrop 2000 (Thermo Scientific, Schwerte, Germany). One microgram of RNA was reverse transcribed using an Omniscript RT Kit (Qiagen, Venlo, Netherlands). mRNA expression levels were determined with predesigned Quantitect Primer Assays for IL-6 (QT00083720), IL-8 (QT00000322), MCP-1 (QT00212730) and β-Actin (QT01680476) and GoTaq® qPCR Master Mix (Promega) on a StepOne Plus real-time PCR system (Applied Biosystems, Carlsbad, CA, USA).

Presentation of data and statistics

Data are expressed as mean ± S.E.M. 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 < 0.05 as statistically significant.

Results

sDPP4-induced ERK1/2 activation leads to increased proliferation of hVSMC

In a previous study, we showed that sDPP4 is involved in hVSMC proliferation [26]. Therefore we investigated the effects of sDPP4 on the MAPK signaling pathway and its involvement in hVSMC proliferation. Using increasing concentrations of sDPP4 (2-500ng/ml), we could observe a concentration-dependent activation of ERK1/2 (3-fold) after 6h (Fig. 1A), which remained stable for up to 24h (Fig. 1B). To demonstrate specificity of this effect, 100 nM of the DPP4-inhibitor K579 was used. The DPP4-inhibitor alone had no effect, but in combination with sDPP4 the ERK1/2 activation could be completely abrogated after 6 h (Fig. 1C). Furthermore, we could show that the sDPP4-induced hVSMC proliferation could totally be prevented by 10 µM of the ERK1/2-inhibitior PD98059 (Fig. 1D).

sDPP4-mediated NF-κB activation results in elevated cytokine and iNOS expression in hVSMC

To determine whether sDPP4 induces inflammation, we analyzed the impact of sDPP4 on the NF-κB signaling pathway. After 6 h of sDPP4 treatment we observed a 1.5-fold enhanced phosphorylation of the NF-κB subunit p-65 (Fig. 2A). Accordingly, we found increased mRNA levels for the known NF-κB-targets IL-6 (Fig. 2B), IL-8 (Fig. 2C) and MCP-1 (Fig. 2D) with the most prominent effect for IL-8 (2-fold induction) after 24 h challenge with sDPP4. To confirm the involvement of NF-κB activation in the sDPP4-induced cytokine expression, we used the highly specific IKK-inhibitor I229 in a concentration of 10 µM. As expected, the IKK-inhibitor itself reduces the basal expression of IL-8 and MCP-1. Moreover, the elevated expression of the pro-inflammatory cytokines could be completely abolished by IKK inhibition (Fig. 2B-D). Since it is known that cytokines upregulate iNOS through NF-κB activation, we tested the impact of sDPP4 on iNOS expression in hVSMC. Using increasing concentrations of sDPP4 (20-500ng/ml), we could observe a concentration-dependent induction of iNOS (up to 3-fold) after 18h (Fig. 2E)

DPP4 inhibition completely prevents sDPP4-induced NF-κB activation and the release of pro-inflammatory cytokines

Consistent with our findings on the ERK signaling pathway, we were able to prevent the sDPP4-induced NF-κB activation after 6 h by inhibition of DPP4 (Fig. 3A), whereas the inhibitor itself showed no effect. Accordingly, we were also able to block the sDPP4-induced expression (Fig. 3B-D) and secretion (Fig. 3E-G) of the pro-inflammatory cytokines IL-6, IL-8 and MCP-1. These data underpin the notion [15] of incretin-independent effects of DPP4-inhibitors.

DPP4 contains a potential PAR2 activating sequence

The signaling signature induced by sDPP4 suggests that sDPP4 might be an agonist for the protease-activated receptor family. These receptors have a unique activation mechanism in that 1) a proteolytically exposed *N*-terminal region acts as a tethered ligand (TL) and 2) a peptide, corresponding to the sequence of the TL, can activate the respective receptor directly. After alignment of the human DPP4 and the different TL sequences for PAR1 (data not shown), PAR2 (Fig. 4A) and PAR4 (data not shown), we only observed for the TL sequence of PAR2 (SLIGKV) an almost perfect match of 75% for the first four amino acids. It is known that these four amino acids (SLIG) are the most important for the activation of PAR2 [1]. The match was found between amino acid 292 and 303 in the cystein-rich region of DPP4 responsible for partner binding. When we aligned the potential TL sequence SLIG (in red) with the sequence of the crystallized DPP4 (in grey), we could locate SLIG on the surface of DPP4 (Fig. 4B), thus potentially enabling binding.

PAR2 silencing prevents sDPP4-induced proliferation and inflammation

It could be shown that PAR2 is highly expressed in epithelial cells and smooth muscle of vascular and nonvascular origin [11]. Here we assessed PAR2 expression in three different primary human cell types and observed a 2-times higher PAR2 expression in hVSMC

compared to *in vitro* differentiated skeletal muscle cells (SkMC) and adipocytes (Fig. 5A). To investigate the impact of PAR2 for the sDPP4-induced effects we transfected hVSMCs with either 20 nM scramble (negative control) or PAR2 siRNA. 24 h after the transfection we could observe a significant downregulation of PAR2 mRNA (by 80%) compared to the control situation (Fig. 5B). sDPP4-induced ERK1/2 activation as well as proliferation could be completely prevented by PAR2 silencing (Fig. 5C,D) while silencing of PAR2 itself has no effect on these parameters. In accordance to these data, sDPP4-induced upregulation of IL-6 and IL-8 could be abolished by PAR2 silencing (Fig. 5E,F).

The PAR2 antagonist GB83 abrogates sDPP4-induced ERK signaling and proliferation of hVSMCs

To confirm our silencing data, we also analyzed the effect of the specific PAR2 antagonist GB83, which is known to block the PAR2 activation both by proteases and the activating peptide (AP) SLIGKV. We could demonstrate that both 10 and 50 µM of the PAR2 antagonist are able to block the PAR2-AP-induced ERK signaling after 10 min, whereas the PAR2 antagonist itself has no effect (Fig. 6A). Using 10 µM of the PAR2 antagonist both sDPP4-induced ERK activation (Fig. 6B) as well as the proliferation (Fig. 6C) was completely blocked.

Discussion

Since central obesity is characterized by an enhanced cardiovascular risk, it has been speculated that adipocyte derived factors might directly contribute to the pathogenesis of atherosclerosis [4,8]. In a previous study, we identified sDPP4 as a novel adipokine, which was markedly higher expressed in and released from visceral compared to subcutaneous fat of obese patients [26,33]. Additionally, we could demonstrate that sDPP4 directly impairs insulin signaling in three different primary cell types, namely, adipocytes, skeletal muscle, and smooth muscle cells and increases hVSMC proliferation [26]. Here we further assessed direct effects of sDPP4 on hVSMC and aimed to identify the underlying receptor signaling pathway for the observed inflammation and proliferation. For our study, we used sDPP4

concentrations reflecting circulating levels that were measured in both lean and obese subjects [26]. Interestingly, high serum levels of sDPP4 have been described in various conditions [17] including atherosclerosis [14]. In the present study, we could show that sDPP4 activates the MAPK and NF-κB signaling cascade resulting in pro-atherogenic changes in hVSMC illustrated by an increased proliferation, the induction of iNOS and elevated expression and secretion of pro-inflammatory cytokines.

Our data on the direct effects of sDPP4 suggest that sDPP4 exerts its signaling properties by receptor interaction. In the literature, it is currently discussed that fibronectin [9], TLR4 [38] and mannose-6-phosphate receptor/IGFII receptor [22] may bind DPP4. Bioinformatic analysis and the signaling signature induced by sDPP4 suggest that sDPP4 might be an agonist for PAR2. Due to the activation of proteases during pathological states such as haemorrhage, tissue damage, and inflammation, protease-activated receptors (PAR) have been suggested to play a crucial role in the development of functional and structural abnormalities in vascular lesions. Especially PAR2 has been reported to be upregulated in the SMCs of vascular lesions in humans [30] and playing an important role in the induction of SMC proliferation and migration *in vitro* [5,28]. PAR2 is activated by proteolytic cleavage of its N-terminus exposing a tethered ligand (TL) that then auto-activates the receptor. In addition, a synthetic peptide corresponding to the TL (SLIGKV) for PAR2 specifically activates PAR2 in the absence of proteases [19]. This activating sequence of PAR2 could be found in the cystein-rich region of DPP4 responsible for partner binding. We observed an almost perfect match for the first four amino acids, which are most important for the activation of PAR2 [1]. Within the tertiary structure of DPP4, the potential PAR2 TL sequence SLIG could be located on the protein surface. It might be speculated, that sDPP4 activates the PAR2 receptor with its TL sequence and that DPP4-inhibitors affect the binding properties of sDPP4 by a conformational change or masking the PAR2 activating sequence. Since sDPP4 is a serine exopeptidase that cleaves dipeptides from proteins carrying proline or alanine in their penultimate position and the cleaving side of PAR2 is located between residue R^{36} and S^{37}, it is most unlikely that sDPP4 cleaves the N-terminus of PAR2 thereby exposing the TL and activating the receptor. In line with our data of the sDPP4-induced signaling, a stimulation with the PAR2-AP leads to NF-κB [7] and ERK1/2 [23] activation resulting in SMCs mitogenesis. Regarding the sDPP4 effects on cytokine secretion, in human neutrophils, it could be shown that the PAR2-AP induces IL-6 and IL-8 secretion [37,37]. In cells expressing

less PAR2 like monocytes, Ikeda *et al.* [21] could show no direct effects of sDPP4 on IL-6 and TNF-α, while the combination of LPS with sDPP4 enhances the expression and secretion of these pro-inflammatory cytokines. This study suggests that sDPP4 is also able to interact with TLR4 signaling cascade but the exact mechanism remains unclear.

DPP4-inhibitors, a class of oral anti-hyperglycemic agents that prolong the bioavailability of the endogenously secreted incretin hormone GLP-1 and the glucose-dependent insulinotropic polypeptide (GIP), are effective in the treatment of type 2 diabetes. DPP4-inhibitors have been validated and approved as drugs that can lower both fasting and postprandial glucose levels and improve islet β-cell function in patients with diabetes [13]. In addition to their anti-diabetic properties, accumulating data indicate that DPP4-inhibitors also have important protective effects on the cardiovascular system. Studies have shown that DPP4-inhibitors could decrease myocardial infarct size, stabilize the cardiac electrophysiological state during myocardial ischemia, reduce ischemia/reperfusion injury, and prevent left ventricular remodeling following myocardial infarction [10,40]. Additionally, DPP4-inhibitors also have vascular protective effects, including anti-inflammatory and anti-atherosclerotic effects and the ability to induce vascular relaxation [29,35]. Although these beneficial effects are ascribed to an increased bioavailability of GLP-1, accumulating data suggest that DPP4-inhibitors exert vascular protective effects independent of GLP-1. Ta et al. [38] could demonstrate that the specific DPP4-inhibitor alogliptin blocked LPS-induced ERK phosphorylation in U937 histiocytes, representing a model of cells involved in the progression of atherosclerosis, such as foam cells. Downstream of ERK, the inhibition of DPP4 prevented the activation of matrix metalloproteinases (MMP) via the Toll-like receptor pathway, which contributes to the destabilization of atherosclerotic plaques leading to acute vascular events. Whether the observed effects could be ascribed to direct effects of sDPP4 or to inhibition of enzymatic degradation of DPP4 substrates remained unknown. Furthermore, it could be demonstrated that sitagliptin prevents TNFα-induced PAI-1, ICAM and VCAM gene and protein expression in vascular endothelial cells independent of GLP-1 [20]. This effect could partially be explained by a reduction of the TNFα-induced NF-κB mRNA expression by sitagliptin. Additionally, incubation of human umbilical vein endothelial cells with the DPP4-inhibitor alogliptin resulted in endothelial nitric oxide synthase (eNOS) and Akt phosphorylation (Ser1177 and Ser473, respectively) paralleled by a rapid increase in nitric oxide [36]. These data suggest that DPP4 inhibition might play a pivotal role in

prevention of endothelial dysfunction. Nevertheless, direct effects of sDPP4 were not taken into consideration in most of these studies. In a recently published study, a sDPP4-induced short-term activation of ERK1/2 in rat vSMC was observed, which could partially be blocked by DPP4 inhibition [14]. However, here we observed a very robust and long-lasting ERK1/2 activation, which can completely be prevented by DPP4 inhibition. Moreover, by using an ERK1/2-inhibitor, we could confirm that the sDPP4-induced proliferation is ERK-dependent. Thus, our data strongly support a direct role for sDPP4 in regulating vascular function independently of GLP-1.

Conclusion

In conclusion, in this study we characterized a novel sDPP4-induced and receptor-mediated signaling cascade in hVSMC. sDPP4 directly and markedly activates the MAPK- and NF-κB signaling pathway in a PAR2 dependent manner leading to pro-atherogenic changes in hVSMC like increased proliferation and inflammation. Thus, this data might contribute to explain the vascular protective effects of DPP4-inhibitors independent of GLP-1. Considering that sDPP4 is an adipokine with increased circulating levels in obesity, it can be speculated that sDPP4 may act in a para- or endocrine fashion on the vascular wall. Our data support the notion that direct effects of sDPP4 may play a role in linking obesity to CVD.

Acknowledgements

This work was supported by the Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen (Ministry of Science and Research of the State of North Rhine-Westphalia), the Bundesministerium für Gesundheit (Federal Ministry of Health), the Deutsche Forschungsgemeinschaft (SE1922/2-2), an EFSD-MSD grant, an EFSD/Janssen Research Fellowship, a DAAD-MICINN PPP (PRI-AIDBE-2011-0811) and Plan Nacional de I+D (SAF2011-28011; SAF2011-24648). TR is the recipient of a FP7 Marie Curie Intra-European fellowship. The technical assistance of Andrea Cramer and Marlis Koenen and the secretarial assistance of Birgit Hurow are gratefully acknowledged.

Conflicts of interest

The authors declare that they have no conflict of interest.

Figures

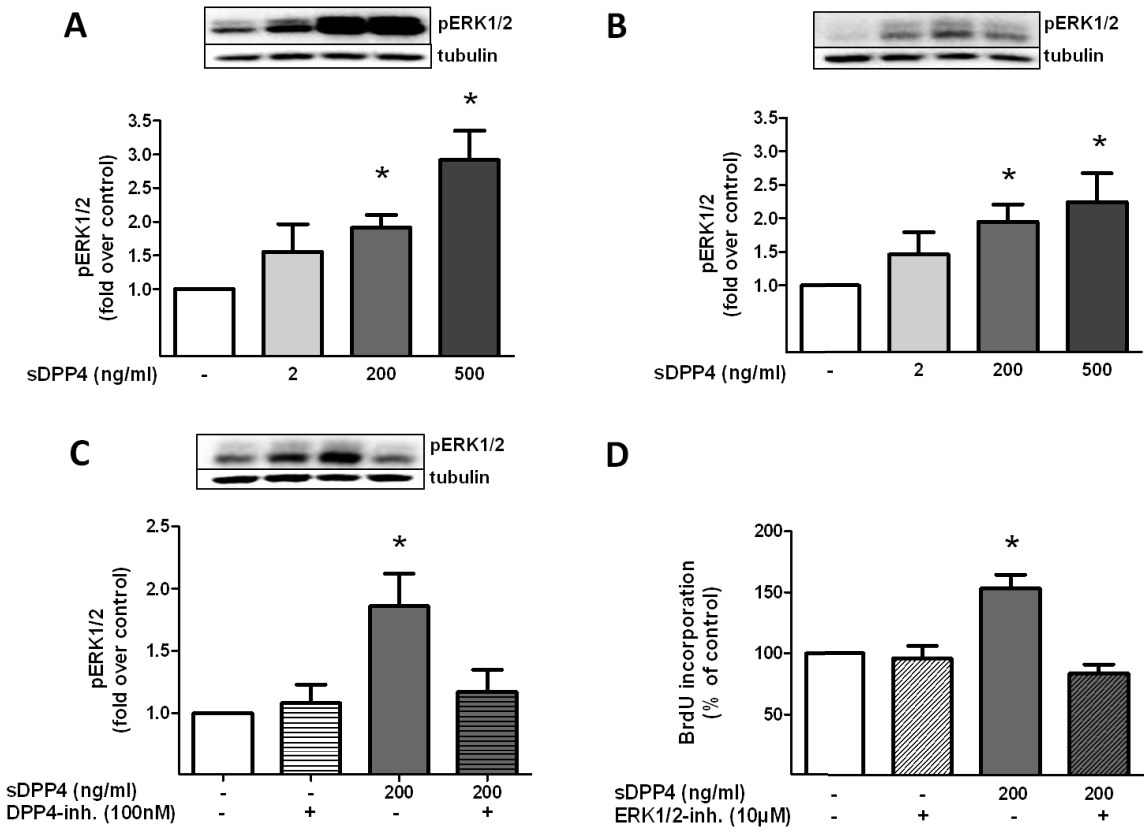


Figure 1 Effect of sDPP4 on the ERK1/2 signaling pathway in hVSMC. Cells were serum-starved for 24 h and subsequently treated with the indicated amounts of sDPP4 with and without concomitant administration of the specific DPP4-inhibitor K579 for 6 **(A,C)** and 24 h **(B)**. **(A-C)** Total cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody to the phosphorylated form of ERK1/2. Data are tubulin normalized mean values ± SEM of four independent experiments. *$p < 0.05$ compared to control hVSMC. Representative Western Blots are presented. **(D)** hVSMC were treated with sDPP4 alone or in combination with the ERK1/2 Inhibitor PD98059 for 24 h. Proliferation of hVSMC was determined by measuring the incorporation of BrdU into DNA. Data are expressed relative to the basal control value, taken as 100%. Data are mean values ± SEM of three independent experiments. *$p < 0.05$ compared to control hVSMC

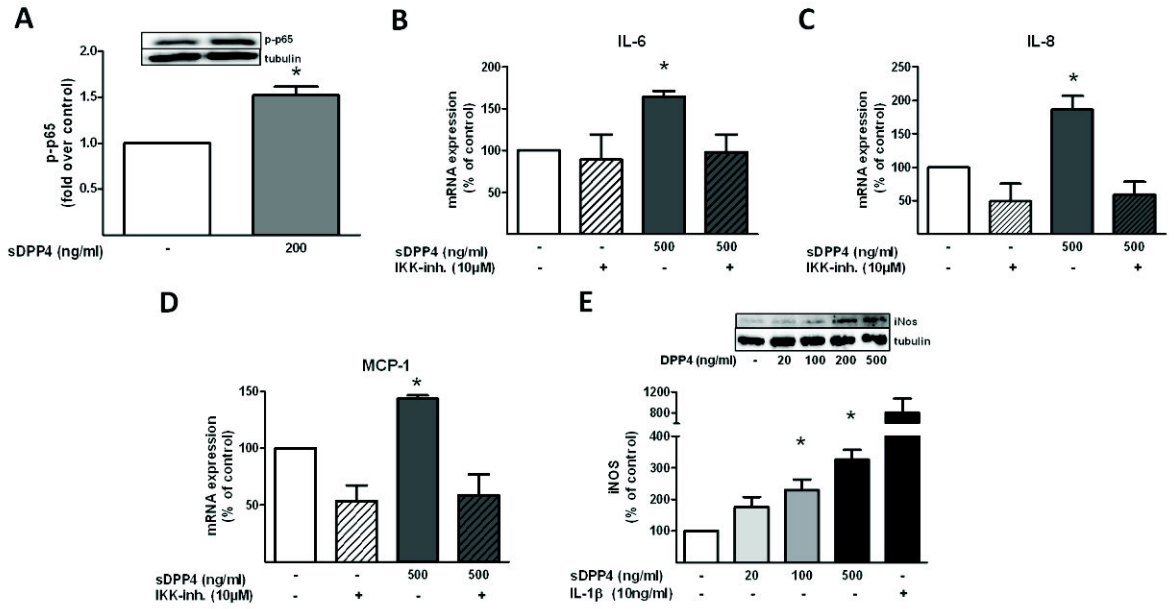


Figure 2 Impact of sDPP4 on the NF-κB signaling pathway and the expression of pro-inflammatory cytokines in hVSMC. (A) After 24h serum-starvation, cells were treated with the indicated amounts of sDPP4 for 6 h. Phosphorylation of the NF-κB subunit p65 was assessed by Western Blot analysis. Data are tubulin normalized mean values ± SEM of four independent experiments. *p < 0.05 compared to control hVSMC. Representative Western Blots are presented. **(B-D)** Cells were serum-starved for 24h and then treated with indicated concentrations of sDPP4 alone or in the combination with the IKK-inhibitor I229 for 24 h. mRNA level of IL-6 **(B)**, IL-8 **(C)** and MCP-1 **(D)** were quantified by Real-time PCR and normalized to the level of β-actin. Data are expressed relative to the basal control value and are means ± SEM of three independent experiments. *p < 0.05 compared to control hVSMC. **(E)** After 24 h serum-starvation, hVSMC were exposed to the indicated amounts of sDPP4 for 18 h. The expression of iNOS was assessed by Western Blot analysis and normalized to tubulin. Representative Western Blot and the respective quantification are presented. IL-1β was used as a positive control. Data are expressed relative to the basal control value and are means ± SEM of four independent experiments. *p < 0.05 compared to control hVSMC

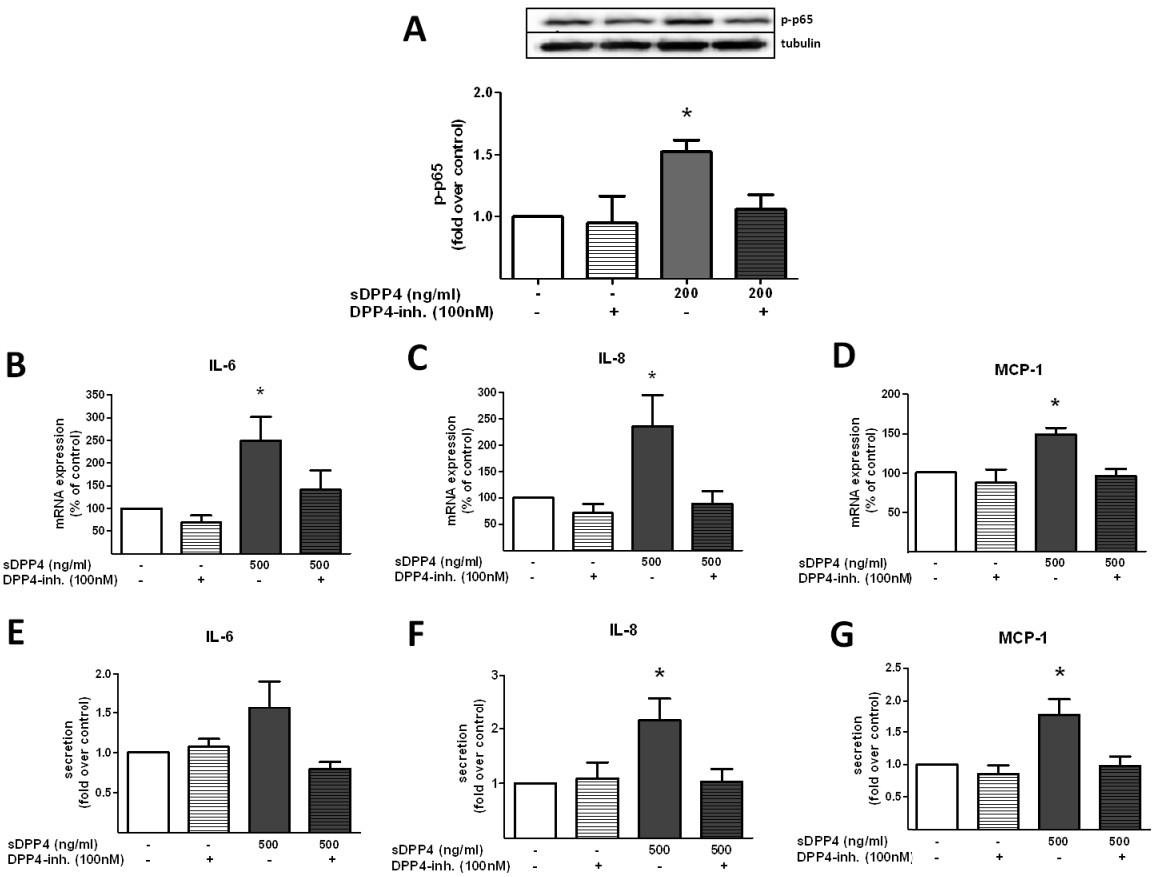


Figure 3 Effect of the DPP4-inhibitor on the sDPP4-induced cytokine expression and secretion. **(A)** After 24h serum-starvation, cells were treated with indicated amounts of sDPP4 alone or in the combination with the DPP4-inhibitor K579 for 6 h. Phosphorylation of the NF-κB subunit p65 was assessed by Western Blot analysis. Data are tubulin normalized mean values ± SEM of four independent experiments. *p < 0.05 compared to control hVSMC. Representative Western Blots are presented. **(B-D)** hVSMC were serum-starved for 24 h and then treated with the indicated amounts of sDPP4 alone or in the combination with the DPP4-inhibitor K579 for 24 h. mRNA level of IL-6 **(B)**, IL-8 **(C)** and MCP-1 **(D)** were quantified by Real-time PCR and normalized to the level of β-actin. Data are expressed relative to the basal control value and are means ± SEM of three independent experiments. *p < 0.05 compared to control hVSMC. **(E-G)** After 24 h exposure to the respective treatments, supernatants were collected and IL-6 **(E)**, IL-8 **(F)** and MCP-1 **(G)** concentration was measured by ELISA assay. Data are expressed relative to the basal control value and are means ± SEM of four independent experiments. *p < 0.05 compared to control hVSMC

A

```
human DPP4   PSYRITWTGKEDIIYNGITDWVYEEEVFSAYSALWWSPNGTFLAYAQFNDTEVPLIEYSF 240
PAR2-TL      ------------------------------------------------------------

human DPP4   YSDESLQYPKTVRVPYPKAGAVNPTVKFFVVNTDSLSSVTNATSIQITAPASMLIGDHYL 300
PAR2-TL      ------------------------------------------------S-LIG---- 4
                                                              * ***

human DPP4   CDVTWATQERISLQWLRRIQNYSVMDICDYDESSGRWNCLVARQHIEMSTTGWVGRFRPS 360
PAR2-TL      -KV-------------------------------------------------------- 6
              .*

human DPP4   EPHFTLDGNSFYKIISNEEGYRHICYFQIDKKDCTFITKGTWEVIGIEALTSDYLYYISN 420
PAR2-TL      ------------------------------------------------------------
```

B

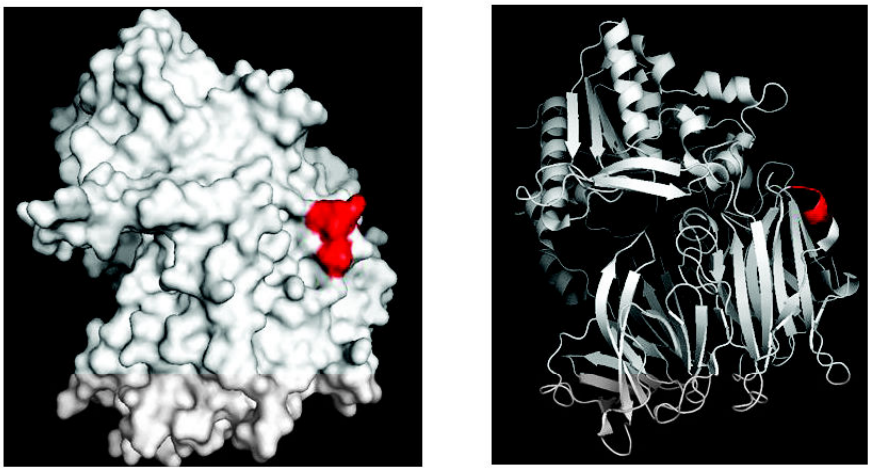


Figure 4 Homology between DPP4 and the tethered ligand (TL) sequence of PAR2. **(A)** CLUSTALW was used to align the sequence of the human DPP4 (P27487 UniProtKB) and the TL sequence of PAR2 (SLIGKV). The TL sequence is also aligned to the sequence of the crystallized DPP4 (PDB ID 4A5S). **(B)** Surface representation (left) and ribbon diagram (right) of the crystal structure human DPP4. The potential TL sequence (SLIG) is highlighted in red. Images were created with PyMol (PyMOL v1.6)

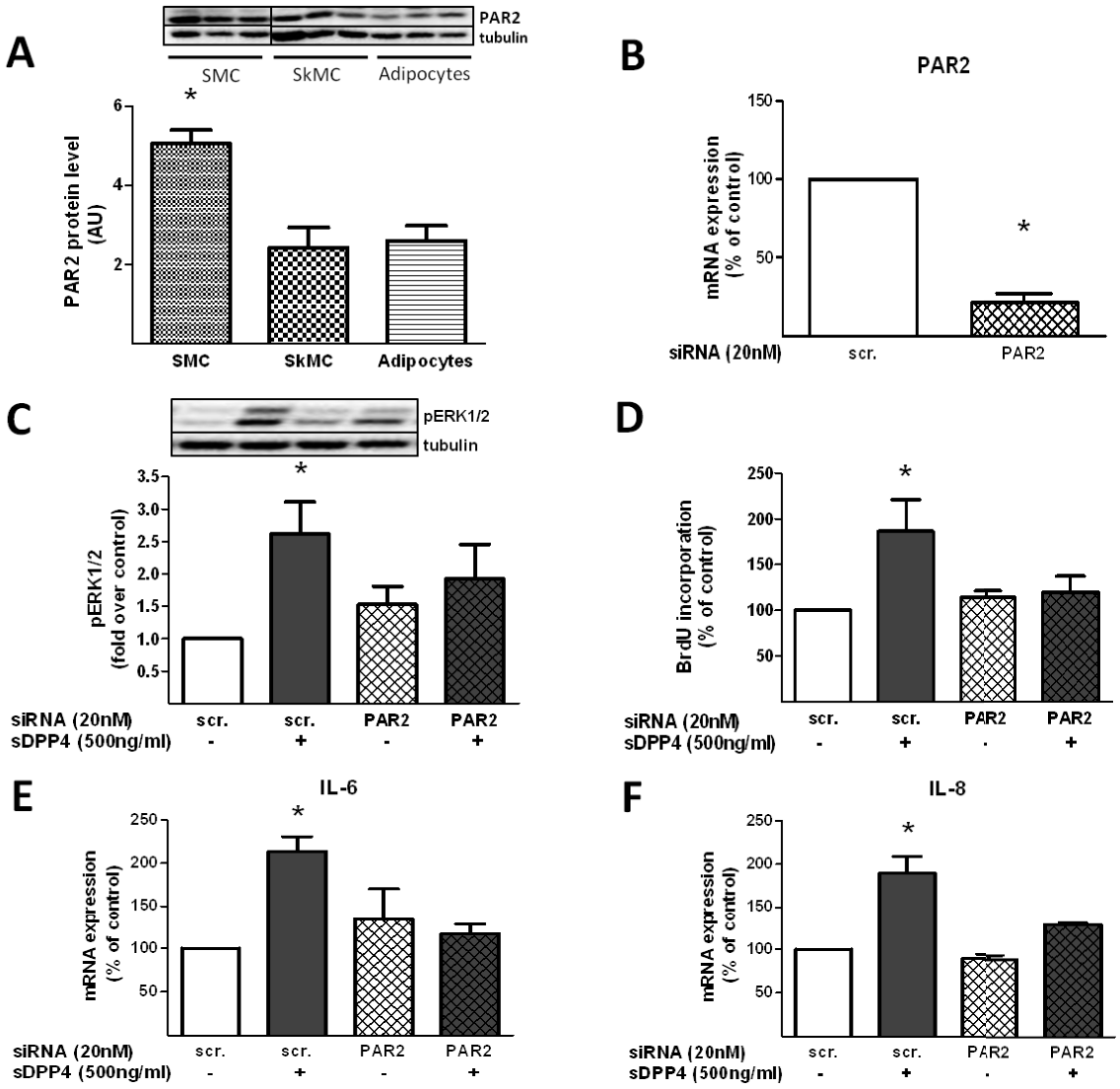


Figure 5 Impact of PAR2 silencing on the sDPP4-induced proliferation and inflammation. **(A)** Expression of PAR2 in three different primary human cell types, namely SMC, *in vitro* differentiated skeletal muscle cells (SkMC) and adipocytes was assessed by Western Blot. For each cell type three different donors were analyzed. Data are tubulin normalized and expressed as mean values ± SEM. *p < 0.05 (n = 3). Representative Western Blots are presented. **(B)** Cells were transfected either with 20 nM scramble or PAR2 siRNA. 24 h after transfection PAR2 mRNA levels were quantified by Real-time PCR and normalized to the level of β-actin. Data are expressed relative to the negative control value and are means ± SEM of three independent experiments. *p < 0.05 compared to control hVSMC. **(C)** 24h after transfection hVSMCs were exposed to sDPP4 for 6 h. Phosphorylation of ERK1/2 was assessed by Western Blot analysis. Data are tubulin normalized mean values ± SEM of six independent experiments. *p < 0.05 compared to control hVSMC. Representative Western Blots are presented. **(D)** 24 h after transfection hVSMCs were exposed to sDPP4 for further 24 h. Proliferation of hVSMC was determined as described before. Data are expressed relative to the negative control value, taken as 100%. Data are mean values ± SEM of three independent experiments. *p < 0.05 compared to control hVSMC. **(E,F)** 24h after transfection hVSMCs were exposed to sDPP4 for further 24 h. IL-6 **(E)** and IL-8 **(F)** mRNA levels were quantified by Real-time PCR and normalized to the level of β-actin. Data are expressed relative to the negative control value and are means ± SEM of three independent experiments. *p < 0.05 compared to control hVSMC

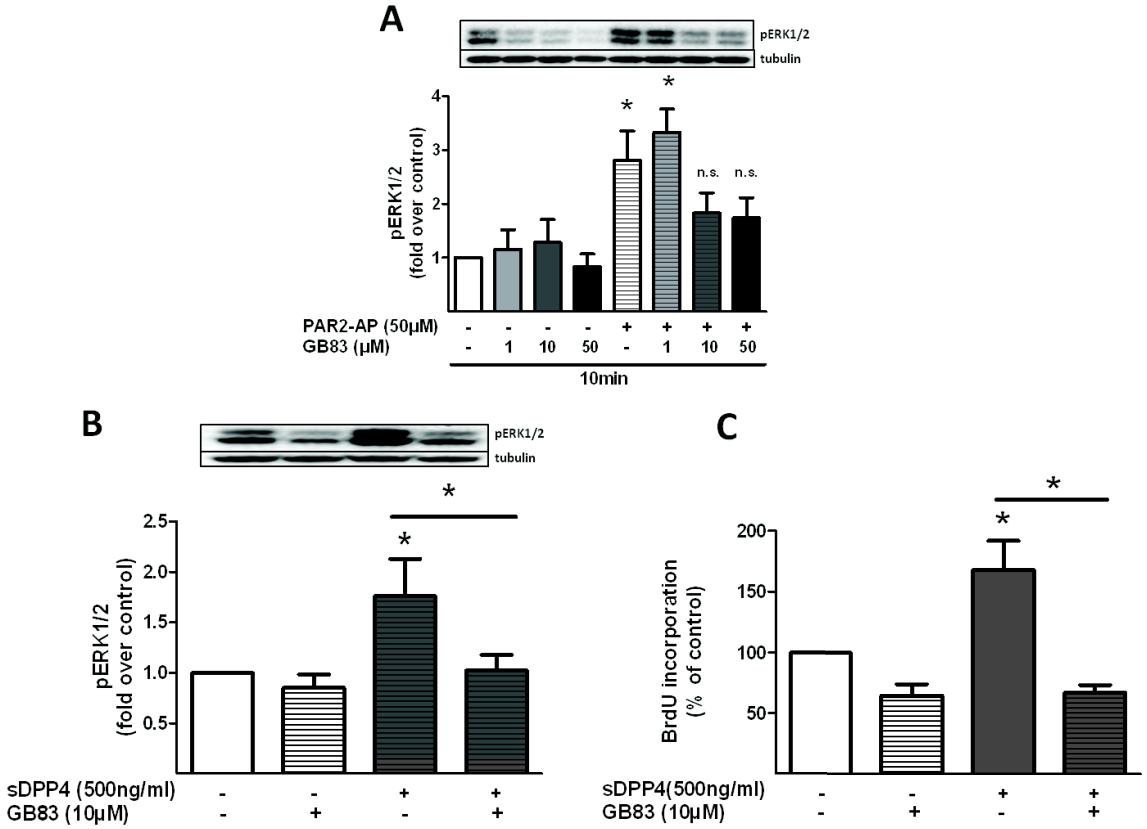


Figure 6 Impact of the PAR2 antagonist GB83 on sDPP4-induced ERK signaling and proliferation. **(A)** Cells were pretreated for 1 h with the indicated amounts of the PAR2 antagonist and then stimulated with the PAR2-activating peptide (AP) (SLIGKV) for 10 min. Phosphorylation of ERK1/2 was assessed by western blot analysis. Data are tubulin normalized mean values ± SEM of three independent experiments. *p < 0.05 compared to control hVSMC. Representative Western Blot is presented. **(B)** After 1 h pre-incubation with the PAR2 antagonist GB83, hVSMC were treated with sDPP4 for 6 h. Phosphorylation of ERK1/2 was assessed by western blot analysis. Data are tubulin normalized mean values ± SEM of three independent experiments. *p < 0.05 compared to control hVSMC. Representative Western Blot is presented. **(C)** Cells were treated 24 h with sDPP4 alone or in combination with the PAR2 antagonist GB83. Proliferation of hVSMC was determined as described before. Data are expressed relative to the negative control value, taken as 100%. Data are mean values ± SEM of four independent experiments. *p < 0.05 compared to control hVSMC

References

[1] M.N. Adams, R. Ramachandran, M.K. Yau, J.Y. Suen, D.P. Fairlie, M.D. Hollenberg, J.D. Hooper, Structure, function and pathophysiology of protease activated receptors, Pharmacol. Ther. 130 (2011) pp. 248-282.

[2] B. Ahren, Dipeptidyl peptidase-4 inhibitors: clinical data and clinical implications, Diabetes Care 30 (2007) pp. 1344-1350.

[3] P. Anagnostis, V.G. Athyros, F. Adamidou, A. Panagiotou, M. Kita, A. Karagiannis, D.P. Mikhailidis, Glucagon-like peptide-1-based therapies and cardiovascular disease: looking beyond glycaemic control, Diabetes Obes. Metab 13 (2011) pp. 302-312.

[4] G. Anfossi, I. Russo, G. Doronzo, M. Trovati, Contribution of insulin resistance to vascular dysfunction, Arch. Physiol Biochem. 115 (2009) pp. 199-217.

[5] F. Bono, I. Lamarche, J.M. Herbert, Induction of vascular smooth muscle cell growth by selective activation of the proteinase activated receptor-2 (PAR-2), Biochem. Biophys. Res. Commun. 241 (1997) pp. 762-764.

[6] M. Braun, P. Pietsch, K. Schror, G. Baumann, S.B. Felix, Cellular adhesion molecules on vascular smooth muscle cells, Cardiovasc. Res. 41 (1999) pp. 395-401.

[7] E. Bretschneider, R. Kaufmann, M. Braun, M. Wittpoth, E. Glusa, G. Nowak, K. Schror, Evidence for proteinase-activated receptor-2 (PAR-2)-mediated mitogenesis in coronary artery smooth muscle cells, Br. J. Pharmacol. 126 (1999) pp. 1735-1740.

[8] P. Calabro and E.T. Yeh, Intra-abdominal adiposity, inflammation, and cardiovascular risk: new insight into global cardiometabolic risk, Curr. Hypertens. Rep. 10 (2008) pp. 32-38.

[9] H.C. Cheng, M. Abdel-Ghany, B.U. Pauli, A novel consensus motif in fibronectin mediates dipeptidyl peptidase IV adhesion and metastasis, J. Biol. Chem. 278 (2003) pp. 24600-24607.

[10] K. Chinda, S. Palee, S. Surinkaew, M. Phornphutkul, S. Chattipakorn, N. Chattipakorn, Cardioprotective effect of dipeptidyl peptidase-4 inhibitor during ischemia-reperfusion injury, Int. J. Cardiol. (2012).

[11] M.R. D'Andrea, C.K. Derian, D. Leturcq, S.M. Baker, A. Brunmark, P. Ling, A.L. Darrow, R.J. Santulli, L.F. Brass, P. Andrade-Gordon, Characterization of protease-activated receptor-2 immunoreactivity in normal human tissues, J. Histochem. Cytochem. 46 (1998) pp. 157-164.

[12] A.D. Dobrian, Q. Ma, J.W. Lindsay, K.A. Leone, K. Ma, J. Coben, E.V. Galkina, J.L. Nadler, Dipeptidyl peptidase IV inhibitor sitagliptin reduces local inflammation in adipose tissue and in pancreatic islets of obese mice, Am. J. Physiol Endocrinol. Metab 300 (2011) p.E410-E421.

[13] D.J. Drucker, Dipeptidyl peptidase-4 inhibition and the treatment of type 2 diabetes: preclinical biology and mechanisms of action, Diabetes Care 30 (2007) pp. 1335-1343.

[14] N. Ervinna, T. Mita, E. Yasunari, K. Azuma, R. Tanaka, S. Fujimura, D. Sukmawati, T. Nomiyama, A. Kanazawa, R. Kawamori, Y. Fujitani, H. Watada, Anagliptin, a DPP-4 inhibitor, suppresses proliferation of vascular smooth muscles and monocyte inflammatory reaction and attenuates atherosclerosis in male apo E-deficient mice, Endocrinology 154 (2013) pp. 1260-1270.

[15] G.P. Fadini and A. Avogaro, Cardiovascular effects of DPP-4 inhibition: beyond GLP-1, Vascul. Pharmacol. 55 (2011) pp. 10-16.

[16] G.P. Fadini, E. Boscaro, M. Albiero, L. Menegazzo, V. Frison, K.S. de, C. Agostini, A. Tiengo, A. Avogaro, The oral dipeptidyl peptidase-4 inhibitor sitagliptin increases circulating endothelial progenitor cells in patients with type 2 diabetes: possible role of stromal-derived factor-1alpha, Diabetes Care 33 (2010) pp. 1607-1609.

[17] M.D. Gorrell, V. Gysbers, G.W. McCaughan, CD26: a multifunctional integral membrane and secreted protein of activated lymphocytes, Scand. J. Immunol. 54 (2001) pp. 249-264.

[18] S.M. Grundy, Obesity, metabolic syndrome, and cardiovascular disease, J. Clin. Endocrinol. Metab 89 (2004) pp. 2595-2600.

[19] M.D. Hollenberg, M. Saifeddine, B. al-Ani, A. Kawabata, Proteinase-activated receptors: structural requirements for activity, receptor cross-reactivity, and receptor selectivity of receptor-activating peptides, Can. J. Physiol Pharmacol. 75 (1997) pp. 832-841.

[20] Y. Hu, H. Liu, R.W. Simpson, A.E. Dear, GLP-1-dependent and independent effects and molecular mechanisms of a dipeptidyl peptidase 4 inhibitor in vascular endothelial cells, Mol. Biol. Rep. 40 (2013) pp. 2273-2279.

[21] T. Ikeda, E. Kumagai, S. Iwata, A. Yamakawa, Soluble CD26/Dipeptidyl Peptidase IV Enhances the Transcription of IL-6 and TNF-alpha in THP-1 Cells and Monocytes, PLoS. One. 8 (2013) p.e66520.

[22] H. Ikushima, Y. Munakata, S. Iwata, K. Ohnuma, S. Kobayashi, N.H. Dang, C. Morimoto, Soluble CD26/dipeptidyl peptidase IV enhances transendothelial migration via its interaction with mannose 6-phosphate/insulin-like growth factor II receptor, Cell Immunol. 215 (2002) pp. 106-110.

[23] B.H. Koo, K.H. Chung, K.C. Hwang, D.S. Kim, Factor Xa induces mitogenesis of coronary artery smooth muscle cell via activation of PAR-2, FEBS Lett. 523 (2002) pp. 85-89.

[24] P. Lacolley, V. Regnault, A. Nicoletti, Z. Li, J.B. Michel, The vascular smooth muscle cell in arterial pathology: a cell that can take on multiple roles, Cardiovasc. Res. 95 (2012) pp. 194-204.

[25] A.M. Lambeir, C. Durinx, S. Scharpe, M. De, I, Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV, Crit Rev. Clin. Lab Sci. 40 (2003) pp. 209-294.

[26] D. Lamers, S. Famulla, N. Wronkowitz, S. Hartwig, S. Lehr, D.M. Ouwens, K. Eckardt, J.M. Kaufman, M. Ryden, S. Muller, F.G. Hanisch, J. Ruige, P. Arner, H. Sell, J. Eckel, Dipeptidyl peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome, Diabetes 60 (2011) pp. 1917-1925.

[27] D. Lamers, R. Schlich, S. Greulich, S. Sasson, H. Sell, J. Eckel, Oleic acid and adipokines synergize in inducing proliferation and inflammatory signalling in human vascular smooth muscle cells, J. Cell Mol. Med. 15 (2011) pp. 1177-1188.

[28] K. Marutsuka, K. Hatakeyama, Y. Sato, A. Yamashita, A. Sumiyoshi, Y. Asada, Protease-activated receptor 2 (PAR2) mediates vascular smooth muscle cell migration induced by tissue factor/factor VIIa complex, Thromb. Res. 107 (2002) pp. 271-276.

[29] J. Matsubara, S. Sugiyama, K. Sugamura, T. Nakamura, Y. Fujiwara, E. Akiyama, H. Kurokawa, T. Nozaki, K. Ohba, M. Konishi, H. Maeda, Y. Izumiya, K. Kaikita, H. Sumida, H. Jinnouchi, K. Matsui, S. Kim-Mitsuyama, M. Takeya, H. Ogawa, A dipeptidyl peptidase-4 inhibitor, des-fluoro-sitagliptin, improves endothelial function and reduces atherosclerotic lesion formation in apolipoprotein E-deficient mice, J. Am. Coll. Cardiol. 59 (2012) pp. 265-276.

[30] C. Napoli, N.F. de, J.L. Wallace, M.D. Hollenberg, G. Tajana, R.G. De, V. Sica, G. Cirino, Evidence that protease activated receptor 2 expression is enhanced in human coronary atherosclerotic lesions, J. Clin. Pathol. 57 (2004) pp. 513-516.

[31] T. Okerson and R.J. Chilton, The cardiovascular effects of GLP-1 receptor agonists, Cardiovasc. Ther. 30 (2012) p.e146-e155.

[32] H.S. Park, J.Y. Park, R. Yu, Relationship of obesity and visceral adiposity with serum concentrations of CRP, TNF-alpha and IL-6, Diabetes Res. Clin. Pract. 69 (2005) pp. 29-35.

[33] H. Sell, M. Blüher, N. Klöting, R. Schlich, M. Willems, F. Ruppe, W. Knoefel, A. Dietrich, P. Arner, K. Frayn, J. Eckel, Adipose Dipeptidyl Peptidase-4 and Obesity: Correlation with insulin resistance and depot-specific release from adipose tissue in vivo and in vitro, Diabetes Care (2013).

[34] H. Sell, J. Laurencikiene, A. Taube, K. Eckardt, A. Cramer, A. Horrighs, P. Arner, J. Eckel, Chemerin is a novel adipocyte-derived factor inducing insulin resistance in primary human skeletal muscle cells, Diabetes 58 (2009) pp. 2731-2740.

[35] Z. Shah, T. Kampfrath, J.A. Deiuliis, J. Zhong, C. Pineda, Z. Ying, X. Xu, B. Lu, S. Moffatt-Bruce, R. Durairaj, Q. Sun, G. Mihai, A. Maiseyeu, S. Rajagopalan, Long-term dipeptidyl-peptidase 4 inhibition reduces atherosclerosis and inflammation via effects on monocyte recruitment and chemotaxis, Circulation 124 (2011) pp. 2338-2349.

[36] Z. Shah, C. Pineda, T. Kampfrath, A. Maiseyeu, Z. Ying, I. Racoma, J. Deiuliis, X. Xu, Q. Sun, S. Moffatt-Bruce, F. Villamena, S. Rajagopalan, Acute DPP-4 inhibition modulates vascular tone through GLP-1 independent pathways, Vascul. Pharmacol. 55 (2011) pp. 2-9.

[37] V.M. Shpacovitch, G. Varga, A. Strey, M. Gunzer, F. Mooren, J. Buddenkotte, N. Vergnolle, C.P. Sommerhoff, S. Grabbe, V. Gerke, B. Homey, M. Hollenberg, T.A. Luger, M. Steinhoff, Agonists of proteinase-activated receptor-2 modulate human neutrophil cytokine secretion, expression of cell adhesion molecules, and migration within 3-D collagen lattices, J. Leukoc. Biol. 76 (2004) pp. 388-398.

[38] N.N. Ta, Y. Li, C.A. Schuyler, M.F. Lopes-Virella, Y. Huang, DPP-4 (CD26) inhibitor alogliptin inhibits TLR4-mediated ERK activation and ERK-dependent MMP-1 expression by U937 histiocytes, Atherosclerosis 213 (2010) pp. 429-435.

[39] K.E. Wellen and G.S. Hotamisligil, Inflammation, stress, and diabetes, J. Clin. Invest 115 (2005) pp. 1111-1119.

[40] Y. Ye, K.T. Keyes, C. Zhang, J.R. Perez-Polo, Y. Lin, Y. Birnbaum, The myocardial infarct size-limiting effect of sitagliptin is PKA-dependent, whereas the protective effect of pioglitazone is partially dependent on PKA, Am. J. Physiol Heart Circ. Physiol 298 (2010) p.H1454-H1465.

[41] W. Zhai, W. Jungraithmayr, M. De, I, I. Inci, K. Augustyns, S. Arni, S. Hillinger, S. Scharpe, W. Weder, S. Korom, Primary graft dysfunction in lung transplantation: the role of CD26/dipeptidylpeptidase IV and vasoactive intestinal peptide, Transplantation 87 (2009) pp. 1140-1146.

[42] D. Zhang, W. Huang, B. Dai, T. Zhao, A. Ashraf, R.W. Millard, M. Ashraf, Y. Wang, Genetically manipulated progenitor cell sheet with diprotin A improves myocardial function and repair of infarcted hearts, Am. J. Physiol Heart Circ. Physiol 299 (2010) p.H1339-H1347.

Journal: *Biochimica et Biophysica Acta – Molecular Basis of Disease*

Impact factor: 4.910

Contribution: Total: 90%

Conceived / designed experiments: 80%

Performed experiments: 100%

Analysed data: 100%

Contributed to discussion: 80%

Wrote the manuscript: 90%

Reviewed / edited manuscript: 90%

Author: 1st author

CHAPTER 4

Study 3

Adipocyte-derived factors impair insulin signaling in differentiated human vascular smooth muscle cells via the upregulation of miR-143†

Marcel Blumensatt[1*], Nina Wronkowitz[2*], Claudia Wiza[1], Andrea Cramer[2], Heidi Mueller[1], Martijn J. Rabelink[3], Rob C. Hoeben[3], Juergen Eckel[2], Henrike Sell[2] and D. Margriet Ouwens[1,4]

[1]*Institute for Clinical Biochemistry and Pathobiochemistry, German Diabetes Center, Duesseldorf, Germany*

[2]*Paul-Langerhans-Group for Integrative Physiology, German Diabetes Center, Duesseldorf, Germany*

[3]*Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands*

[4]*Department of Endocrinology, Ghent University Hospital, Ghent, Belgium*

** both authors contributed equally to this work*

† *Biochimica et Biophysica Acta - Molecular Basis of Disease*, in press

Abstract

Cardiovascular complications are common in patients with type 2 diabetes. Adipokines have been implicated in the induction of proliferative and pro-atherogenic alterations in human vascular smooth muscle cells (hVSMC). Other reports demonstrated the importance of the miRNA cluster miR-143/145 in the regulation of VSMC homeostasis and insulin sensitivity. Here we investigated whether the detrimental effects of adipokines on hVSMC function could be ascribed to alterations in miR-143/145 expression. Exposure of hVSMC to conditioned media (CM) from primary human subcutaneous adipocytes increased the expression of smooth muscle α-actin (SMA), and the miR-143/145 cluster, but markedly impaired the insulin-mediated phosphorylation of Akt and its substrate endothelial nitric oxide synthase (eNOS). Furthermore, CM promoted the phosphorylation of SMAD2 and p38, which have both been linked to miR-143/145 induction. Accordingly, the induction of miR-143/145 as well as the inhibition of insulin-mediated Akt- and eNOS-phosphorylation was prevented when hVSMC were treated with pharmacological inhibitors for Alk-4/5/7 and p38 before the addition of CM. Transfection of hVSMC with precursor miR-143, but not with precursor miR-145, resulted in impaired insulin-mediated phosphorylation of Akt and eNOS. This inhibition of insulin signaling by CM and miR-143 is associated with a reduction in the expression of the oxysterol-binding protein-related protein 8 (ORP8). Finally, knock-down of ORP8 resulted in impaired insulin-mediated phosphorylation of Akt in hVSMC. Thus, the detrimental effects of adipocyte-derived conditioned media on insulin action in primary hVSMC can be ascribed to the Alk- and p38-dependent induction of miR-143 and subsequent downregulation of ORP8.

Highlights

- adipocyte-derived factors impair insulin action in vascular smooth muscle cells
- adipocyte-derived factors induce miR-143 in vascular smooth muscle cells
- miR-143 impairs insulin action via downregulation of ORP8

Keywords

vascular smooth muscle cells, insulin signaling, p38, adipokines, miRNA

Introduction

Cardiovascular complications are common in patients with type 2 diabetes and a major cause of mortality [12]. Accumulating evidence shows that adipose tissue secreted factors, termed adipokines, may participate in the development of cardiovascular complications in patients with type 2 diabetes by affecting the function of amongst others cardiomyocytes and smooth muscle cells (SMC) [11,20,30].

Vascular endothelial cells and SMC represent the major cell types of the artery wall preserving vessel wall homeostasis. SMC are highly plastic and modulate their phenotype in response to physiological and pathological cues. Differentiated SMC are quiescent and contractile. In response to vascular injury or growth factor signaling, SMC de-differentiate and adopt a proliferative, migratory phenotype that contributes to vascular occlusion in a variety of disorders, including atherosclerosis [28]. Alterations in vascular insulin signaling may also participate in the development of cardiovascular dysfunction in type 2 diabetes. The insulin-mediated activation of phosphatidylinositol 3'-kinase (PI3K) results in the activation of Akt, which on its turn promotes the phosphorylation of endothelial nitric oxide synthase (eNOS) [25]. This results in activation of eNOS and an increase in bioavailable nitric oxide (NO) thereby promoting vasodilation [25]. In the vasculature of animal models of insulin resistance, such as obese Zucker (fa/fa) rats, the insulin-mediated activation of the PI3K-pathway is impaired [16]. Intriguingly, this is paralleled by an overactivation of the mitogen activated protein kinase (MAPK) pathway, which on its turn promotes proliferation of vascular cells [16,25]. Studies on mouse models with lacking the insulin receptor in either the endothelium or the entire vasculature have further highlighted the importance of vascular insulin action, and specifically the Akt/eNOS-axis, for the regulation of vasorelaxation *in vivo* [9,31].

Previously we showed that conditioned media (CM) prepared from human adipocytes induces pro-atherogenic changes in primary human vascular smooth muscle cells (hVSMC) as illustrated by an increase in proliferation and migration, thus indicating an involvement of adipokines in the development of atherosclerosis [20,30]. However, until

now the underlying mechanisms, which are responsible for hVSMC dysfunction induced by adipokines remain incompletely understood. In cardiomyocytes, we observed that conditioned media generated from epicardial adipose tissue from patients with type 2 diabetes induce cardiomyocyte dysfunction as illustrated by contractile dysfunction and insulin resistance [11]. Furthermore, we found that the induction of insulin resistance in cardiomyocytes could be ascribed to the increases in miR-143 expression [1]. Also in other tissues, such as the liver and adipose tissue, the induction of miR-143 is associated with obesity and insulin resistance [18,32]. Interestingly, miR-143 and the co-transcribed miR-145 are highly expressed in the smooth muscle cell lineage [2]. Studies on miR143/145-deficient mice showed that this cluster is required for the acquisition of the contractile phenotype [2]. This is established through the activation of a transcriptional network, which promotes the differentiation of smooth muscle cells [8]. Among the genes targeted by the miR-143/145-cluster are the smooth muscle differentiation markers transgelin (TAGLN, also known as SM22), and smooth muscle α-actin (SMA) [8]. These observations suggest a dual role for the miR-143/145 in vascular smooth muscle cells. Therefore the aim of this study is to examine the role of the miR-143/145-cluster in primary human vascular smooth muscle cells (hVSMC) in more detail. This was achieved by studying the effects of CM on the expression of the miR-143/145-cluster and its potential target genes, and whether these effects associate with alterations in insulin action.

Material and Methods

Cell culture and differentiation of smooth muscle cells

Primary human coronary artery smooth muscle cells (hVSMC) from two different donors (Caucasian, female, 55 and 56 years old) were purchased from tebu-bio (Offenbach, Germany) and Lonza (Basel, Switzerland). The hVSMCs were supplied as proliferating cells and cultured according to the manufacturers' instructions. For experiments, differentiation was induced culturing the subconfluent cells of passage 3 for 14 days in smooth muscle cell differentiation medium (tebu-bio, Offenbach, Germany). The transition from the undifferentiated to the differentiated phenotype was judged on the basis of increases in the

protein abundance of smooth muscle cell differentiation markers, such as SMA, the transforming growth factor β (TGFβ) receptor II, and the bone morphogenic protein receptor II [8,13], as well as the lack of proliferation. Following differentiation, hVSMC were incubated for 24 hours in adipocyte-derived conditioned media or maintained in SMC serum-free basal medium (PromoCell, Heidelberg Germany). When indicated cells were treated for 1 hour with 10 µM SB431542 (Sigma Aldrich, St. Louis, MO) or 2.5 µM SB203580 (Promega, Mannheim, Germany) prior to the addition of the media. For insulin signaling, the cultures were stimulated for 10 min with 100 nM insulin.

Adipocyte isolation, culture and generating of conditioned media

Conditioned media (CM) were generated from mature subcutaneous adipocytes that were differentiated from pre-adipocytes isolated from subcutaneous adipose tissue obtained from lean or moderately overweight women (n= 13, body mass index 28.1 ± 1.3, and aged 39.0 ± 3.9 years) undergoing plastic surgery. The procedure was approved by the ethical committee of the Heinrich-Heine-University (Düsseldorf, Germany). All patients were healthy, free of medication and had no evidence of metabolic diseases according to routine laboratory tests. Pre-adipocytes were isolated by collagenase digestion of adipose tissue as described [14]. Isolated pre-adipocytes 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 $NaHCO_3$, pH 7.4) for 15 days with medium change every 2–3 days and addition of 5 µM troglitazone for the first 3 days. For the collection of CM, differentiated adipocytes were maintained for 48 hrs in SMC serum free basal medium (PromoCell) with addition of 50 ng/ml fungizone and 50 µg/ml gentamycin. Then CM was collected and stored as aliquots at -80^oC until further use.

Analysis of protein expression

For analysis of protein expression and phosphorylation, hVSMC were lysed in 50 mM HEPES, pH 7.4, 1% TritonX100 supplemented with protease and phosphatase inhibitor cocktails (Complete, PhosStop; Roche Diagnostics, Mannheim, Germany). After incubation for 2 hrs at 4°C on a rotation shaker, the suspension was centrifuged at 10,000g for 15 min, and protein content was determined using Bradford reagent (Biorad Laboratories, Munich, Germany). Thereafter, five microgram of protein was separated by SDS-PAGE and transferred to polyvenylidene fluorid membranes (Millipore, Schwalbach, Germany) in a semi-dry blotting apparatus [37]. Membranes were blocked with Tris-buffered saline containing 0.1% Tween and 5% non-fat dry milk and subsequently incubated overnight at 4°C with primary antibodies for Akt, phospho-Akt-Ser473, phospho-Akt-Thr308, phospho-proline-rich Akt substrate of 40-kDa (PRAS40) Thr246, phospho-p38-Thr180/Tyr182, phospho-SMAD2-Ser465/467 (all from Cell Signaling Technology, Danvers, MA, USA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), oxysterol-binding protein-related protein 8 (ORP-8) (abcam Cambridge, UK), smooth muscle α-actin (Sigma Aldrich), α-tubulin (Calbiochem Merck Biosciences, Schwalbach, Germany) insulin receptor β-subunit (IRβ), and phospho-eNOS-Ser1177 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, membranes were incubated with corresponding secondary HRP-coupled antibody (Promega). Bound conjugate was detected using enhanced chemiluminescence using Immobilon HRP substrate (Millipore, Billerica, MA, USA). Signals were visualized and quantitated on a Versadoc work station (VersaDoc 4000 MP; BioRad, Munich, Germany) using Quantity One software (BioRad, version 4.6.7).

RNA isolation, cDNA synthesis and qRT-PCR

For analysis of miRNA- and mRNA expression, total RNA was extracted using a miRNeasy mini kit (Qiagen, Hilden, Germany) and reverse transcribed using the miScript RT kit (Qiagen). Then miRNA expression levels were determined with miScript Primer Assays (Qiagen) using miScript SYBR Green (Qiagen) on a StepOne Plus real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Gene expression levels were determined after cDNA synthesis with the GoScript™ Reverse Transcription System (Promega) and GoTaq® qPCR Master Mix (Promega) using QuantiTect Primer Assays (Qiagen) for the amplification of

SMA, TAGLN, PAI-1, MCP-1, activin A, and POLR2A. To amplify RPS28 the following primers were designed using the Primerblast-tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and ordered from Eurogentec (Seraing, Belgium): forward-5'-GGTCTGTCACAGTCTGCTCC-3', and reverse-5'-CATCTCAGTTACGTGTGGCG-3'. Real-time PCR data were analyzed qualitatively with StepOne Plus software (version 2.1; Applied Biosystems) and quantitated using Qbase+ software (version 2.6; Biogazelle, Zwijnaarde, Belgium) in which the Ct-values obtained for RPS28 and POLR2A were used for normalization. The expression of RPS28 and POL2RA was not impacted by the experimental conditions tested as assessed with the GeNorm algorithm within the Qbase+ software [36].

Transfection of hVSMC with miRNA-precursor

To investigate the impact of differentially regulated miRNAs and their potential targets on insulin action, differentiated hVSMCs were transfected in 6-well dishes with 30 nmol/l Cy3™-labeled pre-miR™ (negative control) or pre-miR™ miRNA-precursor (Ambion, Life Technologies, Darmstadt, Germany) using Hiperfect (Qiagen) as transfection reagent. 48h after transfection in SMC serum free basal medium (PromoCell) cells are stimulated with insulin and lysed for protein isolation and western blot analysis.

Lentiviral vector-based Silencing of ORP8

To silence ORP8, three validated MISSION® shRNA constructs (TRCN000014 -6765, -7289 -7487) targeting human ORP8 (NM_020841) (Sigma Aldrich) or empty vector were used to produce infectious virus particles (LV). Therefore, HEK293t were transfected with the shRNA constructs together with helper plasmids encoding HIV-1 gag-pol, HIV-1 rev, and the VSV-G envelope as described previously [4]. For quantification of virus yield in the harvested medium, p24 antigen levels were determined using a HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (ZeptoMetrix Corp., New York, NY, USA). hVSMCs are transduced with a MOI of 2 for 24 hours. Two days after transduction of hVSMCs in serum free basal medium (PromoCell), cells were stimulated with 100 nM insulin for 10 minutes or kept untreated, and harvested.

Statistical analysis

Data are presented as means ± standard error of the mean. Significant differences between experimental conditions were evaluated as described in the legends to the tables and the figures using GraphPad Prism 6 for Mac OS X (GraphPad, LA Jolla, CA) software. *P*-values of <0.05 were considered as statistically significant.

Results

Effects of adipocyte-derived factors on primary human vascular smooth muscle cells

Exposure of hVSMC to CM increased the expression of the miR-143/145 cluster by 1.3-fold (*P*<0.001) as compared to cells kept in control medium (Figure 1 A/B). Furthermore, CM increased the mRNA levels of the miR-143/145 regulated targets SMA and TAGLN by 2.6-fold and 2.0-fold respectively, and of the pro-inflammatory cytokines PAI-1 and MCP-1 by 3.1- and 2.8-fold, respectively (all *P*<0.001) (Figure 1 C/F). Exposing hVSMC to CM inhibited the insulin-mediated phosphorylation of Akt on Thr308 and Ser473, and its substrates eNOS on Ser1177, and PRAS40 on Thr246 by 53%, 44%, 37%, and 36% respectively versus cells kept in control medium (all *P*<0.001) (Figure 2). Incubation with CM did not affect the basal phosphorylation levels of these proteins (Figure 2).

Effect of inhibition of TGFβ receptor- and p38-signaling on the CM-induced expression of miR-143/145 and inhibition of insulin action in hVSMC

A previous report ascribed the induction of the miR-143/145 cluster in hVSMC to the activation of the p38-signaling pathway by TGFβ [23]. Figure 3A/B shows that the phosphorylation of SMAD2, a component of the TGFβ receptor signaling pathway, as well as the phosphorylation of p38 were increased by 3.9- and 5.1-fold, respectively in hVSMC exposed to CM versus cells kept in control medium. To examine whether the TGFβ receptor- and p38-signaling pathway(s) participate in the induction of the miR-143/145 cluster, pharmacological inhibitors were used. Pretreating the hVSMC with either SB431542, which inhibits the TGFβ type I receptors 'Activin Receptor-Like Kinase' (ALK) 4, 5 and -7, or with the p38 inhibitor SB203580, completely abolished the induction the miR-143/145-cluster by CM in hVSMC (Figure 3C/D). Furthermore, the inhibition of insulin-induced phosphorylation of

Akt-Thr308, Akt-Ser473, eNOS-Ser1177, and PRAS40-Thr246 by CM was reversed in hVSMC incubated with either SB431542 or SB203580 before exposure to CM (Figure 4). Both inhibitors also blunted the induction of TAGLN in hVSMC by CM (supplementary Figure 1). However, in contrast to the effects on the miRNA-143/145 cluster and insulin action, the inhibitors did not or only partially prevent the induction of SMA, PAI-1 and MCP-1 in hVSMC by CM (supplementary figure 1).

Effect of miR-143 and miR-145 overexpression on insulin signaling

To investigate the impact of the miR-143/145 cluster on insulin action, hVSMC were transfected with precursors for miR-143 and miR-145, respectively. In line with a previous report [8], SMA protein abundance was increased in cells transfected with pre-miR-143 and pre-miR-145 by 9.0- and 2.0-fold respectively versus cells transfected with control pre-miR (P<0.05) (Figure 5A). Insulin-stimulated phosphorylation of Akt-Thr308, Akt-Ser473, and eNOS-Ser1177 were reduced by 42%, 44%, and 39%, respectively, in cells transfected with pre-miR-143 versus cells transfected with control pre-miR (Figure 5B-D). In contrast, transfection with pre-miR-145 did not affect insulin action (Figure 5B-D).

Several reports have linked miR-143 to an inhibition of insulin signaling through downregulation of ORP8. As shown in Figure 6A, transfection of hVSMC with pre-miR-143 reduced ORP8 protein levels by 25% (P<0.01) versus cells transfected with either control pre-miR or pre-miR-145. Also exposure to CM lowered ORP8 levels by 30% (P<0.01) versus cells kept in control medium, and this reduction in ORP8 abundance was not observed in hVSMC treated with either SB431542 or SB203580 prior to exposure to the CM (Figure 6B). To examine whether the downregulation of ORP8 impairs insulin signaling in hVSMC, cells were transduced with lentiviruses encoding control shRNA or shRNA for ORP8. A 30% decrease in ORP8 abundance, which is comparable to that achieved by expression of miR-143 or exposure to CM, was found to inhibit insulin-mediated Akt-Ser473 phosphorylation by 25% (Figure 6C/D).

Discussion

The present study shows that adipocyte-derived factors impair insulin signaling in hVSMC. Exposing the hVSMC to adipocyte-derived conditioned media was found to increase the expression of miR-143. The resulting decrease in the expression of the validated miR-143 target ORP8 resulted in inhibition of the insulin-mediated phosphorylation of Akt/eNOS-signaling pathway. Furthermore, the activation of the miR-143/ORP8 pathway and the induction of TAGLN by CM were sensitive to inhibition of Alk4/5/7- and p38-signaling. In contrast, the induction of the inflammatory markers PAI-1 and MCP-1 by CM as well as the induction of SMA by CM was not fully reversed by Alk4/5/7- and p38-inhibition, indicating that these effects involve at least in part different pathways.

In contrast to classical target tissues for insulin action, like liver, fat and muscle, the function of proper insulin action in the vasculature is less well understood. Nevertheless, vascular smooth muscle cell function is impaired in patients with type 2 diabetes [24]. *In vitro*, physiological concentrations of insulin have been reported to stimulate the autophosphorylation of the insulin receptor in vascular smooth muscle cells [17,19,25,34,35]. Furthermore, insulin has been found to promote glucose uptake through translocation of the insulin-regulated glucose transported GLUT4 in vascular smooth muscle cells [15]. For these in vitro studies, it remains to be investigated whether these effects can be fully ascribed to activation of the insulin receptor or also to activation of hybrid insulin receptor/ insulin like growth factor 1 receptor. Yet, studies using a vascular insulin receptor knock-out mouse have clearly illustrated the physiological relevance of activation of the Akt2/eNOS-pathway by insulin for vasorelaxation [9,31].

Whether an impaired insulin action in SMC also has detrimental effects on the progression of atherosclerosis is less well understood. One study reported that the presence of high palmitate induces a “selective” inhibition of insulin signaling in hVSMC with a profound abrogation of insulin induced PI3K-activation, whereas the activation of the MAPK-pathway is enhanced [5]. Additionally this study showed that increased activation of MAPK-signaling by insulin is involved in hVSMC proliferation, migration, and inflammation [5]. Alternatively, in advanced plaque progression, inflammation and insulin resistance may promote apoptosis of SMC and therefore thinning of fibrous cap and causing plaque rupture [3]. Finally, insulin-stimulated eNOS-derived NO production has important anti-inflammatory and anti-thrombotic properties through inhibition of leucocyte adhesion, and limiting

platelet adhesion and aggregation, and reduced expression of plasminogen activator inhibitor-1 (PAI-1), a prothrombotic protein [22]. Furthermore, NO has been shown to inhibit DNA synthesis, mitogenesis, and proliferation of vascular smooth muscle cells [10,26,27]. Therefore, impaired insulin signaling with reduced bioavailable NO may predispose vasculature to hyper-inflammatory and thrombotic states.

The present study shows that CM directly impairs insulin action in hVSMC via induction of miR-143 and subsequent downregulation of ORP8. Although the mechanism via which ORP8 regulates insulin action remains unclear, the induction of miR-143 is closely associated with obesity and insulin resistance. Feeding mice a high-fat diet increased the levels of miR-143 in adipose tissue [32], while both miR-143 and miR-145 are upregulated in the liver of mice fed a high-fat diet [18]. Moreover in the liver, heart, skeletal muscle and pancreas from *db/db*-mice, expression of miR-143 was increased as compared to tissues isolated from wild type control animals. Liver specific knock out of the miR-143/145-cluster protects against high-fat diet induced insulin resistance and hepatic Akt-inhibition [18]. The authors of that study further identified ORP8 as a direct miR-143 target, and showed that a decrease in ORP8 protein abundance is responsible for the abrogation of insulin action in the liver [18]. Finally, in cardiomyocytes we recently reported that the induction of miR-143 by activin A secreted from epicardial adipose tissue promotes insulin resistance via ORP8, and that silencing miR-143 expression protects cardiomyocytes against the induction of insulin resistance [1].

The strong association of miR-143 with insulin resistance in multiple tissues seems in contrast to the function ascribed to this miRNA in SMC. In SMC, an anti-proliferative function has been reported for the miR-143/145-cluster [8,23], and especially miR-145 has been found to promote the differentiation to the quiescent contractile phenotype of SMC [23]. Here, CM also induced the expression of miR-145 in quiescent differentiated hVSMC. Accordingly, this was accompanied by the induction of SMA, and TAGLN. In mice, miR-145 overexpression reduces neo-intima formation after vascular injury, but paradoxically miR-145 deficient mice show similar effects [6,38]. In humans, levels of miR-145 are elevated in atherosclerotic plaques [7,29], and were found to correlate with an unstable plaque phenotype [7]. Collectively these findings point toward an important dual role for the miR-143/145 in SMC homeostasis, but also indicate that further studies toward the underlying mechanism(s) are clearly needed, such as comparing the impact of CM on differentiated

versus undifferentiated SMC and elucidation of the targets regulated by the miR-143/145-cluster.

A limitation of the present study is that we could not identify the factor in CM responsible for the induction of the miR-143/145-cluster. In line with previous reports, the induction of miR-143/145 was sensitive to inhibition of Alk4/5/7- and p38-signaling [1,23]. Previous studies identified TGFβ and activin A as inducers of miR-143/145. However, the levels of these factors as determined by enzyme-linked immunosorbent assay were below the limit of detection in the CM used in the present study. Consequently, one may speculate that other members of the TGFβ superfamily, which consists of at least 23 members [33], elicit the effects observed here. Alternatively, CM itself may induce the production of factors promote the induction of the miR-143/145-cluster in an autocrine fashion. In this context, we could demonstrate that CM enhances the expression of activin A in differentiated hVSMC by 1.7-fold (supplementary figure 2). Yet, it remains to be investigated whether this is associated by the release of biologically relevant amounts of activin A from hVSMC. Another limitation is that the CM used in the present study was prepared from adipocytes isolated from subcutaneous adipose tissue biopsies collected from healthy young females. In previous studies, we reported that the adipose tissue secretory profile is affected by type 2 diabetes and different among various adipose tissue depots [1,11,30]. Therefore, one may speculate that conditioned media generated from adipocytes from donors with obesity or type 2 diabetes may a more detrimental impact on the determinants of smooth muscle cell function examined in the present study.

Conclusion

This study shows for the first time that adipocyte-derived factors impair insulin signaling in hVSMC. The inhibition of insulin signaling can be ascribed to the Alk4/5/7- and p38-dependent induction of miR-143 (Figure 7). This miRNA plays a pivotal role in the CM-induced impairment of insulin-induced Akt/eNOS-signaling via downregulation of ORP8. These data further highlight the involvement of adipokines in the pathogenesis of cardiovascular complications in type 2 diabetes.

Acknowledgments

This work was supported by the Federal Ministry of Health, the Ministry of Innovation, Science, Research and Technology of the German State of North-Rhine Westphalia, the Deutsche Forschungsgesellschaft (SE-1922/2-1) and the German Centre for Diabetes Research (Deutsches Zentrum für Diabetesforschung, DZD). The authors further thank professor Dr. Christoph Andree and Dr. Mazen Hagouan (both Sana Hospital, Duesseldorf-Gerresheim, Germany) for their continuous and invaluable support in the collection of adipose tissue samples for adipocyte isolation.

Conflict of interest: none declared.

Figures

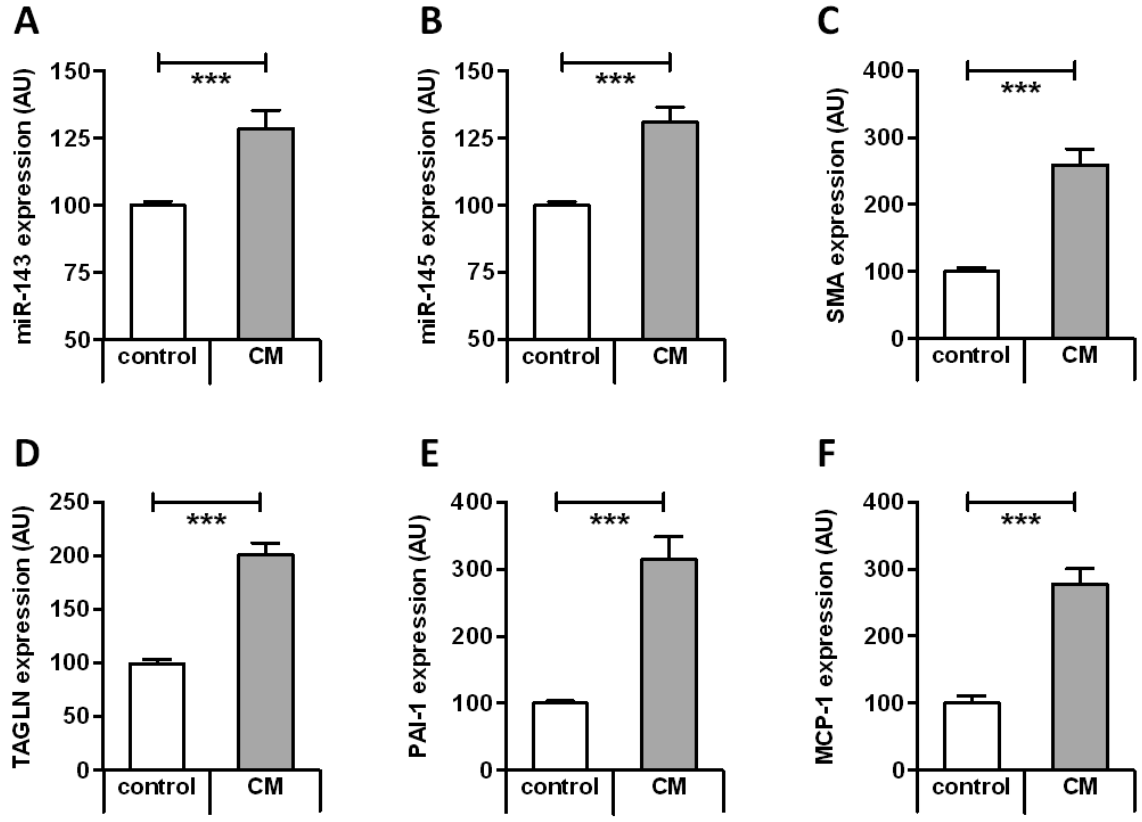


Figure 1. Effect of conditioned media on miRNA and gene expression in primary human vascular smooth muscle cells. Cells were exposed to control media or conditioned media (CM) for 24h and levels of miR-143 (**A)**, miR-145 (**B**), smooth muscle actin (SMA) (**C**), transgelin (TAGLN) (**D**), PAI-1 (**E**), and MCP-1 (**F**) were quantified by real-time PCR. Data were collected in 16 independent experiments

using 2 different donors and CM from 8 different preparations, and are expressed as mean ± standard error of the mean. Differences between the groups were evaluated using student's t-test. ***, indicates *P*<0.001 versus control medium.

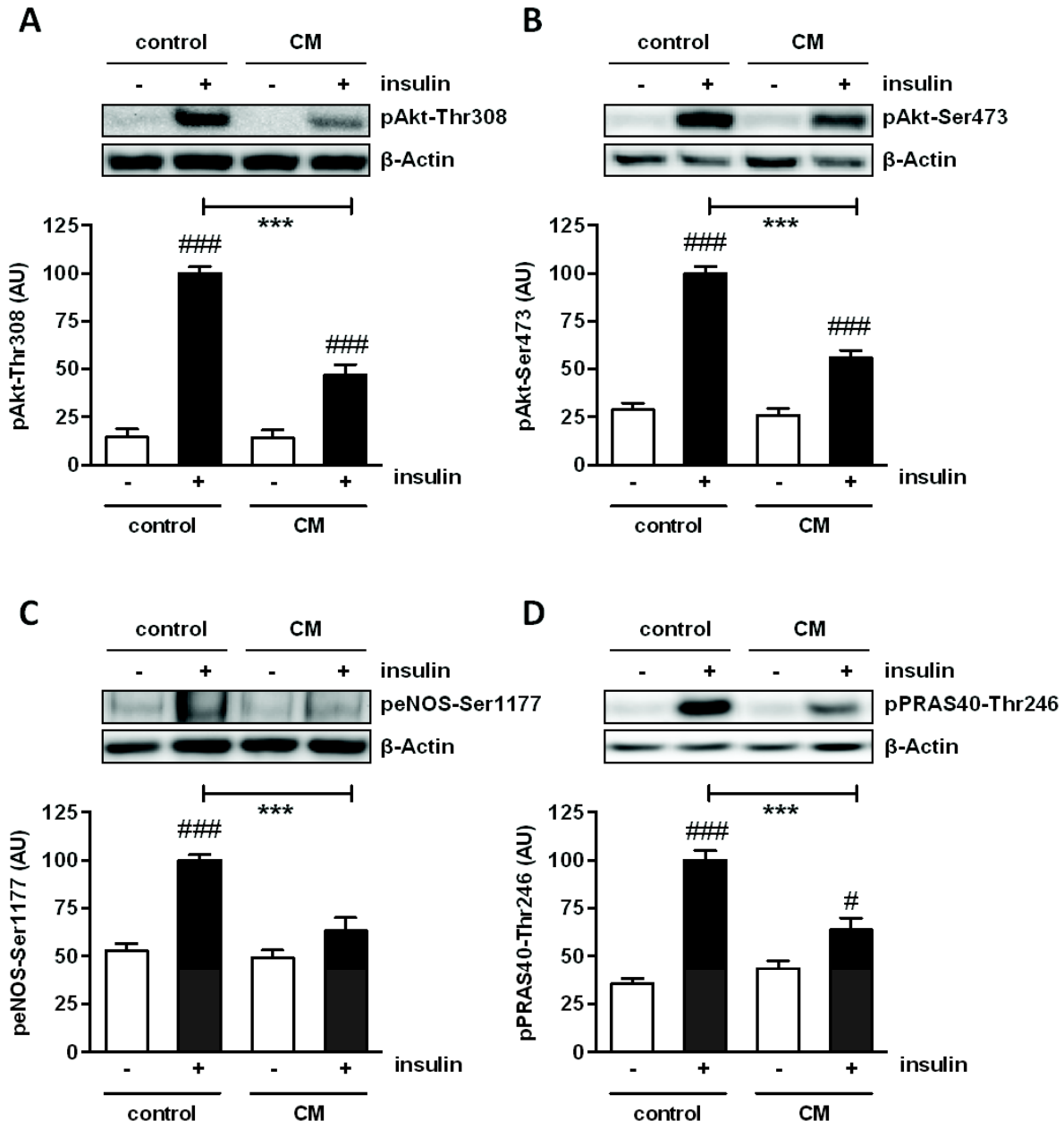


Figure 2. Effect of conditioned media on insulin action in primary human vascular smooth muscle cells. Cells were exposed to control media or conditioned media (CM) for 24h and kept untreated (-) or stimulated for 10 min with 100 nM insulin (+). Shown are representative Western blots and quantifications for the phosphorylation levels of Akt-Thr308 (**A**), Akt-Ser-473 (**B**), eNOS-Ser1177 (**C**), and PRAS40-Thr246 (**D**). Phosphorylation levels normalized for β-actin are expressed as mean ± standard error of the mean of at least 8 independent experiments using cells from 2 different donors and CM from 8 different preparations. Differences among the experimental conditions were evaluated using two-way ANOVA. ###, and # indicate *P*<0.001 and *P*<0.05, respectively, for the effect of insulin (+) versus cells kept untreated (-); *** indicates *P*<0.001 for the effect of CM versus control medium.

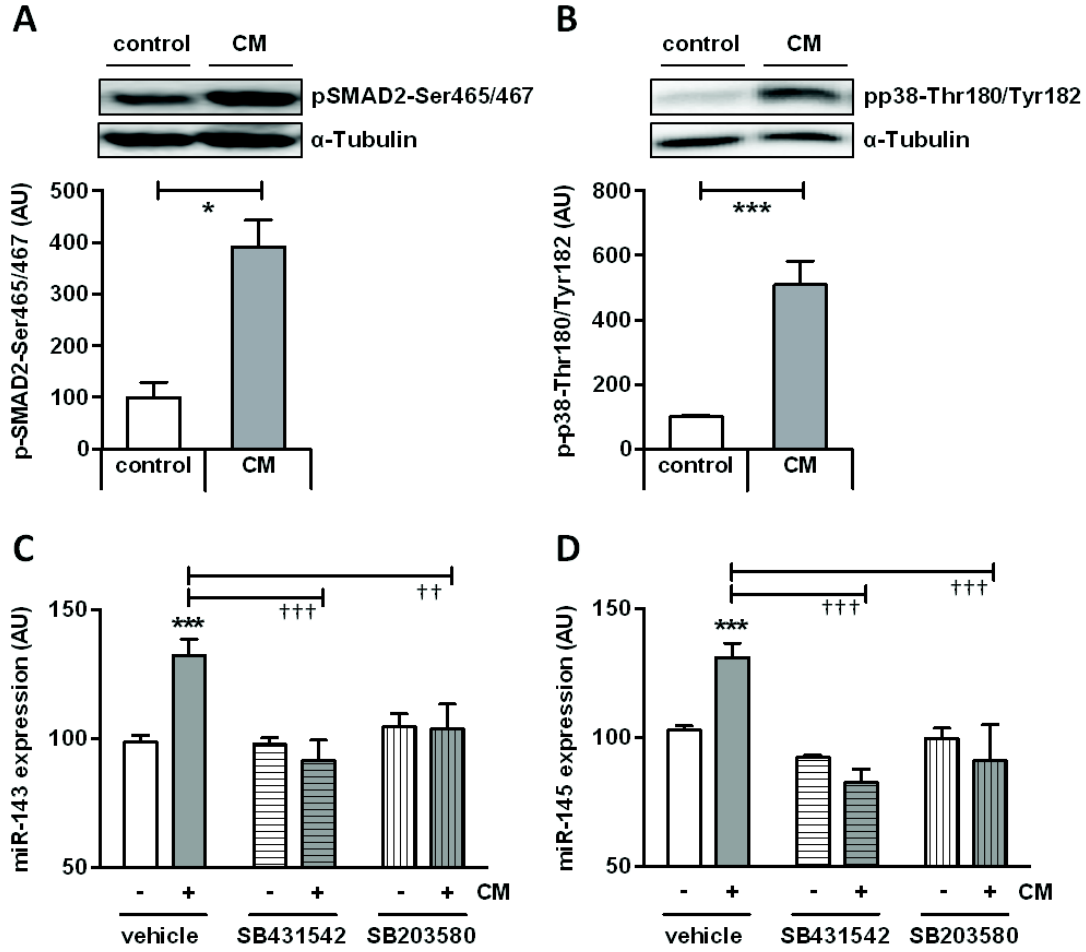
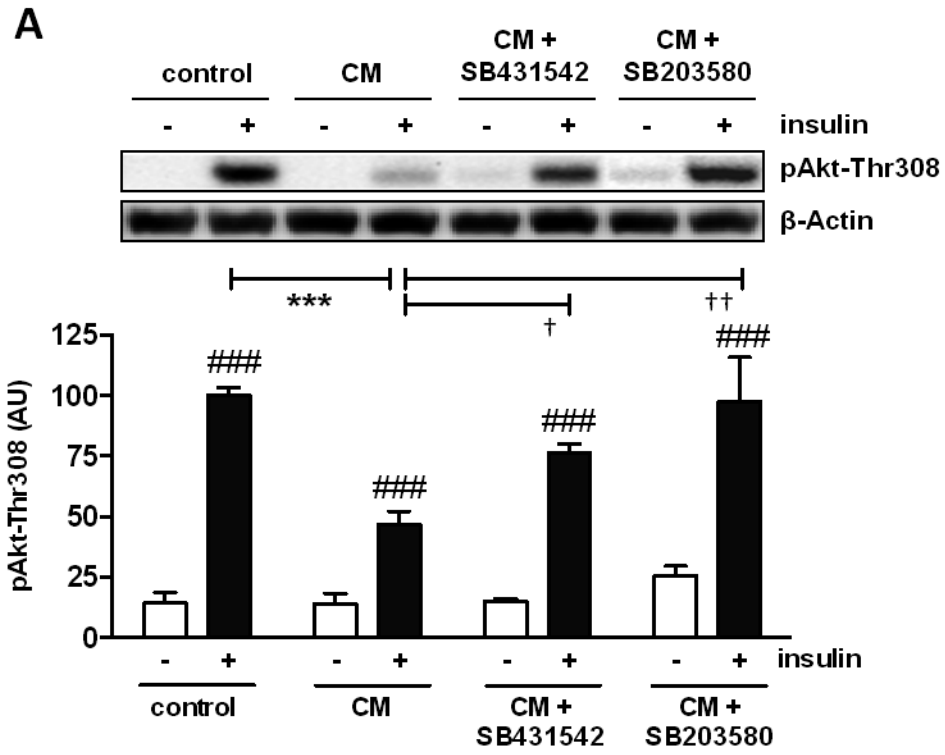
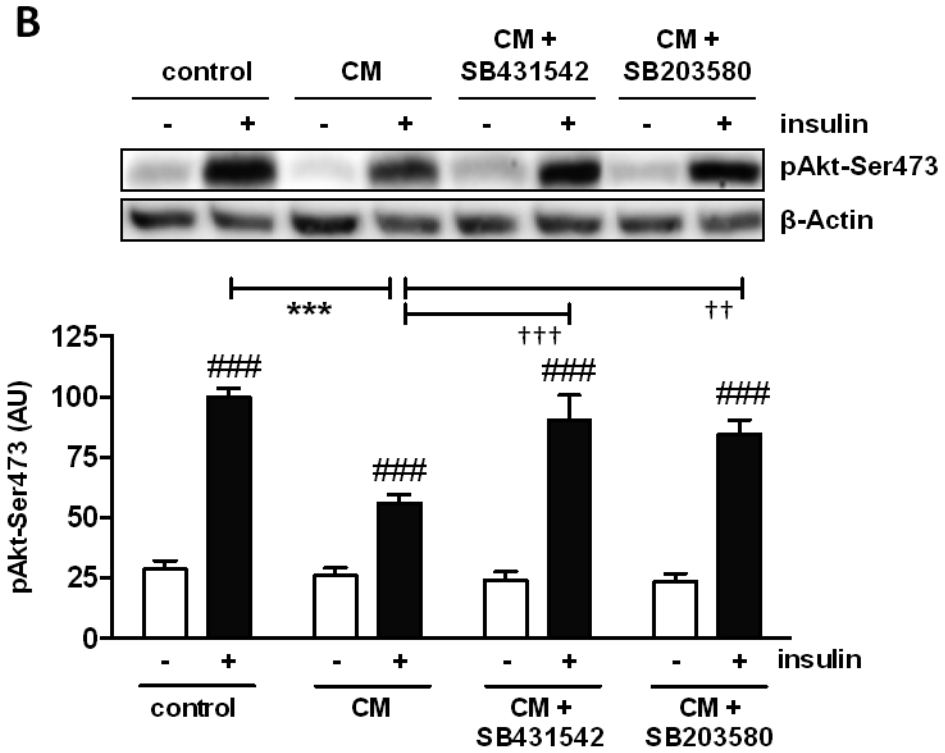


Figure 3. Involvement of TGFβ receptor- and p38-signalling in the induction of the miR-143/145 cluster in primary human vascular smooth muscle cells. Cells were exposed to control media or conditioned media (CM) for 24h. Shown are representative Western blots and quantifications for the phosphorylation levels of SMAD2-Ser465/467 (**A**), and p38-Thr180/Tyr182 (**B**). Phosphorylation levels normalized for α-tubulin are expressed as mean ± standard error of the mean of 14 independent experiments using cells from 2 distinct donors and CM from 7 different preparations. Differences between the groups were evaluated using student's t-test. ***, indicates *P*<0.001; *, *P*<0.05 versus control medium. **C/D**. Cells were exposed to DMSO (vehicle), or pharmacological inhibitors for Alk4/5/7 (SB431542), or p38 (SB203580) for 1 h prior to the addition of control medium (-) or CM (+), whereafter expression of miR-143 (**C**), and miR-145 (**D**) were determined via real-time PCR. Data are expressed as mean ± standard error of the mean of least 8 independent experiments using cells from 2 different donors and CM from 8 different preparations. Differences among the groups were analyzed using two-way ANOVA and Bonferroni multiple comparison analysis. ***, indicates P<0.001 for CM versus control medium; †††, *P*<0.001; ††, *P*<0.01 for CM only versus inhibitors.


A
control
CM
CM + SB431542
CM + SB203580
insulin
pAkt-Thr308
β-Actin
***
†
††
pAkt-Thr308 (AU)
125
100
75
50
25
0
###
control
CM
CM + SB431542
CM + SB203580


B
control
CM
CM + SB431542
CM + SB203580
insulin
pAkt-Ser473
β-Actin
***
†††
††
pAkt-Ser473 (AU)
125
100
75
50
25
0
###
control
CM
CM + SB431542
CM + SB203580

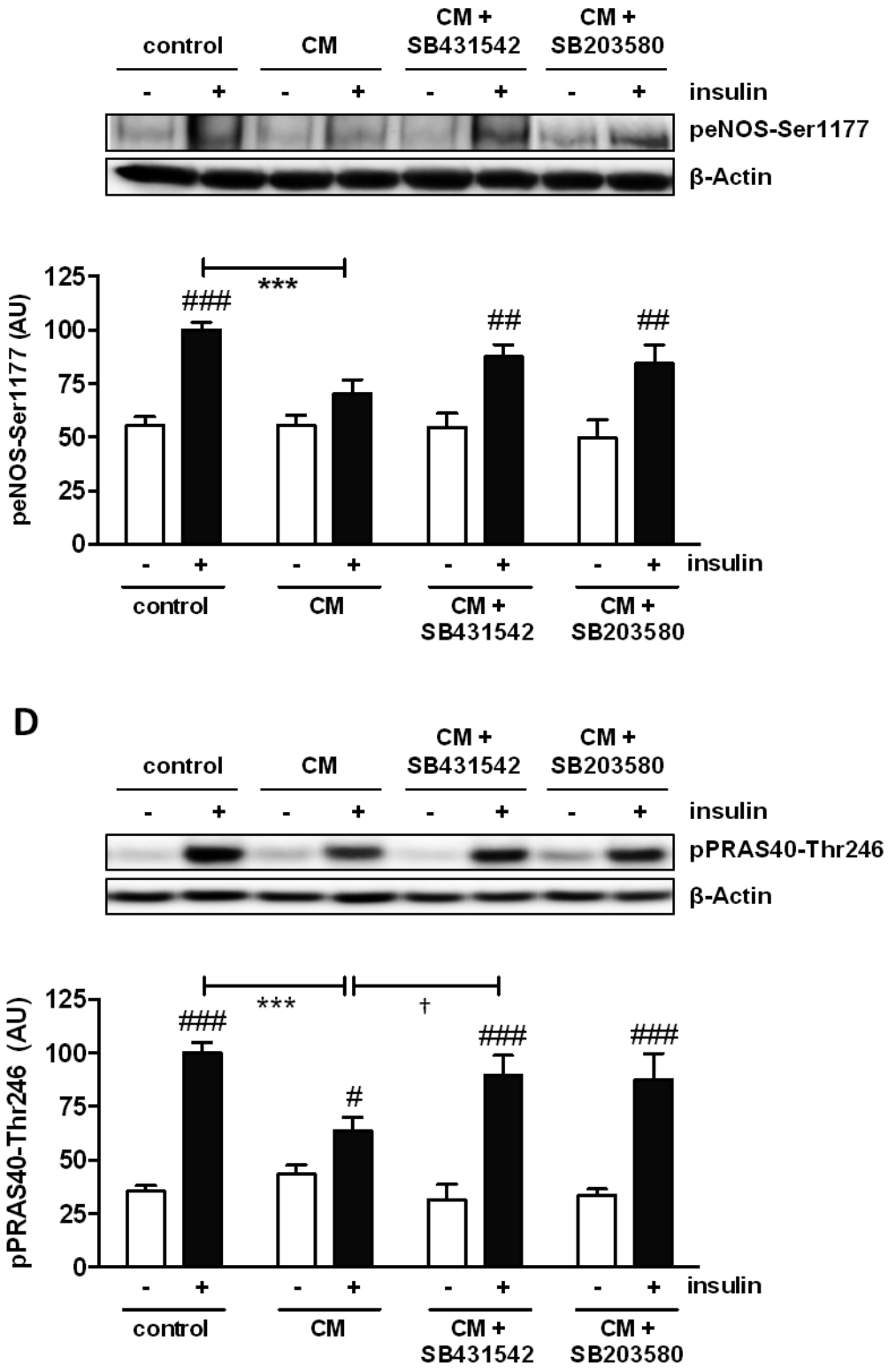


Figure 4. Involvement of TGFβ receptor- and p38-signalling in insulin action in primary human vascular smooth muscle cells. Cells were exposed to DMSO (vehicle), or pharmacological inhibitors for Alk4/5/7 (SB431542), or p38 (SB203580) for 1 h prior to the addition of control medium (-) or conditioned media CM, +). Shown are representative Western blots and quantifications for the phosphorylation levels of Akt-Thr308 (**A**), Akt-Ser-473 (**B**), eNOS-Ser1177 (**C**), and PRAS40-Thr246 (**D**). Phosphorylation levels normalized for β-actin are expressed as mean ± standard error of the mean of at least 4 independent experiments using cells from two distinct donors and CM from 4 different preparations. Differences among the experimental conditions were evaluated using two-way ANOVA.

###, *P*<0.001; ##, *P*<0.01; #, *P*<0.05 for the effect of insulin versus untreated cells; *** indicates *P*<0.001 for the effect of CM versus control medium; †††, *P*<0.001; ††, *P*<0.01; †, *P*<0.05 for CM only versus CM + inhibitors.

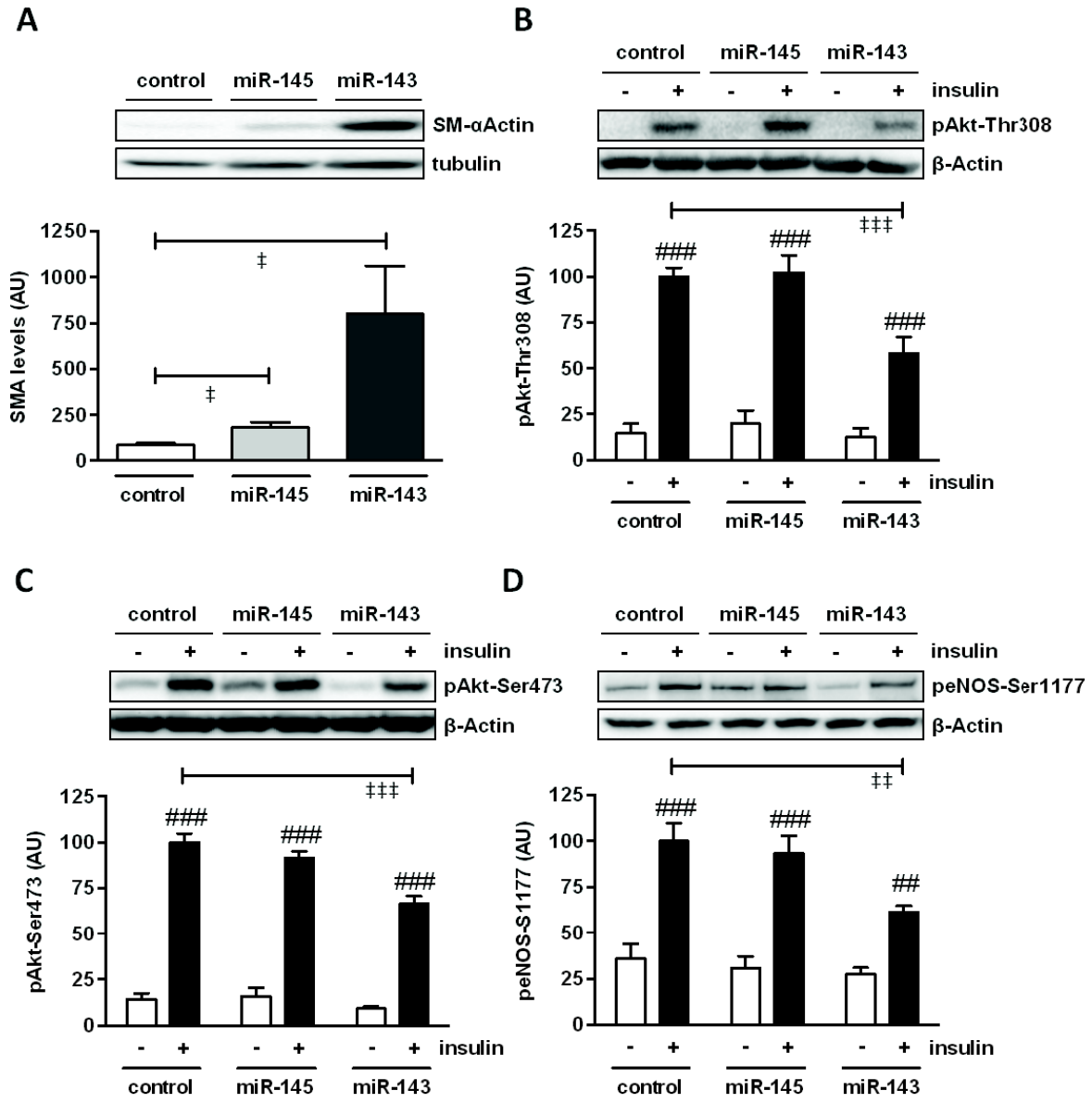


Figure 5. Effect of miR-143 and miR-145 expression on insulin action in primary human vascular smooth muscle cells. Cells were transfected with control precursor miRNA or precursors for miR-143 or miR-145, respectively. Shown are representative blots and quantifications for the protein abundance of smooth muscle α-actin (**A**), and the phosphorylation levels of Akt-Thr308 (**B**), Akt-Ser-473 (**C**), and eNOS-Ser1177 (**D**). Signal normalized for tubulin (**A**) or β-actin (**B-D**) are expressed as mean ± standard error of the mean of 8 independent experiments performed on cells from two different donors. Differences among the experimental conditions were evaluated using ANOVA (**A**) or two-way ANOVA (**B-D**) followed by Bonferroni multiple comparison analysis. ###, indicates *P*<0.001 for the effect of insulin versus untreated cells; ‡‡‡, *P*<0.001; ‡‡, *P*<0.01; ‡, *P*<0.05 for miR-143 or miR-145 versus control pre-miR.

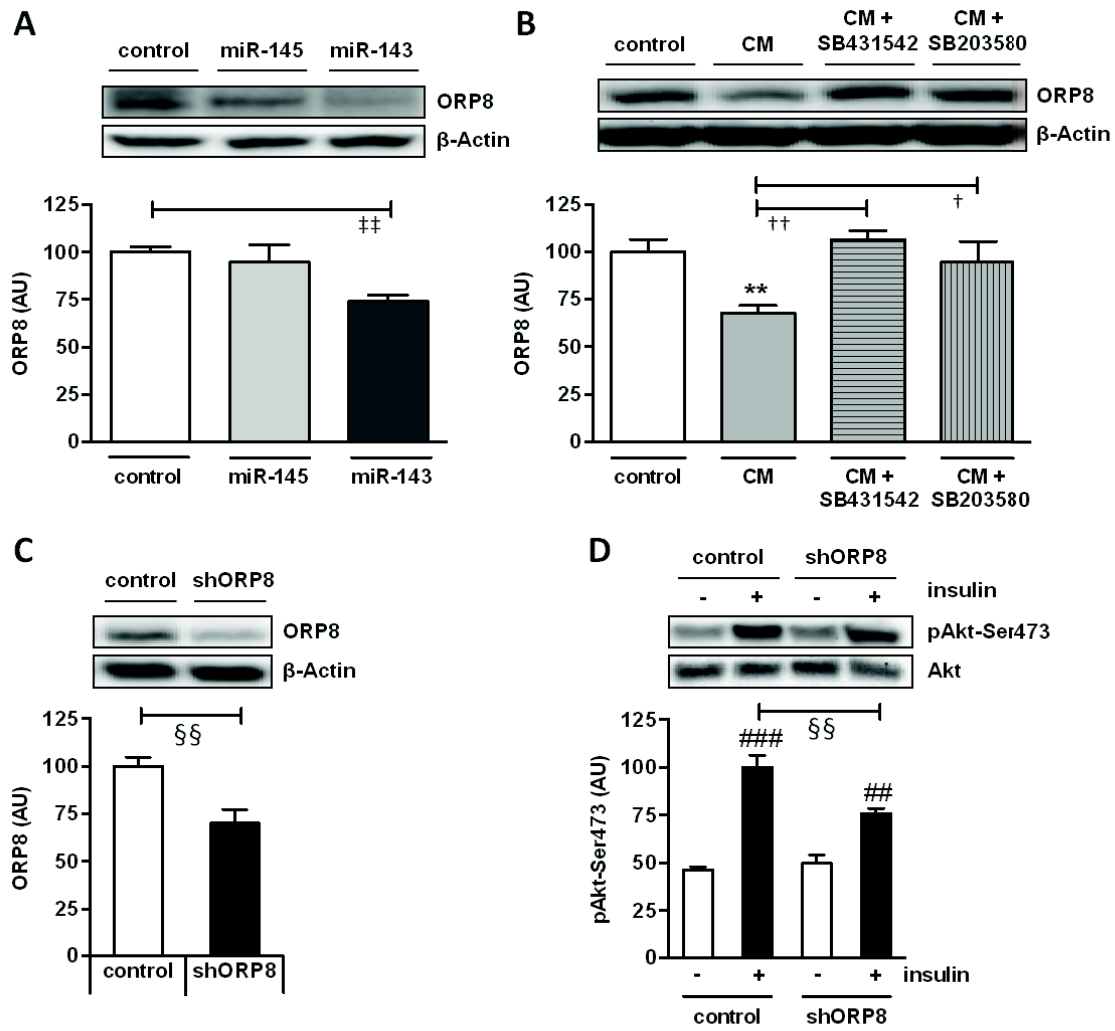


Figure 6. Inhibition of insulin action by conditioned media involves downregulation of the miR-143 target ORP8. Primary human vascular smooth muscle cells were transfected with control precursor miRNA or precursors for miR-143 or miR-145 (**A**), or exposed to conditioned media (CM) in the presence or absence of SB431542 or SB203580 (**B**) whereafter protein levels of ORP8 were examined via Western blot analysis. Shown are representative blots and quantifications. ORP8 abundance normalized for β-actin is expressed as mean ± standard error of the mean of 16 independent experiments using cells from 2 distinct donors and CM from 8 different preparations. Differences among the experimental conditions were evaluated using ANOVA followed by Bonferroni multiple comparison analysis. **, indicates P<0.01 for the effect of CM versus control medium; ††, *P*<0.01; †, *P*<0.05 for CM only versus CM + inhibitors; ; ‡‡, *P*<0.05 for miR-143 or miR-145 versus control pre-miR. **C/D.** Impact of silencing ORP expression in primary human vascular smooth muscle cells. Lysates from cells transduced with lentiviruses coding for control shRNA or ORP8 shRNA were analyzed for ORP8 protein abundance (**C**) and insulin-mediated phosphorylation of Akt-Ser473 (**D**) under untreated conditions (-) or following stimulation with insulin (10 min; 100 nM). Signals were normalized for β-actin (C) or Akt (D) respectively, and expressed as mean ± standard error of the mean of 8 independent experiments using cells from two different donors and 4 distinct shRNA constructs. ### indicates *P*<0.001; ##, *P*<0.01 for the effect of insulin versus untreated cells; §§, *P*<0.01 for the effect of ORP8 shRNA versus control shRNA.

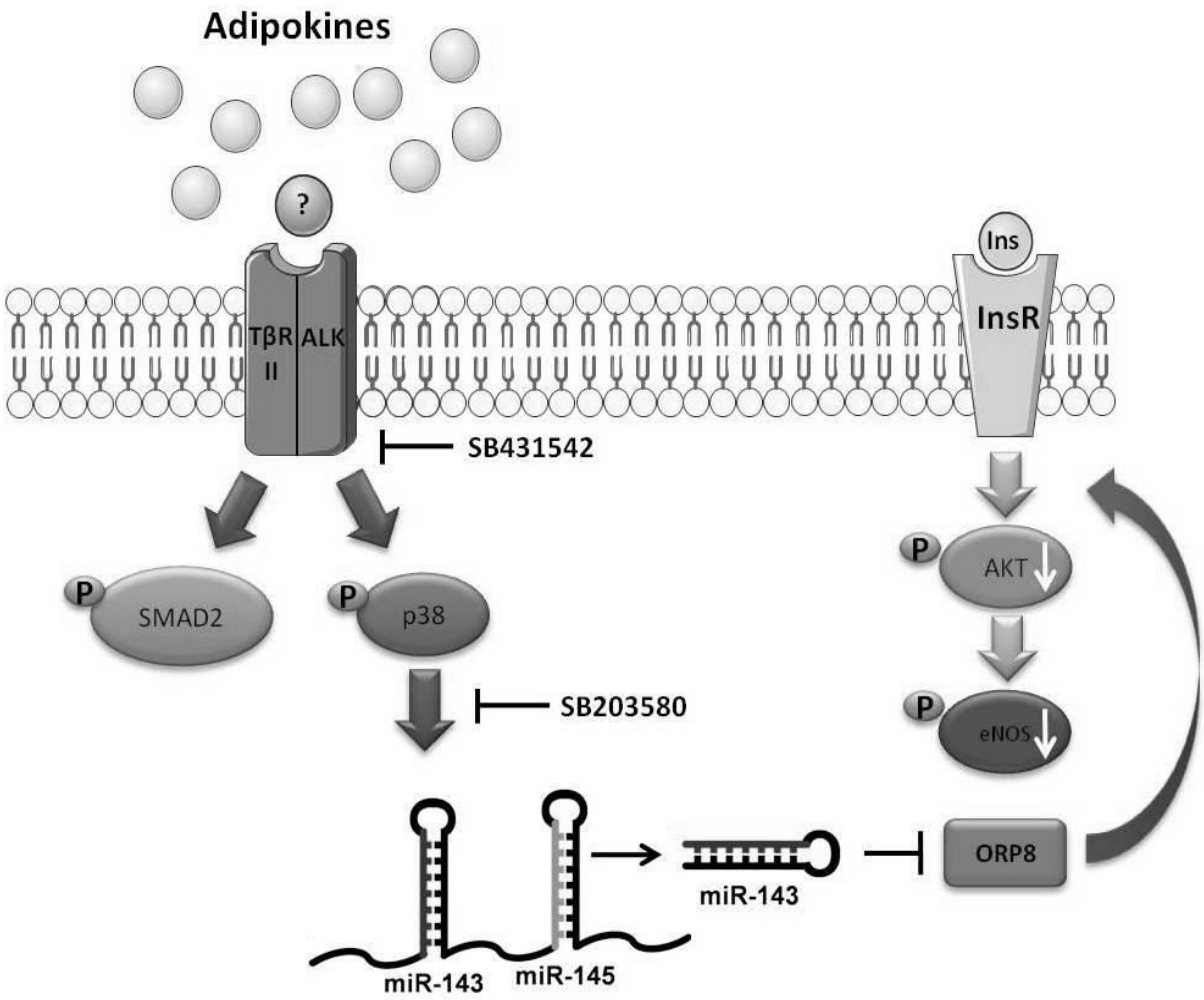
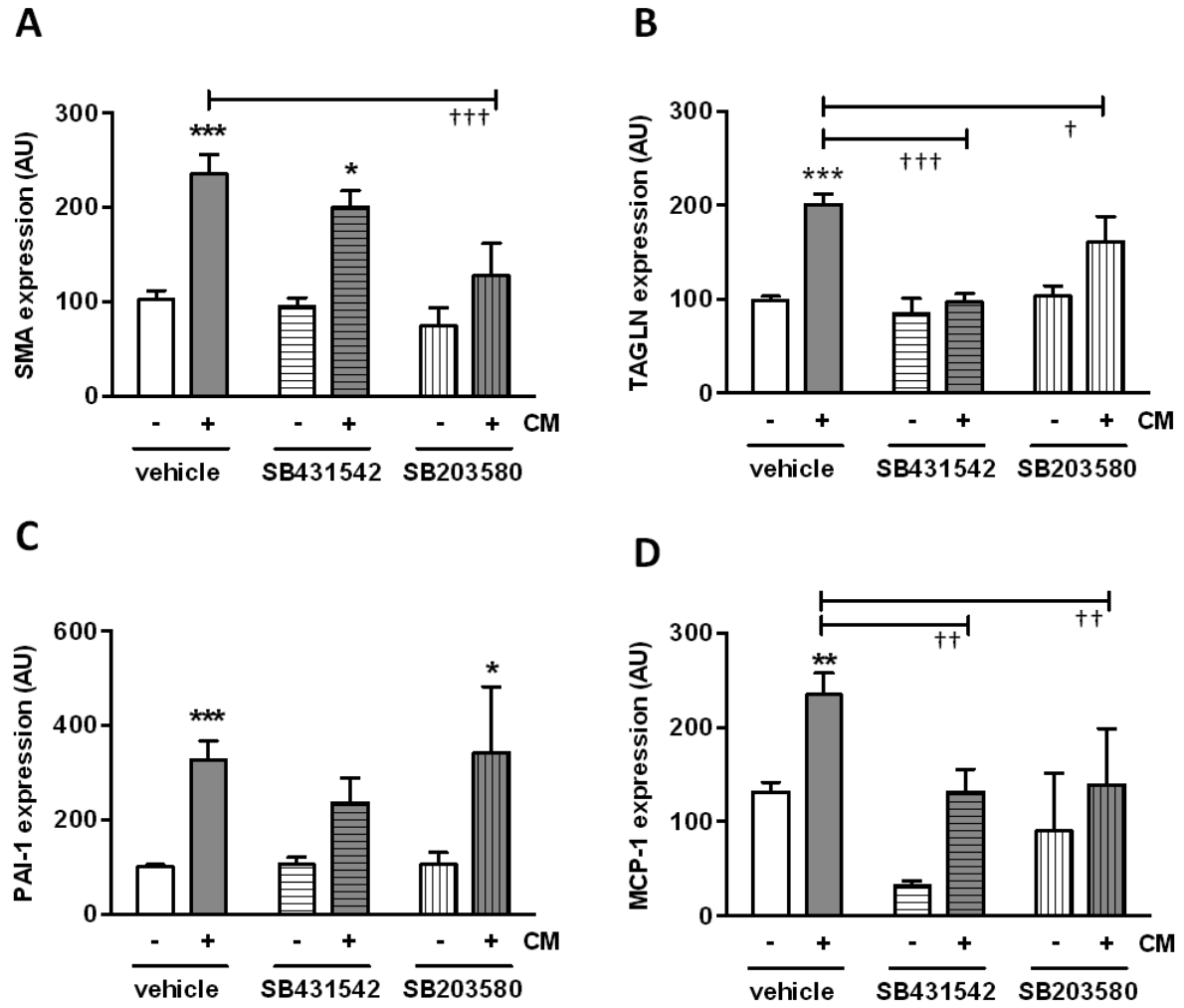
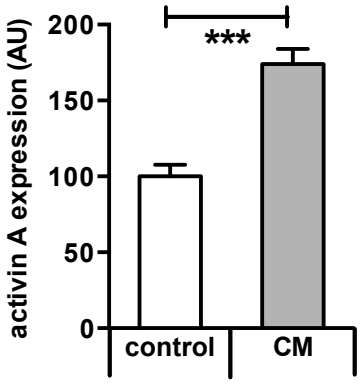


Figure 7. Possible mechanism for CM-induced insulin resistance in hVSMC. Adipocyte-derived factors belonging to the TGFβ-superfamily and binding to ALK induce SMAD2 and p38 phosphorylation. Especially the SMAD-independent Pathway through p38 leads to an upregulation of the miRNA 143/145 cluster. miR-143 is able to reduce the expression of ORP8 resulting in impaired insulin-stimulated Akt and eNOS phosphorylation. ALK: activin receptor-like kinases, Ins: insulin, InsR: insulin receptor, TβR II: transforming growth factor β receptor 2, ORP8: oxysterol-binding protein-related protein 8



Supplementary figure 1. Effect of inhibition of TGFβ receptor- and p38-signalling on the induction of gene expression by conditioned media in primary human vascular smooth muscle cells. Cells were exposed to DMSO (vehicle), or pharmacological inhibitors for Alk4/5/7 (SB431542), or p38 (SB203580) for 1 h prior to the addition of control medium (-) or conditioned media CM, +) for 24 h. Then expression levels of smooth muscle α-actin (SMA), transgelin (TAGLN), PAI-1, and MCP-1 were determined via real-time PCR. Data are expressed as mean ± standard error of the mean of least 8 independent experiments using cells from 2 different donors and CM from 8 different preparations. Differences among the groups were analyzed using two-way ANOVA and Bonferroni multiple comparison analysis. ***, indicates $P<0.001$; **, $P<0.01$; *, $P<0.05$ for CM versus control medium; †††, $P<0.001$; ††, $P<0.01$; †, $P<0.05$ for CM only versus inhibitors.



Supplementary figure 2. Effect of conditioned media on activin A expression in primary human vascular smooth muscle cells. Cells were exposed to control media or conditioned media (CM) for 24h and levels of activin A were quantified by real-time PCR. Data were collected in 16 independent experiments using 2 different donors and CM from 8 different preparations, and are expressed as mean ± standard error of the mean. Differences between the groups were evaluated using student's t-test. ***, indicates *P*<0.001 versus control medium.

References

[1] M. Blumensatt, S. Greulich, D. Herzfeld de Wiza, H. Mueller, B. Maxhera, M.J. Rabelink, R.C. Hoeben, P. Akhyari, H. Al-Hasani, J.B. Ruige, D.M. Ouwens, Activin A impairs insulin action in cardiomyocytes via up-regulation of miR-143, Cardiovasc. Res. 100 (2013) pp. 201-210.

[2] T. Boettger, N. Beetz, S. Kostin, J. Schneider, M. Kruger, L. Hein, T. Braun, Acquisition of the contractile phenotype by murine arterial smooth muscle cells depends on the Mir143/145 gene cluster, J. Clin. Invest 119 (2009) pp. 2634-2647.

[3] K.E. Bornfeldt and I. Tabas, Insulin resistance, hyperglycemia, and atherosclerosis, Cell Metab 14 (2011) pp. 575-585.

[4] F. Carlotti, M. Bazuine, T. Kekarainen, J. Seppen, P. Pognonec, J.A. Maassen, R.C. Hoeben, Lentiviral vectors efficiently transduce quiescent mature 3T3-L1 adipocytes, Mol. Ther. 9 (2004) pp. 209-217.

[5] E. Cersosimo, X. Xu, N. Musi, Potential role of insulin signaling on vascular smooth muscle cell migration, proliferation, and inflammation pathways, Am. J. Physiol Cell Physiol 302 (2012) p.C652-C657.

[6] Y. Cheng, X. Liu, J. Yang, Y. Lin, D.Z. Xu, Q. Lu, E.A. Deitch, Y. Huo, E.S. Delphin, C. Zhang, MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation, Circ. Res. 105 (2009) pp. 158-166.

[7] F. Cipollone, L. Felicioni, R. Sarzani, S. Ucchino, F. Spigonardo, C. Mandolini, S. Malatesta, M. Bucci, C. Mammarella, D. Santovito, L.F. de, A. Marchetti, A. Mezzetti, F. Buttitta, A unique microRNA signature associated with plaque instability in humans, Stroke 42 (2011) pp. 2556-2563.

[8] K.R. Cordes, N.T. Sheehy, M.P. White, E.C. Berry, S.U. Morton, A.N. Muth, T.H. Lee, J.M. Miano, K.N. Ivey, D. Srivastava, miR-145 and miR-143 regulate smooth muscle cell fate and plasticity, Nature 460 (2009) pp. 705-710.

[9] D.J. Fulton, Mechanisms of vascular insulin resistance: a substitute Akt?, Circ. Res. 104 (2009) pp. 1035-1037.

[10] U.C. Garg and A. Hassid, Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells, J. Clin. Invest 83 (1989) pp. 1774-1777.

[11] S. Greulich, B. Maxhera, G. Vandenplas, D.H. de Wiza, K. Smiris, H. Mueller, J. Heinrichs, M. Blumensatt, C. Cuvelier, P. Akhyari, J.B. Ruige, D.M. Ouwens, J. Eckel, Secretory products from epicardial adipose tissue of patients with type 2 diabetes mellitus induce cardiomyocyte dysfunction, Circulation 126 (2012) pp. 2324-2334.

[12] S.M. Grundy, Obesity, metabolic syndrome, and cardiovascular disease, J. Clin. Endocrinol. Metab 89 (2004) pp. 2595-2600.

[13] G. Hansmann, V.A. de Jesus Perez, T.P. Alastalo, C.M. Alvira, C. Guignabert, J.M. Bekker, S. Schellong, T. Urashima, L. Wang, N.W. Morrell, M. Rabinovitch, An antiproliferative BMP-2/PPARgamma/apoE axis in human and murine SMCs and its role in pulmonary hypertension, J. Clin. Invest 118 (2008) pp. 1846-1857.

[14] H. Hauner, T. Petruschke, M. Russ, K. Rohrig, J. Eckel, Effects of tumour necrosis factor alpha (TNF alpha) on glucose transport and lipid metabolism of newly-differentiated human fat cells in cell culture, Diabetologia 38 (1995) pp. 764-771.

[15] Y. Izawa, M. Yoshizumi, Y. Fujita, N. Ali, Y. Kanematsu, K. Ishizawa, K. Tsuchiya, T. Obata, Y. Ebina, S. Tomita, T. Tamaki, ERK1/2 activation by angiotensin II inhibits insulin-induced glucose uptake in vascular smooth muscle cells, Exp. Cell Res. 308 (2005) pp. 291-299.

[16] Z.Y. Jiang, Y.W. Lin, A. Clemont, E.P. Feener, K.D. Hein, M. Igarashi, T. Yamauchi, M.F. White, G.L. King, Characterization of selective resistance to insulin signaling in the vasculature of obese Zucker (fa/fa) rats, J. Clin. Invest 104 (1999) pp. 447-457.

[17] G.S. Johansson and H.J. Arnqvist, Insulin and IGF-I action on insulin receptors, IGF-I receptors, and hybrid insulin/IGF-I receptors in vascular smooth muscle cells, Am. J. Physiol Endocrinol. Metab 291 (2006) p.E1124-E1130.

[18] S.D. Jordan, M. Kruger, D.M. Willmes, N. Redemann, F.T. Wunderlich, H.S. Bronneke, C. Merkwirth, H. Kashkar, V.M. Olkkonen, T. Bottger, T. Braun, J. Seibler, J.C.

Bruning, Obesity-induced overexpression of miRNA-143 inhibits insulin-stimulated AKT activation and impairs glucose metabolism, Nat. Cell Biol. 13 (2011) pp. 434-446.

[19] A.M. Kahn, A. Husid, J.C. Allen, C.L. Seidel, T. Song, Insulin acutely inhibits cultured vascular smooth muscle cell contraction by a nitric oxide synthase-dependent pathway, Hypertension 30 (1997) pp. 928-933.

[20] D. Lamers, R. Schlich, S. Greulich, S. Sasson, H. Sell, J. Eckel, Oleic acid and adipokines synergize in inducing proliferation and inflammatory signalling in human vascular smooth muscle cells, J. Cell Mol. Med. 15 (2011) pp. 1177-1188.

[21] D. Lamers, R. Schlich, A. Horrighs, A. Cramer, H. Sell, J. Eckel, Differential impact of oleate, palmitate, and adipokines on expression of NF-kappaB target genes in human vascular smooth muscle cells, Mol. Cell Endocrinol. 362 (2012) pp. 194-201.

[22] U. Landmesser, B. Hornig, H. Drexler, Endothelial function: a critical determinant in atherosclerosis?, Circulation 109 (2004) p.II27-II33.

[23] X. Long and J.M. Miano, Transforming growth factor-beta1 (TGF-beta1) utilizes distinct pathways for the transcriptional activation of microRNA 143/145 in human coronary artery smooth muscle cells, J. Biol. Chem. 286 (2011) pp. 30119-30129.

[24] D. Montero, G. Walther, A. Perez-Martin, N. Vicente-Salar, E. Roche, A. Vinet, Vascular smooth muscle function in type 2 diabetes mellitus: a systematic review and meta-analysis, Diabetologia 56 (2013) pp. 2122-2133.

[25] R. Muniyappa, M. Montagnani, K.K. Koh, M.J. Quon, Cardiovascular actions of insulin, Endocr. Rev. 28 (2007) pp. 463-491.

[26] T. Nakaki, M. Nakayama, R. Kato, Inhibition by nitric oxide and nitric oxide-producing vasodilators of DNA synthesis in vascular smooth muscle cells, Eur. J. Pharmacol. 189 (1990) pp. 347-353.

[27] Y. Nunokawa and S. Tanaka, Interferon-gamma inhibits proliferation of rat vascular smooth muscle cells by nitric oxide generation, Biochem. Biophys. Res. Commun. 188 (1992) pp. 409-415.

[28] G.K. Owens, Regulation of differentiation of vascular smooth muscle cells, Physiol Rev. 75 (1995) pp. 487-517.

[29] D. Santovito, A. Mezzetti, F. Cipollone, MicroRNAs and atherosclerosis: new actors for an old movie, Nutr. Metab Cardiovasc. Dis. 22 (2012) pp. 937-943.

[30] R. Schlich, M. Willems, S. Greulich, F. Ruppe, W.T. Knoefel, D.M. Ouwens, B. Maxhera, A. Lichtenberg, J. Eckel, H. Sell, VEGF in the Crosstalk between Human Adipocytes and Smooth Muscle Cells: Depot-Specific Release from Visceral and Perivascular Adipose Tissue, Mediators. Inflamm. 2013 (2013) p.982458.

[31] J.D. Symons, S.L. McMillin, C. Riehle, J. Tanner, M. Palionyte, E. Hillas, D. Jones, R.C. Cooksey, M.J. Birnbaum, D.A. McClain, Q.J. Zhang, D. Gale, L.J. Wilson, E.D. Abel, Contribution of insulin and Akt1 signaling to endothelial nitric oxide synthase in the regulation of endothelial function and blood pressure, Circ. Res. 104 (2009) pp. 1085-1094.

[32] R. Takanabe, K. Ono, Y. Abe, T. Takaya, T. Horie, H. Wada, T. Kita, N. Satoh, A. Shimatsu, K. Hasegawa, Up-regulated expression of microRNA-143 in association with obesity in adipose tissue of mice fed high-fat diet, Biochem. Biophys. Res. Commun. 376 (2008) pp. 728-732.

[33] P. ten Dijke and H.M. Arthur, Extracellular control of TGFbeta signalling in vascular development and disease, Nat. Rev. Mol. Cell Biol. 8 (2007) pp. 857-869.

[34] M. Trovati, P. Massucco, L. Mattiello, F. Cavalot, E. Mularoni, A. Hahn, G. Anfossi, Insulin increases cyclic nucleotide content in human vascular smooth muscle cells: a mechanism potentially involved in insulin-induced modulation of vascular tone, Diabetologia 38 (1995) pp. 936-941.

[35] M. Trovati, P. Massucco, L. Mattiello, C. Costamagna, E. Aldieri, F. Cavalot, G. Anfossi, A. Bosia, D. Ghigo, Human vascular smooth muscle cells express a constitutive nitric oxide synthase that insulin rapidly activates, thus increasing guanosine 3':5'-cyclic monophosphate and adenosine 3':5'-cyclic monophosphate concentrations, Diabetologia 42 (1999) pp. 831-839.

[36] J. Vandesompele, P.K. De, F. Pattyn, B. Poppe, R.N. Van, P.A. De, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (2002) p.RESEARCH0034.

[37] A. Wichelhaus, M. Russ, S. Petersen, J. Eckel, G protein expression and adenylate cyclase regulation in ventricular cardiomyocytes from STZ-diabetic rats, Am. J. Physiol 267 (1994) p.H548-H555.

[38] M. Xin, E.M. Small, L.B. Sutherland, X. Qi, J. McAnally, C.F. Plato, J.A. Richardson, R. Bassel-Duby, E.N. Olson, MicroRNAs miR-143 and miR-145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury, Genes Dev. 23 (2009) pp. 2166-2178.

Journal: *Biochimica et Biophysica Acta – Molecular Basis of Disease*

Impact factor: 4.910

Contribution: Total: 50%

Conceived / designed experiments: 40%

Performed experiments: 70%

Analysed data: 40%

Contributed to discussion: 60%

Wrote the manuscript: 50%

Reviewed / edited manuscript: 40%

Author: 1st author (shared)

CHAPTER 5

Study 4

Adipose tissue dysfunction and inflammation in cardiovascular disease†

Running head: Adipose tissue and vascular dysfunction

Nina Wronkowitz, Tania Romacho, Henrike Sell, Juergen Eckel

Paul-Langerhans-Group for Integrative Physiology, German Diabetes Center, Düsseldorf, Germany

† *Frontiers of Hormone Research*, in press 2014

Abstract

Adipose tissue was long seen as a passive lipid storage depot but it is now considered as an endocrine organ that produces a large number of mediators that affect metabolism, inflammation and coagulation. In obesity, the increased size of adipocytes and the chronic low-grade inflammation within adipose tissue alter its normal physiological function. Adipose tissue dysfunction results in an altered production and secretion of adipokines, which in turn affects several tissues in a para- or endocrine manner like liver, skeletal muscle and the vasculature. Numerous circulating pro-inflammatory mediators participate in the development of cardiovascular disease (CVD) are released directly from adipocytes, thereby linking obesity to an increased cardiovascular risk. In the current chapter we focused on the one hand on a small selection of novel adipokines with a potentially strong link to cardiovascular disease, like soluble DPP4, visfatin and lipocalin-2. And on the other hand we summarized the most recent findings on the novel cardioprotective adipokines omentin and apelin.

Introduction

In the context of obesity, adipose tissue has gained considerable interest as a key player in the pathogenesis of several major chronic diseases like type 2 diabetes and cardiovascular disease (CVD), and its associated complications including endothelial and myocardial dysfunction, hypertension, dyslipidemia and atherosclerosis. Accumulating knowledge about the secretory and endocrine function of adipocytes and the adipose organ have led to a paradigm shift in adipose tissue biology, allocating to this tissue a major role in regulating energy homeostasis and food intake, reproduction, immunity, and cardiovascular function [1]. Thus, adipose tissue communicates with other organs in a complex network of inter-organ crosstalk by releasing a multitude of bioactive proteins and peptides, collectively termed adipokines and/or adipocytokines [2]. Originally, these molecules represented a small group of either hormone-like substances like leptin or adiponectin, or of pro-inflammatory cytokines like TNFalpha. However, more recent studies have shown the complexity of the human adipokinome consisting of hundreds of different factors [3], with many novel adipokines still being identified.

In adddition, it has been recognized that the adipokinome is substantially different between different adipose tissue depots. The visceral and the subcutaneous compartment are the two most abundant fat depots and it has been shown that they produce unique profiles of adipokines. 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 disease [4]. Importantly, specific smaller depots of visceral adipose tissue located around blood vessels and the heart, namely perivascular and epicardial fat, have been shown to affect cardiovascular and myocardial function in a paracrine fashion. The secretory output of these local depots may directly affect the underlying vasculature and induce pathological alterations like thickening of the vessel wall, fatty streak formation, or promotion of atherosclerotic plaques.

It is well accepted that the adipokine profile is altered in pathological conditions such as obesity, and that plasma levels of pro-inflammatory adipocytokines are increased, contributing to a chronic state of low-grade inflammation. Obesity-associated inflammation within adipose tissue is a complex scenario involving adipocyte hypertrophy and hyperplasia, cellular stress, hypoxia, and increasing infiltration of pro-inflammatory immune cells [5]. Obesity-associated enlargement of the adipocyte is also known to be associated with a shift of the secretome to a more pro-inflammatory phenotype, with positive correlations to adipocyte size being reported for TNFalpha, IL-6, IL-8, MCP-1, leptin and many others. The vast majority of adipokines exhibits an increased expression and secretion in obesity, whereas adiponectin is decreased. This molecule has an established cardioprotective function due to its anti-inflammatory and antiproliferative effects. Very recently, novel cardioprotective adipokines have been studied in more detail like omentin [6] and apelin. These novel players will be discussed in depth in this chapter.

The adipo-vascular axis

Obesity represents a major risk factor for cardiovascular diseases, which are characterized by a variety of vessel alterations, with atherosclerosis and vascular inflammation mediated by adipocytokines being the key event. This generated a deep interest in the adipo-vascular axis and the effects of various adipokines on cells of the vascular wall. Since augmented proliferation and migration of smooth muscle cells represents a central event in atherosclerosis, adipokines able to induce these processes have

been studied in detail. Further, the activation of pro-inflammatory signaling pathways by adipocytokines like TNFalpha, leptin and many others is considered to contribute significantly to the development of cardiovascular disease. In this context, the transcription factor NFkappaB plays a dominant role, since its downstream target genes represent adhesion molecules, pro-inflammatory cytokines and mediators of proliferation. We have recently reported a comprehensive analysis of the adipo-vascular axis and identified more than 30 adipokines being associated with cardiovascular disease [7]. In the current chapter we decided to focus on a small selection of novel adipokines with a potentially very strong link to cardiovascular disease, like soluble DPP4, visfatin and lipocalin-2. In addition, the most recent findings on the novel cardioprotective adipokines omentin and apelin are summarized here.

DPP4 and DPP4 inhibitors

By comprehensive proteomic profiling of the human adipocyte secretome we could identify DPP4 as a novel adipokine [8]. DPP4 is a ubiquitously expressed cell-surface protease, which is widely known for its role in regulation of glycemia through cleavage and inactivation of incretin hormones. Due to shedding from the cell membrane, a soluble form of DPP4 (sDPP4) lacking the cytoplasmatic tail and the transmembrane region can be found in plasma and other body fluids. DPP4 serum concentrations positively correlate with various parameters of the metabolic syndrome like BMI, adipocyte surface, leptin and insulin levels [8] and are increased in inflammatory disease and atherosclerosis. Interestingly, comparison of different fat depots showed the highest DPP4 expression in visceral adipose tissue of obese patients [8,9]. The development of DPP4 inhibitors and a central role for these agents in glycemic control have brought into focus the importance of DPP4 and its potential role in cardiovascular physiology and pathology. The functional role of DPP4 is related to both its enzymatic and non-catalytic function. DPP4 is a serine exopeptidase, which cleaves X-proline dipeptides from the N-terminus of several substrates, including chemokines, neuropeptides and vasoactive peptides. In addition to its peptidase activity, another important function of DPP4 is interaction with a range of ligands, including ADA, caveolin-1, thromboxane A2 receptor and fibronectin [10]. The binding of DPP4 to its ligands plays a role in various processes including immune regulatory function. Recent studies indicated that in addition to

regulation of post-prandial glycemia, DPP4 may exert several functions on the cardiovascular system, both directly and indirectly by its inhibition.

DPP4-inhibitors - Indirect effects of DPP4

Since DPP4-inhibitors are available drugs approved for the treatment of type 2 diabetes mellitus, research was more focusing on the effects of DPP4 inhibition than of direct effects of sDPP4. These inhibitors are able to prolong the bioavailability of the endogenously secreted incretin hormone GLP-1 and the glucose-dependent insulinotropic polypeptide (GIP). In addition to their anti-diabetic properties, accumulating data indicate that DPP4-inhibitors also have important protective effects on the cardiovascular system, through endothelial repair, anti-inflammatory effects and blunting of ischemic injury [11]. Due to various experimental and preliminary clinical data suggesting that GLP-1 itself has favorable cardiovascular effects, most of the beneficial effects of DPP4-inhibitors are ascribed to increased GLP-1 bioavailabilitiy and signaling. In vitro it could be shown that the GLP-1 analogue exendin-4 stimulates proliferation of human coronary artery endothelial cells through endothelial nitric oxide synthase (eNOS)-, protein kinase A (PKA)- and phosphoinositol-3 kinase (PI3K/Akt)-dependent pathways. Using an in vivo model of vascular injury it could be shown that continuous infusion of exendin-4 reduces neointimal formation at 4 weeks after injury without altering body weight or various metabolic parameters. From in vitro studies, the authors suggest that this effect was mediated by the ability of GLP-1 to suppress PDGF-induced proliferation of vascular smooth muscle cells. As a clinical readout, a large retrospective analysis indicates that patients prescribed with the GLP-1 analogue exenatide had a significant 20% reduction of CVD events compared with patients on other glucose-lowering agents. Nevertheless, studies showing vascular benefits of GLP-1 were carried out using either native GLP-1 or recombinant GLP-1 analogues at high concentrations or in a way that induced supraphysiological GLP-1 signaling. Considering that DPP4 inhibition restores GLP-1 signaling within the physiological range, beneficial effects of DPP4-inhibitors might be different to those of GLP-1 analogues.

But beside GLP-1 there are further substrates of DPP4, which might play a role in the favorable cardiovascular effects of DPP4-inhibitors. Two of the most promising candidates are stromal derived factor (SDF)-1α and Neuropeptide Y (NPY). SDF-1α is one of the most important soluble regulator of endothelial progenitor cells (EPC), which acts by binding to its

receptor CXCR4. EPCs are derived from the bone marrow and can be mobilized into the circulation in response to many stimuli. EPCs adhere to endothelium at sites of hypoxia or vascular damage and are able to participate in new vessel formation as well as to reconstitute the anatomical integrity of the intimal layer. In mice it could be shown that genetic deletion or pharmacologic inhibition of DPP4 is able to increase the homing of CXCR4+ EPCs at sites of myocardial damage, resulting in a reduced cardiac remodeling and improved heart function and survival [11]. Also in a human study Fadini et al. could demonstrate, that type 2 diabetic patients receiving a 4 week course of therapy with the DPP4-inhibitor sitagliptin, show increased SDF-1α plasma concentrations and circulating EPC levels. These effects were not related to changes in nitrite/nitrate levels or to reduction in plasma glucose, suggesting that they are GLP-1-independent [11]. These studies implicate a rationale to use DPP4-inhibitors for vascular repair through stimulation of EPCs. Additionally, the effects of DPP4 inhibition on angiogenesis may also be mediated by modulation of the Neuropeptide Y (NPY) signaling. DPP4 converts NPY1–36 to its shorter form (NPY3–36), thus shifting its activity from Y1-mediated vasoconstriction and vascular smooth muscle cell growth to Y2/Y5-mediated angiogenesis [11]. Considering the NPY signaling modulation the direct effect of DPP4 may be even detrimental, regarding vascular smooth muscle proliferation, and intimal hyperplasia [11].

Nevertheless, there are also various studies showing direct effects of DPP4-inhibitors, which could not be explained by an increased bioavailability of DPP4 substrates. Ta et al. could demonstrate that the specific DPP4-inhibitor alogliptin blocked LPS-induced ERK phosphorylation in U937 histiocytes, representing a model of cells involved in the progression of atherosclerosis, such as foam cells [12]. Downstream of ERK, the inhibition of DPP4 prevented the activation of matrixmetalloproteinases (MMP) via the Toll-like receptor pathway, which contributes to the destabilization of atherosclerotic plaques leading to acute vascular events. Furthermore, it could be demonstrated that sitagliptin prevents TNFα-induced PAI-1, ICAM and VCAM gene and protein expression in vascular endothelial cells independent of GLP-1 [13]. This effect could partially be explained by a reduction of the TNFα-induced NF-κB1 mRNA expression by sitagliptin. Additionally, incubation of human umbilical vein endothelial cells with the DPP4-inhibitor alogliptin resulted in endothelial nitric oxide synthase (eNOS) and Akt phosphorylation (Ser1177 and Ser473, respectively) paralleled by a rapid increase in nitric oxide [14]. In accordance to these data alogliptin

causes acute vascular relaxation in a non-diabetic mouse model through GLP-1 independent pathways that are both nitric oxide and endothelium-derived hyperpolarizing factor (EDHF) dependent. All in all these studies might implicate that DPP4 itself play a role in the beneficial effects of DPP4-inhibitors.

Direct effects of sDPP4

Since research was focusing on the effects of DPP4 inhibition on post-prandial glycemia and CVD there are only few available studies about direct effects of soluble DPP4 (sDPP4) on vascular cells. Our group could show for the first time direct effects of sDPP4 on human vascular smooth muscle cells (hVSMC), illustrated by increased proliferation and the induction of inflammation. Using DPP4 concentrations which reflect serum levels of lean and obese patients, sDPP4 induced a marked activation of the MAPK signaling pathway resulting in increased proliferation of hVSMCs [15,16]. In addition to the stress signaling, DPP4 directly activates the pro-inflammatory NF-κB signaling pathway leading to an increased expression and secretion of the pro-inflammatory cytokines IL-6, IL-8 and MCP-1. Since all DPP4-induced effects could be completely prevented by DPP4 inhibition, it seemed that direct effects of sDPP4 were dependent on its enzymatic activity. However, to identify the exact mechanisms how DPP4 exerts its signaling properties further investigations will be required. Another study analyzed direct effects of DPP4 on vascular function with special focus on vascular reactivity [17]. Therefore mouse mesenteric microvascular fragments were isolated and mounted on a small vessel wire myograph. DPP4 impaired the endothelium-dependent relaxation to acetylcholine in a concentration-dependent manner by up to 75%, without modifying endothelium-independent relaxations to sodium nitroprusside. Again the co-incubation of DPP4 with its enzymatic inhibitor prevented the impaired endothelium-dependent relaxation by DPP4. Similarly, the cyclooxygenase inhibitor indomethacin and the thromboxane A2 receptor antagonist SQ29548 abrogated the impairing action of DPP4. These data suggest that DPP-4 directly impairs endothelium-dependent relaxation, through a mechanism that involves cyclooxygenase activation and likely the release of a vasoconstrictor prostanoid.

Furthermore, DPP4 has long been considered as having a role in immunomodulation. Since atherosclerosis is an immune-inflammatory disease, it may be speculated that the inhibition of DPP4 modulates responses occurring within early or late atherosclerotic lesions.

In low-density lipoprotein receptor-deficient (LDLR-/-) mice Shah et al. could demonstrate that exogenously injected DPP4 increases monocyte migration in vivo [18]. These promigratory properties of DPP4 could be completely inhibited by sitagliptin. Moreover, in a recent published study it could be shown that sDPP4 enhances the LPS-induced TNFα and IL-6 secretion in monocytes [19]. These data suggest an interaction of sDPP4 with the TLR4 signaling cascade but the exact mechanism remains unclear. All in all, these studies on direct effects of sDPP4 might contribute to explain the vascular protective effects of DPP4-inhibitors independent of GLP-1.

In conclusion, DPP4 is a promising candidate linking adipose tissue dysfunction to cardiovascular disease (Fig. 1). However, the actual relationship between DPP4 inhibition and concrete cardiovascular outcomes remains unknown and requires further investigations.

Lipocalin-2 and visfatin

Lipocalin-2 (or neutrophil gelatinase-associated lipocalin – NGAL) is a small lipid binding protein with yet unidentified ligands [20]. It is expressed in many tissues including adipose tissue where it is highly expressed in mature adipocytes compared to undifferentiated preadipocytes. In addition to lipocalin's well-characterized function in the innate immune response to bacterial infection, some evidence indicate that it also plays a role as a pro-inflammatory adipokine in obesity and in obesity-associated metabolic diseases.

Circulating concentrations of lipocalin-2 are increased in the obese state and in patients with atherosclerosis, hypertension, coronary artery disease and coronary heart disease [5]. Elevated circulating concentrations are predictive of increased mortality after myocardial infarction and correlate with severity of coronary heart disease. Experimental data from rodents showing selective induction of lipocalin-2 in adipose tissue and liver indicate that adipose tissue might be a source of lipocalin-2 in obesity. In addition, a high expression of lipocalin-2 can be found in atheromatous human plaques in association with increased matrix metalloproteinase-9 activity. In experimental vessel damage, lipocalin is increased via inflammatory pathways such as the NF-κB pathway in smooth muscle cells. A

role of lipocalin-2 in endothelial dysfunction preceding cardiovascular diseases has not fully been established. Lipocalin-2 knockout mice are protected from aging- and dietary obesity-associated endothelial dysfunctions [21]. Functioning as a lipid carrier, lipocalin-2 might elevate endothelium-dependent vasoconstriction and attenuate relaxation by modulating eNOS activity and increased COX expression in intact arteries and primary endothelial cells. Lipocalin-2 expression can be induced by various pro-inflammatory stimuli including IL-1b, TNF, dexamethasone and LPS. Conversely, lipocalin-2 induces inflammation in different cell types including cardiomyocytes. Treatment with recombinant lipocalin-2 causes cardiomyocyte apoptosis in parallel to macrophage infiltration in the myocardium Lipocalin-2 might also play a role in the initiation of inflammation in the context of ischemia reperfusion and it has been suggested that this adipokine may mediate post-ischaemic inflammatory and remodelling responses. Also in damaged vessel, lipocalin-2 is increased in parallel to inflammation for example in smooth muscle cells. It should however been noted that lipocalin-2 may also exert anti-inflammatory effects since it reduces LPS-induced cytokine production in macrophages and counteracts TNF in the context of insulin signaling in adipocytes and macrophages. In summary, lipocalin-2 is a mediator of inflammation and cardiovascular diseases with a potential to be used as a biomarker and a therapeutic target in the context of obesity (Fig. 1).

Visfatin was first described as an adipokine binding and activating the insulin receptor and promoting adipogenesis, stimulating glucose uptake *in vitro* and exerting glucose-lowering effects in mice *in vivo* [22], although this later affirmation was retracted due to lack of reproducibility [23]. However, the participation of the insulin receptor in visfatin-mediated actions still remains unclear and insulin-mimetic properties have not been reproduced *in vitro* and *in vivo* [24]. The term visfatin refers to visceral fat, as this depot was proposed its main source in adipose tissue (AT) in both mice and humans. However, later works have reported similar visfatin levels in human subcutaneous and visceral fat. Importantly, visfatin is also found in perivascular and epicardial fat where it might exert a paracrine cardiovascular action. Activated macrophages in human visceral AT have been proposed to release higher amounts of visfatin that adipocytes. (as reviewed in [25]).

In both human and murine adipocytes visfatin expression and release is upregulated during differentiation. However, it only known that this adipokine is secreted by a yet

unknown non-classical mechanism [24]. Besides being an adipokine, visfatin is ubiquitously expressed and exerts multiple biological actions beyond the adipose tissue. Thus, visfatin is identical to pre-B cell colony-enhancing factor (PBEF), a previously described cytokine promoting maturation on early B-lineage precursor cells. Moreover, visfatin displays intrinsic enzymatic activity as a nicotinamide phosphoribosyltransferase (Nampt), catalyzing the rate-limiting step leading to the synthesis of nicotinamide adenine dinucleotide (NAD) [25].

In the context of metabolic diseases, although there are conflicting results, several studies have reported elevated circulating levels of visfatin in obesity, type 2 diabetes mellitus and the metabolic syndrome [25,26]. Moreover, different studies have established positive associations between enhanced circulating visfatin levels and athero-thrombotic diseases, suggesting that visfatin may represent a biomarker of metabolic-related cardiovascular complications. In this line, visfatin has been proposed as a marker of endothelial dysfunction in type 2 diabetic patients and patients with renal disease. In both patients with the metabolic syndrome and type 2 diabetes, circulating visfatin have been proposed as a marker of advanced carotid atherosclerosis for type 2 diabetic patients. In morbid obese patients, epicardial fat thickness correlates with enhanced visfatin and plasminogen activator inhibitor-1 (PAI-1) levels as well and with visceral obesity [25,27].

On the other hand, visfatin levels are positively associated to circulating inflammatory markers, such as IL-6, C-reactive protein (CRP) and MCP-1. Hence, it has been proposed that high visfatin levels reflect changes in the systemic inflammation, independently of the insulin resistance state [25].

The potential pharmacological regulation of visfatin circulating levels is not fully understood, since there are discrepant clinical and in vitro studies. Dexamethasone upregulates visfatin expression in vitro in 3T3-L1 adipocytes but does not alter serum levels in humans. Regarding anti-diabetic drugs, troglitazone suppresses visfatin expression in 3T3-L1 adipocytes while rosiglitazone increases visfatin secretion in human adipocytes. Furthermore, there are conflicting results on the effects of weight loss, exercise and anti-obesity drugs on visfatin levels. Visfatin expression and circulating levels can be regulated by fat distribution/area, inflammatory state and hormones among other factors. This fact, combined with the differences between cell types, species and reported discrepancies among commercial available ELISAS, require that the impact of visfatin levels in metabolic diseases are further investigated [27].

Increasing *in vitro* evidence supports a detrimental role for visfatin in the cardiovascular system. Visfatin is arising as a pro-atherogenic factor promoting vascular cell proliferation and inflammation. Thus, PVAT-derived visfatin enhances proliferation in rat aortic smooth muscle cells. In endothelial cells, the adipokine also promotes proliferation, migration and capillary tube formation contributing to angiogenesis. Indeed, the inhibitors of visfatin Nampt enzymatic activity, FK866 (or APO866) and CSH are under investigation as anti-cancer agents. Importantly, visfatin exerts its proliferative effects beyond the vascular wall, since both adenoviral delivery and exogenous administration of visfatin increased proliferation *in vitro* in rat cardiomyocytes. On the contrary, visfatin pro-angiogenic effects have been proven beneficial in a murine model of unilateral hindlimb ischemia [25].

Visfatin also displays pro-inflammatory properties on the vascular wall. The adipokine directly activates the ERK1/2-NF-κB axis in human vascular smooth muscle cells (VSMC) leading to iNOS induction. Moreover, visfatin activates endothelial cells triggering NF-κB–mediated induction of the adhesion molecules VCAM-1, ICAM-1 and E-Selectin, upregulating IL-6, IL-8 and MCP-1 secretion and CCR2 expression as well as NADPH oxidase activation. Moreover, visfatin exerts immunomodulatory actions on monocytes by enhancing both TNF-α and IL-8 secretion, inhibiting apoptosis in neutrophils and promoting macrophage survival. Visfatin enhances the expression and activity of MMP-2 and 9 in both endothelial cells and monocytes and thus has been proposed to promote plaque destabilization. Besides these effects on the extracellular matrix remodeling in the vascular wall, visfatin has been proposed to promote cardiac and renal fibrosis through the induction of classical pro-fibrotic mediators such as TGF-*β* or PAI-1, or by directly upregulating procollagen and MMPs secretion [25].

Regarding the regulation of the vascular tone by visfatin, there are some conflicting results. On one hand, visfatin induces the relaxation of noradrenaline (NA)-precontracted rat aortic rings. On the other hand, other works have shown that visfatin impairs endothelium-dependent relaxation in both human and murine microvessels as well as in bovine coronary arteries, via NADPH oxidase activation [25].

One limitation to explore visfatin actions in the CV system *in vivo* is that the visfatin/Nampt knockout genotype is lethal. It has been reported that insulin secretion is impaired in heterozygous mice in the visfatin/Nampt gene resulting in glucose intolerance. Interestingly, this impairment in insulin secretion is prevented by nicotinamide

mononucleotide (NMN), the product of visfatin enzymatic activity [24]. On the other hand, visfatin heterozygous knockout mice are protected against agonist-mediated cardiac hypertrophy while visfatin cardiac-specific overexpressing transgenic mice show increased cardiac fibrosis [25].

Although initially described as a promising physiological insulin-mimetic derived from visceral fat, clinical studies rather point toward a role for visfatin as a biomarker or even a predictor of CV diseases related to metabolic diseases where AT dysfunction is present. Importantly*, in vitro* and *ex vivo* studies have demonstrated that visfatin exerts direct deleterious actions on the CV system (Fig. 1). Thus, visfatin might represent a novel therapeutic target in metabolic-related cardiovascular complications. On the other hand, in ischemia visfatin may represent a beneficial pharmacological tool. Therefore, further investigation is still required to unveil the real impact of visfatin in different clinical conditions and to validate it as a therapeutical target in metabolic-related vascular diseases.

Omentin and apelin

Omentin (also called intelectin) has first been known as a protein expressed by Paneth cells in the intestine where it locates within carbohydrate complexes of bacterial walls and plays a role in the defense against pathogenic bacteria. A homologue to omentin that shares about 83% of its primary structure has been identified and named omentin-2. However, omentin is the predominant circulating form and is therefore mainly studied. As an adipokines, omentin was first described in 2005 when it was identified by a cDNA library screening of omental adipose tissue (reviewed in [28]). In accordance to its name, omentin is more abundantly expressed in visceral adipose tissue as compared to subcutaneous fat. Furthermore, this adipokine is predominantly expressed in the stroma-vascular fraction within adipose tissue and its expression is significantly depressed in adipose tissue from obese patients. As a result, serum omentin is decreased in obesity and increased in patients after weight loss. This is a feature that omentin shares with adiponectin as one of the few adipokines that are decreased in the obese state. Similar to adiponectin, omentin is also regarded as an anti-inflammatory factor with beneficial effects in the cardiovascular system.

The first function attributed to omentin as an adipokines was that it enhances insulin-stimulated glucose uptake in adipocytes in an auto-/paracrine way. Subsequently, omentin

has also been characterized as a vasoactive and cardioprotective factor [29]. On isolated vessels, omentin inhibits noradrenaline-induced contraction. Furthermore, this adipokine induces an endothelium-dependent vasorelaxation in both aorta and mesenteric arteries that is mediated by NO synthesis via phosphorylation of eNOS. Accordingly, omentin has been described as a novel marker for endothelial dysfunction in vivo that correlates with endothelium-dependent vasodilation even after adjustment for CRP [30]. A recent publication also indicates that circulating omentin is lower in type 2 diabetic patients and positively correlating with left ventricular diastolic function [31]. In non-diabetic controls, omentin is also highly expressed in epicardial adipose tissue as compared to pericardial and subcutaneous fat while omentin abundance is significantly depressed particularly in epicardial adipose tissue of type 2 diabetic patients. On isolated cardiomyocytes, omentin exerts cardioprotective effects as it prevents adipokines-induced contractile dysfunction and improves cardiomyocyte insulin signaling. Epicardial fat directly lies on the heart without any facial barrier and is regarded as a special visceral fat depot potentially modulating cardiac function. The reduction in omentin expression in epicardial adipose tissue might directly contribute to the development of cardiac dysfunction in type 2 diabetic patients since epicardial adipose tissue is not separated by a fascia from the myocardium directly draining its secretory output to adjacent cardiomyocytes. Anti-diabetic treatments such as metformin and TZDs significantly increase omentin serum concentrations which may contribute to their anti-diabetic action and beneficial effects on the cardiovascular system. As for metformin, it has been shown that increased omentin levels following the treatment might contribute to lower inflammatory and angiogenic potential of patient serum on endothelial cells.

In vitro, omentin exerts anti-inflammatory action on different cell types including endothelial cells and smooth muscle cells (Fig. 2). In TNFα-stimulated endothelial cells, omentin inhibits TNFα-induced expression of adhesion molecules potentially via an inhibition of the ERK and NF-κB pathway. Furthermore, omentin prevents TNFα-induced COX-2 induction through activation of AMPK and eNOS while preventing JNK activation concomitantly. In smooth muscle cells, omentin prevents TNFα-induced oxidative stress and expression of adhesion molecules also via inhibition of JNK activation. Further research on the role of ometin as an anti-inflammatory adipokines should resolve the issue of what cell type within the stroma-vascular fraction is contributing to omentin expression and release. Additionally, effects of omentin on adipose tissue-resident macrophages are not yet known

but might play a role in the observed anti-inflammatory effects of omentin also within adipose tissue.

Similar to omentin, the adipocytokine apelin has been shown to exert cardioprotective effects in addition to its effects on glucose and lipid metabolism [32]. In addition to adipose tissue, apelin is expressed in a number of tissues including the vascular endothelium, the myocardium and the brain. Apelin was first described in 1998 and binds to the G-protein coupled APJ receptor. Apelin can be considered as a “good” adipokine with direct effects on both cardiovascular cells and cardiomyocytes. Thus, apelin induces endothelium-dependent nitric oxide-mediated vasodilation, protects against atheroma, limits myocardial infarction and represents one of the most potent endogenous positive inotropic agents [32]. This is confirmed by observations in apelin knockout mice [Kuba et al., 2007]. These animals exhibit augmented cardiac dysfunction and altered cardiac remodeling under different conditions. Currently, the apelin-APJ pathway is considered as a potentially novel target for prevention of heart failure, myocardial ischemia and vascular disease. More work will be needed to fully define the role of apelin in cardiovascular pathology.

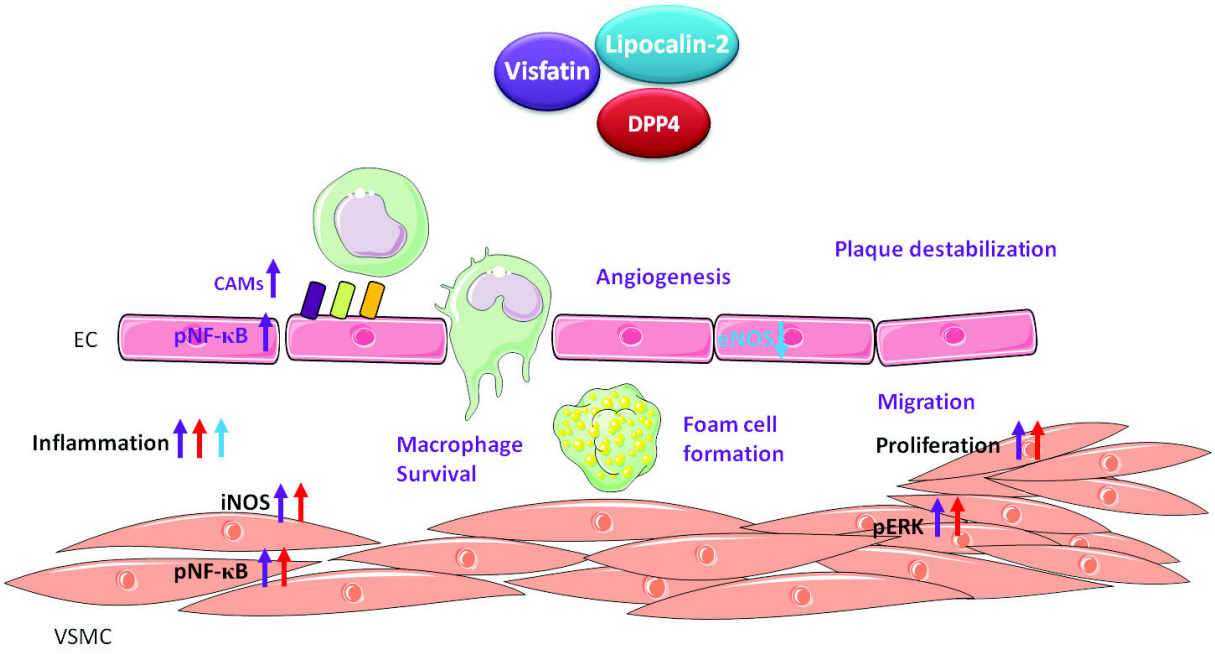


Figure 1: Detrimental effects of sDPP4, Visfatin and Lipocalin on the development of atherosclerosis. sDPP4 exerts its function mostly on VSMC by the induction of inflammatory- and stress-signaling pathways resulting in an increased proliferation (in red). Visfatin shows similar effects on VSMC but it also plays a pivotal role in endothelial dysfunction and in the ongoing low-grade inflammation within arteriosclerotic lesions (in violet). Although the role of Lipocalin-2 in atherosclerosis is not fully established, there are hints that Lipocalin-2 is involved in endothelial dysfunction and inflammation (in blue). **EC** : endothelial cells; **VSMC** . vascular smooth muscle cells;

CAM : cellular adhesion molecule; **eNOS** : endothelial nitric oxide synthase; **iNOS** : inducible nitric oxide synthase

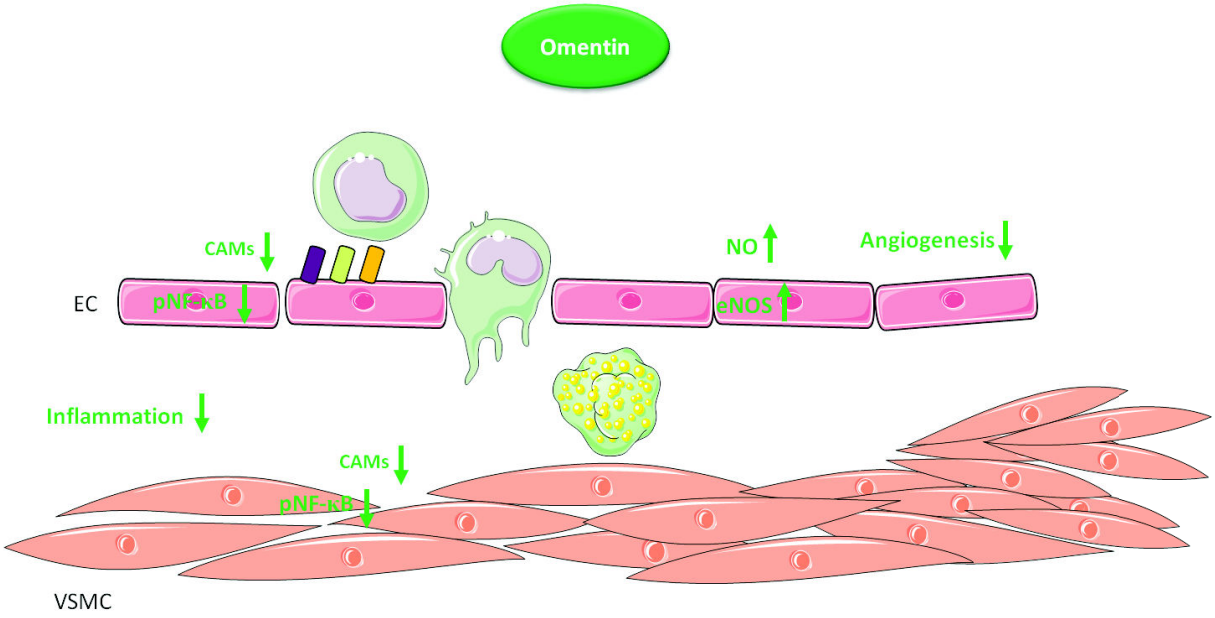


Figure 2**: **Beneficial effects of omentin on vascular cells. Omentin exerts anti-inflammatory action on both EC and VSMC through a reduced NF-κB Signaling resulting in a decreased expression of CAMs. Additionally, Omentin induces endothelial-dependent vasodilatation through a increased bioavailability of NO thereby reducing endothelial dysfunction. **EC** : endothelial cells; **VSMC** . vascular smooth muscle cells; **CAM** : cellular adhesion molecule; **eNOS** : endothelial nitric oxide synthase.

References

1. Gualillo O, González-Juanatey JR, Lago F: The emerging role of adipokines as mediators of cardiovascular function: physiologic and clinical perspectives. Trends Cardiovasc Med 2007; 17:275-83

2. Trayhurn P, Drevon CA, Eckel J: Secreted proteins from adipose tissue and skeletal muscle - adipokines, myokines and adipose/muscle cross-talk. Arch Physiol Biochem 2011; 117:47-56

3. Lehr S, Hartwig S, Lamers D, Famulla S, Müller S, Hanisch FG, Cuvelier C, Ruige J, Eckardt K, Ouwens DM, Sell H, Eckel J: Identification and validation of novel adipokines released from primary human adipocytes. Mol Cell Proteomics 2012; 11:M111.010504

4. Bosello O, Zamboni M: Visceral obesity and metabolic syndrome. Obes Rev 2000; 1:47-56

5. Sell H, Habich C, Eckel J: Adaptive immunity in obesity and insulin resistance. Nat Rev Endocrinol 2012; 8:709-16

6. Greulich S, Chen WJ, Maxhera B, Rijzewijk LJ, van der Meer RW, Jonker JT, Mueller H, de Wiza DH, Floerke RR, Smiris K, Lamb HJ, de Roos A, Bax JJ, Romijn JA, Smit JW, Akhyari P, Lichtenberg A, Eckel J, Diamant M, Ouwens DM: Cardioprotective properties of omentin-1 in type 2 diabetes: evidence from clinical and in vitro studies. PLoS One 2013; 8:e59697

7. Taube A, Schlich R, Sell H, Eckardt K, Eckel J: Inflammation and metabolic dysfunction: links to cardiovascular diseases. Am J Physiol Heart Circ Physiol 2012; 302:H2148-65

8. Lamers,D, Famulla,S, Wronkowitz,N, Hartwig,S, Lehr,S, Ouwens,DM, Eckardt,K, Kaufman,JM, Ryden,M, Muller,S, Hanisch,FG, Ruige,J, Arner,P, Sell,H, Eckel,J: Dipeptidyl peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome. Diabetes 60:1917-1925, 2011

9. Sell H, Blüher M, Klöting N, Schlich R, Willems M, Ruppe F, Knoefel W, Dietrich A, Arner P, Frayn K, Eckel J: Adipose DPP4 and obesity: correlation with insulin resistance and depot-specific release from adipose tissue in vivo and in vitro. Diabetes Care 2013; (in Press)

10. Zhong J, Rao X, Rajagopalan S: An emerging role of dipeptidyl peptidase 4 (DPP4) beyond glucose control: potential implications in cardiovascular disease. Atherosclerosis 2013; 226:305-14

11. Fadini GP, Avogaro A: Cardiovascular effects of DPP-4 inhibition: beyond GLP-1. Vascul Pharmacol 2011; 55:10-6

12. Ta NN, Li Y, Schuyler CA, Lopes-Virella MF, Huang Y: DPP-4 (CD26) inhibitor alogliptin inhibits TLR4-mediated ERK activation and ERK-dependent MMP-1 expression by U937 histiocytes. Atherosclerosis 2010; 213:429-35

13. Hu Y, Liu H, Simpson RW, Dear AE: GLP-1-dependent and independent effects and molecular mechanisms of a dipeptidyl peptidase 4 inhibitor in vascular endothelial cells. Mol Biol Rep 2013; 40:2273-79

14. Shah Z, Pineda C, Kampfrath T, Maiseyeu A, Ying Z, Racoma I, Deiuliis J, Xu X, Sun Q, Moffatt-Bruce S, Villamena F, Rajagopalan S: Acute DPP-4 inhibition modulates vascular tone through GLP-1 independent pathways. Vascul Pharmacol 2011; 55:2-9

15. Wronkowitz N, Villalobos L, Sanchez-Ferrer C, Peiro C, Sell H, Eckel J: Dipeptidyl Peptidase 4 (DPP4) induces inflammation and proliferation of human smooth muscle cells by activation of the ERK- signaling pathway. Diabetes 2013; 62 Suppl.1:A52

16. Ervinna N, Mita T, Yasunari E, Azuma K, Tanaka R, Fujimura S, Sukmawati D, Nomiyama T, Kanazawa A, Kawamori R, Fujitani Y, Watada H: Anagliptin, a DPP-4 inhibitor, suppresses proliferation of vascular smooth muscles and monocyte inflammatory reaction and attenuates atherosclerosis in male apo E-deficient mice. Endocrinology 2013; 154:1260-70

17. Sanchez-Ferrer C, Vallejo S, Romacho T, Villalobos L, Wronkowitz N, Sell H, Eckel J, Peiro C: Dipeptidyl Peptidase-4 impairs microvascular endothelial-dependent relaxation : the role of cyclooxygenase. Diabetes 2013; 62 Suppl.1:A128

18. Shah Z, Kampfrath T, Deiuliis JA, Zhong J, Pineda C, Ying Z, Xu X, Lu B, Moffatt-Bruce S, Durairaj R, Sun Q, Mihai G, Maiseyeu A, Rajagopalan S: Long-term dipeptidyl-peptidase 4 inhibition reduces atherosclerosis and inflammation via effects on monocyte recruitment and chemotaxis. Circulation 2011; 124:2338-49

19. Ikeda T, Kumagai E, Iwata S, Yamakawa A: Soluble CD26/Dipeptidyl Peptidase IV Enhances the Transcription of IL-6 and TNF-alpha in THP-1 Cells and Monocytes. PLoS One 2013; 8:e66520

20. Wang Y: Small lipid-binding proteins in regulating endothelial and vascular functions: focusing on adipocyte fatty acid binding protein and lipocalin-2. Br J Pharmacol 2012; 165:603-21

21. Liu JT, Song E, Xu A, Berger T, Mak TW, Tse HF, Law IK, Huang B, Liang Y, Vanhoutte PM, Wang Y: Lipocalin-2 deficiency prevents endothelial dysfunction associated with dietary obesity: role of cytochrome P450 2C inhibition. Br J Pharmacol 2012; 165:520-31.

22. Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K: Visfatin: A protein secreted by visceral fat that mimics the effects of insulin. Science 2005; 307:426-30

23. Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K, Matsuki Y, Murakami M, Ichisaka T, Murakami H, Watanabe E, Takagi T, Akiyoshi M, Ohtsubo T, Kihara S, Yamashita S, Makishima M, Funahashi T, Yamanaka S, Hiramatsu R, Matsuzawa Y, Shimomura I: Retraction. Science 2007; 318:565

24. Revollo JR, Korner A, Mills KF, Satoh A, Wang T, Garten A, Dasgupta B, Sasaki Y, Wolberger C, Townsend RR, Milbrandt J, Kiess W, Imai S: Nampt/pbef/visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. Cell Metab 2007; 6:363-75

25. Romacho T, Sánchez-Ferrer CF, Peiró C: Visfatin/Nampt: an adipokine with cardiovascular impact. Mediators Inflamm 2013; 2013:946427

26. Sommer G, Garten A, Petzold S, Beck-Sickinger AG, Bluher M, Stumvoll M, Fasshauer M: Visfatin/pbef/nampt: Structure, regulation and potential function of a novel adipokine. Clin Sci 2008; 115:13-23

27. Filippatos TD, Randeva HS, Derdemezis CS, Elisaf MS, Mikhailidis DP: Visfatin/pbef and atherosclerosis-related diseases. Curr Vasc Pharmacol 2010; 8:12-28

28. Tan BK, Adya R, Randeva HS: Omentin: a novel link between inflammation, diabesity, and cardiovascular disease. Trends Cardiovasc Med 2010; 20:143-48.

29. Yamawaki H: Vascular effects of novel adipocytokines: focus on vascular contractility and inflammatory responses. Biol Pharm Bull 2011; 34:307-10.

30. Moreno-Navarrete JM, Ortega F, Castro A, Sabater M, Ricart W, Fernández-Real JM: Circulating omentin as a novel biomarker of endothelial dysfunction. Obesity (Silver Spring). 2011; 19:1552-59.

31. Greulich S, Chen WJ, Maxhera B, Rijzewijk LJ, van der Meer RW, Jonker JT, Mueller H, de Wiza DH, Floerke RR, Smiris K, Lamb HJ, de Roos A, Bax JJ, Romijn JA, Smit JW, Akhyari P, Lichtenberg A, Eckel J, Diamant M, Ouwens DM: Cardioprotective properties of omentin-1 in type 2 diabetes: evidence from clinical and in vitro studies. PLoS One 2013; 8:e59697

32. Barnes G, Japp AG, Newby DE: Translational promise of the apelin-APJ system. Heart 2010; 96:1011-1016.

33. Kuba K, Zhang L, Imai Y, Arab S, Chen M, Maekawa Y, Leschnik M, Leibbrandt A, Markovic M, Schwaighofer J, Beetz N, Musialek R, Neely GG, Komnenovic V, Kolm U, Metzler B, Ricci R, Hara H, Meixner A, Nghiem M, Chen X, Dawood F, Wong KM, Sarao R, Cukerman E, Kimura A, Hein L, Thalhammer J, Liu PP, Penninger JM. Impaired heart contractility in Apelin gene-deficient mice associated with aging and pressure overload. Circ Res 2007; 101(4):e32-42.

Journal: *Frontiers of Hormone Research*

Impact factor: 4.875

Contribution: Total: 50%

Conceived / designed experiments: -

Performed experiments: -

Analysed data: -

Contributed to discussion: 50%

Wrote the manuscript: 40%

Reviewed / edited manuscript: 60%

Author: 1st author

CHAPTER 6: General Discussion

6.1. Characterization and validation of the novel adipokine soluble DPP4

The concept that AT acts as an endocrine gland affecting whole-body energy homeostasis was a major break-through toward a better molecular understanding of obesity and its co-morbidities. Numerous studies have demonstrated that excess of AT mass is responsible for metabolic and inflammatory disturbances that cause T2D and CVD (110). AT communicates with other central and peripheral organs by secreting a variety of molecules known as adipokines (156). The levels of some adipokines correlate with specific metabolic states and have the ability to directly affect the regulation of metabolic homeostasis. Thus, a deregulation of adipokines has been implicated in obesity, T2D, hypertension, CVD, and a growing list of pathological situations. There is an ever-increasing number of adipokines described in the literature which until today has risen to more than 700 proteins (204-212). However, the secretome of adipocytes remains still incompletely characterized and the discovery and validation of new adipocyte-derived factors is still in progress. In a previous study, our group investigated the secretome of human adipocytes by a comprehensive proteomic approach and identified 347 proteins, 263 of which were predicted to be secreted (213).

Among others, DPP4 was identified as a novel adipokine released by fully differentiated human adipocytes. DPP4 is an ubiquitously expressed cell-surface protease belonging to the prolyloligopeptidase family. It was first discovered in 1966 as glycyl-prolyl-β-naphthylamidase1 and later has been named dipeptidyl aminopeptidase IV, post-proline dipeptidyl aminopeptidase, and X-Pro dipeptidyl aminopeptidase. The current name is dipeptidyl-peptidase 4, as recommended by the Enzyme Commission (214). Due to shedding from the cell membrane, a soluble form of DPP4 (sDPP4) lacking the cytoplasmatic tail and the transmembrane region can be found in plasma and other body fluids. Although the origin of sDPP4 is considered to be most likely from endothelial cells, liver epithelium and from circulating leukocytes (214), the major source of circulating DPP4 and its regulation remains unknown. Low levels of DPP4 occur concomitantly with impaired immune status,

whereas increased levels emerge in inflammatory and infectious diseases (enhanced immune status), hematological tumors, and liver diseases (215;216). The functional role of DPP4 is related to both its enzymatic and non-catalytic function. DPP4 is a serine exopeptidase, which cleaves X-proline/alanine dipeptides from the N-terminus of several substrates, including chemokines, neuropeptides and vasoactive peptides. In addition to its peptidase activity, another important function of DPP4 is the interaction with a range of ligands, including ADA, caveolin-1, thromboxane A2 receptor and fibronectin (217). The binding of DPP4 to its ligands plays a role in various processes including immune regulatory function, such as enhancing T-cell activation and functional modulation of antigen-presenting cells (217). An increasing number of publications describe its diverse properties and multiple functions in fields ranging from immunology and inflammation to glucose homeostasis, oncology, and neuroendocrinology (214). However, due to its degradation of the incretin hormones DPP4 has gained most interest as a therapeutic target for T2D treatment, and a variety of DPP4-inhibitors that prolong the insulinotropic effect of GLP-1 are now in clinical use (218). Thus, indicating that the novel adipokine sDPP4 is of broad interest.

In the present study, we demonstrated that sDPP4 release is increased considerably during adipocyte differentiation. The comparison with preadipocytes and adipose tissue macrophages show that adipocytes most likely represent the major source of sDPP4, which is released from the intact organ into the circulation. The promoter of the DPP4 gene has the characteristics of a housekeeping gene promoter but can nevertheless initiate the transcription in a tissue-specific fashion (214). The promoter contains several potential binding sites for transcription factors (214;219-221) and a variety of cytokines (e.g. IL-12, Interferon γ, TGFβ2) have been shown to increase DPP4 expression in a cell type-specific way (222-226). In contrast, the regulation of DPP4 shedding or the responsible protease is presently unknown. Interestingly, we demonstrated that both insulin and TNFα augment the shedding of DPP4 by approximately 50% despite an unaltered expression. Thus, factors related to insulin resistance and AT inflammation enhance the shedding of this novel adipokine from the fat cell.

6.1.1. Direct effects of soluble DPP4

So far, the direct effects of sDPP4 on isolated cells have not been investigated, although it binds to the extracellular matrix and may exert signaling functions (227). In the present study, we demonstrated for the first time that sDPP4 consistently impairs insulin signaling at the level of Akt in human primary cell types, including adipocytes and skeletal muscle cells. Enzymatic activity of sDPP4 seems to be involved in this process, but DPP4 inhibitors may also affect the binding properties of sDPP4 to a putative receptor (further discussed in 5.2.1.). In our study, we used sDPP4 concentrations reflecting circulating levels that were measured in both lean and obese subjects. Furthermore, recent studies in obese mouse models proposed a role of DPP4 in AT inflammation, since DPP4 inhibition leads to reduced expression of inflammatory markers as well as immune cell infiltration (228;229). Beside its role in insulin signaling and inflammation, sDPP4 may also be involved in AT lipolysis. DPP4 recruits adenosine deaminase (ADA), a monomeric enzyme catalyzing deamination of adenosine to inosine and ammonia, to the cell surface (230;231). DPP4-bound ADA has 1000-fold greater activity than free ADA (232), which in turn may modulate the well established antilipolytic effects of adenosine. Moreover, DPP4 is a strong inhibitor of the antilipolytic activity of neuropeptide Y (NPY) (233), which is one of the best peptide substrates of the enzyme (234). Therefore, enhanced abundance of DPP4 within AT of obese subjects may substantially augment the lipolytic activity of enlarged adipocytes. Finally, the biological activity and specificity of a large number of chemokines, adipokines and neuropeptides is modified by DPP4–mediated cleavage. This supports the notion that sDPP4 plays a yet undefined functional role in the intraorgan crosstalk among macrophages, adipocytes, and other components of the stromal-vascular fraction.

6.1.2. Role in obesity and the metabolic syndrome

Serum levels of sDPP4 are altered in many pathophysiologic conditions, including different types of cancer, allergic asthma, or hepatitis C (235). In our study, we analyzed for the first time circulating sDPP4 in the context of obesity and the metabolic syndrome. DPP4 serum levels of morbidly obese men are elevated compared with lean controls and

significantly correlated with BMI, the size of adipocytes in subcutaneous and visceral fat, and the adipocyte hormones adiponectin (negatively) and leptin. This data suggest that sDPP4 is related not only to increased body weight but also to other important parameters of AT physiology. In addition to circulating sDPP4, the protein expression of this adipokine is significantly different not only between lean and obese subjects but also between their fat depots. Regarding DPP4 expression there are conflicting data in the literature, describing both decreased and increased mRNA expression of DPP4 in AT of obese men (233;236). We now clearly demonstrate at the protein level that obesity leads to a prominent induction of DPP4 abundance in both subcutaneous and visceral AT and that the visceral fat exhibits the highest DPP4 level in obese subjects. Furthermore, we found that the increased expression of DPP4 in obese compared to lean individuals is paralleled by an increased release of sDPP4 in AT explants of obese patients compared to lean controls. Similar to circulating sDPP4, its release from AT correlates with various classical markers for the metabolic syndrome, namely BMI, waist circumference and plasma triglycerides, and HOMA as an index of insulin resistance, as well as with fat cell volume and the adipokine leptin. In addition, sDPP4 release and serum concentration can be reversed to normal levels by surgery-induced weight loss. Thus, in obesity, both circulating levels of sDPP4 and sDPP4 release by AT are increased and correlate strongly with the metabolic syndrome but can be reduced to control levels by substantial weight loss. Therefore, we conclude that enlargement of visceral adipocytes in obesity may substantially contribute to the augmented level of circulating sDPP4 in obese patients.

It is noteworthy that we measured DPP4 serum concentration and not its activity. It has been shown that there is no direct correlation between serum sDPP4 protein concentrations and serum DPP4 enzymatic activity, for several reasons. On the one hand, there are some circulating proteins other than DPP4 with DPP4 activity (226). Additionally, sialylation, a type of glycosylation, of sDPP4 is strongly enhanced in elderly individuals (214;226;237;238), and the finding that a certain type of hypersialylation can inhibit DPP4 activity is consistent with the fact that serum/plasma DPP4 enzymatic activity tends to decrease with age (214;239).

Considering the increased circulating levels of sDPP4 in obesity and its detrimental effects on insulin signaling *in vitro*, it may be speculated that sDPP4 may participate in the induction of insulin resistance. Therefore, DPP4 knockout models offer novel insights into the potential role of DPP4 in obesity and the metabolic syndrome. Inactivation of the DPP4

gene through homologous recombination produces surprisingly normal mice. Despite the putative importance of DPP4 for immune function, $DPP4^{-/-}$ mice develop normally with no major immune phenotype reported in the absence of immunological challenge. However, glycaemic excursion is significantly reduced in association with increased levels of glucose-stimulated insulin and decreased degradation of both GLP-1 and GIP (240). Furthermore, in rats, DPP4 deficiency leads to reduced weight gain under high-caloric dietary conditions (241). F344/DuCrlCrlj rats, a substrain of the inbred Fischer 344 (F344) strain, express *DPP4* mRNA but lack DPP4 enzyme activity due to the translation of abnormal isoforms that fail to be processed to the biologically active mature glycosylated enzyme (242-244). A couple of studies have previously reported that high-fat diet-induced insulin resistance and glucose intolerance were less prevalent in F344/DuCrlCrlj rats than wild-type F344/Jcl rats (242;245;246). Furthermore, in a streptozotocin (STZ)-induced diabetic model DPP4 deficiency in F344/DuCrlCrlj rats delayed the onset of diabetes, but worsened dyslipidaemia and renal dysfunction induced by STZ (242). Whether the observed effects could be ascribed to direct effects of sDPP4 or to inhibition of enzymatic degradation of DPP4 substrates remained unknown.

In summary, we showed that sDPP4 is a novel adipokine that is substantially overexpressed in visceral fat from obese subjects and exhibits an augmented release in obesity. sDPP4 exerts autocrine and paracrine effects as shown by impaired insulin signaling in adipocytes and skeletal muscle cells. We further observed a tight correlation of sDPP4 release to adipocyte cell size, and plasma levels of DPP4 strongly correlate with the risk of having the metabolic syndrome. Thus, our data indicate that sDPP4 might be a novel biomarker and a potential link between obesity and the metabolic syndrome. Future work will be needed to address the mechanism and the functional role of sDPP4 in the pathogenesis of insulin resistance and obesity-associated complications.

6.2. Crosstalk between adipose tissue and vascular smooth muscle cells

As previously outlined, in the context of obesity AT has gained considerable interest as a key player in the pathogenesis of several metabolic diseases like T2D and cardiovascular disease (CVD). AT was long seen as a passive lipid storage depot but it is now considered as an endocrine organ that produces a large number of mediators that affect metabolism, inflammation and coagulation. In obesity, adipocyte hypertrophy and the chronic low-grade inflammation within AT affects its normal physiological function. AT dysfunction results in an altered production and secretion of adipokines, which in turn affect several peripheral tissues in an endocrine manner such as liver, skeletal muscle and vasculature. Thus, numerous circulating pro-inflammatory mediators participate in the development of CVD and are released directly from adipocytes, thereby linking obesity to an increased cardiovascular risk. Furthermore, almost all blood vessels are surrounded by PVAT, which has not received attention in research until recently. Since perivascular adipocytes encroach into the outer adventitial region without an anatomical barrier, mediators secreted by PVAT may readily gain access into the blood vessel wall, and PVAT may function as a paracrine organ that transduces metabolic signals to blood vessels (247;248). However, the crosstalk of AT with cells of the arterial wall is not yet completely understood.

6.2.1. Role of soluble DPP4

With comprehensive proteomic profiling of the human adipocyte secretome we identified sDPP4 as a novel adipokine that turned out to be a promising candidate potentially linking obesity to the metabolic syndrome. Interestingly, high serum levels of sDPP4 have been described in various pathological conditions (249) including atherosclerosis (250). Additionally, DPP4-inhibitors, a class of oral anti-hyperglycaemic agents, hold interesting promise for cardiovascular protection; however, the underlying mechanisms of these beneficial effects remain poorly understood. Since the DPP4 substrate GLP-1 itself has favorable cardiovascular effects (251;252) most of these protective effects of DPP4 inhibition are ascribed to the increased bioavailability of GLP-1. Nevertheless, there are accumulating

data suggesting that DPP4-inhibitors mediate vascular protection independent of GLP-1, involving endothelial repair (253), anti-inflammatory effects (253-255) and blunting of ischemic injury (256;257). Whether sDPP4 directly participates in the development of CVD has not yet been elucidated. Additionally, the signaling pathways mediated by sDPP4 are not completely understood and what is known varies depending on the cell type, context and the microenvironment.

In the present study (CHAPTER 3), we demonstrated that sDPP4 directly induces VSMC proliferation via the MAPK pathway. VSMCs are not terminally differentiated and modulate their phenotype in response to physiological and pathological cues. Differentiated VSMC are characterized by low rates of proliferation and secretion and express a unique repertoire of contractile proteins, ion channels, and signaling molecules required for the cell's contractile function (258-260). In response to vascular injury or growth factor signaling, VSMC de-differentiate and adopt a proliferative, migratory phenotype that contributes to vascular occlusion in a variety of disorders, including atherosclerosis (261). This phenotypic switching is accompanied by reduced expression of contractile proteins (e.g. α-smooth muscle actin (αSMA)) as well as an increased expression and release of inflammatory cytokines and matrix metalloproteinases (MMP) (262). Accordingly, the sDPP4-induced proliferation was paralleled by a reduced protein abundance of well known differentiation markers, such as αSMA and bone morphogenic protein receptor II (BMPR2) (Figure 4). Moreover, sDPP4 treatment leads to markedly increased expression of MMP-1, which is largely upregulated in VSMCs of atherosclerotic plaques (263). *In vitro*, it has been demonstrated that MMP-1 expression is stimulated by a host of growth factors and cytokines, including IL-1, transforming growth factor-β (TGFβ), and TNFα (264). Furthermore, we observed an elevated release of vascular endothelial growth factor (VEGF) after exposure of VSMC to sDPP4 (Figure 4). Since our group has recently published that VEGF plays a major role in the induction of VSMC proliferation (265), one may speculate that sDPP4 contributes to increased VSMC proliferation both by activating the MAPK pathway and by increased VEGF release.

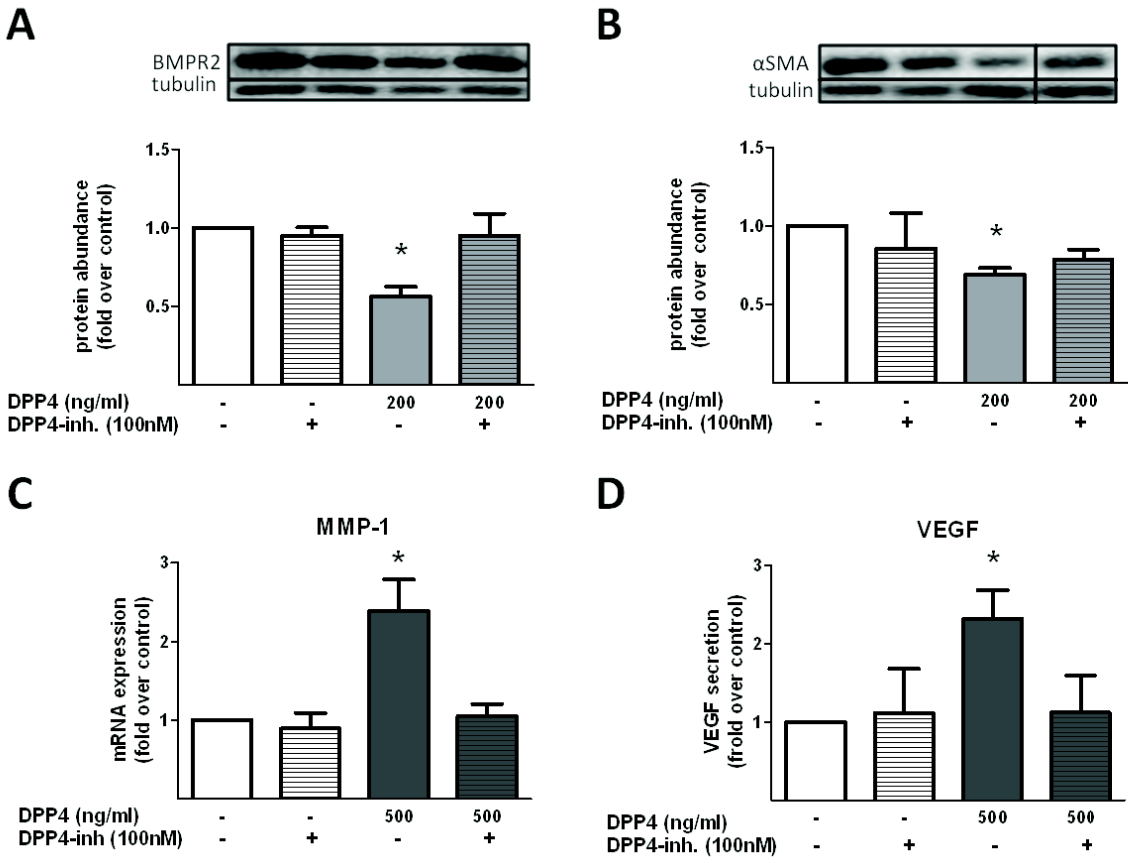


Figure 4:. Impact of sDPP4 on VSMC phenotype.
Cells were serum-starved for 24 h and subsequently treated with indicated amounts of sDPP4 alone or in combination with its specific enzymatic inhibitor K579 for 24 h. (A-B) Total cell lysates were resolved by SDS-PAGE and immunoblotted with indicated antibodies. Data are tubulin normalized mean values ± SEM. *p < 0.05 vs. to control (n=3-4). Representative Western Blots are presented. (C) mRNA level of MMP-1 was quantified by Real-time PCR and normalized to the level of β-actin. Data are expressed relative to the basal control value and are means ± SEM. *p < 0.05 vs. control (n=3-4). (D) After 24 h exposure to the respective treatments, supernatants were collected and VEGF concentration was measured by ELISA assay. Data are expressed relative to the basal control value and are means ± SEM. *p < 0.05 vs. control VSMC (n=4)

As a further indicator for VSMC phenotype switch, we showed that sDPP4 induces the expression and the release of pro-inflammatory cytokines via NF-κB activation as well as increases inducible nitric oxide synthase (iNOS) protein abundance. iNOS, a nitric oxide- and peroxynitrite-forming enzyme, is overproduced in atherosclerosis and plays a key role in vascular inflammation and endothelial dysfunction (266). The expression of iNOS is induced by cytokines and requires the sequential upstream activation of ERK1/2 and NF-κB (267). In contrast to acute induction of NO production by endothelial nitric oxide synthase (eNOS), iNOS produces large amounts of NO in a chronic way (268-270). Although NO initiate responses such as vasodilation (271), inhibition of platelet aggregation and adhesion to the

vessel wall (272), and inhibition of VSMC proliferation (273), in higher concentrations NO has other effects, some of which are cytotoxic. This indicates that sDPP4 by inducing pro-inflammatory cytokines and iNOS also participates in the chronic low-grade inflammation within the vascular wall.

Similar to the results in adipocytes and skeletal muscle cells, we showed that sDPP4 impairs insulin signaling on the level of Akt in VSMC. In contrast to classical target tissues for insulin action such as liver, fat and muscle, the function of proper insulin action in the vasculature is less well understood. In the literature there are two major possibilities discussed how the inhibition of insulin action could have detrimental effects on vascular cells (Figure 5).

Firstly, the impaired activation of the Akt/eNOS-pathway by insulin results in decreased NO production. NO is a powerful vasodilator leading to VSMC relaxation and thereby regulating vascular tone. It is well accepted, that decreased bioavailability of NO and the impairment of vasodilatation is involved in hypertension and the development of atherosclerosis. Although a major part of NO is released by endothelial cells, it has been shown *in vitro* that insulin inhibits contraction at the level of the individual VSMC in the absence of any other cell type (274). Thus suggesting that insulin-induced vasodilation *in vivo* is not entirely endothelium-mediated. Furthermore, insulin-stimulated NO production has important anti-inflammatory and anti-thrombotic properties through inhibition of leucocyte adhesion, and limiting platelet adhesion and aggregation, and reduced expression of PAI-1, a pro-thrombotic protein (275). Furthermore, NO has been shown to inhibit DNA synthesis, mitogenesis and proliferation of VSMC (276-278). Conversely, in advanced plaque progression, disruption of Akt cell-survival signaling may promote apoptosis of VSMC and therefore thinning of fibrous cap and causing plaque rupture (279). Therefore, sDPP4 may reduce bioavailable NO thereby predisposing vasculature to hyper-inflammatory and thrombotic states.

Secondly, numerous studies reported a “selective” insulin resistance in VSMC with a profound inhibition of the insulin-induced PI3K signaling, whereas the activation of the MAPK-pathway is enhanced (280;281). The shift from the insulin-mediated metabolic (Akt) to the mitogenic (MAPK) pathway leads to increased proliferation and migration in VSMC. Thus, the sDPP4-induced proliferation might also partially be a result of selective insulin resistance in VSMC.

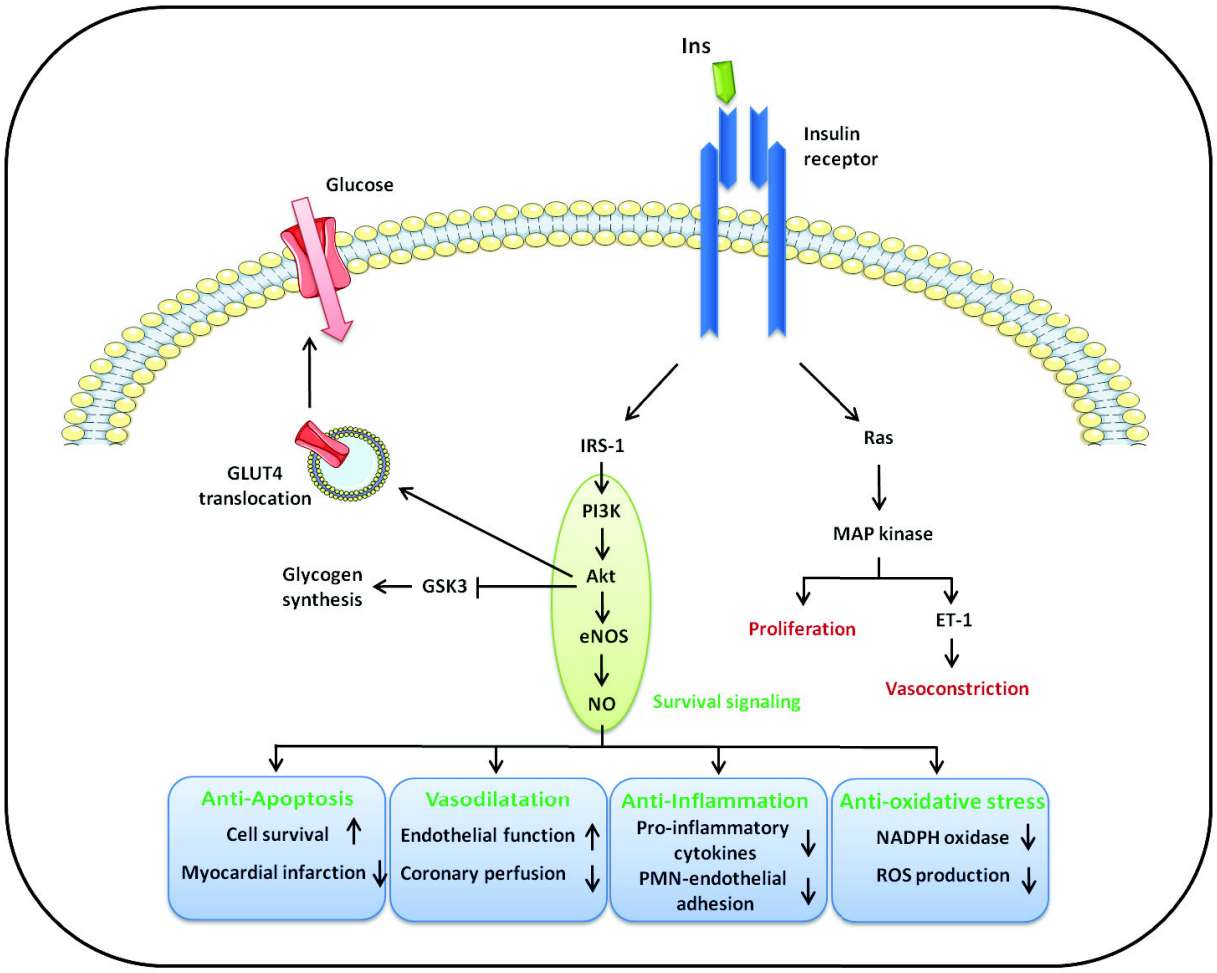


Figure 5: Insulin signaling in cardiovascular cells.
Insulin binds to the cell membrane insulin receptor, resulting in the activation of mainly two signaling pathways: Ras-MAPK, which leads to proliferation, and PI3K-Akt-eNOS, which results in metabolic modulation and cardiovascular protection. Akt, protein kinase B; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; GLUT4, glucose transporter 4, GSK, glycogen synthase kinase; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositol 3´-kinase; PMN, polymorphonuclear neutrophil; ROS, reactive oxygen species (modified from (282))

Our data on the direct effects of sDPP4 indicate that sDPP4 exerts its signaling properties by receptor interaction. In the literature, it has currently been discussed that DPP4 may bind to fibronectin (283), toll-like receptor 4 (TLR4) (284) and mannose-6-phosphate receptor/IGF-2 receptor (M6P/IGF2R) (285). DPP4 binding to fibronectin seemed to have a role in the interaction of cells with the extracellular matrix (ECM) and may promote cell migration and metastasis (283). The ECM-binding domain of DPP4 is located in the cystein-rich region of DPP4 and is distinct from its exoprotease catalytic site (214;283;286). However, specific inhibitors of DPP4 noticeably enhanced the DPP4-ECM interaction, possibly as a result of an altered conformation of DPP4 (286). DPP4 may also interfere with

TLR 4-mediated signaling. In cultured macrophages (U937 cells), Ta *et al.* have recently shown that the ERK phosphorylation induced by the TLR4-ligand lipopolysaccharide (LPS) can be inhibited by the specific DPP4-inhibitor alogliptin (284). Moreover, LPS-induced MMP-1, -9, -12 and -15 expression was also suppressed by alogliptin (284). Furthermore, binding of sDPP4 to M6P/IGF2R plays an important role in DPP4-mediated co-stimulation (287) as well as transendothelial migration of T cells (285). Additionally, a recent study in human umbilical vein endothelial cells (HUVEC) observed a sDPP4-induced increase in superoxide generation which was completely prevented by inhibition of sDPP4-M6P/IGF2R interaction (288).

In the present study (CHAPTER 3), bioinformatic analysis and the signaling signature induced by sDPP4 suggest that sDPP4 might be an agonist for the protease-activated receptor 2 (PAR2). PARs have been suggested to play a crucial role in the development of functional and structural abnormalities in vascular lesions due to the activation of proteases during pathological states such as hemorrhage, tissue damage, and inflammation. The mechanism of PARs activation is unique. Instead of binding to the receptor and activate it as a conventional ligand, proteases cleave the N-terminus, thereby exposing a tethered ligand (TL) that auto-activates the receptor. However, synthetic peptides corresponding to the TL specifically activate PAR in the absence of proteases (289), (exemplary shown for PAR2 Figure 6). Among the four so far identified members, PAR1 and PAR2 were found to play an important role in the vascular system, whereas the functional roles of PAR3 and PAR4 are mainly limited to platelets (290). Especially PAR2 seems to play an important role in VSMC homeostasis. This receptor is upregulated in VSMC of vascular lesions in humans (291) and induces VSMC proliferation and migration *in vitro* (292;293). Physiological agonists for PAR2 are proteases, such as mast cell tryptase and coagulation factor Xa and VIIa. Trypsin is also a PAR2 agonist, but it does not belong to the physiological agonist, since it is a digestive and its activation in the vascular system is unusual.

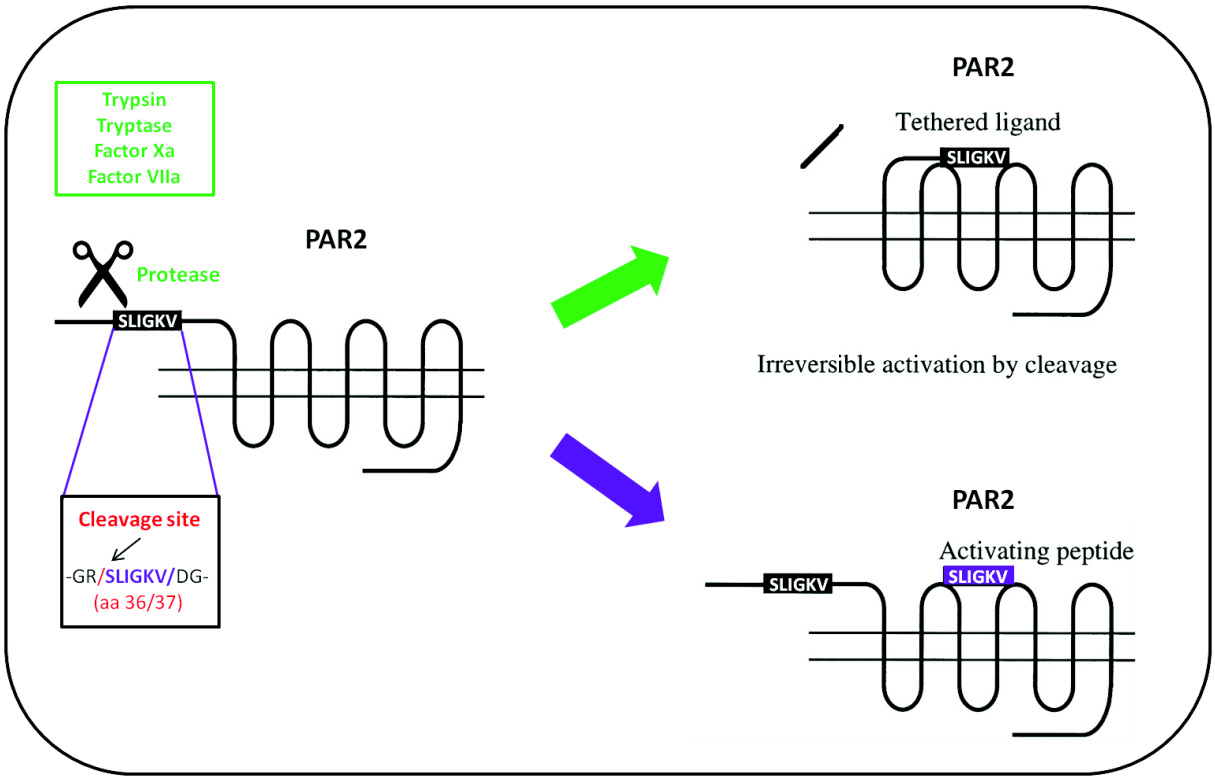


Figure 6: Different mechanisms of PAR2 activation.
Proteases cleave the R36/S37 peptide bond, liberating the new N terminus SLIGKV, which then serves as a tethered ligand that binds to the receptor thereby triggering transmembrane signaling. In addition, a synthetic peptide corresponding to the TL (SLIGKV) for PAR2 specifically activates PAR2 in the absence of proteases. PAR2, protease-activated receptor; aa, amino acid (modified from (294))

By bioinformatic analysis, we searched for the different TL sequences of all PAR members within the DPP4 sequence. However, we found only the TL sequence SLIGKV of PAR2 in the cystein-rich region of DPP4 responsible for partner binding. We showed an almost perfect match for the first four amino acids, which are most important for PAR2 activation (295). Within the tertiary structure of DPP4, the potential PAR2 TL sequence SLIG was located on the protein surface. One may speculate that sDPP4 activates the PAR2 receptor with its TL sequence and that DPP4-inhibitors affect the binding properties of sDPP4 by a conformational change or masking the PAR2 TL sequence. Since sDPP4 is a serine exopeptidase that cleaves dipeptides from proteins carrying proline or alanine in their penultimate position and the cleaving side of PAR2 is located between residue R36 and S37, it is most unlikely that sDPP4 cleaves the N-terminus of PAR2 thereby exposing the TL and activating the receptor.

In accordance to the sDPP4-induced signaling, a stimulation with the PAR2-activating peptide (AP) SLIGKV leads to NF-κB (296) and ERK1/2 (297) activation resulting in VSMC mitogenesis. Regarding the sDPP4 effects on cytokine secretion, in human neutrophils, the PAR2-AP induces IL-6 and IL-8 secretion (298). Accordingly, in cells expressing less PAR2 like monocytes, Ikeda *et al.* (299) showed no direct effects of sDPP4 on IL-6 and TNF-α expression and secretion. However, the combination of LPS with sDPP4 enhances the expression and secretion of these pro-inflammatory cytokines. This study further indicates that sDPP4 is able to interact with TLR4 signaling cascade; however, the exact mechanism remains unclear.

Other cell types participating in the development of atherosclerosis, such as macrophages and endothelial cells are also affected by sDPP4. DPP4 has long been considered as having an important role in immunomodulation. Since atherosclerosis is an inflammatory disease, it may be speculated that sDPP4 modulates responses occurring within early or late atherosclerotic lesions. Accordingly, in macrophages, DPP4 (soluble or membrane-bound) was reported to interact with caveolin-1, resulting in the phosphorylation of this protein (300). Subsequently, caveolin-1 induces the dissociation of the toll interacting protein (Tollip) and interleukin-1 receptor-associated kinase 1 (IRAK1) complex, leading to the downstream activation of NF-κB via IRAK-1-dependent mechanisms (301;302). Additionally, in low-density lipoprotein receptor-deficient (LDLR-/-) mice Shah *et al.* demonstrated that exogenously injected DPP4 increases monocyte migration *in vivo* (303). These pro-migratory properties of sDPP4 could be completely inhibited by sitagliptin. Moreover, in collaboration with Sánchez-Ferrer and colleagues, we analyzed direct effects of sDPP4 on vascular function with special focus on vascular reactivity (304). Therefore, mouse mesenteric microvascular fragments were isolated and mounted on a small vessel wire myograph. sDPP4 impaired the endothelium-dependent relaxation to acetylcholine in a concentration-dependent manner, without modifying endothelium-independent relaxations to sodium nitroprusside. Co-incubation of sDPP4 with its enzymatic inhibitor prevented impaired endothelium-dependent relaxation by sDPP4. Similarly, Inhibition of cyclooxygenase or thromboxane A2 receptor signaling abrogated the impairing action of sDPP4. These data suggest that sDPP4 directly impairs endothelium-dependent relaxation, through a mechanism that involves cyclooxygenase activation and likely the release of a vasoconstrictor prostanoid. Collectively, these studies point to the notion that sDPP4 may

contribute to the chronic low-grade inflammation within the vascular wall and participate in the impairment of vasodilatation in atherosclerosis.

In conclusion, we characterized a novel sDPP4-induced and receptor-mediated signaling cascade in VSMC. sDPP4 directly activates the MAPK- and NF-κB signaling pathway in a PAR2 dependent manner leading to pro-atherogenic changes in VSMC like increased proliferation and inflammation. Thus, this data might contribute to explain the vascular protective effects of DPP4-inhibitors independent of GLP-1. Considering that sDPP4 is an adipokine with increased circulating levels in obesity, it can be speculated that sDPP4 may act in a para- or endocrine fashion on the vascular wall. Our data support the notion that direct effects of sDPP4 may play a pivotal role in linking obesity to CVD.

6.2.2. Role of the whole secretory output of adipocytes

It is well-accepted that specific adipocyte-derived factors such as leptin, resistin and visfatin induce VSMC proliferation and migration thereby participating in VSMC dysfunction and the development of atherosclerosis (305). However, there are only few studies investigating the impact of the whole secretory output of human adipocytes on VSMC. In a recent published study from our working group Lamers *et al.* showed that adipocyte conditioned medium (CM) induces pro-atherogenic changes in VSMC with an increase in proliferation and migration (306). However, the underlying mechanism of this switch from the contractile to the synthetic phenotype of VSMC is not yet completely understood. In the present study, we used *in vitro* differentiated VSMC to create a more physiological cell model in comparison to widely used proliferating smooth muscle cells. The transition from the undifferentiated to the differentiated phenotype was judged on the basis of increases in the protein abundance of smooth muscle cell differentiation markers, such as αSMA, the transforming growth factor β receptor II (TGFβR2), and the BMPR2 (307;308), as well as the lack of proliferation in response to adipocyte CM (Figure 7).

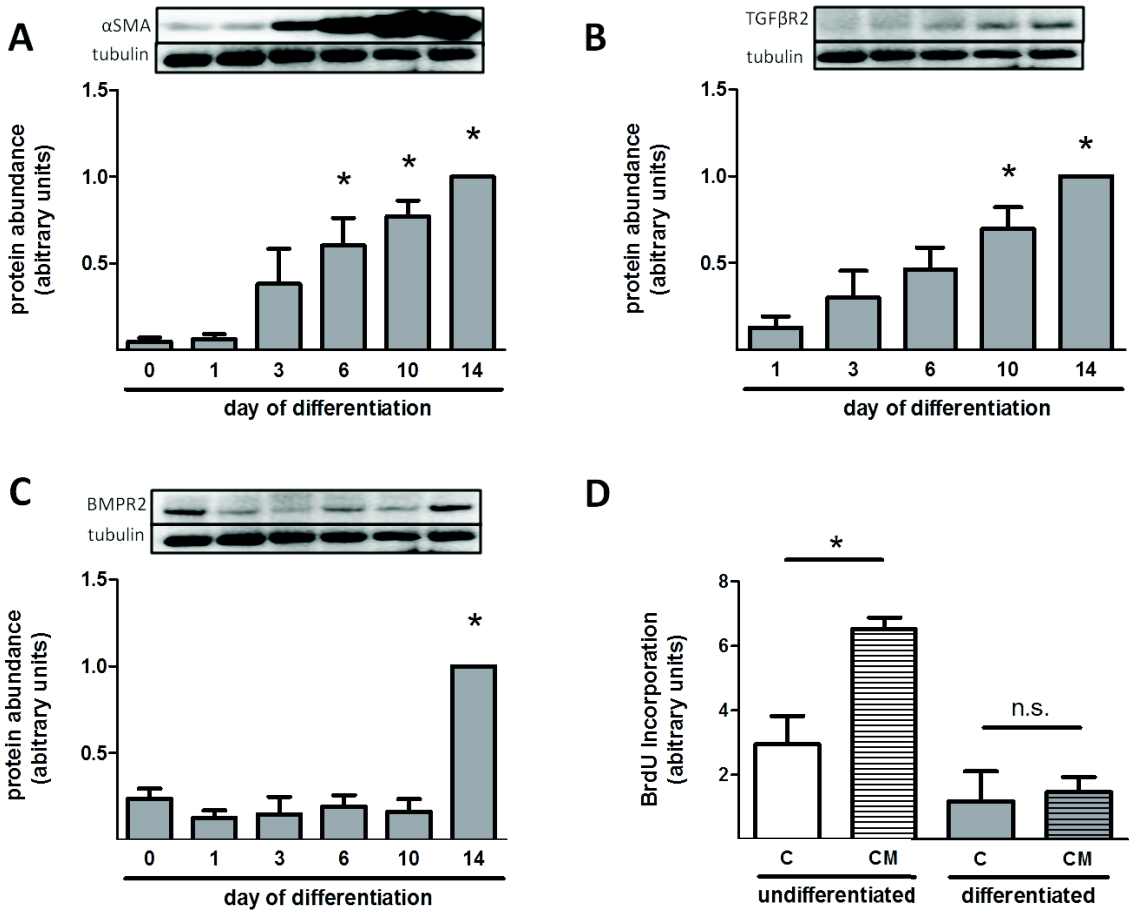


Figure 7:. Characterization of in vitro differentiated VSMC.
Human VSMC were differentiated and protein level of αSMC, TGFβR2 and BMPR2 during differentiation were analyzed by SDS-PAGE and Western Blot. Data were normalized to the protein level of tubulin and are expressed relative to day 14. Data are mean values± SEM, *p < 0.05 vs. day 0 or 1, respectively (n=3-5). Proliferation of hVSMC was determined by measuring the incorporation of BrdU into DNA. Data are mean values ± SEM. *p < 0.05 as indicated (n=15).

Accumulating evidence suggests that insulin resistance may play a pivotal role in the development of smooth muscle cell dysfunction, especially in patients with T2D (309). In the present study, we demonstrated for the first time that adipocyte-derived factors impair insulin signaling in *in vitro* differentiated human VSMC via p38-signaling. As previously discussed proper insulin action may play an important role in NO bioavailability and the induction of VSMC proliferation. However, in the present study, we observed CM-induced insulin resistance on the level of Akt and its target protein eNOS, but we did not see an increase in VSMC proliferation. The reason for that could be that in studies observing the selective insulin resistance, VSMC were exposed to one specific adipokine instead of the whole secretory output of human adipocytes. Due to the high amount of different

adipokines, we cannot exclude that anti-proliferative and anti-inflammatory adipokines such as adiponectin (310) prevent an increase in proliferation. Furthermore, the fact that we used *in vitro* differentiated VSMC might also have an impact on the CM-induced effects. Our working group has already demonstrated that CM induces proliferation in synthetic VSMC (310). However, during VSCM differentiation, we observed an increased expression of transforming growth factor β receptor 2 (TGFβR2) and BMPR2, both receptors belonging to the TGFβ signaling pathway and being involved in the inhibition of VSMC proliferation (311;312). Especially, the TGFβ signaling pathway via p38 has been shown to inhibit VSMC growth (311). In-line with this notion we found an increased expression of the differentiation marker αSMA. αSMA is a contractile protein that is one of the earliest markers of smooth muscle lineage in cell differentiation and the most abundant contractile protein present in VSMC (260;313;314). Conversely, αSMA concentrations are decreased in proliferating VSMC *in vitro* (314-317). On the other hand, a recent published study suggests that αSMA could also have detrimental effects on VSMC by playing a key role in the enhancement of VSMC stiffness during aging (318). Additionally, some groups have speculated that VSMC migration and proliferation may be an important component in vascular repair, and that VSMC quiescence or senescence contributes to the thin-walled fibrous cap of unstable atheroma (319-321). Whether enhanced VSMC mitogenic activity in insulin-resistance is a reparative or a maladaptive response to vascular injury or both remains debatable (321).

Finally, we showed the induction of the inflammatory markers PAI-1 and MCP-1 by CM. PAI-1 is the major physiologic regulator of the plasmin-based pericellular proteolytic cascade, a modulator of VSMC migration and a causative factor in CVD and restenosis (322). Inhibition of the fibrinolytic system by PAI-1 overexpression in transgenic mice results in the spontaneous development of thrombi and perivascular fibrosis (323-325). Additionally, transgenic overexpression of PAI-1 in VSMC promotes proliferation through activation of the ERK1/2 and NF-κB pathways (326). Furthermore, there is considerable evidence that PAI-1 expression protects VSMC from plasmin-induced apoptosis (322;327;328), suggesting that PAI-1 modulation of neointimal growth is a consequence of both increased proliferation and reduced apoptosis. In contrast, PAI-$1^{-/-}$ mice exhibit significantly reduced VSMC density in the neointima compared to wild type mice in response to oxidative stress-induced vessel injury, in the balloon-catheterized carotid artery as well as in atherosclerosis prone $ApoE^{-/-}$ mice

(329-331). Thus, plasminogen activation goes beyond fibrinolytic control and extends to processes including cell migration and proliferation.

MCP-1, acting through the C-C chemokine receptor type 2 (CCR2), is the prototype of the C-C chemokine β subfamily and exhibits its most potent chemotactic activity toward monocytes (332). Moreover, recent evidence suggests that MCP-1 contributes to thrombin generation and thrombus formation by generating tissue factor (333). In vascular cells, MCP-1 expression is induced in response to pro-inflammatory factors such as IL-1 and TNFα, as well as oxLDL (334-336). In bone marrow transplantation studies, overexpression of MCP-1 in vessel wall macrophages led to increased foam cell formation and increased atherosclerosis (334;337). Conversely, deletion of MCP-1 in $LDLR^{-/-}$mice attenuated the progression of dietary-induced atherosclerosis (334;338). Thus, MCP-1 appears to play a pivotal role in the recruitment of monocytes to atherosclerotic lesions, in the formation of intimal hyperplasia after arterial injury and in aspects of thrombosis.

In summary, we have shown that adipocyte-derived factors impair insulin signaling and induce the expression of pro-inflammatory cytokines PAI-1 and MCP-1 in differentiated VSMC. We therefore propose that the elevated release of adipokines in the obese state may link AT to vascular inflammation, VSMC dysfunction, and the development of atherosclerosis.

6.2.1.1. Role of micro RNAs

Micro RNAs (miRNAs) are relatively recently discovered negative regulators of gene expression and act at the posttranscriptional level (Figure 8). Biogenesis of mature miRNA begins in the nucleus with transcription of the miRNA gene, generating the primary miRNA (pri-miRNA) that contains the mature miRNA as an RNA hairpin (339-341). Transcription of miRNA genes is regulated by multiple mechanisms including transcription factors (342;343), epigenetic silencing (344), and even other miRNA (345). After transcription, the primary miRNA is cleaved by Drosha, an endonuclease, to create an approximately 70-base pairs precursor miRNA (pre-miRNA) (339). The pre-miRNA is then exported out of the nucleus to the cytoplasm via exportin-5. In the cytoplasm, the pre-miRNA is further processed by Dicer, a second endonuclease, resulting in a miRNA duplex (miRNA:miRNA*). The miRNA:miRNA*

duplex contains the miRNA strand identified by the RNA-induced silencing complex (RISC) and the complementary strand (miRNA*) that is generally degraded. The mature miRNA consist of approximately 20-25 nucleotides. The miRNA loaded in the complex targets the RISC to specific binding sites in the 3′ untranslated region (UTR) of mRNA transcripts via a target recognition sequence known as a 'seed sequence' (346-348). The binding of a miRNA to its target can occur by perfect or imperfect base paring resulting in translational inhibition or mRNA degradation (349-352). There are more than 1000 human miRNAs that have currently been identified and which may target up to 60% of gene transcripts (339;353;354). Recently, miRNAs have also been implicated in the regulation of VSMC phenotype by modulation of transcription factors and other signaling molecules involved in proliferation and migration (352;355-360).

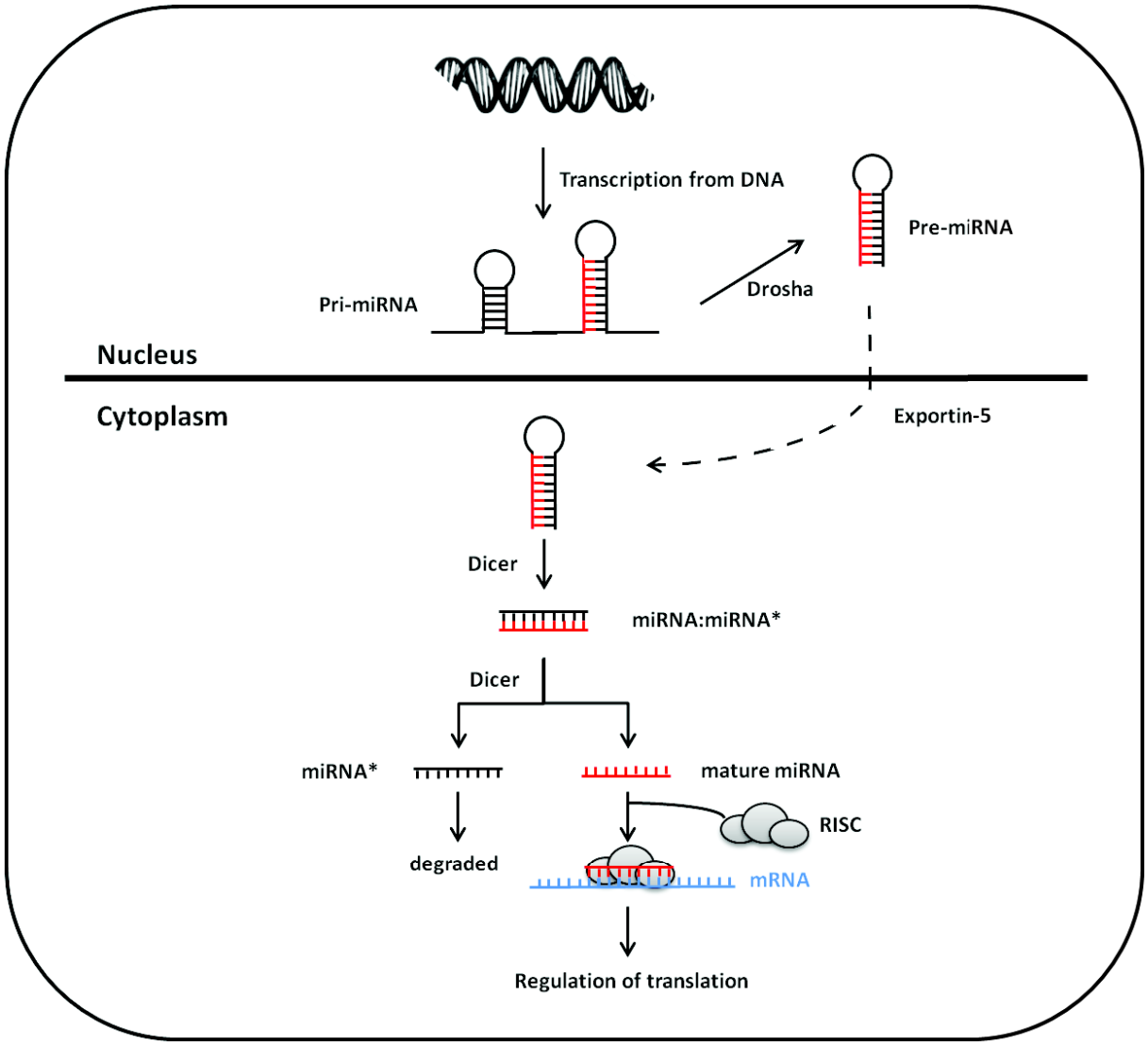


Figure 8: miRNA biogenesis pathway.
miRNAs are initially transcribed as long pri-miRNA. The Drosha complex crops the pri-miRNA into a hairpin-shaped pre-miRNA. Next, Exportin-5 promotes the nuclear translocation of the pre-miRNA which is further processed by the Dicer complex. Following Dicing, the resulting miRNA:miRNA* is dissociated and the mature miRNA is incorporated into the RISC where it functions to mediate gene silencing either by translational inhibition or by promoting the degradation of target mRNAs. (modified from (361))

In the present study (CHAPTER 4), we demonstrated that adipocyte-derived factors directly impair insulin action in VSMC via induction of miRNA-143 and subsequent downregulation of its target oxysterol-binding-protein-related protein (ORP8). It is well known that miRNA-143 is co-transcribed with miRNA-145 from the same gene and both are highly expressed in VSMCs (359;362;363). The induction of miR-143 is closely associated with obesity and insulin resistance. In mice fed a high fat diet it has been shown that the

miRNA143/145 cluster is upregulated in AT and liver (364;365). Additionally, expression of miR-143 was increased in the liver, heart, skeletal muscle and pancreas from *db/db*-mice as compared to tissues isolated from wild type control animals (364). Conversely, liver specific knock out of the miR-143/145-cluster protects against high-fat diet induced insulin resistance and hepatic Akt-inhibition (364). In accordance to our data, the authors of this study further identified ORP8 as a direct miR-143 target, and showed that a decrease in ORP8 protein abundance is responsible for the abrogation of insulin action in the liver (364). Finally, it was recently reported in cardiomyocytes that the induction of miRNA-143 by activin A secreted from epicardial AT promotes insulin resistance via ORP8, and that silencing miRNA-143 expression protects cardiomyocytes against the induction of insulin resistance (366).

In general, ORPs act as sterol sensors that relay information to diverse cellular processes such as intracellular sterol transport, integration of sterol and sphingomyelin metabolism, regulation of neutral lipid metabolism, secretory vesicle generation and microtubule-based motility of endo-lysosomes (367). ORP8 is expressed at high levels in macrophages, followed by substantial expression in liver and brain and little or no expression in heart, skeletal muscle and white AT (364). Jordan *et al.* showed that ORP8 downregulation has no impact on insulin receptor-mediated IRS-1 activation in liver, indicating that ORP8 acts closely upstream of or directly at Akt phosphorylation (364). On the other hand, it has been demonstrated that ORP8 binds 25-OH-cholesterol, which in turn regulate Akt signaling during apoptosis by promoting proteasomal degradation of Akt and/or inhibition of PI3K in macrophages (368;369). However, the exact mechanism via which ORP8 regulates insulin action remains unclear.

The strong association of miRNA-143 with insulin resistance in multiple tissues seems to be in contrast to the function ascribed to this miRNA in VSMC. The miRNA-143/145 cluster is very well characterized in the vasculature and is known as a critical regulator of VSMC differentiation. Studies on miRNA-143/145-deficient mice showed that this cluster is required for the acquisition of the contractile phenotype (370). Multiple targets of these miRNASs have been identified, including krüppel-like factor (KLF) 4, KLF5, ELK1, versican, several actin remodeling proteins, and angiotensin-converting enzyme (371;372). Interestingly, all these proteins inhibit VSMC differentiation; thus, repression of these targets by miRNA-143/145 facilitates VSMC differentiation (358;360;362;372;373). As a

consequence, the induction of miR-143/145-cluster leads to increased expression of the smooth muscle differentiation markers transgelin (TAGLN, also known as SM22), and αSMA (307). In this context, an anti-proliferative function has been reported for the miRNA-143/145-cluster (360;373), and especially miRNA-145 has been found to promote the differentiation to the quiescent contractile phenotype of VSMC (373). Accordingly, CM also induced the expression of miRNA-145 in differentiated VSMC, accompanied by the induction of αSMA. In mice, miRNA-145 overexpression reduces neo-intima formation after vascular injury, but paradoxically miRNA-145 deficient mice show similar effects (358;374). In humans, levels of miRNA-145 are elevated in atherosclerotic plaques (375;376), and were found to correlate with an unstable plaque phenotype (375). Collectively these findings point toward an important dual role for the miRNA-143/145-cluster in VSMC homeostasis.

However, a limitation of our study was that we could not identify the factor in CM responsible for the induction of the miRNA-143/145-cluster. Previous studies showing that the induction of miR-143/145 was sensitive to inhibition of the TGFβ signaling (358;366;373) identified TGFβ and activin A as responsible factors. However, levels of these factors were below the limit of detection in our CM indicating that other members of the TGFβ family elicit the effects observed here. Alternatively, CM itself may induce the production of TGFβ or activin A in VSMC thereby promoting the induction of the miR-143/145-cluster in an autocrine fashion. In this context, we could demonstrate that CM enhances the expression of activin A in differentiated VSMC by 1.7-fold. However, it remains to be investigated whether the release of activin A from VSMC is within biologically relevant range.

In conclusion, miRNA-143 plays a pivotal role in the crosstalk between adipocytes and VSMC by affecting insulin signaling. However, the identification of the responsible factor leading to the CM-induced upreglation of miRNA-143 requires further investigations. Nevertheless, these data further highlight the involvement of adipokines in linking obesity to CVD.

6.3. Cardiovascular effects of DPP4-inhibitors

Our data on the direct effects of sDPP4 on human VSMC and the fact that all sDPP4-induced effects were prevented by DPP4 inhibition substantially extend the current view of DPP4 as a target for T2D treatment. Accordingly, accumulating studies demonstrated that DPP4-inhibitors also exert important protective effects on the cardiovascular system. It has been shown that DPP4-inhibitors decrease myocardial infarct size, stabilize the cardiac electrophysiological state during myocardial ischemia, reduce ischemia/reperfusion injury, and prevent left ventricular remodeling following myocardial infarction (377;378). Additionally, DPP4-inhibitors exert also vascular protective effects, including anti-inflammatory and anti-atherosclerotic effects and the ability to induce vascular relaxation (303;379).

Accumulating experimental data suggest that the DPP4 substrate GLP-1 itself has favorable cardiovascular effects. Thus, most of the vascular protective effects of DPP4-inhibitors are ascribed to increased GLP-1 bioavailability and signaling. mRNA transcripts of the GLP-1 receptor (GLP1R) have been detected in the heart of rodents (380;381) and humans (382). Furthermore, GLP-1R has also been localized to mouse aortic smooth muscle and endothelial cells, as well as monocytes and macrophages (383).

Regarding the vascular system in non-diabetic C57BL/6 and *ApoE*$^{-}$/$^{-}$ mice, continuous infusion of the GLP-1 analogue exendin-4 reduced monocyte adhesion to aortic endothelial cells, associated with a reduction in atherosclerotic lesion size. Furthermore, treatment for 1 h with exendin-4 reduced the expression of the pro-inflammatory cytokines TNFα and MCP-1 in response to LPS (383). Moreover, exendin-4 stimulates proliferation of human coronary artery endothelial cells through eNOS-, protein kinase A (PKA)- and PI3K/Akt-dependent pathways (384;385). Accordingly, in humans, preliminary data confirm the ability of GLP-1 to protect from high glucose-induced endothelial dysfunction in the post-meal phase (386). In a model of vascular injury, it has been shown that continuous infusion of exendin-4 reduces neointimal formation at 4 weeks after injury without altering body weight or various metabolic parameters (384;387). From *in vitro* studies, Goto *et al.* suggest that this effect was mediated by the ability of GLP-1 to suppress platelet derived growth factor (PDGF)-induced proliferation of VSMC.

Additionally, in a rat model of chronic heart failure, GLP-1 analogues were able to improve cardiac function and morphology, with a concomitant amelioration of hyperglycemia and hyperinsulinemia (388). Similarly, in patients with heart failure, pilot studies also suggest cardio-protection by GLP-1 infusion (389;390).

Accordingly, a large retrospective analysis indicates that patients prescribed with the GLP-1 analogue exenatide had a significant 20% reduction of CVD events compared with patients on other glucose-lowering agents (384;391). Nevertheless, studies showing cardiovascular protective effects of GLP-1 were carried out using either native GLP-1 or recombinant GLP-1 analogues at high concentrations or in a way that induced supraphysiological GLP-1 signaling. Considering that DPP4 inhibition restores GLP-1 signaling within the physiological range, beneficial effects of DPP4-inhibitors might be different to those of GLP-1 analogues.

But beside GLP-1, there are further substrates of DPP4, which might play a role in the favorable cardiovascular effects of DPP4-inhibitors. Two of the most promising candidates are stromal derived factor (SDF)-1α and brain natriuretic peptide (BNP). SDF-1 is a chemokine that promotes angiogenesis and attracts endothelial progenitor cells (EPC) by binding to its receptor CXCR4. EPC are derived from the bone marrow and are known to promote vascular repair and neoangiogenesis. When vascular damage occurs, local growth factors and cytokines signal the bone marrow to release EPC targeted to the injured sites. EPC then differentiate into mature endothelial cells and assist in the reconstruction of the vasculature (384). In mice, genetic deletion or pharmacologic inhibition of DPP4 is able to increase the homing of CXCR4+ EPC at sites of myocardial damage, resulting in a reduced cardiac remodeling and improved heart function and survival (392). In a human study, Fadini *et al.* demonstrated that type 2 diabetic patients receiving a 4 week course of therapy with the DPP4-inhibitor sitagliptin, show increased SDF-1α plasma concentrations and circulating EPC levels (393). Additionally, SDF-1 engineered to be resistant to DPP4 cleavage, and delivered by nanofibers, improves blood flow in a model of peripheral artery disease (394). Collectively, these studies implicate a rationale to use DPP4-inhibitors for vascular repair through stimulation of EPC and neovascularization.

BNP, another substrate of DPP4, plays an important role in regulating body fluid homeostasis and vascular tone through binding and subsequent activation of the cGMP-coupled natriuretic peptide receptor type A (NPR-A) (395;396). BNP is secreted predominantly by ventricular cardiomyocytes in response to increased wall stress. Thus,

elevated BNP is a sensitive marker of heart failure and appears to play a role in cardiac remodeling and healing after acute myocardial infarction (396-398). DPP4 cleavage of the physiologically active BNP(1–32) to BNP(3–32) effectively lowers plasma cGMP levels, reduces diuresis and natriuresis, and inhibits vasodilatation (399;400).

Nevertheless, there are also various studies showing direct effects of DPP4-inhibitors, which could not be explained by an increased bioavailability of DPP4 substrates. Ta *et al*. demonstrated that the specific DPP4-inhibitor alogliptin blocks LPS-induced ERK phosphorylation in a macrophage cell-line, (284). Downstream of ERK, the inhibition of DPP4 prevents the activation of MMP-1 via TLR pathway. These data might indicate that DPP4 is involved in foam cells formation and contributes to the destabilization of atherosclerotic plaques. Additionally, sitagliptin prevents TNFα-induced PAI-1, ICAM and VCAM gene and protein expression in vascular endothelial cells both dependent and independent of GLP-1 (401). This effect could partially be explained by a reduction of the TNFα-induced NF-κB1 mRNA expression by sitagliptin. Furthermore, DPP4 inhibition by alogliptin in HUVEC resulted in eNOS and Akt phosphorylation (Ser1177 and Ser473, respectively) paralleled by a rapid increase in nitric oxide (402). Accordingly, alogliptin causes acute vascular relaxation in a non-diabetic mouse model through GLP-1-independent pathways that are both nitric oxide- and endothelium-derived hyperpolarizing factor (EDHF)-dependent. All in all, these studies might implicate that sDPP4 itself plays a role in the beneficial effects of DPP4-inhibitors.

In summary, although DPP4-inhibitors exert cardiovascular-protective effects, the actual relationship between DPP4 inhibition and concrete cardiovascular outcomes remains unknown and requires further investigations.

6.4. Perspectives

In the first part of this work (CHAPTER 2), sDPP4 was characterized and further validated as novel adipokine. sDPP4 was markedly released by adipocytes and shows the highest expression in visceral AT of obese patients. Accordingly, circulating levels of sDPP4 were increased in obesity and correlate with parameters of the metabolic syndrome. However, the major source of circulating sDPP4 is still unknown. To address this issue, an adipose tissue-specific DPP4 knockout mouse was generated. After challenging these transgenic mice with high-fat diet, glucose tolerance, energy expenditure and body composition will be analyzed. Thus, future work will help to define the impact of adipose tissue-released sDPP4 in obesity.

In our organ crosstalk model, the impact of sDPP4 on VSMC was investigated (CHAPTER 3). Thereby, a novel sDPP4-induced and receptor-mediated signaling cascade was characterized. sDPP4 directly and markedly activates the MAPK- and NF-κB pathway in a PAR2-dependent manner leading to increased proliferation and inflammation in VSMC (Figure 9). Since endothelial cells are mainly exposed to circulating sDPP4, future work should translate our knowledge of this novel DPP4-induced signaling to other cells types, especially endothelial cells. In collaboration with Sánchez-Ferrer and colleagues, we already showed that sDPP4 impairs endothelium-dependent relaxation. However, the underlying mechanism of this effect remains unknown. To contribute to a better understanding of vascular protective properties of DPP4-inhibitors, further studies on direct effects of sDPP4 on vascular cells are required.

In another part of this work (CHAPTER 4), the impact of the whole secretory output of adipocytes in terms of CM on differentiated VSMC was investigated. Adipocyte-derived factors directly impair insulin action in VSMC via induction of miRNA-143 and subsequent downregulation of its target ORP8. miRNAs in cardiovascular research are a newly emerging powerful biomolecules, which demonstrate several unique opportunities for microRNAs-based therapeutics. Accumulating studies show the importance of miRNAs in the regulation of VSMC development and phenotypic modification. Our cross-talk model provides a promising tool to study the effect of adipocyte-derived factors on miRNA expression in

VSMC, and thereby contributing to a better understanding how adipokines affect VSMC function.

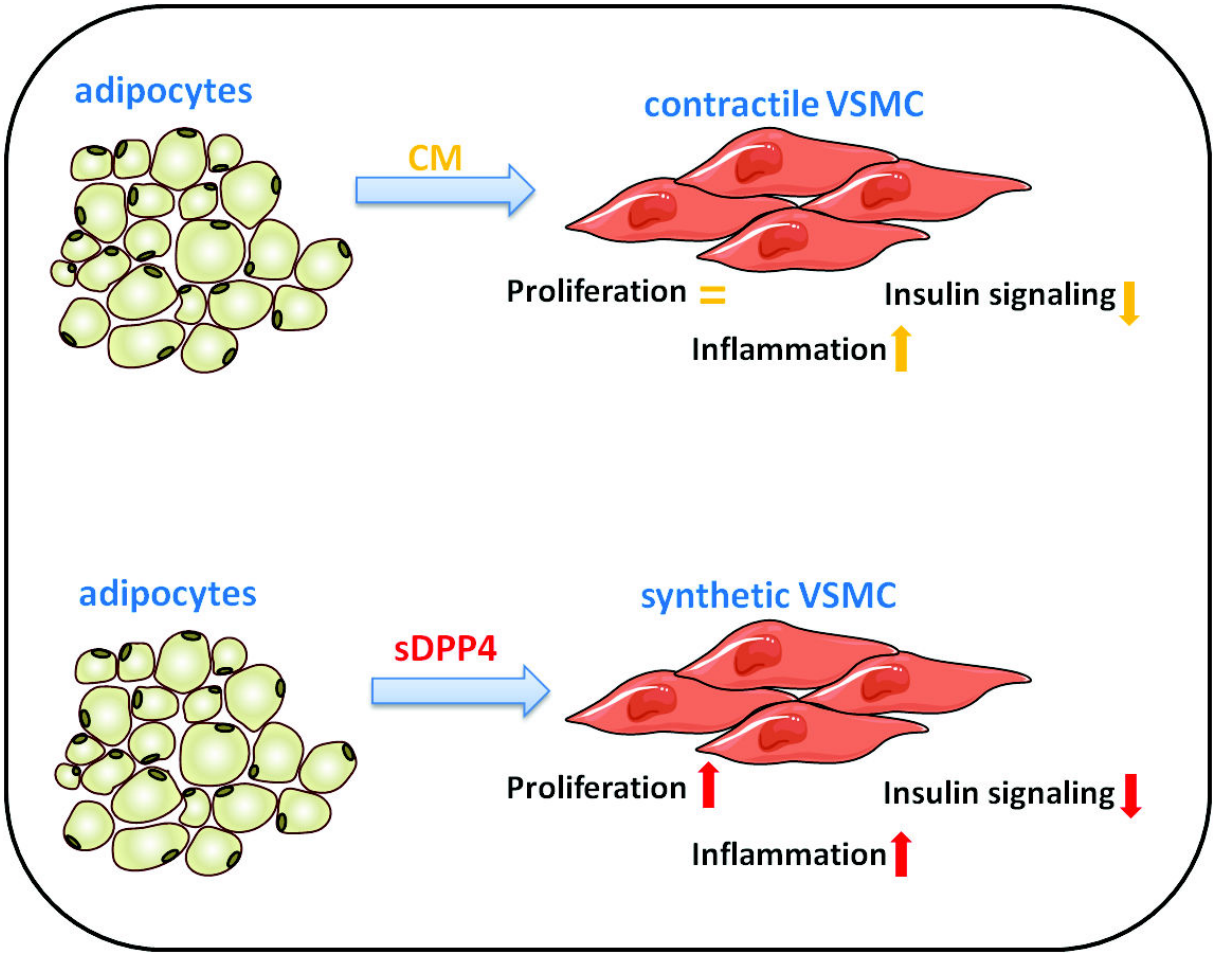


Figure 9: Schematic summary of the presented work.
The whole secretory output of human adipocytes in terms of CM impairs insulin signaling and induces inflammation in *in vitro* differentiated VSMC. The novel adipokine sDPP4 leads to pro-atherogenic changes of VSMC, such as increased proliferation, inflammation and impaired insulin signaling.

Bibliography

1. Obesity: preventing and managing the global epidemic. Report of aWHOconsultation. World Health Organ Tech Rep Ser 2000;894:i-253,
2. Must,A, Strauss RS: Risks and consequences of childhood and adolescent obesity. Int J Obes Relat Metab Disord 1999;23 Suppl 2:S2-11,
3. Guo,SS, Chumlea WC: Tracking of body mass index in children in relation to overweight in adulthood. Am J Clin Nutr 1999;70:145S-148S,
4. Reaven,GM: Pathophysiology of insulin resistance in human disease. Physiol Rev 1995;75:473-486,
5. Abate,N, Garg A, Peshock RM, Stray-Gundersen J, Grundy SM: Relationships of generalized and regional adiposity to insulin sensitivity in men. J Clin Invest 1995;96:88-98,
6. Despres,JP, Lemieux I: Abdominal obesity and metabolic syndrome. Nature 2006;444:881-887,
7. Van Gaal,LF, Mertens IL, De Block CE: Mechanisms linking obesity with cardiovascular disease. Nature 2006;444:875-880,
8. Kloting,N, Fasshauer M, Dietrich A, Kovacs P, Schon MR, Kern M, Stumvoll M, Bluher M: Insulin-sensitive obesity. Am J Physiol Endocrinol Metab 2010;299:E506-E515,
9. Bogardus,C, Lillioja S, Mott DM, Hollenbeck C, Reaven G: Relationship between degree of obesity and in vivo insulin action in man. Am J Physiol 1985;248:E286-E291,
10. Ferrannini,E, Natali A, Bell P, Cavallo-Perin P, Lalic N, Mingrone G: Insulin resistance and hypersecretion in obesity. European Group for the Study of Insulin Resistance (EGIR). J Clin Invest 1997;100:1166-1173,
11. Kahn,SE, Prigeon RL, McCulloch DK, Boyko EJ, Bergman RN, Schwartz MW, Neifing JL, Ward WK, Beard JC, Palmer JP, .: Quantification of the relationship between insulin sensitivity and beta-cell function in human subjects. Evidence for a hyperbolic function. Diabetes 1993;42:1663-1672,
12. Polonsky,KS, Given BD, Van CE: Twenty-four-hour profiles and pulsatile patterns of insulin secretion in normal and obese subjects. J Clin Invest 1988;81:442-448,
13. Perley,M, Kipnis DM: Plasma insulin responses to glucose and tolbutamide of normal weight and obese diabetic and nondiabetic subjects. Diabetes 1966;15:867-874,
14. Seidell,JC: The impact of obesity on health status: some implications for health care costs. Int J Obes Relat Metab Disord 1995;19 Suppl 6:S13-S16,
15. Maes,HH, Neale MC, Eaves LJ: Genetic and environmental factors in relative body weight and human adiposity. Behav Genet 1997;27:325-351,
16. Hill,JO, Peters JC: Environmental contributions to the obesity epidemic. Science 1998;280:1371-1374,
17. Jakicic,JM: Exercise in the treatment of obesity. Endocrinol Metab Clin North Am 2003;32:967-980,
18. Hajer,GR, van Haeften TW, Visseren FL: Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. Eur Heart J 2008;29:2959-2971,

19. Bays,HE, Gonzalez-Campoy JM, Bray GA, Kitabchi AE, Bergman DA, Schorr AB, Rodbard HW, Henry RR: Pathogenic potential of adipose tissue and metabolic consequences of adipocyte hypertrophy and increased visceral adiposity. Expert Rev Cardiovasc Ther 2008;6:343-368,
20. Geloen,A, Roy PE, Bukowiecki LJ: Regression of white adipose tissue in diabetic rats. Am J Physiol 1989;257:E547-E553,
21. van,H, V, Skurk T, Rohrig K, Lee YM, Halbleib M, Aprath-Husmann I, Hauner H: Effect of BMI and age on adipose tissue cellularity and differentiation capacity in women. Int J Obes Relat Metab Disord 2003;27:889-895,
22. Marques,BG, Hausman DB, Martin RJ: Association of fat cell size and paracrine growth factors in development of hyperplastic obesity. Am J Physiol 1998;275:R1898-R1908,
23. Hausman,DB, DiGirolamo M, Bartness TJ, Hausman GJ, Martin RJ: The biology of white adipocyte proliferation. Obes Rev 2001;2:239-254,
24. Karagiannides,I, Tchkonia T, Dobson DE, Steppan CM, Cummins P, Chan G, Salvatori K, Hadzopoulou-Cladaras M, Kirkland JL: Altered expression of C/EBP family members results in decreased adipogenesis with aging. Am J Physiol Regul Integr Comp Physiol 2001;280:R1772-R1780,
25. Dubois,SG, Heilbronn LK, Smith SR, Albu JB, Kelley DE, Ravussin E: Decreased expression of adipogenic genes in obese subjects with type 2 diabetes. Obesity (Silver Spring) 2006;14:1543-1552,
26. Lebovitz,HE, Banerji MA: Point: visceral adiposity is causally related to insulin resistance. Diabetes Care 2005;28:2322-2325,
27. Smith,SR, Lovejoy JC, Greenway F, Ryan D, deJonge L, de la Bretonne J, Volafova J, Bray GA: Contributions of total body fat, abdominal subcutaneous adipose tissue compartments, and visceral adipose tissue to the metabolic complications of obesity. Metabolism 2001;50:425-435,
28. Bergman,RN, Kim SP, Catalano KJ, Hsu IR, Chiu JD, Kabir M, Hucking K, Ader M: Why visceral fat is bad: mechanisms of the metabolic syndrome. Obesity (Silver Spring) 2006;14 Suppl 1:16S-19S,
29. Sims,EA: Are there persons who are obese, but metabolically healthy? Metabolism 2001;50:1499-1504,
30. Karelis,AD, St-Pierre DH, Conus F, Rabasa-Lhoret R, Poehlman ET: Metabolic and body composition factors in subgroups of obesity: what do we know? J Clin Endocrinol Metab 2004;89:2569-2575,
31. Yamauchi,T, Nio Y, Maki T, Kobayashi M, Takazawa T, Iwabu M, Okada-Iwabu M, Kawamoto S, Kubota N, Kubota T, Ito Y, Kamon J, Tsuchida A, Kumagai K, Kozono H, Hada Y, Ogata H, Tokuyama K, Tsunoda M, Ide T, Murakami K, Awazawa M, Takamoto I, Froguel P, Hara K, Tobe K, Nagai R, Ueki K, Kadowaki T: Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. Nat Med 2007;13:332-339,
32. Ran,J, Hirano T, Fukui T, Saito K, Kageyama H, Okada K, Adachi M: Angiotensin II infusion decreases plasma adiponectin level via its type 1 receptor in rats: an implication for hypertension-related insulin resistance. Metabolism 2006;55:478-488,
33. Chu,NF, Spiegelman D, Hotamisligil GS, Rifai N, Stampfer M, Rimm EB: Plasma insulin, leptin, and soluble TNF receptors levels in relation to obesity-related

atherogenic and thrombogenic cardiovascular disease risk factors among men. Atherosclerosis 2001;157:495-503,
34. Yoda-Murakami,M, Taniguchi M, Takahashi K, Kawamata S, Saito K, Choi-Miura NH, Tomita M: Change in expression of GBP28/adiponectin in carbon tetrachloride-administrated mouse liver. Biochem Biophys Res Commun 2001;285:372-377,
35. Cook,KS, Min HY, Johnson D, Chaplinsky RJ, Flier JS, Hunt CR, Spiegelman BM: Adipsin: a circulating serine protease homolog secreted by adipose tissue and sciatic nerve. Science 1987;237:402-405,
36. Bado,A, Levasseur S, Attoub S, Kermorgant S, Laigneau JP, Bortoluzzi MN, Moizo L, Lehy T, Guerre-Millo M, Le Marchand-Brustel Y, Lewin MJ: The stomach is a source of leptin. Nature 1998;394:790-793,
37. Rajala,MW, Obici S, Scherer PE, Rossetti L: Adipose-derived resistin and gut-derived resistin-like molecule-beta selectively impair insulin action on glucose production. J Clin Invest 2003;111:225-230,
38. Fukuhara,A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K, Matsuki Y, Murakami M, Ichisaka T, Murakami H, Watanabe E, Takagi T, Akiyoshi M, Ohtsubo T, Kihara S, Yamashita S, Makishima M, Funahashi T, Yamanaka S, Hiramatsu R, Matsuzawa Y, Shimomura I: Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. Science 2005;307:426-430,
39. Arner,P: Visfatin--a true or false trail to type 2 diabetes mellitus. J Clin Endocrinol Metab 2006;91:28-30,
40. Zhang,Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM: Positional cloning of the mouse obese gene and its human homologue. Nature 1994;372:425-432,
41. Flier,JS: Obesity wars: molecular progress confronts an expanding epidemic. Cell 2004;116:337-350,
42. Wajchenberg,BL: Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. Endocr Rev 2000;21:697-738,
43. Fain,JN, Madan AK, Hiler ML, Cheema P, Bahouth SW: Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology 2004;145:2273-2282,
44. Long,YC, Zierath JR: AMP-activated protein kinase signaling in metabolic regulation. J Clin Invest 2006;116:1776-1783,
45. Cheung,CC, Clifton DK, Steiner RA: Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. Endocrinology 1997;138:4489-4492,
46. Tartaglia,LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore KJ, Smutko JS, Mays GG, Wool EA, Monroe CA, Tepper RI: Identification and expression cloning of a leptin receptor, OB-R. Cell 1995;83:1263-1271,
47. Munzberg,H, Myers MG, Jr.: Molecular and anatomical determinants of central leptin resistance. Nat Neurosci 2005;8:566-570,
48. Leshan,RL, Bjornholm M, Munzberg H, Myers MG, Jr.: Leptin receptor signaling and action in the central nervous system. Obesity (Silver Spring) 2006;14 Suppl 5:208S-212S,

49. Bjorbaek,C, Kahn BB: Leptin signaling in the central nervous system and the periphery. Recent Prog Horm Res 2004;59:305-331,
50. Lord,GM: Leptin as a proinflammatory cytokine. Contrib Nephrol 2006;151:151-164,
51. Kershaw,EE, Flier JS: Adipose tissue as an endocrine organ. J Clin Endocrinol Metab 2004;89:2548-2556,
52. Schraw,T, Wang ZV, Halberg N, Hawkins M, Scherer PE: Plasma adiponectin complexes have distinct biochemical characteristics. Endocrinology 2008;149:2270-2282,
53. Pajvani,UB, Du X, Combs TP, Berg AH, Rajala MW, Schulthess T, Engel J, Brownlee M, Scherer PE: Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications fpr metabolic regulation and bioactivity. J Biol Chem 2003;278:9073-9085,
54. Barre,L, Richardson C, Hirshman MF, Brozinick J, Fiering S, Kemp BE, Goodyear LJ, Witters LA: Genetic model for the chronic activation of skeletal muscle AMP-activated protein kinase leads to glycogen accumulation. Am J Physiol Endocrinol Metab 2007;292:E802-E811,
55. Fonseca-Alaniz,MH, Takada J, Alonso-Vale MI, Lima FB: Adipose tissue as an endocrine organ: from theory to practice. J Pediatr (Rio J) 2007;83:S192-S203,
56. Steppan,CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA: The hormone resistin links obesity to diabetes. Nature 2001;409:307-312,
57. Patel,L, Buckels AC, Kinghorn IJ, Murdock PR, Holbrook JD, Plumpton C, Macphee CH, Smith SA: Resistin is expressed in human macrophages and directly regulated by PPAR gamma activators. Biochem Biophys Res Commun 2003;300:472-476,
58. Rajala,MW, Obici S, Scherer PE, Rossetti L: Adipose-derived resistin and gut-derived resistin-like molecule-beta selectively impair insulin action on glucose production. J Clin Invest 2003;111:225-230,
59. Muse,ED, Obici S, Bhanot S, Monia BP, McKay RA, Rajala MW, Scherer PE, Rossetti L: Role of resistin in diet-induced hepatic insulin resistance. J Clin Invest 2004;114:232-239,
60. Banerjee,RR, Rangwala SM, Shapiro JS, Rich AS, Rhoades B, Qi Y, Wang J, Rajala MW, Pocai A, Scherer PE, Steppan CM, Ahima RS, Obici S, Rossetti L, Lazar MA: Regulation of fasted blood glucose by resistin. Science 2004;303:1195-1198,
61. Yang,Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L, Kahn BB: Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. Nature 2005;436:356-362,
62. Kloting,N, Graham TE, Berndt J, Kralisch S, Kovacs P, Wason CJ, Fasshauer M, Schon MR, Stumvoll M, Bluher M, Kahn BB: Serum retinol-binding protein is more highly expressed in visceral than in subcutaneous adipose tissue and is a marker of intra-abdominal fat mass. Cell Metab 2007;6:79-87,
63. Ost,A, Danielsson A, Liden M, Eriksson U, Nystrom FH, Stralfors P: Retinol-binding protein-4 attenuates insulin-induced phosphorylation of IRS1 and ERK1/2 in primary human adipocytes. FASEB J 2007;21:3696-3704,

64. Weisberg,SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr.: Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 2003;112:1796-1808,
65. Lumeng,CN, Bodzin JL, Saltiel AR: Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest 2007;117:175-184,
66. Kriegler,M, Perez C, DeFay K, Albert I, Lu SD: A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. Cell 1988;53:45-53,
67. Moller,DE: Potential role of TNF-alpha in the pathogenesis of insulin resistance and type 2 diabetes. Trends Endocrinol Metab 2000;11:212-217,
68. Trujillo,ME, Scherer PE: Adipose tissue-derived factors: impact on health and disease. Endocr Rev 2006;27:762-778,
69. Mohamed-Ali,V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, Klein S, Coppack SW: Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. J Clin Endocrinol Metab 1997;82:4196-4200,
70. Harwood,HJ, Jr.: The adipocyte as an endocrine organ in the regulation of metabolic homeostasis. Neuropharmacology 2012;63:57-75,
71. Lazar,MA: How obesity causes diabetes: not a tall tale. Science 2005;307:373-375,
72. Urs,S, Smith C, Campbell B, Saxton AM, Taylor J, Zhang B, Snoddy J, Jones VB, Moustaid-Moussa N: Gene expression profiling in human preadipocytes and adipocytes by microarray analysis. J Nutr 2004;134:762-770,
73. Trujillo,ME, Sullivan S, Harten I, Schneider SH, Greenberg AS, Fried SK: Interleukin-6 regulates human adipose tissue lipid metabolism and leptin production in vitro. J Clin Endocrinol Metab 2004;89:5577-5582,
74. Elliot Reimers. 2013. http://muscle-primer.com/wp-content/uploads/2013/09/1-s2.0-S0303720709003992-gr1.jpg
75. Kalderon,B, Mayorek N, Berry E, Zevit N, Bar-Tana J: Fatty acid cycling in the fasting rat. Am J Physiol Endocrinol Metab 2000;279:E221-E227,
76. Rosen,ED, Spiegelman BM: Adipocytes as regulators of energy balance and glucose homeostasis. Nature 2006;444:847-853,
77. Frayn,KN: Adipose tissue and the insulin resistance syndrome. Proc Nutr Soc 2001;60:375-380,
78. Goossens,GH: The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. Physiol Behav 2008;94:206-218,
79. Brook,CG, Lloyd JK, Wolf OH: Relation between age of onset of obesity and size and number of adipose cells. Br Med J 1972;2:25-27,
80. Helmlinger,G, Yuan F, Dellian M, Jain RK: Interstitial pH and pO2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. Nat Med 1997;3:177-182,
81. Skurk,T, Alberti-Huber C, Herder C, Hauner H: Relationship between adipocyte size and adipokine expression and secretion. J Clin Endocrinol Metab 2007;92:1023-1033,
82. Bahceci,M, Gokalp D, Bahceci S, Tuzcu A, Atmaca S, Arikan S: The correlation between adiposity and adiponectin, tumor necrosis factor alpha, interleukin-6 and high sensitivity C-reactive protein levels. Is adipocyte size associated with inflammation in adults? J Endocrinol Invest 2007;30:210-214,

83. Trayhurn,P, Wang B, Wood IS: Hypoxia and the endocrine and signalling role of white adipose tissue. Arch Physiol Biochem 2008;114:267-276,
84. Maachi,M, Pieroni L, Bruckert E, Jardel C, Fellahi S, Hainque B, Capeau J, Bastard JP: Systemic low-grade inflammation is related to both circulating and adipose tissue TNFalpha, leptin and IL-6 levels in obese women. Int J Obes Relat Metab Disord 2004;28:993-997,
85. Kern,PA, Ranganathan S, Li C, Wood L, Ranganathan G: Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. Am J Physiol Endocrinol Metab 2001;280:E745-E751,
86. Hotamisligil,GS, Spiegelman BM: Tumor necrosis factor alpha: a key component of the obesity-diabetes link. Diabetes 1994;43:1271-1278,
87. Hotamisligil,GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM: Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J Clin Invest 1995;95:2409-2415,
88. Mohamed-Ali,V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, Klein S, Coppack SW: Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. J Clin Endocrinol Metab 1997;82:4196-4200,
89. Fried,SK, Bunkin DA, Greenberg AS: Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. J Clin Endocrinol Metab 1998;83:847-850,
90. Alessi,MC, Bastelica D, Morange P, Berthet B, Leduc I, Verdier M, Geel O, Juhan-Vague I: Plasminogen activator inhibitor 1, transforming growth factor-beta1, and BMI are closely associated in human adipose tissue during morbid obesity. Diabetes 2000;49:1374-1380,
91. Mohamed-Ali,V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, Klein S, Coppack SW: Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. J Clin Endocrinol Metab 1997;82:4196-4200,
92. Ostlund,RE, Jr., Yang JW, Klein S, Gingerich R: Relation between plasma leptin concentration and body fat, gender, diet, age, and metabolic covariates. J Clin Endocrinol Metab 1996;81:3909-3913,
93. Klein,S, Coppack SW, Mohamed-Ali V, Landt M: Adipose tissue leptin production and plasma leptin kinetics in humans. Diabetes 1996;45:984-987,
94. Considine,RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL, .: Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 1996;334:292-295,
95. Weyer,C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, Tataranni PA: Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. J Clin Endocrinol Metab 2001;86:1930-1935,
96. Hotta,K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, Matsuzawa Y: Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. Arterioscler Thromb Vasc Biol 2000;20:1595-1599,

97. Arita,Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y: Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem Biophys Res Commun 1999;257:79-83,
98. Jernas,M, Palming J, Sjoholm K, Jennische E, Svensson PA, Gabrielsson BG, Levin M, Sjogren A, Rudemo M, Lystig TC, Carlsson B, Carlsson LM, Lonn M: Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression. FASEB J 2006;20:1540-1542,
99. Hamilton,BS, Paglia D, Kwan AY, Deitel M: Increased obese mRNA expression in omental fat cells from massively obese humans. Nat Med 1995;1:953-956,
100. Guo,KY, Halo P, Leibel RL, Zhang Y: Effects of obesity on the relationship of leptin mRNA expression and adipocyte size in anatomically distinct fat depots in mice. Am J Physiol Regul Integr Comp Physiol 2004;287:R112-R119,
101. Skurk,T, Alberti-Huber C, Herder C, Hauner H: Relationship between adipocyte size and adipokine expression and secretion. J Clin Endocrinol Metab 2007;92:1023-1033,
102. Baggiolini,M: Chemokines and leukocyte traffic. Nature 1998;392:565-568,
103. Christiansen,T, Richelsen B, Bruun JM: Monocyte chemoattractant protein-1 is produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects. Int J Obes (Lond) 2005;29:146-150,
104. Bruun,JM, Lihn AS, Pedersen SB, Richelsen B: Monocyte chemoattractant protein-1 release is higher in visceral than subcutaneous human adipose tissue (AT): implication of macrophages resident in the AT. J Clin Endocrinol Metab 2005;90:2282-2289,
105. Bullo,M, Garcia-Lorda P, Megias I, Salas-Salvado J: Systemic inflammation, adipose tissue tumor necrosis factor, and leptin expression. Obes Res 2003;11:525-531,
106. Ramos,EJ, Xu Y, Romanova I, Middleton F, Chen C, Quinn R, Inui A, Das U, Meguid MM: Is obesity an inflammatory disease? Surgery 2003;134:329-335,
107. Weisberg,SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr.: Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 2003;112:1796-1808,
108. Lacasa,D, Taleb S, Keophiphath M, Miranville A, Clement K: Macrophage-secreted factors impair human adipogenesis: involvement of proinflammatory state in preadipocytes. Endocrinology 2007;148:868-877,
109. Permana,PA, Menge C, Reaven PD: Macrophage-secreted factors induce adipocyte inflammation and insulin resistance. Biochem Biophys Res Commun 2006;341:507-514,
110. Hotamisligil,GS: Inflammation and metabolic disorders. Nature 2006;444:860-867,
111. Nesto,RW: The relation of insulin resistance syndromes to risk of cardiovascular disease. Rev Cardiovasc Med 2003;4 Suppl 6:S11-S18,
112. Alberti,KG, Zimmet PZ: Should we dump the metabolic syndrome? No. BMJ 2008;336:641,

113. Stern,MP, Williams K, Gonzalez-Villalpando C, Hunt KJ, Haffner SM: Does the metabolic syndrome improve identification of individuals at risk of type 2 diabetes and/or cardiovascular disease? Diabetes Care 2004;27:2676-2681,
114. Whiting,DR, Guariguata L, Weil C, Shaw J: IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. Diabetes Res Clin Pract 2011;94:311-321,
115. Robertson,RP: Antagonist: diabetes and insulin resistance--philosophy, science, and the multiplier hypothesis. J Lab Clin Med 1995;125:560-564,
116. Kahn,CR: Banting Lecture. Insulin action, diabetogenes, and the cause of type II diabetes. Diabetes 1994;43:1066-1084,
117. Kahn,SE: The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. Diabetologia 2003;46:3-19,
118. Coccheri,S: Approaches to prevention of cardiovascular complications and events in diabetes mellitus. Drugs 2007;67:997-1026,
119. Kwak,SH, Park KS: Genetics of type 2 diabetes and potential clinical implications. Arch Pharm Res 2013;36:167-177,
120. Lindgren,CM, Heid IM, Randall JC, Lamina C, Steinthorsdottir V, et al.: Genome-wide association scan meta-analysis identifies three Loci influencing adiposity and fat distribution. PLoS Genet 2009;5:e1000508,
121. Scherag,A, Dina C, Hinney A, Vatin V, Scherag S, et al.: Two new Loci for body-weight regulation identified in a joint analysis of genome-wide association studies for early-onset extreme obesity in French and german study groups. PLoS Genet 2010;6:e1000916,
122. Bogardus,C: Missing heritability and GWAS utility. Obesity (Silver Spring) 2009;17:209-210,
123. Venables,MC, Jeukendrup AE: Physical inactivity and obesity: links with insulin resistance and type 2 diabetes mellitus. Diabetes Metab Res Rev 2009;25 Suppl 1:S18-S23,
124. Bailey,CJ: The challenge of managing coexistent type 2 diabetes and obesity. BMJ 2011;342:d1996,
125. Saltiel,AR, Kahn CR: Insulin signalling and the regulation of glucose and lipid metabolism. Nature 2001;414:799-806,
126. Patzelt,C, Labrecque AD, Duguid JR, Carroll RJ, Keim PS, Heinrikson RL, Steiner DF: Detection and kinetic behavior of preproinsulin in pancreatic islets. Proc Natl Acad Sci U S A 1978;75:1260-1264,
127. Huang,XF, Arvan P: Intracellular transport of proinsulin in pancreatic beta-cells. Structural maturation probed by disulfide accessibility. J Biol Chem 1995;270:20417-20423,
128. Wilcox,G: Insulin and insulin resistance. Clin Biochem Rev 2005;26:19-39,
129. Patti,ME, Kahn CR: The insulin receptor--a critical link in glucose homeostasis and insulin action. J Basic Clin Physiol Pharmacol 1998;9:89-109,
130. Kido,Y, Nakae J, Accili D: Clinical review 125: The insulin receptor and its cellular targets. J Clin Endocrinol Metab 2001;86:972-979,
131. White,MF: The IRS-signalling system: a network of docking proteins that mediate insulin action. Mol Cell Biochem 1998;182:3-11,
132. Pessin,JE, Saltiel AR: Signaling pathways in insulin action: molecular targets of insulin resistance. J Clin Invest 2000;106:165-169,

133. Myers,MG, Jr., Backer JM, Sun XJ, Shoelson S, Hu P, Schlessinger J, Yoakim M, Schaffhausen B, White MF: IRS-1 activates phosphatidylinositol 3'-kinase by associating with src homology 2 domains of p85. Proc Natl Acad Sci U S A 1992;89:10350-10354,
134. Pessin,JE, Thurmond DC, Elmendorf JS, Coker KJ, Okada S: Molecular basis of insulin-stimulated GLUT4 vesicle trafficking. Location! Location! Location! J Biol Chem 1999;274:2593-2596,
135. Boulton,TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH, Yancopoulos GD: ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 1991;65:663-675,
136. Kahn,BB, Flier JS: Obesity and insulin resistance. J Clin Invest 2000;106:473-481,
137. Kwon,H, Pessin JE: Adipokines mediate inflammation and insulin resistance. Front Endocrinol (Lausanne) 2013;4:71,
138. Gavrilova,O, Marcus-Samuels B, Graham D, Kim JK, Shulman GI, Castle AL, Vinson C, Eckhaus M, Reitman ML: Surgical implantation of adipose tissue reverses diabetes in lipoatrophic mice. J Clin Invest 2000;105:271-278,
139. Rabe,K, Lehrke M, Parhofer KG, Broedl UC: Adipokines and insulin resistance. Mol Med 2008;14:741-751,
140. Kadowaki,T, Yamauchi T, Kubota N, Hara K, Ueki K, Tobe K: Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. J Clin Invest 2006;116:1784-1792,
141. Yue,P, Jin H, Aillaud M, Deng AC, Azuma J, Asagami T, Kundu RK, Reaven GM, Quertermous T, Tsao PS: Apelin is necessary for the maintenance of insulin sensitivity. Am J Physiol Endocrinol Metab 2010;298:E59-E67,
142. Wada,J: Vaspin: a novel serpin with insulin-sensitizing effects. Expert Opin Investig Drugs 2008;17:327-333,
143. Yamauchi,T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, Kadowaki T: Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med 2002;8:1288-1295,
144. Hoffstedt,J, Arvidsson E, Sjolin E, Wahlen K, Arner P: Adipose tissue adiponectin production and adiponectin serum concentration in human obesity and insulin resistance. J Clin Endocrinol Metab 2004;89:1391-1396,
145. Weyer,C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, Tataranni PA: Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. J Clin Endocrinol Metab 2001;86:1930-1935,
146. Arner,P: Insulin resistance in type 2 diabetes -- role of the adipokines. Curr Mol Med 2005;5:333-339,
147. Spranger,J, Kroke A, Mohlig M, Bergmann MM, Ristow M, Boeing H, Pfeiffer AF: Adiponectin and protection against type 2 diabetes mellitus. Lancet 2003;361:226-228,
148. Tabak,AG, Carstensen M, Witte DR, Brunner EJ, Shipley MJ, Jokela M, Roden M, Kivimaki M, Herder C: Adiponectin trajectories before type 2 diabetes diagnosis: Whitehall II study. Diabetes Care 2012;35:2540-2547,

149. Rui,L, Yuan M, Frantz D, Shoelson S, White MF: SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. J Biol Chem 2002;277:42394-42398,
150. Boura-Halfon,S, Zick Y: Phosphorylation of IRS proteins, insulin action, and insulin resistance. Am J Physiol Endocrinol Metab 2009;296:E581-E591,
151. Aguirre,V, Uchida T, Yenush L, Davis R, White MF: The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). J Biol Chem 2000;275:9047-9054,
152. Hirosumi,J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS: A central role for JNK in obesity and insulin resistance. Nature 2002;420:333-336,
153. Karin,M: The beginning of the end: IkappaB kinase (IKK) and NF-kappaB activation. J Biol Chem 1999;274:27339-27342,
154. Yuan,M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M, Shoelson SE: Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science 2001;293:1673-1677,
155. Kim,JK, Kim YJ, Fillmore JJ, Chen Y, Moore I, Lee J, Yuan M, Li ZW, Karin M, Perret P, Shoelson SE, Shulman GI: Prevention of fat-induced insulin resistance by salicylate. J Clin Invest 2001;108:437-446,
156. Antuna-Puente,B, Feve B, Fellahi S, Bastard JP: Adipokines: the missing link between insulin resistance and obesity. Diabetes Metab 2008;34:2-11,
157. Zick,Y: Ser/Thr phosphorylation of IRS proteins: a molecular basis for insulin resistance. Sci STKE 2005;2005:e4,
158. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group. Lancet 1998;352:854-865,
159. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. Lancet 1998;352:837-853,
160. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. N Engl J Med 1993;329:977-986,
161. Executive summary: standards of medical care in diabetes--2011. Diabetes Care 2011;34 Suppl 1:S4-10,
162. Nathan,DM, Buse JB, Davidson MB, Ferrannini E, Holman RR, Sherwin R, Zinman B: Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes. Diabetes Care 2009;32:193-203,
163. Nathan,DM, Buse JB, Davidson MB, Heine RJ, Holman RR, Sherwin R, Zinman B: Management of hyperglycemia in type 2 diabetes: A consensus algorithm for the initiation and adjustment of therapy: a consensus statement from the American Diabetes Association and the European Association for the Study of Diabetes. Diabetes Care 2006;29:1963-1972,

164. Patel,A, MacMahon S, Chalmers J, Neal B, Billot L, Woodward M, Marre M, Cooper M, Glasziou P, Grobbee D, Hamet P, Harrap S, Heller S, Liu L, Mancia G, Mogensen CE, Pan C, Poulter N, Rodgers A, Williams B, Bompoint S, de Galan BE, Joshi R, Travert F: Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes. N Engl J Med 2008;358:2560-2572,
165. Gerstein,HC, Miller ME, Byington RP, Goff DC, Jr., Bigger JT, Buse JB, Cushman WC, Genuth S, Ismail-Beigi F, Grimm RH, Jr., Probstfield JL, Simons-Morton DG, Friedewald WT: Effects of intensive glucose lowering in type 2 diabetes. N Engl J Med 2008;358:2545-2559,
166. Collier,CA, Bruce CR, Smith AC, Lopaschuk G, Dyck DJ: Metformin counters the insulin-induced suppression of fatty acid oxidation and stimulation of triacylglycerol storage in rodent skeletal muscle. Am J Physiol Endocrinol Metab 2006;291:E182-E189,
167. DeFronzo,RA, Goodman AM: Efficacy of metformin in patients with non-insulin-dependent diabetes mellitus. The Multicenter Metformin Study Group. N Engl J Med 1995;333:541-549,
168. Ismail-Beigi,F: Clinical practice. Glycemic management of type 2 diabetes mellitus. N Engl J Med 2012;366:1319-1327,
169. Fontbonne,A, Charles MA, Juhan-Vague I, Bard JM, Andre P, Isnard F, Cohen JM, Grandmottet P, Vague P, Safar ME, Eschwege E: The effect of metformin on the metabolic abnormalities associated with upper-body fat distribution. BIGPRO Study Group. Diabetes Care 1996;19:920-926,
170. Mather,KJ, Verma S, Anderson TJ: Improved endothelial function with metformin in type 2 diabetes mellitus. J Am Coll Cardiol 2001;37:1344-1350,
171. Johansen,K: Efficacy of metformin in the treatment of NIDDM. Meta-analysis. Diabetes Care 1999;22:33-37,
172. Bailey,CJ, Turner RC: Metformin. N Engl J Med 1996;334:574-579,
173. Inzucchi,SE, Maggs DG, Spollett GR, Page SL, Rife FS, Walton V, Shulman GI: Efficacy and metabolic effects of metformin and troglitazone in type II diabetes mellitus. N Engl J Med 1998;338:867-872,
174. Pavo,I, Jermendy G, Varkonyi TT, Kerenyi Z, Gyimesi A, Shoustov S, Shestakova M, Herz M, Johns D, Schluchter BJ, Festa A, Tan MH: Effect of pioglitazone compared with metformin on glycemic control and indicators of insulin sensitivity in recently diagnosed patients with type 2 diabetes. J Clin Endocrinol Metab 2003;88:1637-1645,
175. Scherbaum,WA, Goke B: Metabolic efficacy and safety of once-daily pioglitazone monotherapy in patients with type 2 diabetes: a double-blind, placebo-controlled study. Horm Metab Res 2002;34:589-595,
176. Olokoba,AB, Obateru OA, Olokoba LB: Type 2 diabetes mellitus: a review of current trends. Oman Med J 2012;27:269-273,
177. Simonson,DC, Kourides IA, Feinglos M, Shamoon H, Fischette CT: Efficacy, safety, and dose-response characteristics of glipizide gastrointestinal therapeutic system on glycemic control and insulin secretion in NIDDM. Results of two multicenter, randomized, placebo-controlled clinical trials. The Glipizide Gastrointestinal Therapeutic System Study Group. Diabetes Care 1997;20:597-606,

178. Schade,DS, Jovanovic L, Schneider J: A placebo-controlled, randomized study of glimepiride in patients with type 2 diabetes mellitus for whom diet therapy is unsuccessful. J Clin Pharmacol 1998;38:636-641,
179. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. Lancet 1998;352:837-853,
180. Mazzola,N: Review of current and emerging therapies in type 2 diabetes mellitus. Am J Manag Care 2012;18:S17-S26,
181. Drucker,DJ, Nauck MA: The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet 2006;368:1696-1705,
182. Triplitt,CL: New technologies and therapies in the management of diabetes. Am J Manag Care 2007;13 Suppl 2:S47-S54,
183. Stonehouse,AH, Darsow T, Maggs DG: Incretin-based therapies. J Diabetes 2012;4:55-67,
184. Lovshin,JA, Drucker DJ: Incretin-based therapies for type 2 diabetes mellitus. Nat Rev Endocrinol 2009;5:262-269,
185. Martin,JH, Deacon CF, Gorrell MD, Prins JB: Incretin-based therapies--review of the physiology, pharmacology and emerging clinical experience. Intern Med J 2011;41:299-307,
186. Pratley,RE, Salsali A: Inhibition of DPP-4: a new therapeutic approach for the treatment of type 2 diabetes. Curr Med Res Opin 2007;23:919-931,
187. Nathan,DM, Buse JB, Davidson MB, Heine RJ, Holman RR, Sherwin R, Zinman B: Management of hyperglycemia in type 2 diabetes: A consensus algorithm for the initiation and adjustment of therapy: a consensus statement from the American Diabetes Association and the European Association for the Study of Diabetes. Diabetes Care 2006;29:1963-1972,
188. Jadzinsky,M, Pfutzner A, Paz-Pacheco E, Xu Z, Allen E, Chen R: Saxagliptin given in combination with metformin as initial therapy improves glycaemic control in patients with type 2 diabetes compared with either monotherapy: a randomized controlled trial. Diabetes Obes Metab 2009;11:611-622,
189. Richter,B, Bandeira-Echtler E, Bergerhoff K, Lerch CL: Dipeptidyl peptidase-4 (DPP-4) inhibitors for type 2 diabetes mellitus. Cochrane Database Syst Rev 2008;CD006739,
190. Camejo,G, Hurt-Camejo E, Wiklund O, Bondjers G: Association of apo B lipoproteins with arterial proteoglycans: pathological significance and molecular basis. Atherosclerosis 1998;139:205-222,
191. Hansson,GK, Robertson AK, Soderberg-Naucler C: Inflammation and atherosclerosis. Annu Rev Pathol 2006;1:297-329,
192. Lerman,A, Burnett JC, Jr.: Intact and altered endothelium in regulation of vasomotion. Circulation 1992;86:III12-III19,
193. Rudijanto,A: The role of vascular smooth muscle cells on the pathogenesis of atherosclerosis. Acta Med Indones 2007;39:86-93,
194. Eckel,RH, Kahn R, Robertson RM, Rizza RA: Preventing cardiovascular disease and diabetes: a call to action from the American Diabetes Association and the American Heart Association. Circulation 2006;113:2943-2946,

195. Gu,P, Xu A: Interplay between adipose tissue and blood vessels in obesity and vascular dysfunction. Rev Endocr Metab Disord 2013;14:49-58,
196. Ketonen,J, Shi J, Martonen E, Mervaala E: Periadventitial adipose tissue promotes endothelial dysfunction via oxidative stress in diet-induced obese C57Bl/6 mice. Circ J 2010;74:1479-1487,
197. Greenstein,AS, Khavandi K, Withers SB, Sonoyama K, Clancy O, Jeziorska M, Laing I, Yates AP, Pemberton PW, Malik RA, Heagerty AM: Local inflammation and hypoxia abolish the protective anticontractile properties of perivascular fat in obese patients. Circulation 2009;119:1661-1670,
198. Cassis,LA, Police SB, Yiannikouris F, Thatcher SE: Local adipose tissue renin-angiotensin system. Curr Hypertens Rep 2008;10:93-98,
199. Nguyen Dinh,CA, Briones AM, Callera GE, Yogi A, He Y, Montezano AC, Touyz RM: Adipocyte-derived factors regulate vascular smooth muscle cells through mineralocorticoid and glucocorticoid receptors. Hypertension 2011;58:479-488,
200. Li,FY, Cheng KK, Lam KS, Vanhoutte PM, Xu A: Cross-talk between adipose tissue and vasculature: role of adiponectin. Acta Physiol (Oxf) 2011;203:167-180,
201. Libby,P, Okamoto Y, Rocha VZ, Folco E: Inflammation in atherosclerosis: transition from theory to practice. Circ J 2010;74:213-220,
202. Takaoka,M, Nagata D, Kihara S, Shimomura I, Kimura Y, Tabata Y, Saito Y, Nagai R, Sata M: Periadventitial adipose tissue plays a critical role in vascular remodeling. Circ Res 2009;105:906-911,
203. Wang,P, Xu TY, Guan YF, Su DF, Fan GR, Miao CY: Perivascular adipose tissue-derived visfatin is a vascular smooth muscle cell growth factor: role of nicotinamide mononucleotide. Cardiovasc Res 2009;81:370-380,
204. Pardo,M, Roca-Rivada A, Seoane LM, Casanueva FF: Obesidomics: contribution of adipose tissue secretome analysis to obesity research. Endocrine 2012;41:374-383,
205. Tsuruga,H, Kumagai H, Kojima T, Kitamura T: Identification of novel membrane and secreted proteins upregulated during adipocyte differentiation. Biochem Biophys Res Commun 2000;272:293-297,
206. Molina,H, Yang Y, Ruch T, Kim JW, Mortensen P, Otto T, Nalli A, Tang QQ, Lane MD, Chaerkady R, Pandey A: Temporal profiling of the adipocyte proteome during differentiation using a five-plex SILAC based strategy. J Proteome Res 2009;8:48-58,
207. Kim,J, Choi YS, Lim S, Yea K, Yoon JH, Jun DJ, Ha SH, Kim JW, Kim JH, Suh PG, Ryu SH, Lee TG: Comparative analysis of the secretory proteome of human adipose stromal vascular fraction cells during adipogenesis. Proteomics 2010;10:394-405,
208. Alvarez-Llamas,G, Szalowska E, de Vries MP, Weening D, Landman K, Hoek A, Wolffenbuttel BH, Roelofsen H, Vonk RJ: Characterization of the human visceral adipose tissue secretome. Mol Cell Proteomics 2007;6:589-600,
209. Kratchmarova,I, Kalume DE, Blagoev B, Scherer PE, Podtelejnikov AV, Molina H, Bickel PE, Andersen JS, Fernandez MM, Bunkenborg J, Roepstorff P, Kristiansen K, Lodish HF, Mann M, Pandey A: A proteomic approach for identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes. Mol Cell Proteomics 2002;1:213-222,

210. Rosenow,A, Arrey TN, Bouwman FG, Noben JP, Wabitsch M, Mariman EC, Karas M, Renes J: Identification of novel human adipocyte secreted proteins by using SGBS cells. J Proteome Res 2010;9:5389-5401,
211. Klimcakova,E, Moro C, Mazzucotelli A, Lolmede K, Viguerie N, Galitzky J, Stich V, Langin D: Profiling of adipokines secreted from human subcutaneous adipose tissue in response to PPAR agonists. Biochem Biophys Res Commun 2007;358:897-902,
212. Chen,X, Cushman SW, Pannell LK, Hess S: Quantitative proteomic analysis of the secretory proteins from rat adipose cells using a 2D liquid chromatography-MS/MS approach. J Proteome Res 2005;4:570-577,
213. Lehr,S, Hartwig S, Lamers D, Famulla S, Muller S, Hanisch FG, Cuvelier C, Ruige J, Eckardt K, Ouwens DM, Sell H, Eckel J: Identification and validation of novel adipokines released from primary human adipocytes. Mol Cell Proteomics 2012;11:M111,
214. Lambeir,AM, Durinx C, Scharpe S, De M, I: Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. Crit Rev Clin Lab Sci 2003;40:209-294,
215. Cordero,OJ, Salgado FJ, Nogueira M: On the origin of serum CD26 and its altered concentration in cancer patients. Cancer Immunol Immunother 2009;58:1723-1747,
216. Cordero,OJ, Imbernon M, Chiara LD, Martinez-Zorzano VS, Ayude D, de la Cadena MP, Rodriguez-Berrocal FJ: Potential of soluble CD26 as a serum marker for colorectal cancer detection. World J Clin Oncol 2011;2:245-261,
217. Zhong,J, Rao X, Rajagopalan S: An emerging role of dipeptidyl peptidase 4 (DPP4) beyond glucose control: potential implications in cardiovascular disease. Atherosclerosis 2013;226:305-314,
218. Ahren,B: Dipeptidyl peptidase-4 inhibitors: clinical data and clinical implications. Diabetes Care 2007;30:1344-1350,
219. Antczak,C, De M, I, Bauvois B: Ectopeptidases in pathophysiology. Bioessays 2001;23:251-260,
220. Bohm,SK, Gum JR, Jr., Erickson RH, Hicks JW, Kim YS: Human dipeptidyl peptidase IV gene promoter: tissue-specific regulation from a TATA-less GC-rich sequence characteristic of a housekeeping gene promoter. Biochem J 1995;311 (Pt 3):835-843,
221. Abbott,CA, Baker E, Sutherland GR, McCaughan GW: Genomic organization, exact localization, and tissue expression of the human CD26 (dipeptidyl peptidase IV) gene. Immunogenetics 1994;40:331-338,
222. Willheim,M, Ebner C, Baier K, Kern W, Schrattbauer K, Thien R, Kraft D, Breiteneder H, Reinisch W, Scheiner O: Cell surface characterization of T lymphocytes and allergen-specific T cell clones: correlation of CD26 expression with T(H1) subsets. J Allergy Clin Immunol 1997;100:348-355,
223. Stefanovic,V, Ardaillou N, Vlahovic P, Placier S, Ronco P, Ardaillou R: Interferon-gamma induces dipeptidylpeptidase IV expression in human glomerular epithelial cells. Immunology 1993;80:465-470,
224. Riemann,D, Kehlen A, Langner J: Stimulation of the expression and the enzyme activity of aminopeptidase N/CD13 and dipeptidylpeptidase IV/CD26 on human renal cell carcinoma cells and renal tubular epithelial cells by T

cell-derived cytokines, such as IL-4 and IL-13. Clin Exp Immunol 1995;100:277-283,
225. Yamabe,T, Takakura K, Sugie K, Kitaoka Y, Takeda S, Okubo Y, Teshigawara K, Yodoi J, Hori T: Induction of the 2B9 antigen/dipeptidyl peptidase IV/CD26 on human natural killer cells by IL-2, IL-12 or IL-15. Immunology 1997;91:151-158,
226. Cordero,OJ, Salgado FJ, Vinuela JE, Nogueira M: Interleukin-12-dependent activation of human lymphocyte subsets. Immunol Lett 1998;61:7-13,
227. Gorrell,MD: Dipeptidyl peptidase IV and related enzymes in cell biology and liver disorders. Clin Sci (Lond) 2005;108:277-292,
228. Shirakawa,J, Fujii H, Ohnuma K, Sato K, Ito Y, Kaji M, Sakamoto E, Koganei M, Sasaki H, Nagashima Y, Amo K, Aoki K, Morimoto C, Takeda E, Terauchi Y: Diet-induced adipose tissue inflammation and liver steatosis are prevented by DPP-4 inhibition in diabetic mice. Diabetes 2011;60:1246-1257,
229. Dobrian,AD, Ma Q, Lindsay JW, Leone KA, Ma K, Coben J, Galkina EV, Nadler JL: Dipeptidyl peptidase IV inhibitor sitagliptin reduces local inflammation in adipose tissue and in pancreatic islets of obese mice. Am J Physiol Endocrinol Metab 2011;300:E410-E421,
230. Pacheco,R, Martinez-Navio JM, Lejeune M, Climent N, Oliva H, Gatell JM, Gallart T, Mallol J, Lluis C, Franco R: CD26, adenosine deaminase, and adenosine receptors mediate costimulatory signals in the immunological synapse. Proc Natl Acad Sci U S A 2005;102:9583-9588,
231. Schrader,WP, West CA, Miczek AD, Norton EK: Characterization of the adenosine deaminase-adenosine deaminase complexing protein binding reaction. J Biol Chem 1990;265:19312-19318,
232. Link,DC: Neutrophil homeostasis: a new role for stromal cell-derived factor-1. Immunol Res 2005;32:169-178,
233. Kos,K, Baker AR, Jernas M, Harte AL, Clapham JC, O'Hare JP, Carlsson L, Kumar S, McTernan PG: DPP-IV inhibition enhances the antilipolytic action of NPY in human adipose tissue. Diabetes Obes Metab 2009;11:285-292,
234. Mentlein,R: Dipeptidyl-peptidase IV (CD26)--role in the inactivation of regulatory peptides. Regul Pept 1999;85:9-24,
235. Cordero,OJ, Salgado FJ, Nogueira M: On the origin of serum CD26 and its altered concentration in cancer patients. Cancer Immunol Immunother 2009;58:1723-1747,
236. Bouchard,L, Tchernof A, Deshaies Y, Marceau S, Lescelleur O, Biron S, Vohl MC: ZFP36: a promising candidate gene for obesity-related metabolic complications identified by converging genomics. Obes Surg 2007;17:372-382,
237. Smith,RE, Talhouk JW, Brown EE, Edgar SE: The significance of hypersialylation of dipeptidyl peptidase IV (CD26) in the inhibition of its activity by Tat and other cationic peptides. CD26: a subverted adhesion molecule for HIV peptide binding. AIDS Res Hum Retroviruses 1998;14:851-868,
238. Boonacker,E, Van Noorden CJ: The multifunctional or moonlighting protein CD26/DPPIV. Eur J Cell Biol 2003;82:53-73,

239. Christopherson,KW, Hangoc G, Mantel CR, Broxmeyer HE: Modulation of hematopoietic stem cell homing and engraftment by CD26. Science 2004;305:1000-1003,
240. Marguet,D, Baggio L, Kobayashi T, Bernard AM, Pierres M, Nielsen PF, Ribel U, Watanabe T, Drucker DJ, Wagtmann N: Enhanced insulin secretion and improved glucose tolerance in mice lacking CD26. Proc Natl Acad Sci U S A 2000;97:6874-6879,
241. Stephan,M, Radicke A, Leutloff S, Schmiedl A, Pabst R, von HS, Dettmer S, Lotz J, Nave H: Dipeptidyl peptidase IV (DPP4)-deficiency attenuates diet-induced obesity in rats: possible implications for the hypothalamic neuropeptidergic system. Behav Brain Res 2011;216:712-718,
242. Kirino,Y, Sato Y, Kamimoto T, Kawazoe K, Minakuchi K, Nakahori Y: Interrelationship of dipeptidyl peptidase IV (DPP4) with the development of diabetes, dyslipidaemia and nephropathy: a streptozotocin-induced model using wild-type and DPP4-deficient rats. J Endocrinol 2009;200:53-61,
243. Thompson,NL, Hixson DC, Callanan H, Panzica M, Flanagan D, Faris RA, Hong WJ, Hartel-Schenk S, Doyle D: A Fischer rat substrain deficient in dipeptidyl peptidase IV activity makes normal steady-state RNA levels and an altered protein. Use as a liver-cell transplantation model. Biochem J 1991;273 (Pt 3):497-502,
244. Erickson,RH, Suzuki Y, Sedlmayer A, Kim YS: Biosynthesis and degradation of altered immature forms of intestinal dipeptidyl peptidase IV in a rat strain lacking the enzyme. J Biol Chem 1992;267:21623-21629,
245. Yasuda,N, Nagakura T, Yamazaki K, Inoue T, Tanaka I: Improvement of high fat-diet-induced insulin resistance in dipeptidyl peptidase IV-deficient Fischer rats. Life Sci 2002;71:227-238,
246. Mitani,H, Takimoto M, Hughes TE, Kimura M: Dipeptidyl peptidase IV inhibition improves impaired glucose tolerance in high-fat diet-fed rats: study using a Fischer 344 rat substrain deficient in its enzyme activity. Jpn J Pharmacol 2002;88:442-450,
247. Rajsheker,S, Manka D, Blomkalns AL, Chatterjee TK, Stoll LL, Weintraub NL: Crosstalk between perivascular adipose tissue and blood vessels. Curr Opin Pharmacol 2010;10:191-196,
248. Miao,CY, Li ZY: The role of perivascular adipose tissue in vascular smooth muscle cell growth. Br J Pharmacol 2012;165:643-658,
249. Gorrell,MD, Gysbers V, McCaughan GW: CD26: a multifunctional integral membrane and secreted protein of activated lymphocytes. Scand J Immunol 2001;54:249-264,
250. Ervinna,N, Mita T, Yasunari E, Azuma K, Tanaka R, Fujimura S, Sukmawati D, Nomiyama T, Kanazawa A, Kawamori R, Fujitani Y, Watada H: Anagliptin, a DPP-4 inhibitor, suppresses proliferation of vascular smooth muscles and monocyte inflammatory reaction and attenuates atherosclerosis in male apo E-deficient mice. Endocrinology 2013;154:1260-1270,
251. Anagnostis,P, Athyros VG, Adamidou F, Panagiotou A, Kita M, Karagiannis A, Mikhailidis DP: Glucagon-like peptide-1-based therapies and cardiovascular disease: looking beyond glycaemic control. Diabetes Obes Metab 2011;13:302-312,

252. Okerson,T, Chilton RJ: The cardiovascular effects of GLP-1 receptor agonists. Cardiovasc Ther 2012;30:e146-e155,
253. Fadini,GP, Boscaro E, Albiero M, Menegazzo L, Frison V, de KS, Agostini C, Tiengo A, Avogaro A: The oral dipeptidyl peptidase-4 inhibitor sitagliptin increases circulating endothelial progenitor cells in patients with type 2 diabetes: possible role of stromal-derived factor-1alpha. Diabetes Care 2010;33:1607-1609,
254. Ta,NN, Li Y, Schuyler CA, Lopes-Virella MF, Huang Y: DPP-4 (CD26) inhibitor alogliptin inhibits TLR4-mediated ERK activation and ERK-dependent MMP-1 expression by U937 histiocytes. Atherosclerosis 2010;213:429-435,
255. Dobrian,AD, Ma Q, Lindsay JW, Leone KA, Ma K, Coben J, Galkina EV, Nadler JL: Dipeptidyl peptidase IV inhibitor sitagliptin reduces local inflammation in adipose tissue and in pancreatic islets of obese mice. Am J Physiol Endocrinol Metab 2011;300:E410-E421,
256. Zhai,W, Jungraithmayr W, De M, I, Inci I, Augustyns K, Arni S, Hillinger S, Scharpe S, Weder W, Korom S: Primary graft dysfunction in lung transplantation: the role of CD26/dipeptidylpeptidase IV and vasoactive intestinal peptide. Transplantation 2009;87:1140-1146,
257. Zhang,D, Huang W, Dai B, Zhao T, Ashraf A, Millard RW, Ashraf M, Wang Y: Genetically manipulated progenitor cell sheet with diprotin A improves myocardial function and repair of infarcted hearts. Am J Physiol Heart Circ Physiol 2010;299:H1339-H1347,
258. Rzucidlo,EM, Martin KA, Powell RJ: Regulation of vascular smooth muscle cell differentiation. J Vasc Surg 2007;45 Suppl A:A25-A32,
259. Cecchettini,A, Rocchiccioli S, Boccardi C, Citti L: Vascular smooth-muscle-cell activation: proteomics point of view. Int Rev Cell Mol Biol 2011;288:43-99,
260. Owens,GK: Regulation of differentiation of vascular smooth muscle cells. Physiol Rev 1995;75:487-517,
261. Owens,GK: Regulation of differentiation of vascular smooth muscle cells. Physiol Rev 1995;75:487-517,
262. Campbell,GR, Campbell JH: Smooth muscle phenotypic changes in arterial wall homeostasis: implications for the pathogenesis of atherosclerosis. Exp Mol Pathol 1985;42:139-162,
263. Galis,ZS, Sukhova GK, Lark MW, Libby P: Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. J Clin Invest 1994;94:2493-2503,
264. Fabunmi,RP, Baker AH, Murray EJ, Booth RF, Newby AC: Divergent regulation by growth factors and cytokines of 95 kDa and 72 kDa gelatinases and tissue inhibitors or metalloproteinases-1, -2, and -3 in rabbit aortic smooth muscle cells. Biochem J 1996;315 (Pt 1):335-342,
265. Schlich,R, Willems M, Greulich S, Ruppe F, Knoefel WT, Ouwens DM, Maxhera B, Lichtenberg A, Eckel J, Sell H: VEGF in the crosstalk between human adipocytes and smooth muscle cells: depot-specific release from visceral and perivascular adipose tissue. Mediators Inflamm 2013;2013:982458,
266. Pacher,P, Obrosova IG, Mabley JG, Szabo C: Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies. Curr Med Chem 2005;12:267-275,

267. Jiang,B, Xu S, Hou X, Pimentel DR, Brecher P, Cohen RA: Temporal control of NF-kappaB activation by ERK differentially regulates interleukin-1beta-induced gene expression. J Biol Chem 2004;279:1323-1329,
268. Forstermann,U, Schmidt HH, Pollock JS, Sheng H, Mitchell JA, Warner TD, Nakane M, Murad F: Isoforms of nitric oxide synthase. Characterization and purification from different cell types. Biochem Pharmacol 1991;42:1849-1857,
269. Moncada,S, Palmer RM, Higgs EA: Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991;43:109-142,
270. Nathan,C: Nitric oxide as a secretory product of mammalian cells. FASEB J 1992;6:3051-3064,
271. Waldman,SA, Murad F: Biochemical mechanisms underlying vascular smooth muscle relaxation: the guanylate cyclase-cyclic GMP system. J Cardiovasc Pharmacol 1988;12 Suppl 5:S115-S118,
272. Radomski,MW, Palmer RM, Moncada S: The role of nitric oxide and cGMP in platelet adhesion to vascular endothelium. Biochem Biophys Res Commun 1987;148:1482-1489,
273. Garg,UC, Hassid A: Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. J Clin Invest 1989;83:1774-1777,
274. Kahn,AM, Seidel CL, Allen JC, O'Neil RG, Shelat H, Song T: Insulin reduces contraction and intracellular calcium concentration in vascular smooth muscle. Hypertension 1993;22:735-742,
275. Landmesser,U, Hornig B, Drexler H: Endothelial function: a critical determinant in atherosclerosis? Circulation 2004;109:II27-II33,
276. Garg,UC, Hassid A: Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. J Clin Invest 1989;83:1774-1777,
277. Nakaki,T, Nakayama M, Kato R: Inhibition by nitric oxide and nitric oxide-producing vasodilators of DNA synthesis in vascular smooth muscle cells. Eur J Pharmacol 1990;189:347-353,
278. Nunokawa,Y, Tanaka S: Interferon-gamma inhibits proliferation of rat vascular smooth muscle cells by nitric oxide generation. Biochem Biophys Res Commun 1992;188:409-415,
279. Bornfeldt,KE, Tabas I: Insulin resistance, hyperglycemia, and atherosclerosis. Cell Metab 2011;14:575-585,
280. Cersosimo,E, Xu X, Musi N: Potential role of insulin signaling on vascular smooth muscle cell migration, proliferation, and inflammation pathways. Am J Physiol Cell Physiol 2012;302:C652-C657,
281. Ketsawatsomkron,P, Stepp DW, Fulton DJ, Marrero MB: Molecular mechanism of angiotensin II-induced insulin resistance in aortic vascular smooth muscle cells: roles of Protein Tyrosine Phosphatase-1B. Vascul Pharmacol 2010;53:160-168,
282. Yu,Q, Gao F, Ma XL: Insulin says NO to cardiovascular disease. Cardiovasc Res 2011;89:516-524,
283. Cheng,HC, Abdel-Ghany M, Pauli BU: A novel consensus motif in fibronectin mediates dipeptidyl peptidase IV adhesion and metastasis. J Biol Chem 2003;278:24600-24607,

284. Ta,NN, Li Y, Schuyler CA, Lopes-Virella MF, Huang Y: DPP-4 (CD26) inhibitor alogliptin inhibits TLR4-mediated ERK activation and ERK-dependent MMP-1 expression by U937 histiocytes. Atherosclerosis 2010;213:429-435,
285. Ikushima,H, Munakata Y, Iwata S, Ohnuma K, Kobayashi S, Dang NH, Morimoto C: Soluble CD26/dipeptidyl peptidase IV enhances transendothelial migration via its interaction with mannose 6-phosphate/insulin-like growth factor II receptor. Cell Immunol 2002;215:106-110,
286. Piazza,GA, Callanan HM, Mowery J, Hixson DC: Evidence for a role of dipeptidyl peptidase IV in fibronectin-mediated interactions of hepatocytes with extracellular matrix. Biochem J 1989;262:327-334,
287. Ikushima,H, Munakata Y, Ishii T, Iwata S, Terashima M, Tanaka H, Schlossman SF, Morimoto C: Internalization of CD26 by mannose 6-phosphate/insulin-like growth factor II receptor contributes to T cell activation. Proc Natl Acad Sci U S A 2000;97:8439-8444,
288. Ishibashi,Y, Matsui T, Maeda S, Higashimoto Y, Yamagishi S: Advanced glycation end products evoke endothelial cell damage by stimulating soluble dipeptidyl peptidase-4 production and its interaction with mannose 6-phosphate/insulin-like growth factor II receptor. Cardiovasc Diabetol 2013;12:125,
289. Hollenberg,MD, Saifeddine M, al-Ani B, Kawabata A: Proteinase-activated receptors: structural requirements for activity, receptor cross-reactivity, and receptor selectivity of receptor-activating peptides. Can J Physiol Pharmacol 1997;75:832-841,
290. Hirano,K, Kanaide H: Role of protease-activated receptors in the vascular system. J Atheroscler Thromb 2003;10:211-225,
291. Napoli,C, de NF, Wallace JL, Hollenberg MD, Tajana G, De RG, Sica V, Cirino G: Evidence that protease activated receptor 2 expression is enhanced in human coronary atherosclerotic lesions. J Clin Pathol 2004;57:513-516,
292. Marutsuka,K, Hatakeyama K, Sato Y, Yamashita A, Sumiyoshi A, Asada Y: Protease-activated receptor 2 (PAR2) mediates vascular smooth muscle cell migration induced by tissue factor/factor VIIa complex. Thromb Res 2002;107:271-276,
293. Bono,F, Lamarche I, Herbert JM: Induction of vascular smooth muscle cell growth by selective activation of the proteinase activated receptor-2 (PAR-2). Biochem Biophys Res Commun 1997;241:762-764,
294. Dery,O, Corvera CU, Steinhoff M, Bunnett NW: Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. Am J Physiol 1998;274:C1429-C1452,
295. Adams,MN, Ramachandran R, Yau MK, Suen JY, Fairlie DP, Hollenberg MD, Hooper JD: Structure, function and pathophysiology of protease activated receptors. Pharmacol Ther 2011;130:248-282,
296. Bretschneider,E, Kaufmann R, Braun M, Wittpoth M, Glusa E, Nowak G, Schror K: Evidence for proteinase-activated receptor-2 (PAR-2)-mediated mitogenesis in coronary artery smooth muscle cells. Br J Pharmacol 1999;126:1735-1740,
297. Koo,BH, Chung KH, Hwang KC, Kim DS: Factor Xa induces mitogenesis of coronary artery smooth muscle cell via activation of PAR-2. FEBS Lett 2002;523:85-89,

298. Shpacovitch,VM, Varga G, Strey A, Gunzer M, Mooren F, Buddenkotte J, Vergnolle N, Sommerhoff CP, Grabbe S, Gerke V, Homey B, Hollenberg M, Luger TA, Steinhoff M: Agonists of proteinase-activated receptor-2 modulate human neutrophil cytokine secretion, expression of cell adhesion molecules, and migration within 3-D collagen lattices. J Leukoc Biol 2004;76:388-398,
299. Ikeda,T, Kumagai E, Iwata S, Yamakawa A: Soluble CD26/Dipeptidyl Peptidase IV Enhances the Transcription of IL-6 and TNF-alpha in THP-1 Cells and Monocytes. PLoS One 2013;8:e66520,
300. Ohnuma,K, Yamochi T, Uchiyama M, Nishibashi K, Yoshikawa N, Shimizu N, Iwata S, Tanaka H, Dang NH, Morimoto C: CD26 up-regulates expression of CD86 on antigen-presenting cells by means of caveolin-1. Proc Natl Acad Sci U S A 2004;101:14186-14191,
301. Ohnuma,K, Takahashi N, Yamochi T, Hosono O, Dang NH, Morimoto C: Role of CD26/dipeptidyl peptidase IV in human T cell activation and function. Front Biosci 2008;13:2299-2310,
302. Ohnuma,K, Yamochi T, Uchiyama M, Nishibashi K, Iwata S, Hosono O, Kawasaki H, Tanaka H, Dang NH, Morimoto C: CD26 mediates dissociation of Tollip and IRAK-1 from caveolin-1 and induces upregulation of CD86 on antigen-presenting cells. Mol Cell Biol 2005;25:7743-7757,
303. Shah,Z, Kampfrath T, Deiuliis JA, Zhong J, Pineda C, Ying Z, Xu X, Lu B, Moffatt-Bruce S, Durairaj R, Sun Q, Mihai G, Maiseyeu A, Rajagopalan S: Long-term dipeptidyl-peptidase 4 inhibition reduces atherosclerosis and inflammation via effects on monocyte recruitment and chemotaxis. Circulation 2011;124:2338-2349,
304. Sanchez-Ferrer,C, Vallejo S, Romacho T, Villalobos L, Wronkowitz N, Sell H, Eckel J, Peiro C: Dipeptidyl Peptidase-4 impairs microvascular endothelial-dependent relaxation : the role of cyclooxygenase. Diabetes 2013;62 Suppl.1:A128,
305. Northcott,JM, Yeganeh A, Taylor CG, Zahradka P, Wigle JT: Adipokines and the cardiovascular system: mechanisms mediating health and disease. Can J Physiol Pharmacol 2012;90:1029-1059,
306. Lamers,D, Schlich R, Greulich S, Sasson S, Sell H, Eckel J: Oleic acid and adipokines synergize in inducing proliferation and inflammatory signalling in human vascular smooth muscle cells. J Cell Mol Med 2011;15:1177-1188,
307. Cordes,KR, Sheehy NT, White MP, Berry EC, Morton SU, Muth AN, Lee TH, Miano JM, Ivey KN, Srivastava D: miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. Nature 2009;460:705-710,
308. Hansmann,G, de Jesus Perez VA, Alastalo TP, Alvira CM, Guignabert C, Bekker JM, Schellong S, Urashima T, Wang L, Morrell NW, Rabinovitch M: An antiproliferative BMP-2/PPARgamma/apoE axis in human and murine SMCs and its role in pulmonary hypertension. J Clin Invest 2008;118:1846-1857,
309. Montero,D, Walther G, Perez-Martin A, Vicente-Salar N, Roche E, Vinet A: Vascular smooth muscle function in type 2 diabetes mellitus: a systematic review and meta-analysis. Diabetologia 2013;56:2122-2133,
310. Lamers,D, Schlich R, Greulich S, Sasson S, Sell H, Eckel J: Oleic acid and adipokines synergize in inducing proliferation and inflammatory signalling in human vascular smooth muscle cells. J Cell Mol Med 2011;15:1177-1188,

311. Seay,U, Sedding D, Krick S, Hecker M, Seeger W, Eickelberg O: Transforming growth factor-beta-dependent growth inhibition in primary vascular smooth muscle cells is p38-dependent. J Pharmacol Exp Ther 2005;315:1005-1012,
312. Hansmann,G, de Jesus Perez VA, Alastalo TP, Alvira CM, Guignabert C, Bekker JM, Schellong S, Urashima T, Wang L, Morrell NW, Rabinovitch M: An antiproliferative BMP-2/PPARgamma/apoE axis in human and murine SMCs and its role in pulmonary hypertension. J Clin Invest 2008;118:1846-1857,
313. Skalli,O, Ropraz P, Trzeciak A, Benzonana G, Gillessen D, Gabbiani G: A monoclonal antibody against alpha-smooth muscle actin: a new probe for smooth muscle differentiation. J Cell Biol 1986;103:2787-2796,
314. Wang,J, Zohar R, McCulloch CA: Multiple roles of alpha-smooth muscle actin in mechanotransduction. Exp Cell Res 2006;312:205-214,
315. Desmouliere,A, Rubbia-Brandt L, Gabbiani G: Modulation of actin isoform expression in cultured arterial smooth muscle cells by heparin and culture conditions. Arterioscler Thromb 1991;11:244-253,
316. Aikawa,M, Sivam PN, Kuro-o M, Kimura K, Nakahara K, Takewaki S, Ueda M, Yamaguchi H, Yazaki Y, Periasamy M, .: Human smooth muscle myosin heavy chain isoforms as molecular markers for vascular development and atherosclerosis. Circ Res 1993;73:1000-1012,
317. Shanahan,CM, Weissberg PL, Metcalfe JC: Isolation of gene markers of differentiated and proliferating vascular smooth muscle cells. Circ Res 1993;73:193-204,
318. Qiu,H, Zhu Y, Sun Z, Trzeciakowski JP, Gansner M, Depre C, Resuello RR, Natividad FF, Hunter WC, Genin GM, Elson EL, Vatner DE, Meininger GA, Vatner SF: Short communication: vascular smooth muscle cell stiffness as a mechanism for increased aortic stiffness with aging. Circ Res 2010;107:615-619,
319. Gorenne,I, Kavurma M, Scott S, Bennett M: Vascular smooth muscle cell senescence in atherosclerosis. Cardiovasc Res 2006;72:9-17,
320. Dzau,VJ, Braun-Dullaeus RC, Sedding DG: Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. Nat Med 2002;8:1249-1256,
321. Rajwani,A, Cubbon RM, Wheatcroft SB: Cell-specific insulin resistance: implications for atherosclerosis. Diabetes Metab Res Rev 2012;28:627-634,
322. Samarakoon,R, Higgins PJ: Integration of non-SMAD and SMAD signaling in TGF-beta1-induced plasminogen activator inhibitor type-1 gene expression in vascular smooth muscle cells. Thromb Haemost 2008;100:976-983,
323. Eren,M, Painter CA, Atkinson JB, Declerck PJ, Vaughan DE: Age-dependent spontaneous coronary arterial thrombosis in transgenic mice that express a stable form of human plasminogen activator inhibitor-1. Circulation 2002;106:491-496,
324. Erickson,LA, Fici GJ, Lund JE, Boyle TP, Polites HG, Marotti KR: Development of venous occlusions in mice transgenic for the plasminogen activator inhibitor-1 gene. Nature 1990;346:74-76,
325. Simone,TM, Higgins PJ: Low Molecular Weight Antagonists of Plasminogen Activator Inhibitor-1: Therapeutic Potential in Cardiovascular Disease. Mol Med Ther 2012;1:101,

326. Chen,Y, Budd RC, Kelm RJ, Jr., Sobel BE, Schneider DJ: Augmentation of proliferation of vascular smooth muscle cells by plasminogen activator inhibitor type 1. Arterioscler Thromb Vasc Biol 2006;26:1777-1783,
327. Michel,JB: Anoikis in the cardiovascular system: known and unknown extracellular mediators. Arterioscler Thromb Vasc Biol 2003;23:2146-2154,
328. Meilhac,O, Ho-Tin-Noe B, Houard X, Philippe M, Michel JB, Angles-Cano E: Pericellular plasmin induces smooth muscle cell anoikis. FASEB J 2003;17:1301-1303,
329. DeYoung,MB, Tom C, Dichek DA: Plasminogen activator inhibitor type 1 increases neointima formation in balloon-injured rat carotid arteries. Circulation 2001;104:1972-1,
330. Zhu,Y, Farrehi PM, Fay WP: Plasminogen activator inhibitor type 1 enhances neointima formation after oxidative vascular injury in atherosclerosis-prone mice. Circulation 2001;103:3105-3110,
331. Ploplis,VA, Cornelissen I, Sandoval-Cooper MJ, Weeks L, Noria FA, Castellino FJ: Remodeling of the vessel wall after copper-induced injury is highly attenuated in mice with a total deficiency of plasminogen activator inhibitor-1. Am J Pathol 2001;158:107-117,
332. Rollins,BJ: JE/MCP-1: an early-response gene encodes a monocyte-specific cytokine. Cancer Cells 1991;3:517-524,
333. Charo,IF, Taubman MB: Chemokines in the pathogenesis of vascular disease. Circ Res 2004;95:858-866,
334. Charo,IF, Taubman MB: Chemokines in the pathogenesis of vascular disease. Circ Res 2004;95:858-866,
335. Strieter,RM, Wiggins R, Phan SH, Wharram BL, Showell HJ, Remick DG, Chensue SW, Kunkel SL: Monocyte chemotactic protein gene expression by cytokine-treated human fibroblasts and endothelial cells. Biochem Biophys Res Commun 1989;162:694-700,
336. Cushing,SD, Berliner JA, Valente AJ, Territo MC, Navab M, Parhami F, Gerrity R, Schwartz CJ, Fogelman AM: Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. Proc Natl Acad Sci U S A 1990;87:5134-5138,
337. Aiello,RJ, Bourassa PA, Lindsey S, Weng W, Natoli E, Rollins BJ, Milos PM: Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 1999;19:1518-1525,
338. Gu,L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ: Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. Mol Cell 1998;2:275-281,
339. McDaneld,TG: MicroRNA: mechanism of gene regulation and application to livestock. J Anim Sci 2009;87:E21-E28,
340. Murchison,EP, Hannon GJ: miRNAs on the move: miRNA biogenesis and the RNAi machinery. Curr Opin Cell Biol 2004;16:223-229,
341. Lee,Y, Jeon K, Lee JT, Kim S, Kim VN: MicroRNA maturation: stepwise processing and subcellular localization. EMBO J 2002;21:4663-4670,
342. Rosenberg,MI, Georges SA, Asawachaicharn A, Analau E, Tapscott SJ: MyoD inhibits Fstl1 and Utrn expression by inducing transcription of miR-206. J Cell Biol 2006;175:77-85,

343. Liu,N, Williams AH, Kim Y, McAnally J, Bezprozvannaya S, Sutherland LB, Richardson JA, Bassel-Duby R, Olson EN: An intragenic MEF2-dependent enhancer directs muscle-specific expression of microRNAs 1 and 133. Proc Natl Acad Sci U S A 2007;104:20844-20849,
344. Lujambio,A, Esteller M: CpG island hypermethylation of tumor suppressor microRNAs in human cancer. Cell Cycle 2007;6:1455-1459,
345. Tuccoli,A, Poliseno L, Rainaldi G: miRNAs regulate miRNAs: coordinated transcriptional and post-transcriptional regulation. Cell Cycle 2006;5:2473-2476,
346. Huntzinger,E, Izaurralde E: Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat Rev Genet 2011;12:99-110,
347. Ambros,V: The functions of animal microRNAs. Nature 2004;431:350-355,
348. Ambros,V: microRNAs: tiny regulators with great potential. Cell 2001;107:823-826,
349. Flynt,AS, Lai EC: Biological principles of microRNA-mediated regulation: shared themes amid diversity. Nat Rev Genet 2008;9:831-842,
350. Czech,B, Hannon GJ: Small RNA sorting: matchmaking for Argonautes. Nat Rev Genet 2011;12:19-31,
351. Tomari,Y, Zamore PD: Perspective: machines for RNAi. Genes Dev 2005;19:517-529,
352. Robinson,HC, Baker AH: How do microRNAs affect vascular smooth muscle cell biology? Curr Opin Lipidol 2012;23:405-411,
353. van,RE: Introduction to the series on microRNAs in the cardiovascular system. Circ Res 2012;110:481-482,
354. van,RE: The art of microRNA research. Circ Res 2011;108:219-234,
355. Ji,R, Cheng Y, Yue J, Yang J, Liu X, Chen H, Dean DB, Zhang C: MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. Circ Res 2007;100:1579-1588,
356. Liu,X, Cheng Y, Zhang S, Lin Y, Yang J, Zhang C: A necessary role of miR-221 and miR-222 in vascular smooth muscle cell proliferation and neointimal hyperplasia. Circ Res 2009;104:476-487,
357. Chen,J, Yin H, Jiang Y, Radhakrishnan SK, Huang ZP, Li J, Shi Z, Kilsdonk EP, Gui Y, Wang DZ, Zheng XL: Induction of microRNA-1 by myocardin in smooth muscle cells inhibits cell proliferation. Arterioscler Thromb Vasc Biol 2011;31:368-375,
358. Xin,M, Small EM, Sutherland LB, Qi X, McAnally J, Plato CF, Richardson JA, Bassel-Duby R, Olson EN: MicroRNAs miR-143 and miR-145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury. Genes Dev 2009;23:2166-2178,
359. Cheng,Y, Liu X, Yang J, Lin Y, Xu DZ, Lu Q, Deitch EA, Huo Y, Delphin ES, Zhang C: MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation. Circ Res 2009;105:158-166,
360. Cordes,KR, Sheehy NT, White MP, Berry EC, Morton SU, Muth AN, Lee TH, Miano JM, Ivey KN, Srivastava D: miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. Nature 2009;460:705-710,

361. McDaneld,TG: MicroRNA: mechanism of gene regulation and application to livestock. J Anim Sci 2009;87:E21-E28,
362. Boettger,T, Beetz N, Kostin S, Schneider J, Kruger M, Hein L, Braun T: Acquisition of the contractile phenotype by murine arterial smooth muscle cells depends on the Mir143/145 gene cluster. J Clin Invest 2009;119:2634-2647,
363. Elia,L, Quintavalle M, Zhang J, Contu R, Cossu L, Latronico MV, Peterson KL, Indolfi C, Catalucci D, Chen J, Courtneidge SA, Condorelli G: The knockout of miR-143 and -145 alters smooth muscle cell maintenance and vascular homeostasis in mice: correlates with human disease. Cell Death Differ 2009;16:1590-1598,
364. Jordan,SD, Kruger M, Willmes DM, Redemann N, Wunderlich FT, Bronneke HS, Merkwirth C, Kashkar H, Olkkonen VM, Bottger T, Braun T, Seibler J, Bruning JC: Obesity-induced overexpression of miRNA-143 inhibits insulin-stimulated AKT activation and impairs glucose metabolism. Nat Cell Biol 2011;13:434-446,
365. Takanabe,R, Ono K, Abe Y, Takaya T, Horie T, Wada H, Kita T, Satoh N, Shimatsu A, Hasegawa K: Up-regulated expression of microRNA-143 in association with obesity in adipose tissue of mice fed high-fat diet. Biochem Biophys Res Commun 2008;376:728-732,
366. Blumensatt,M, Greulich S, Herzfeld de WD, Mueller H, Maxhera B, Rabelink MJ, Hoeben RC, Akhyari P, Al-Hasani H, Ruige JB, Ouwens DM: Activin A impairs insulin action in cardiomyocytes via up-regulation of miR-143. Cardiovasc Res 2013;100:201-210,
367. Olkkonen,VM, Johansson M, Suchanek M, Yan D, Hynynen R, Ehnholm C, Jauhiainen M, Thiele C, Lehto M: The OSBP-related proteins (ORPs): global sterol sensors for co-ordination of cellular lipid metabolism, membrane trafficking and signalling processes? Biochem Soc Trans 2006;34:389-391,
368. Rusinol,AE, Thewke D, Liu J, Freeman N, Panini SR, Sinensky MS: AKT/protein kinase B regulation of BCL family members during oxysterol-induced apoptosis. J Biol Chem 2004;279:1392-1399,
369. Vejux,A, Guyot S, Montange T, Riedinger JM, Kahn E, Lizard G: Phospholipidosis and down-regulation of the PI3-K/PDK-1/Akt signalling pathway are vitamin E inhibitable events associated with 7-ketocholesterol-induced apoptosis. J Nutr Biochem 2009;20:45-61,
370. Boettger,T, Beetz N, Kostin S, Schneider J, Kruger M, Hein L, Braun T: Acquisition of the contractile phenotype by murine arterial smooth muscle cells depends on the Mir143/145 gene cluster. J Clin Invest 2009;119:2634-2647,
371. Davis-Dusenbery,BN, Wu C, Hata A: Micromanaging vascular smooth muscle cell differentiation and phenotypic modulation. Arterioscler Thromb Vasc Biol 2011;31:2370-2377,
372. Rangrez,AY, Massy ZA, Metzinger-Le M, V, Metzinger L: miR-143 and miR-145: molecular keys to switch the phenotype of vascular smooth muscle cells. Circ Cardiovasc Genet 2011;4:197-205,
373. Long,X, Miano JM: Transforming growth factor-beta1 (TGF-beta1) utilizes distinct pathways for the transcriptional activation of microRNA 143/145 in

human coronary artery smooth muscle cells. J Biol Chem 2011;286:30119-30129,
374. Cheng,Y, Liu X, Yang J, Lin Y, Xu DZ, Lu Q, Deitch EA, Huo Y, Delphin ES, Zhang C: MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation. Circ Res 2009;105:158-166,
375. Cipollone,F, Felicioni L, Sarzani R, Ucchino S, Spigonardo F, Mandolini C, Malatesta S, Bucci M, Mammarella C, Santovito D, de LF, Marchetti A, Mezzetti A, Buttitta F: A unique microRNA signature associated with plaque instability in humans. Stroke 2011;42:2556-2563,
376. Santovito,D, Mezzetti A, Cipollone F: MicroRNAs and atherosclerosis: new actors for an old movie. Nutr Metab Cardiovasc Dis 2012;22:937-943,
377. Chinda,K, Palee S, Surinkaew S, Phornphutkul M, Chattipakorn S, Chattipakorn N: Cardioprotective effect of dipeptidyl peptidase-4 inhibitor during ischemia-reperfusion injury. Int J Cardiol 2013;167:451-457,
378. Ye,Y, Keyes KT, Zhang C, Perez-Polo JR, Lin Y, Birnbaum Y: The myocardial infarct size-limiting effect of sitagliptin is PKA-dependent, whereas the protective effect of pioglitazone is partially dependent on PKA. Am J Physiol Heart Circ Physiol 2010;298:H1454-H1465,
379. Matsubara,J, Sugiyama S, Sugamura K, Nakamura T, Fujiwara Y, Akiyama E, Kurokawa H, Nozaki T, Ohba K, Konishi M, Maeda H, Izumiya Y, Kaikita K, Sumida H, Jinnouchi H, Matsui K, Kim-Mitsuyama S, Takeya M, Ogawa H: A dipeptidyl peptidase-4 inhibitor, des-fluoro-sitagliptin, improves endothelial function and reduces atherosclerotic lesion formation in apolipoprotein E-deficient mice. J Am Coll Cardiol 2012;59:265-276,
380. Bullock,BP, Heller RS, Habener JF: Tissue distribution of messenger ribonucleic acid encoding the rat glucagon-like peptide-1 receptor. Endocrinology 1996;137:2968-2978,
381. Ban,K, Noyan-Ashraf MH, Hoefer J, Bolz SS, Drucker DJ, Husain M: Cardioprotective and vasodilatory actions of glucagon-like peptide 1 receptor are mediated through both glucagon-like peptide 1 receptor-dependent and -independent pathways. Circulation 2008;117:2340-2350,
382. Wei,Y, Mojsov S: Distribution of GLP-1 and PACAP receptors in human tissues. Acta Physiol Scand 1996;157:355-357,
383. Arakawa,M, Mita T, Azuma K, Ebato C, Goto H, Nomiyama T, Fujitani Y, Hirose T, Kawamori R, Watada H: Inhibition of monocyte adhesion to endothelial cells and attenuation of atherosclerotic lesion by a glucagon-like peptide-1 receptor agonist, exendin-4. Diabetes 2010;59:1030-1037,
384. Fadini,GP, Avogaro A: Cardiovascular effects of DPP-4 inhibition: beyond GLP-1. Vascul Pharmacol 2011;55:10-16,
385. Erdogdu,O, Nathanson D, Sjoholm A, Nystrom T, Zhang Q: Exendin-4 stimulates proliferation of human coronary artery endothelial cells through eNOS-, PKA- and PI3K/Akt-dependent pathways and requires GLP-1 receptor. Mol Cell Endocrinol 2010;325:26-35,
386. Ceriello,A, Esposito K, Testa R, Bonfigli AR, Marra M, Giugliano D: The possible protective role of glucagon-like peptide 1 on endothelium during the meal and evidence for an "endothelial resistance" to glucagon-like peptide 1 in diabetes. Diabetes Care 2011;34:697-702,

387. Goto,H, Nomiyama T, Mita T, Yasunari E, Azuma K, Komiya K, Arakawa M, Jin WL, Kanazawa A, Kawamori R, Fujitani Y, Hirose T, Watada H: Exendin-4, a glucagon-like peptide-1 receptor agonist, reduces intimal thickening after vascular injury. Biochem Biophys Res Commun 2011;405:79-84,
388. Liu,Q, Adams L, Broyde A, Fernandez R, Baron AD, Parkes DG: The exenatide analogue AC3174 attenuates hypertension, insulin resistance, and renal dysfunction in Dahl salt-sensitive rats. Cardiovasc Diabetol 2010;9:32,
389. Nikolaidis,LA, Mankad S, Sokos GG, Miske G, Shah A, Elahi D, Shannon RP: Effects of glucagon-like peptide-1 in patients with acute myocardial infarction and left ventricular dysfunction after successful reperfusion. Circulation 2004;109:962-965,
390. Sokos,GG, Nikolaidis LA, Mankad S, Elahi D, Shannon RP: Glucagon-like peptide-1 infusion improves left ventricular ejection fraction and functional status in patients with chronic heart failure. J Card Fail 2006;12:694-699,
391. Best,JH, Hoogwerf BJ, Herman WH, Pelletier EM, Smith DB, Wenten M, Hussein MA: Risk of cardiovascular disease events in patients with type 2 diabetes prescribed the glucagon-like peptide 1 (GLP-1) receptor agonist exenatide twice daily or other glucose-lowering therapies: a retrospective analysis of the LifeLink database. Diabetes Care 2011;34:90-95,
392. Zaruba,MM, Theiss HD, Vallaster M, Mehl U, Brunner S, David R, Fischer R, Krieg L, Hirsch E, Huber B, Nathan P, Israel L, Imhof A, Herbach N, Assmann G, Wanke R, Mueller-Hoecker J, Steinbeck G, Franz WM: Synergy between CD26/DPP-IV inhibition and G-CSF improves cardiac function after acute myocardial infarction. Cell Stem Cell 2009;4:313-323,
393. Fadini,GP, Boscaro E, Albiero M, Menegazzo L, Frison V, de KS, Agostini C, Tiengo A, Avogaro A: The oral dipeptidyl peptidase-4 inhibitor sitagliptin increases circulating endothelial progenitor cells in patients with type 2 diabetes: possible role of stromal-derived factor-1alpha. Diabetes Care 2010;33:1607-1609,
394. Segers,VF, Revin V, Wu W, Qiu H, Yan Z, Lee RT, Sandrasagra A: Protease-resistant stromal cell-derived factor-1 for the treatment of experimental peripheral artery disease. Circulation 2011;123:1306-1315,
395. Boerrigter,G, Costello-Boerrigter LC, Harty GJ, Lapp H, Burnett JC, Jr.: Des-serine-proline brain natriuretic peptide 3-32 in cardiorenal regulation. Am J Physiol Regul Integr Comp Physiol 2007;292:R897-R901,
396. Kuhn,M: Endothelial actions of atrial and B-type natriuretic peptides. Br J Pharmacol 2012;166:522-531,
397. Kawakami,R, Saito Y, Kishimoto I, Harada M, Kuwahara K, Takahashi N, Nakagawa Y, Nakanishi M, Tanimoto K, Usami S, Yasuno S, Kinoshita H, Chusho H, Tamura N, Ogawa Y, Nakao K: Overexpression of brain natriuretic peptide facilitates neutrophil infiltration and cardiac matrix metalloproteinase-9 expression after acute myocardial infarction. Circulation 2004;110:3306-3312,
398. Palazzuoli,A, Gallotta M, Quatrini I, Nuti R: Natriuretic peptides (BNP and NT-proBNP): measurement and relevance in heart failure. Vasc Health Risk Manag 2010;6:411-418,

399. Brandt,I, Lambeir AM, Ketelslegers JM, Vanderheyden M, Scharpe S, De M, I: Dipeptidyl-peptidase IV converts intact B-type natriuretic peptide into its des-SerPro form. Clin Chem 2006;52:82-87,
400. Boerrigter,G, Costello-Boerrigter LC, Harty GJ, Lapp H, Burnett JC, Jr.: Des-serine-proline brain natriuretic peptide 3-32 in cardiorenal regulation. Am J Physiol Regul Integr Comp Physiol 2007;292:R897-R901,
401. Hu,Y, Liu H, Simpson RW, Dear AE: GLP-1-dependent and independent effects and molecular mechanisms of a dipeptidyl peptidase 4 inhibitor in vascular endothelial cells. Mol Biol Rep 2013;40:2273-2279,
402. Shah,Z, Pineda C, Kampfrath T, Maiseyeu A, Ying Z, Racoma I, Deiuliis J, Xu X, Sun Q, Moffatt-Bruce S, Villamena F, Rajagopalan S: Acute DPP-4 inhibition modulates vascular tone through GLP-1 independent pathways. Vascul Pharmacol 2011;55:2-9,

Danksagung

Ein großer Dank gilt Herrn Prof. Eckel für die Bereitstellung des Themas, das fortwährende Interesse an dieser Arbeit und für viele konstruktive und motivierende Diskussionen, welche maßgeblich zum Gelingen dieser Arbeit beigetragen haben. Darüber hinaus haben die von ihm ermöglichten nationalen und internationalen Kongressteilnahmen sowie ein Forschungsaufenthalt im Ausland wertvolle Beiträge und Anregungen zum Fortkommen dieser Arbeit zugelassen.

Herrn Prof. Wagner danke ich für die Übernahme des Korreferats und das Interesse an meiner Arbeit.

Besonders möchte ich mich bei allen gegenwärtigen und ehemaligen Mitgliedern der Paul-Langerhans-Gruppe für eine unvergessliche Zeit bedanken, die im Nachhinein wie im Fluge verging. Dank euch war geteiltes Leid nur halb so schlimm und geteilte Freude doppelt so schön :). Hierbei möchte ich mich speziell bei Henrike bedanken, die jederzeit ein offenes Ohr für allerlei Fragen und Nöte hatte und mich stets unterstützt hat. Ebenso gilt ein riesen Dank Diana für einen unvergesslichen Abend in Barcelona, Manuela für einen Kakao zum richtigen Zeitpunkt, Philipp für viele Freudentränen auch wenn es nicht immer zum Lachen war :), Kristin für ihre stete Hilfsbereitschaft und Silja für ein YES ;). Lieben Dank auch an unseren Labor-Engel Andrea, dafür dass du immer für mich da warst sowohl innerhalb als auch außerhalb des Labors. Danke an Birgit für die Bewältigung aller bürokratischen Probleme und die netten Gespräche zwischen Tür und Angel.

Danke auch an Marcel für die erfolgreiche und gute Zusammenarbeit unter erschwerten Bedingungen ;). Danke Claudia, dass du so oft mit mir gelitten hast, sei es auf der Arbeit, beim Sport, Privat oder beim Karaoke Singen ;).

Ein ebenso großer Dank gilt meinen Freunden und allen Menschen, die mich auf diesen Weg begleitet und unterstützt haben. Hierbei seien besonders Daniel K., Daniel S., Ingo, Nastia und Jörn erwähnt.

Besonders möchte ich mich auch bei meinem Freund Sven bedanken. Du warst zur richtigen Zeit am richtigen Ort, hast Düsseldorf zu meinem Zuhause gemacht und du hast nie aufgehört an mich zu glauben. Danke für all deine Liebe.

Der größte Dank gilt meinen Eltern, die mich immer unterstützt und an mich geglaubt haben und die mir diese Ausbildung erst ermöglicht haben.

,, ENDE

Erklärung

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

Düsseldorf, 16. Dezember 2013

Nina Wronkowitz