DPP4 and its role in vascular dysfunction

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“Imagination is more important than knowledge”

- A. Einstein –

Zusammenfassung

Adipositas, stellt ein wesentliches globales Gesundheitsproblem dar und ist mit der Entstehung von metabolischen Erkrankungen wie Typ 2 Diabetes assoziiert. Zusätzlich, wird Adipositas auch mit einem erhöhten Risiko für die Entwicklung von kardiovaskulären Erkrankungen, momentan die Hauptursache für Mortalität weltweit, in Zusammenhang gebracht. In diesem Kontext erlangte das Fettgewebe in den letzten Jahren besondere Aufmerksamkeit. Weißes Fettgewebe spielt eine wichtige Rolle in der Regulation des Energiestoffwechsels und stellt ein endokrines Organ dar, das eine Vielzahl verschiedener bioaktiver Proteine, sogenannte Adipokine, freisetzt. Infolge des beständigen, multidirektionalen Crosstalks zwischen den Organen, beeinflussen sekretierte Adipokine das Fettgewebe und periphere Organe wie die Leber, den Skelettmuskel oder das Gefäßsystem auf para- und endokrine Weise. Während des Verlaufs der Adipositas ändert sich die Funktion und Struktur des Fettgewebes sowie dessen Sekretionsprofil. Das nun durch pro-inflammatorische Adipokine geprägte Profil, kann zur Schädigung entfernter Organe führen und repräsentiert somit eine potentielle Verknüpfung zwischen Adipositas und metabolischen sowie kardiovaskulären Erkrankungen.

Erst kürzlich konnte unsere Arbeitsgruppe Dipeptidyl Peptidase 4 (DPP4) als neues Adipokin, dessen Expression im Fettgewebe und der Zirkulation im Zustand der Adipositas hochreguliert wird, identifizieren und charakterisieren. Zu den Substraten von DPP4, ein Glykoprotein, welches endständige Dipeptide von diversen Proteinen enzymatisch abspaltet, gehören unter anderem die Inkretinhormone Glucagon-like Peptid 1 (GLP-1) und Gastroinhibitorisches Peptid (GIP). Unter physiologischen Bedingungen stimulieren diese Hormone die Insulinsekretion und inhibieren die Bildung von Glukagon, wodurch die Konzentration des Blutzuckers gesenkt wird. Aufgrund der vermehrten Spaltung dieser Proteine durch hohe Konzentrationen von DPP4 erfolgt eine Inaktivierung, die eine Störung des Glukosehaushalts nach sich zieht. Daher werden derzeit DPP4 Inhibitoren (DPP4i) für die Behandlung von Stoffwechselerkrankungen wie Typ 2 Diabetes genutzt. Zusätzlich ist der Gehalt an DPP4 im Blut adipöser Individuen erhöht und korreliert mit verschiedenen Charakteristika des Metabolischen Syndroms, so dass DPP4 als Link zwischen Adipositas und metabolischen als auch kardiovaskulären Störungen in Betracht gezogen wird. In Übereinstimmung dieser Erkenntnis konnten bereits mehrere in vivo und in vitro Studien einen kardio-protektiven Effekt von DPP4i aufdecken. Klinische Studien allerdings zeigten kontroverse Resultate und konnten bisherige Daten nicht vollständig bestätigen. Trotz der

intensiven Analysen von DPP4i ist die Funktion des Proteins auf periphere Organe bisher noch nicht genau bekannt.

Um die Rolle von DPP4 während diätinduzierter Adipositas zu evaluieren, wurde eine Fettgewebe-spezifische DPP4 Knockout (AT-DPP4-KO) Maus generiert, da das Fettgewebe während Adipositas eine wichtige Quelle von zirkulierendem DPP4 darstellt. Das Fehlen von DPP4 im Fettgewebe resultierte in einer vorteilhaften Umstrukturierung des Fettgewebes und einer verbesserten Insulinsensitivität der Leber, welche vermutlich auf die reduzierte Freisetzung von IGFBP3 aus dem Fettgewebe zurückzuführen ist. Dieser Mechanismus stellt eine neue Möglichkeit der Verknüpfung von Adipositas und Leberfehlfunktionen dar. Ex vivo wurde außerdem der direkte Effekt von DPP4 auf die Funktion der Endothelzellen untersucht. Der der negativen Wirkung von DPP4 auf den vaskulären Tonus zugrunde liegende Mechanismus involvierte die Erhöhung der Cyclooxygenase2 Expression und der Thromboxan A Sekretion sowie die Aktivierung des Protease-aktivierten Rezeptor (PAR) 2. Die Inhibierung von DPP4 mittels Linagliptin führte zu einer Zunahme der vaskulären Reaktionsfähigkeit von murinen, mesenterischen Gefäßen.

Es ist bekannt, dass Adipositas schädliche Auswirkungen auf das Gefäßsystem hat. Eine wichtige Rolle im Gefäßsystem spielt der G-protein-gekoppelte Rezeptor PAR2, der sowohl in glatten Muskelzellen als auch in Endothelzellen exprimiert wird. PAR2 ist für die Regulation des vaskulären Tonus sowie für den Koagulationsprozess in der Gefäßwand verantwortlich. Unter pathologischen Konditionen wird PAR2 mit entzündlichen Prozessen und vaskulärer Fehlfunktion in Verbindung gebracht. Unsere Gruppe konnte in früheren Veröffentlichungen zeigen, dass die durch DPP4 induzierte Proliferation von glatten Muskelzellen PAR2 abhängig war. Des Weiteren konnte nachgewiesen werden, dass die Expression von PAR2 im Fettgewebe durch die Entwicklung von Adipositas zunimmt. Das Wissen über PAR2 im Gefäßsystem mit Assoziation zur Adipositas ist jedoch sehr limitiert. Unter Verwendung humaner glatter Muskelzellen konnte gezeigt werden, dass PAR2 als Mediator von pro-atherogenen Prozessen fungiert, die durch das Sekretom von aus dem Fettgewebe übergewichtiger Patienten isolierten Adipozyten in Form von konditioniertem Medium hervorgerufen wurden. Überdies wurde nachgewiesen, dass die Proliferation von glatten Muskelzellen und die Induktion von entzündungsfördernden Faktoren die Transaktivierung des VEGFR2 benötigt. Daher stellt PAR2 ein potenzielles therapeutisches Ziel dar, das in der Behandlung von durch Adipositas induzierten vaskulären Erkrankungen genutzt werden kann.

Zusammenfassend konnte gezeigt werden, dass unter diätinduzierter Adipositas das aus dem Fettgewebe stammende DPP4 die Funktion des Fettgewebes und der Leber beeinflusst. Der genetische Knockout von DPP4 im Fettgewebe führte entsprechend zu einer Umgestaltung des Gewebes und milderte die hervorgerufene hepatische Insulinresistenz. Diese Effekte wurden der verminderten Expression und Freisetzung von IGFBP3 zugeschrieben. Zusätzlich konnte ex vivo eine Verbesserung der vaskulären Reaktivität aufgrund der Inhibition von DPP4 beobachtet werden. Hierbei wurde die Involvierung des Rezeptors PAR2 aufgedeckt. In einem weiteren Element der Arbeit konnte die proatherogene Funktion von PAR2 im Gefäßsystem während Adipositas demonstriert werden. In diesem Zusammenhang spielt die Transaktivierung des VEGFR2 eine spezielle Rolle.

Summary

Obesity, a principal health problem worldwide, is strongly associated with the risk for developing metabolic disorders such as type 2 diabetes mellitus. Moreover, obesity is related to cardiovascular diseases, the major global cause for mortality. In this context adipose tissue (AT) biology gained considerable interest in the recent years. White AT has been recognized as an organ for energy storage and importantly as an endocrine organ, releasing a variety of adipocyte derived factors, named adipokines. In a permanent multi-directional inter-organ crosstalk, adipokines affect the function of AT and peripheral organs such as liver, skeletal muscle or the vasculature in a para- and endocrine manner. The function and structure of AT alters with the onset of obesity and so does the adipokine secretion pattern. In this state, a change towards a more pro-inflammatory adipose secretome can exert harmful effects on distant organs and might provide a link between obesity and its related diseases.

Recently our group identified dipeptidyl peptidase 4 (DPP4) as a novel adipokine which is upregulated during obesity locally, in AT, and the circulation. DPP4, a glycoprotein cleaving dipeptides from the N-terminus of multiple proteins, targets the incretin system, particularly glucagon like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP). Under physiological conditions, these hormones stimulate insulin secretion and inhibit the generation of glucagon, thereby lowering the concentration of glucose in the blood. Augmented truncation of these substrates by abundant DPP4 entails their inactivation and thus impairment of glucose homeostasis. Therefore, DPP4 inhibitors (DPP4i) are currently used to treat metabolic diseases such as type 2 diabetes mellitus. Moreover, circulating DPP4 levels are elevated in obesity and correlate with several markers of the metabolic syndrome, thereby potentially linking obesity and metabolic and cardiovascular disorders. In line, several *in vitro* and *in vivo* studies suggest a cardio-protective effect of DPP4i. However, clinical approaches do not totally confirm these data. Although DPP4i are extensively studied, the function of DPP4 on peripheral organs is yet not fully understood.

In order to characterize the role of DPP4 in diet-induced obesity we generated a novel AT-specific DPP4 knockout (AT-DPP4-KO) mouse model, since AT was found to be an important source of DPP4 during obesity. Depletion of DPP4 resulted in beneficial AT remodeling and improvement of liver insulin sensitivity potentially through reduced release of adipose IGFBP3, thereby providing a novel mechanism linking obesity and hepatic insulin resistance. Additionally, we characterized the direct actions of DPP4 on endothelial function which involved cyclooxygenase2 activity and thromboxane A release, as well as activation of

protease-activated receptor (PAR) 2 e*x vivo.* The inhibition of DPP4 by linagliptin increased vascular reactivity in murine mesenteric vessels.

It is well known that obesity negatively affects the vascular wall. In the vascular system the G-protein coupled receptor PAR2 plays an essential role, thus the receptor has been found to be expressed in smooth muscle and endothelial cells. In the vascular wall its function is the regulation of vascular tone and the process of coagulation. Under pathophysiological conditions PAR2 is associated with inflammation and vascular dysfunction. Previous data from our group demonstrate that DPP4-mediated smooth muscle cell proliferation was PAR2-dependent. During obesity PAR2 levels are strongly upregulated in AT. However, the knowledge about PAR2 in the vasculature under obesogenic conditions is very limited. In an *in vitro* approach using human smooth muscle cells, PAR2 was recognized as a mediator of pro-atherogenic conditions induced by conditioned medium, which was obtained from isolated adipocytes of AT from overweight and obese subjects. Furthermore, the mechanism leading to smooth muscle cell proliferation and induction of pro-inflammatory factors comprised the transactivation of the VEGFR2. Therefore PAR2 might serve as a therapeutic target in the treatment of obesity-associated vascular diseases.

In conclusion, AT-derived DPP4 was revealed to impact AT as well as liver function during diet-induced obesity. *In vivo,* DPP4 deletion was found to promote AT remodeling and to attenuate liver insulin resistance, involving diminished IGFBP3 expression and release. Moreover, *ex vivo* DPP4 led to impaired vascular reactivity including participation of PAR2. In this context, the last study provides data about the pro-atherogenic function of PAR2 in the vascular wall under obesogenic conditions. Here the transactivation of the VEGFR2 by activation of PAR2 is highlighted.

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AC	Adenylyl cyclase
AP	Activating peptide
ADA	Adenine deaminase
AT	Adipose tissue
BAT	Brown adipose tissue
BMAT	Bone marrow adipose tissue
BMI	Body mass index
CLS	Crown-like structures
CM	Conditioned medium
cAMP	Cyclic adenosine monophosphate
COX	Cyclooxygenase
CVD	Cardiovascular disease
DIO	Diet-induced obesity
DPP4	Dipeptidyl peptidase 4
DPP4i	Dipeptidyl peptidase 4 inhibitors
EC	Endothelial cells
ECL	Extracellular loop
ECM	Extracellular matrix
EGP	Endogenous glucose production
GLUT4	Glucose transporter type 4
GIP	Gastric inhibitory peptide
GLP-1	Glucagon-like peptide-1
GPCR	G-protein coupled receptor
GTT	Glucose tolerance test
HDL	High density lipoprotein
HFD	High fat diet
ICAM-1	Intracellular adhesion molecule 1
ICL	Intracellular loop
IGF	Insulin like growth factor
IGFBP3	Insulin like growth factor binding protein 3
IL	Interleukin

List of Abbreviations

IR	Insulin resistance
IRS-1	Insulin receptor substrate 1
ITT	Insulin tolerance test
KO	Knock out
MCP-1	Monocyte chemoattractant protein 1
NAFLD	Non-alcoholic fatty liver disease
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
oxLDL	Oxidized low density lipoprotein
PAR	Protease-activated receptor
PAI-1	Plasminogen activator inhibitor-1
PGC1α	Peroxisome proliferator-activated receptor-gamma coactivator 1-alpha
PLC	Phospholipase C
PPARγ	Peroxisome proliferator-activated receptor-gamma
PVAT	Perivascular adipose tissue
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
sDPP4	Soluble DPP4
SMC	Smooth muscle cells
SVF	Stromal vascular fraction
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TNFα	Tumor necrosis factor alpha
TRAP	Thrombin receptor activating peptide
TXA_2	Thromboxane 2
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
WAT	White adipose tissue
WHO	World health organization
WT	Wild type

1 INTRODUCTION

1.1 Adipose tissue in health and disease

1.1.1 Obesity

Obesity is characterized by excessive fat accumulation provoked by an imbalance in lifestyle due to higher levels of energy intake than energy expenditure [1]. Obesity has currently reached epidemic proportions and is a major health problem worldwide [2]. In 2014, according to the world health organization, approximately 39% of the world's adult population was overweight and 13% obese and the prevalence is still increasing [1]. Thus global prevalence for obesity has doubled from 1980 until 2014, with 5% of men and 8% of women being obese in 1980, to 11% of men and 15% of women in 2014 [1]. The body mass index (BMI) is used as a criteria for determining the state of overweight and obesity (Table1). It is calculated on the basis of weight and height and is expressed as kilograms divided by square meters (kg/m^2). Overweight is defined by a BMI greater than or equal to 25 kg/m^2 while obesity is defined by a BMI greater than or equal to 30 kg/m^2. Nonetheless, BMI is only an estimator for obesity since the distribution of fat mass or differentiation between fat and lean mass is not taken into account. Therefore, other measurements for determination of obesity such as waist to hip-ratio are additionally consulted.

Obesity is strongly associated with comorbidities including insulin resistance (IR), dyslipidemia, type 2 diabetes mellitus (T2DM) and cardiovascular diseases (CVD), the latter representing obesity-associated mortality [3, 4].

Table 1. Weight classification by BMI according to the WHO

Category	BMI (kg/m^2)
Underweight	<18.50
Severe thinness	<16.00
Moderate thinness	16.00-16.99
Mild thinness	17.00-18.49
Normal range	**18.50-24.99**
Overweight	25.00-30.00
Moderately Obese	30.00-35.00
Severly obese	35.00-40.00
Very severly obese	≥40.00

1.1.2 Adipose tissue distribution and functionality

Adipose tissue (AT), a loose connective tissue, represents one of the largest body compartments, especially under obesogenic conditions [5]. Lipid-laden adipocytes represent around 35% to 70% of the components in AT [6]. Besides adipocytes there are several other cell types existing in AT referred as the stromal vascular fraction (SVF) [7], which contains pre-adipocytes, fibroblasts, vascular endothelial cells (EC), immune cells, nerve fibers and others [7].

There are two types of AT, referred as white adipose tissue (WAT) and brown adipose tissue (BAT), which possess divergent morphological characteristics, anatomical location and function [8]. These main depots are further subdivided based on anatomical distribution. WAT comprises two major AT depots: the subcutaneous and visceral depot [9]. Subcutaneous AT comprises approximately 80% of total body fat and is mainly located under the skin [6]. It can be distinguished into deep subcutaneous and superficial subcutaneous AT which are separated by a layer of connective tissue (*Scarpa fascia*). The visceral AT can be found in the inner parts of the body where it surrounds organs. It includes the following AT depots: (1) omental, (2) mesenteric and (3) retroperitoneal AT [10]. Additionally, visceral AT comprises smaller depots such as perivascular AT (PVAT) or epigastric and appears at none AT but ectopic storage sites. In contrast, BAT is divided into the supraclavicular and cervical depot, located in the deep neck region [11].

BAT and WAT substantially regulate energy homeostasis by energy consumptions and energy storage in form of triglycerides, respectively. BAT has been described to play a crucial role in energy conversion into heat by a process called non-shivering thermogenesis [12]. During the attempt of maintaining body temperature, BAT generates heat mediated by uncoupling protein-1 in the membrane of the highly abundant mitochondria in this type of AT [13]. Energy homeostasis controlled by WAT involves the processing, storage and oxidation of lipids. As a storage organ for excessive energy, circulating free fatty acids enter the adipocytes and are deposited as triglycerides in lipid droplets. Triglycerides make up 95% of lipid content stored in adipocytes, whereas the remaining 5% consist of phospholipids, un-esterified fatty acids and cholesterol [6]. In times of food deprivation triglycerides are available to be released as free fatty acids, a process called lipolysis, to ensure sufficient energy supply [14]. During positive energy balance or growth, adipose tissue plasticity plays a pivotal role, resulting in an increase in adipocyte size and an elevation of adipocyte number, termed hypertrophy and hyperplasia, respectively [15]. With growth, both, hypertrophy and hyperplasia occurs in AT. However, along with age the capacity of pre-adipocytes to undergo

hyperplasia declines and thus modulation of AT is shifted in favor of hypertrophy [16]. Beyond its role in fat storage, AT functions as a regulator of temperature homeostasis, protection for organs [17] and importantly as an dynamic endocrine and secretory organ [3, 18].

1.1.3 Adipose tissue as an endocrine organ

1.1.3.1 Adipokines

As an endocrine organ AT releases a variety of bioactive substances comprising lipids, lactate [19] and proteins [18]. Proteins expressed and released from adipocytes are referred to as adipokines and are actively involved in physiological processes in an auto-, para- and endocrine manner [20]. However, other cell types present in the SVF contribute to the AT secretome. Apart from adipocytes also immune cells, such as macrophages, have been determined to produce tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), thereby affecting local and systemic metabolism [21]. Adipocyte-derived factors act as signals with influence on AT itself in an auto- and paracrine manner [22]. Moreover, at systemic levels, circulating factors serve as mediators of communication with distant organs such as liver, skeletal muscle, the vascular system or brain [23]. AT thereby critically regulates biological processes such as angiogenesis, adipogenesis, extracellular matrix dissolution and reformation, energy metabolism, immune function and steroid metabolism [24]. Adipokines such as adiponectin, leptin, resistin or visfatin, have been of interest in recent years, as the prototypes of adipokines, leptin and adiponectin were discovered in 1994 and 1995, respectively [25]. Both are predominantly expressed and secreted by adipocytes. While leptin has been described to regulate systemic energy balance and food intake [26] adiponectin reduces glucose plasma levels and thus exerts insulin sensitizing effects [27].

Currently the number of characterized adipokines is estimated to be higher than 600 [28]. Expression of only a few adipokines, for example adiponectin, is consistently restricted to adipocytes, others, however, can also be secreted by skeletal muscle, liver or other organs and are thus not adipocyte-specific [29, 30]. Moreover AT is targeted by circulating factors released from distinct organs such as heart (cardiokines) and skeletal muscle (myokines). Thus, the inter-organ crosstalk takes place in a bidirectional manner (Figure 1).

1.1.3.2 Adipose tissue crosstalk to skeletal muscle and vasculature

The adipo-myocyte axis

Skeletal muscle plays a major role in glucose metabolism due to insulin-stimulated glucose clearance from the blood and glucose storage [31]. Additionally, skeletal muscle represents a true endocrine organ releasing so called myokines [32]. Muscle insulin sensitivity has been related to growing AT mass and release of certain adipokines [3]. Leptin has been described to be not only expressed by adipocytes but also by skeletal muscle, therefore referred as an adipomyokine [33]. However, our group found that it is rather an adipokine than a myokine [32]. Circulating leptin acts on skeletal muscle cells by inducing fatty acid oxidation and decreasing triglyceride accumulation, thus promoting insulin sensitivity [34]

E*x vivo* and *in vitro* leptin improves insulin-stimulated glucose uptake, oxidation and glycogen synthesis in rats [35]. In line, it has been reported that leptin administration in ob/ob mice enhances intracellular trafficking of glucose transporter 4 (GLUT4) in skeletal muscle [36].

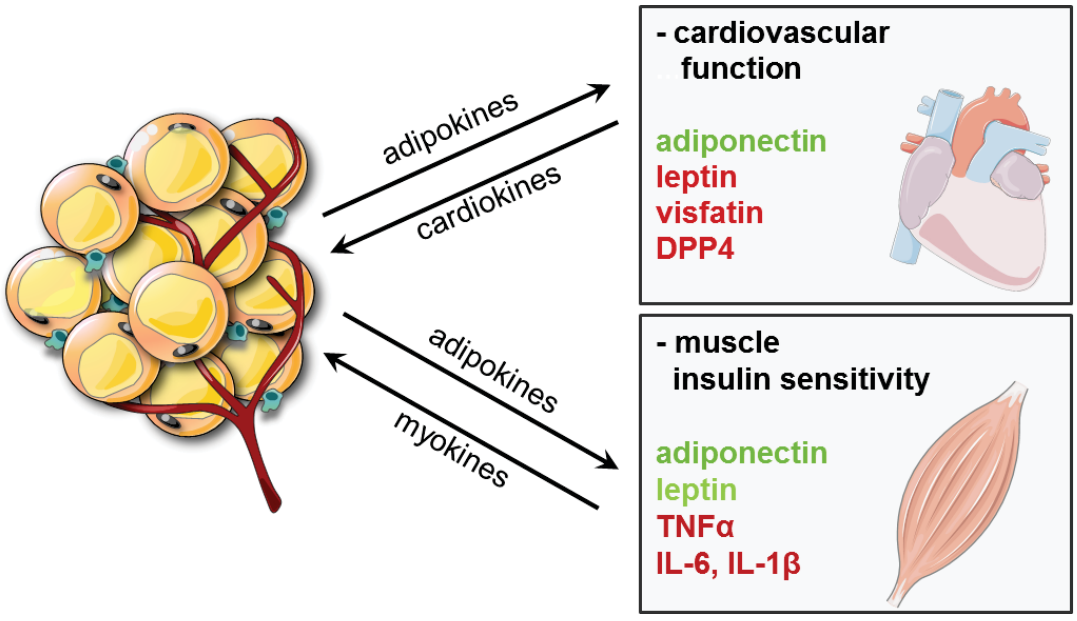

Figure 1. Crosstalk between AT and peripheral organs. AT expresses and secretes a variety of bioactive substances termed adipokines. Due to the release of adipokines into the circulation, AT communicates with other organs on a systemic level in a bidirectional crosstalk. During obesity, the adipocyte secretome shifts towards more pro-inflammatory adipokines (red) while release of anti-inflammatory adipokines (green) decreases, thereby contributing to metabolic and vascular diseases. Modified from Romacho et al. Acta Physiol (2014). Dipeptidyl peptidase 4 (DPP4), monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor alpha (TNFα).

Circulating adiponectin levels are diminished during obesity and are negatively correlated with insulin sensitivity [37]. In skeletal muscle cells, adiponectin increases GLUT4 translocation to the cell surface and glucose uptake [38]. Moreover, in myocytes co-cultured with adipocytes adiponectin directly prevented IR [39]. Another study using co-cultures from adipocytes and skeletal muscle cells supports the role of adiponectin in the control of skeletal muscle glucose uptake [40]. *In vivo* and *in vitro*, adiponectin stimulates fatty acid uptake and oxidation [41-43].

DPP4 has been validated as an adipokine as well as a myokine [44, 45]. Inhibition of DPP4 by sitagliptin increased GLUT4 expression in rat skeletal muscle [46]. However, knowledge about the impact of AT-derived DPP4 on skeletal muscle is not available. In addition, high release of pro-inflammatory adipokines such as TNF-α, IL-6 or interleukin-1β (IL-1β), by both immune cells and adipocytes, provokes IR in skeletal muscle through a variety of mechanisms, for instance activation of Ser/Thr kinases and diminution of IRS-1 and GLUT4 expression [47-49].

The adipo-vascular axis

In the circulation, released adipokines mediate AT crosstalk with the vascular wall. In addition, perivascular adipose tissue in close proximity to vessels highly influences the vascular system due to the lack of a facial layer or elastic lamina [50]. Secretion of pro-inflammatory adipokines has been implicated in vascular inflammation and EC dysfunction [51]. For instance, leptin has pro-inflammatory impact on EC, stimulates platelet aggregation and increases reactive oxygen species (ROS) [52]. In vascular SMC, leptin abundance triggers migration and proliferation [52]. Accordingly, leptin levels are positively correlated with pivotal cardiovascular events [53]. However, the relevance of leptin in obesity-related cardiovascular disorders remains elusive since study outcomes differ with certain pathological conditions [3].

In contrast, previous publications describe the positive effects of adiponectin, such as decreasing ROS, inflammation and expression of adhesion molecules on EC. Moreover, adiponectin binds to growth factors that stimulate vascular SMC proliferation and adiponectin levels are negatively correlated with cardiovascular events [54]. In accordance, knockdown of adiponectin is associated with pro-atherogenic effects [55].

Our group previously showed that the adipokine DPP4 induced vascular SMC proliferation [44]. In addition, Ishibashi et al. demonstrated that advanced glycation endproducts-induced ROS generation drives the secretion of DPP4 from EC, thereby

potentiating the detrimental effects of advanced glycation endproducts [56]. Moreover, DPP4 inhibition has been associated with protective cardiovascular consequences, which will be addressed in an upcoming section. However, there are many other adipokines such as TNF-α or visfatin, that cause the activation of pro-inflammatory signaling cascades in vascular cells [57] and thereby promote cardiovascular complications. There is also evidence that high amounts of pro-inflammatory adipokines contribute mechanically to increased vascular stiffness during obesity [58, 59].

1.1.4 Obesity-associated disorders

1.1.4.1 Adipose tissue distribution

During obesity, AT distribution is shifted towards visceral adiposity, which is associated with an increased risk for multiple morbidities including the metabolic syndrome and CVD [60]. According to the international diabetes federation, the metabolic syndrome is a combination of elevated waist circumference and at least two other parameters, such as IR, hypertension, hyperglycemia, high serum triglycerides, and reduced high-density lipoprotein (HDL) levels [61], leading to the development of T2DM and cardiovascular diseases.

The observed difference in disease risk between the visceral and subcutaneous depot may be due to (1) the differences in the depot-specific pattern of receptor expression and (2) differences in endocrine function with an unique adipokine expression profile for each depot. It has been reported that steroid receptor density such as androgen and estrogen receptor α and β as well as glucocorticoid receptors are higher at mRNA levels within visceral depots compared to subcutaneous counterparts [62, 63]. Since distinct depots exhibit different receptor densities, they hence have different options to respond to afferent signals. Moreover, expression and secretion of pro-inflammatory adipokines are higher in visceral adipose tissue, whereas anti-inflammatory adipokines are more abundant in subcutaneous adipose tissue [28]. Adipokines derived from visceral AT are released into the portal system [64]. Therefore they have a direct connection to the liver where they influence especially hepatic metabolism and are strongly associated with cardiovascular diseases [60].

1.1.4.2 Adipose tissue inflammation

Adipose tissue mass is altered with the onset of obesity, mainly due to a combination of adipocyte hyperplasia and hypertrophy [6]. Furthermore, chronic inflammation of AT occurs during obesity [65], accompanied by activation of the transcription factor nuclear factor-kappa B (NFκB) [7]. Subsequently, the AT secretome switches towards a prominent release

of pro-inflammatory adipokines while the expression and release of anti-inflammatory adipokines is strongly decreased. As a consequence, the recruitment of immune cells such as macrophages takes place [7]. In obesity concentrations of leptin, which promotes atherogenesis and IR, are elevated [59] while adiponectin levels, as an adipokine with anti-atherogenic, anti-inflammatory and anti-diabetic properties, are reduced [66]. Other adipokines such as TNFα, IL-6, RBP4, resistin or DPP4, have been associated with IR and are upregulated in obesity [49, 67-70]. In the vasculature, especially in the vessels within AT, pro-inflammatory adipokines upregulate the expression of cell adhesion molecules, thereby recruiting monocytes [70]. Moreover, enhanced secretion of MCP-1 during obesity facilitates the direct infiltration of macrophages into the AT [71]. Furthermore, immune cells, such as neutrophils and T-cells, migrate into the AT and contribute to macrophage recruitment [72].

Macrophages accumulate in "crown-like structures" around dead adipocytes, which commonly appear in inflamed AT, in order to scavenge adipocyte debris [7]. Besides macrophage recruitment, obesity induces a switch in macrophage polarization from the M2 phenotype, producing high levels of anti-inflammatory IL-10, to M1 macrophages, producing more pro-inflammatory cytokines, including TNFα and IL-6 [7]. The infiltration of AT by inflamed macrophages contributes to a bi-directional crosstalk to the AT in a vicious cycle, exacerbating the production of pro-inflammatory adipokines. Elevated levels of these circulating pro-inflammatory adipokines found in diseases such as obesity or T2DM [64] have been shown to be risk factors for metabolic and cardiovascular diseases [65].

1.1.4.3 Obesity-associated T2DM

Type 1 diabetes mellitus (T1DM) is a metabolic disorder based on a dysfunction of the body to produce insulin as a consequence of an auto-immune reaction which results in destruction of the pancreatic β-cells [73]. Differently, in T2DM the body develops a resistance to insulin. Approximately 5-10 % of the diabetic patients suffer from T1DM while T2DM accounts for around 90% of all cases of diabetes [74]. To diagnose diabetes there are different approaches all on basis of measuring glucose abundance. Glucose levels can be analyzed either randomly, in the fasting state, after glucose load or covalently bound to hemoglobin (HbA1c level) [73]. In healthy subjects measured values of glucose amount should not exceed a certain threshold (Table 2). Estimated by the international diabetes federation, 415 million people worldwide had diabetes in 2015 and 642 million are expected to have diabetes in 2040. To counteract this disease countries spend 5% to 20% of their total health expenditure on diabetes [74].

Obesity is closely related to a group of overlapping diseases, including T2DM [75]. T2DM, the most common form of diabetes, is characterized by an inefficiency of insulin [74]. Insulin is a peptide hormone produced by pancreatic β-cells and released in response to high glucose levels in the circulation to stimulate glucose transport into the cells [74]. The inability of the organism to respond to insulin causes reduced uptake of glucose from the blood and devoted to that chronic hyperglycemia [76]. To compensate IR, pancreatic β-cells increase their insulin release [77]. However, the constant hyperinsulinaemic state initiates β-cell dysfunction and death entailing decreased levels of insulin and hyperglycemia. Disturbance of the glucose metabolism drives elevation of hepatic gluconeogenesis and circulating lipids, which in turn promote inflammation and damage multiple organs. Thus, IR contributes to the development of many complications linked to T2DM [76]. T2DM is often but not necessarily always associated with obesity. However, obesity itself can cause IR and high levels of blood glucose [74].

The number of people with T2DM is growing rapidly and is associated with lifestyle of the modern society (physical inactivity and high caloric food consumption), aging population and increasing urbanization [78]. Therefore a change in lifestyle such as weight reduction or physical activity initially helps to treat T2DM. However, over time anti-hyperglycaemic agents will be required.

Table 2: Criteria for the diagnosis of diabetes according to the american diabetes association

Diagnostic criteria	Concentration mg/dl
Random plasma glucose	≥200
Fasting plasma glucoe	<126
2 h after 75 g oral glucose load	≥200
HbA1c	≥6.5%

1.1.4.4 Obesity-associated cardiovascular diseases

AT-derived factors in the blood stream can directly influence vascular functions [3, 57]. In obesity, there is a strong relationship to major cardiovascular risk factors such as hypertension, glucose intolerance, T2DM and dyslipidaemia [75]. CVD are the leading cause for deaths globally, accounting for 31% of worldwide death in 2012 [1], and cause a significant increase in obesity-associated mortality [4]. CVD comprises different disorders regarding heart and blood vessels, among which cerebrovascular (stroke) and coronary heart disease (myocardial infarction) are the most common ones [79]. These complications evolve from other underlying cardiovascular dysfunctions, mainly atherosclerosis.

Atherosclerosis

Atherosclerosis is an inflammatory process in the wall of medium- and large-sized vessels initiated by metabolic disorders such as the disturbance of lipid metabolism and immune response resulting in low-grade inflammation of the arterial wall. During the initiation of atherosclerosis the endothelium plays a major role. Endothelial dysfunction is an early marker of atherogenesis [80]. Various stimuli such as elevated cytokines, high glucose, lipoproteins, cholesterol or free radical levels, modulate the endothelium (endothelial activation) as a regulator of vascular wall homeostasis [81]. In response to these stimuli and the deposition of fatty material, EC change into an inflammatory state [82], thereby recruiting T-cells, monocyte and macrophages, becoming more permeable and increase their adhesiveness [80].

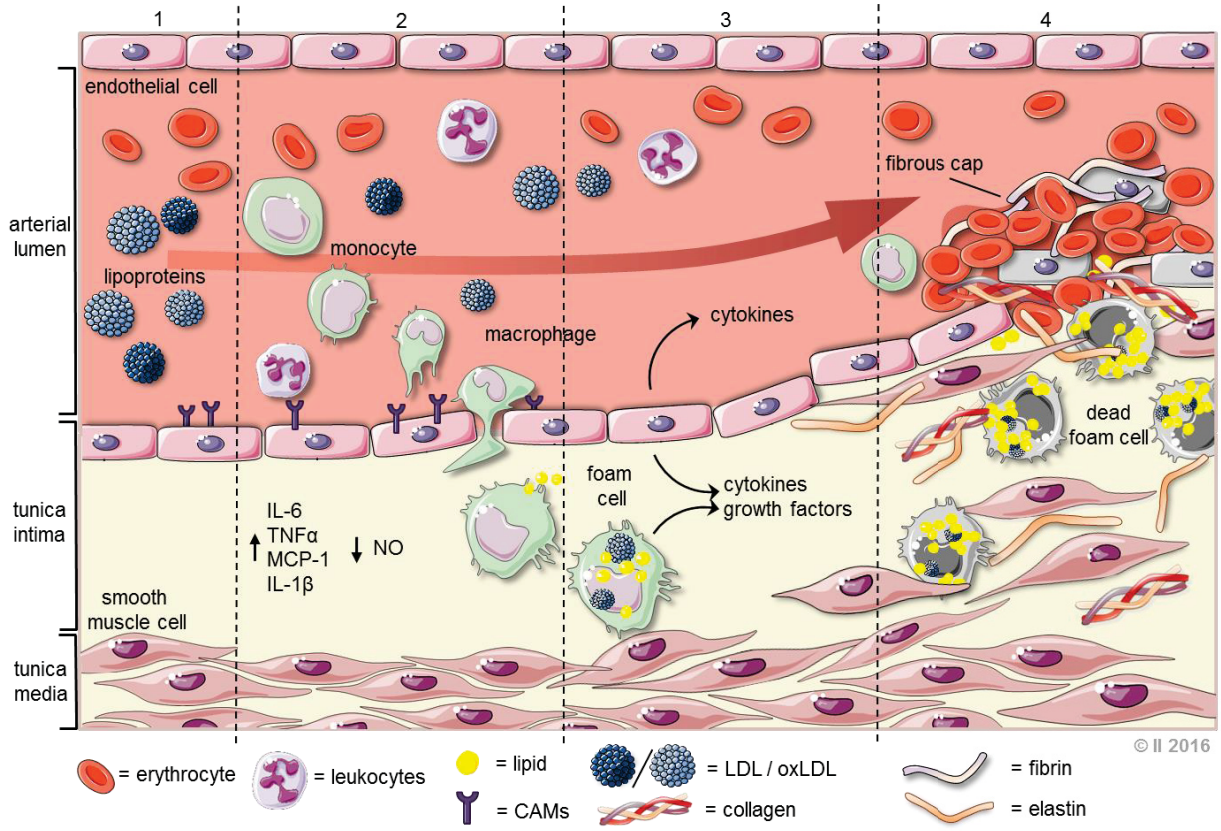

Figure 2. The development of atherosclerosis. During the onset of atherosclerosis high levels of lipoproteins, glucose and pro-inflammatory cytokines increase the expression of CAMs (1). Recruited monocytes and macrophages migrate into the vessel wall and transform into foam cells (2). Elevated levels of cytokines and growth factors lead to SMC proliferation and migration into the *tunica intima* (3). In a later stage cell apoptosis and synthesis of fibrin and collagen result in the formation of a fibrous cap (4). Rupture of the fibrous cap possibly lead to thrombus formation and promotes the occurrence of strokes and heart attacks. Cell adhesion molecule (CAM), endothelial cell (EC), smooth muscle cell (SMC).

In the early stage of endothelial dysfunction, activated EC highly express intracellular adhesion molecules 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) [81] which bind to circulating leukocytes as well as to monocytes and T-lymphocytes, respectively [82]. Additionally, the oxidized form of low-density lipoproteins (oxLDL) in the circulation amplifies the activation of EC and expression of adhesion molecules [81]. Recruited monocytes differentiate into macrophages, infiltrate the endothelial layer, migrate into the *tunica intima* and transform into foam cells [83]. Transformation from macrophages into foam cells comprises the accumulation of lipids [84], which are internalized by scavenger receptor-mediated endocytosis or phagocytosis [85]. Moreover, during the progression of atherosclerosis EC produce more vasoactive substances, cytokines and growth factors and less nitric oxide (NO) [86], thereby diminishing the dilatory features of the vessel [82]. With ongoing inflammatory process, cytokines and growth factors elicit an alteration of SMC phenotype from the contractile to the active state [87]. SMC start to proliferate, and migrate from the *tunica media* to the *tunica intima*, where they synthesize collagen, elastin, growth factors and cytokines [88].

In a later stage of the disease, accumulation of lipoproteins into macrophages elicit apoptosis and necrosis, followed by the formation of a necrotic core, termed plaque, by these dying cells [89, 90]. Upon growing, due to lipid and cell clustering the plaque begins to bulge into the vessel lumen reducing vessel elasticity [1]. Towering into the lumen it is prone to rupture. Rupture of the plaque releases lipid fragments, cellular debris and collagen to the circulation where it is exposed to thrombogenic proteins, thereby potentially facilitating the formation of a thrombus [91]. Blocking of arteries in the brain or the heart by thrombi causes ischaemic stroke or myocardial infarction, respectively. Behavioral risk factors are responsible for about 80% of CVD [1], hence prevention seems to be easy by addressing risk factors such as smoking, unhealthy diet, obesity and physical inactivity.

1.2 DPP4 and its role in organ crosstalk

1.2.1 Functionality and structure of DPP4

Since the discovery of leptin and adiponectin, the two prototypes of adipokines, several new adipokines have been identified [92, 93], among them a glycoprotein called dipeptidyl peptidase 4 (DPP4).

Recently our group described DPP4, expressed and released from adipocytes, as a novel adipokine [44]. DPP4, a 110 kDa sized glycoprotein discovered by Hopsu-Havu and Glenner

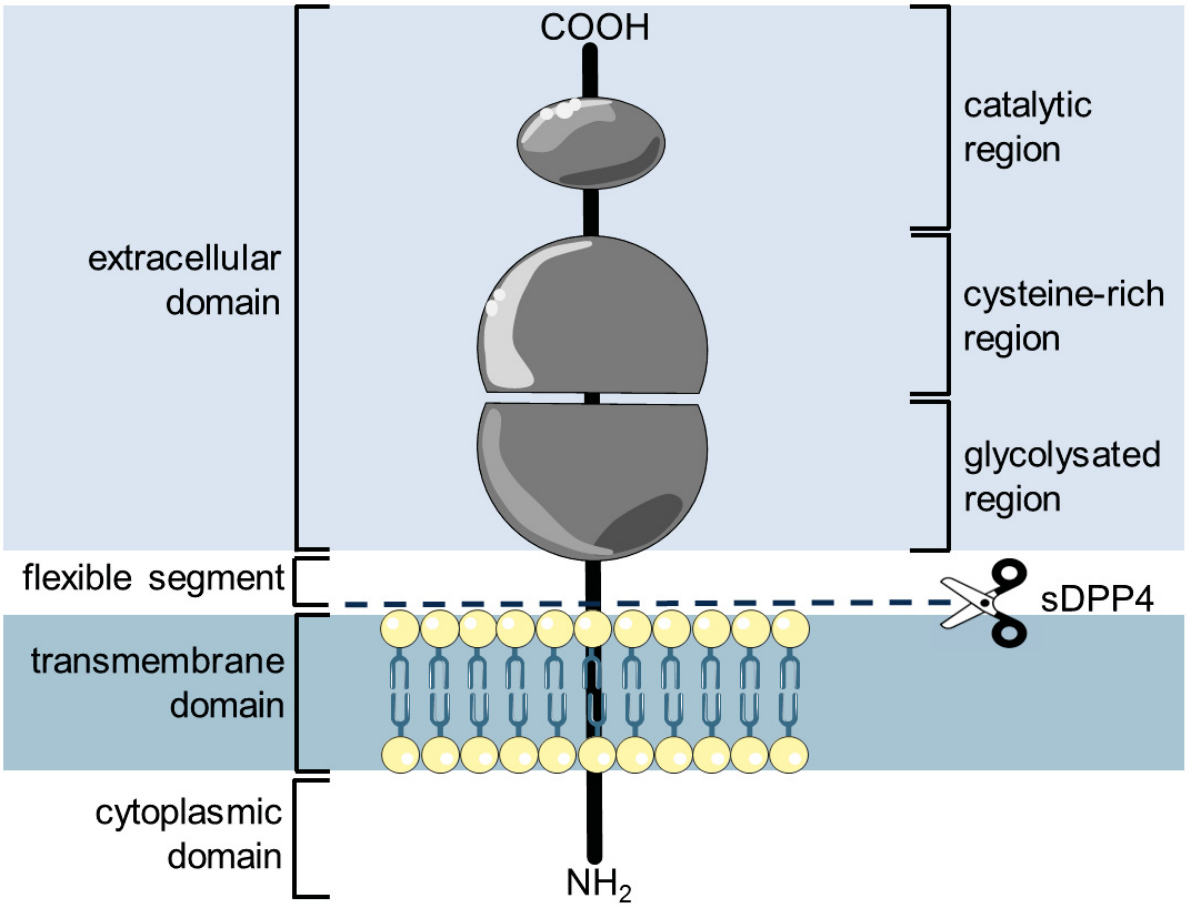

Figure 3. Structure of DPP4. DPP4 comprises 4 domains, namely the extracellular domain, the flexible segment, the transmembrane domain and the cytoplasmic domain. The extracellular domain of DPP4 includes the cysteine-rich region which interacts with binding partners such as PAR2 and the highly glycosylated part of DPP4 which plays a role in the trafficking process. Its enzymatic function is implemented by the catalytic region. Due to shedding the membrane bound DPP4, a soluble form of DPP4 emerges, lacking the transmembrane and cytoplasmic domain. Modified from Röhrborn et al. Front Immunol (2015). Dipeptidyl peptidase 4 (DPP4).

[94], is an exoprotease belonging to the subfamily of S9B which also includes the other dipeptidyl peptidases 1-10 and FAPα [95]. These exopeptides clip the terminal dipeptides of various substrates [96]. DPP4 can be found as monomers, dimers, which represent the dominant form, or tetramers on the cell surface of different cell types [97], including adipocytes, EC and SMC. Due to a process called shedding, a soluble form of DPP4 (sDPP4) is released into the circulation and thus influences multiple organs [98]. DPP4 consists of 4 domains, first the cytoplasmic domain, second the transmembrane domain, third the flexible segment and forth the extracellular domain (Figure 3) [96]. The latter can be further divided into the catalytic domain, the cysteine-rich domain and the highly glycosylated region. While glycosylation is important for trafficking of the protein [99, 100], the catalytic domain

contains serine in the active center, through which DPP4 exerts its enzymatic action, cleaving off the dipeptides from the N-terminal position of its substrates [101]. Among others, the incretins, particularly glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP), serve as substrates for the enzymatic activities of DPP4 [102]. GLP-1 and GIP are secreted into the intestine and in turn trigger insulin release from pancreatic β-cells via G-protein coupled receptors (GPCR) signaling in a glucose-dependent fashion [103]. Around 50%-70% of insulin secretion after oral glucose load is ascribed to the incretins [104]. Moreover the incretins control satiety through the hypothalamus, fat metabolism in adipocytes and regulate β-cell mass. The cysteine-rich region allows DPP4 to also interact with several binding partners. So does adenosine deaminase (ADA), an enzyme converting adenosine to inosine and thus playing an important role in the purine metabolism [105]. Upon binding of DPP4, ADA has a 1000 fold higher activity than free ADA [106]. *In vitro*, the complex of ADA and DPP4 enhances activation and proliferation of T-cells [107]. However, T-cell proliferation has been described to be promoted by DPP4 independently of ADA [108].

A proposed receptor for DPP4 is protease-activated receptor (PAR) 2. A sequence in the cysteine-rich region of DPP4 has been found to display homology with the tethered ligand of PAR2 which binds at the second loop of the receptor, thereby auto-activating it [109].

1.2.2 Role of DPP4 deficiency in animal models

DPP4 deficient animal models have been studied to unravel the role of DPP4 in health and disease. Most of these studies have been performed in rats. Under high fat diet (HFD) feeding, F344/DuCrj rats with a naturally occurring inactivating mutation in the DPP4 gene showed improved HOMA-IR, whereas controls were more insulin resistant [110]. Furthermore, glucose and insulin plasma levels as well as active GLP-1 levels were enhanced in F344/DuCrj rats compared to controls suggesting that the lack of DPP4 leads to improved glucose metabolism due to elevated GLP-1 levels [110]. Similarly, in a congenic strain it has been demonstrated that whole body DPP4 deficiency enhanced glucose tolerance during diet-induced obesity [111]. In DPP4 deficient rats from another study, insulin sensitivity was augmented illustrated by higher phosphorylation of AKT during an insulin tolerance test (ITT) [112]. AT expression levels of several adipokines was altered too. Leptin, adiponectin and peroxisome proliferator-activated receptor gamma (PPARγ) were increased whereas TNFα, IL-6 and plasminogen activator inhibitor-1 (PAI-1) were diminished in rats with destruction of DPP4 compared to controls indicating less AT inflammation. Moreover, ICAM-1 and VCAM-1 were reduced in AT as a consequence of DPP4 degradation. These

effects have been found to be GIP dependent [104]. In streptozotocin-induced diabetic rats knock down of DPP4 delayed the initiation of hyperglycemia. However, these DPP4 deficient rats were prone to develop dyslipidaemia [113].

The effects of DPP4 deficiency in mice are comparable. Conarello et al. explored the role of DPP4 in C57BL/6 mice and their DPP4 KO counterparts under HFD. KO mice gained less weight than WT mice since they exhibited reduced food intake and increased energy expenditure [114]. Additionally, KO mice were more resistant to hyperinsulinemia and showed improved islet and liver function, analyzed by lipid content and marker gene expression [114]. It is uncertain whether these effects are rather evoked by the reduced weight gain in DPP4 KO animals or might be a direct impact of DPP4 depletion. Nonetheless, an earlier study proved that whole body DPP4-KO animals displayed improved glucose tolerance, lower plasma glucose and higher GLP-1 as well as insulin levels upon glucose load [115].

1.2.3 DPP4 inhibitors as oral therapeutic agents

Knockdown of DPP4 entails beneficial effects on glucose and energy metabolism, often related to enhanced levels of the incretin hormones GLP-1 and GIP, the major substrates of DPP4. Therefore DPP4 inhibitors (DPP4i) are now widely used in the treatment of metabolic diseases such as T2DM. As sDPP4 exerts also pleiotropic impact on other organs the inhibition of these effects might be an advantageous side-effect of DPP4i.

Inhibition of DPP4 causes prolonged activity of GLP-1 and GIP, thereby stimulating secretion of insulin from β-cells and suppressing secretion of glucagon from α-cells in the pancreas [103]. At present there are several competitive, reversible inhibitors of DPP4, also known as gliptins, with 5 of them approved in Europe and the United States so far [116]. These DPP4i are grouped into 3 classes based on their binding characteristics in the active center of DPP4 [117]. Class 1, comprising vilda- and saxagliptin, form a covalent bond with the nitril group and Ser630 of the S_1 subsite of DPP4. The hydroxy adamantyl groups bind to the S_2 subsite. However, inhibitory potency of saxagliptin is 5-fold stronger than that of vildagliptin [118]. Class 2 includes alo- and linagliptin, which induce a conformational change in the Tyr547 of DPP4 due to binding of their uracil rings. The latter displays an 8-fold higher inhibitory activity compared to alogliptin [118]. However, alogliptin next to sitagliptin, is the most selective gliptin. Sitagliptin belongs to class 3 and binds to the S_2-extensive subsite of the active center. Class 3 also comprises teneligliptin (approved only in Japan and Korea). Due to their unique binding mode these inhibitors have strongest inhibitory

function. Owing to their binding mode, gliptins are different in their pharmacokinetic and reveal different EC50 ranging from 1 nM (Linagliptin) to 62 nM (Vildagliptin) [117]. Since gliptins prolong insulin release in a physiological range, the risk for hypoglycemia is estimated very low [119]. In clinical studies, a combination of DPP4i and metformin lead to increased insulin sensitivity and glucose-dependent insulin secretion as well as suppression of hepatic gluconeogenesis in patients with early T2DM [120]. Treatment with saxagliptin and metformin in combination compared to treatment restricted to only one agent, improved glycaemic control [121].

Additionally, numerous experimental studies suggest that DPP4i positively influence vascular disorders such as high blood pressure, arterial stiffness, the onset of atherosclerosis or thrombosis [122-125].

Treating diabetic rats with vildagliptin resulted in minimized oxidative stress and reduced expression of ICAM-1 and PAI-1 [126]. In contrast, lack of DPP4 in HUVECs elicited a pro-thrombotic status in response to induction of tissue factor and platelet adhesion [127]. Since DPP4i are used for the therapy of T2DM which increases the risk for cardiovascular complications [128], cardio-protective actions of these drugs would be advantageous. In accordance, cardiovascular outcome trials have been implemented to address the cardiovascular protective properties of DPP4i. Contrary to expectations several studies did not confirm cardiac and vascular benefits of DPP4i [129], instead they are rather controversially discussed, also in respect to their low number of cardiovascular adverse events [129]. However, a recent meta-analysis provides evidence that DPP4i are safe and even might be beneficial from a cardiovascular point of view [130].

1.3 Protease-activated receptor 2

1.3.1 Family of PARs

The PAR family, comprises 4 members (PAR1, PAR2, PAR3, PAR4), which belong to the 7 transmembrane cell surface protein GPCR superfamily [131]. These receptors possess a unique activation mechanism due to irreversible cleavage of the extracellular N-terminus of the receptor by certain proteases [132]. Cleavage of the amino terminus results in the exposure of a so called tethered ligand which in turn targets the binding pocket at the extracellular loop (ECL) 2 of the receptor [133], thereby activating the receptor and subsequently inducing intracellular signal cascades [134]. Despite overlapping expression patterns in cells and tissues, functions of these receptors are distinct.

PAR1 was identified in 1991 as the receptor for thrombin signaling [135]. Additionally, activation by trypsin is possible but less potent than thrombin [132]. Thrombin, which is present in close proximity of EC, SMC, neurons, leukocytes and others, is a highly potent activator of PAR1 due to two different cleavage sites, in particular the N-terminus cleavage domain and the hirudin-like domain [135]. Moreover, activation of PAR1 can be triggered by synthetic thrombin receptor activating peptides (TRAP). Although PAR1 is ubiquitously expressed, it is mainly involved in vascular processes such as wound healing by platelet aggregation and revascularization [136]. For example, TRAP and thrombin induce endothelium-dependent dilation of blood vessels via release of cyclooxygenase (COX) products or by NO [132]. Since the effect of thrombin could not be fully mimicked by TRAPs, the existence of a second receptor has been postulated. In 1996 Connolly et al. found PAR3 by targeted disruption of the thrombin receptor gene. The human isoform was 27% and 28% homologous to human PAR1 and human PAR2, respectively [132]. Similar to PAR1, it also comprises a hirudin-like domain which facilitates responses to thrombin. PAR3 was found to be expressed in several tissues such as heart, bone marrow, airway SMC and endothelium [137-139] but not, as PAR1, in platelets [132]. However, since synthetic peptides to study this receptor, were not active [139], the function of PAR3 is almost not known. PAR4 has been identified 1998 by Kahn and colleagues [140]. The fourth family member with 33% homology to the other PARs, shows differences in N- and C-terminus as well as in lacking a hirudin-like thrombin binding site [141]. PAR4 expression is ubiquitously but highest in lung followed by pancreas, testes and small intestines. The receptor is affine to thrombin and trypsin but requires higher concentrations than PAR-1 to be activated [140, 142]. In the late phase of the platelet aggregation, when PAR1 is inactivated and internalized, PAR4 is involved in sustaining this process [143, 144]. In line, observation from Shapiro et al. revealed a slow phosphorylation and desensitization kinetics of PAR4 [145].

1.3.2 Structure and signaling of PAR2

As the second member of the PAR family, PAR2 was discovered in 1994 by isolation from a mouse genomic library [146] and one year later isolated from a human genomic DNA library [147]. The receptor is deficient of a hirudin-like binding site and has an N-terminus shorter than that of PAR1 [132]. The gene encoding PAR2 is located at chromosome 5q13 close to the genes for PAR1 and PAR3 [133]. PAR2 is activated by serine proteases such as tryptase, trypsin, factor Xa, tissue kallikreins, the TF-FVIIa complex or acrosin, depending on the

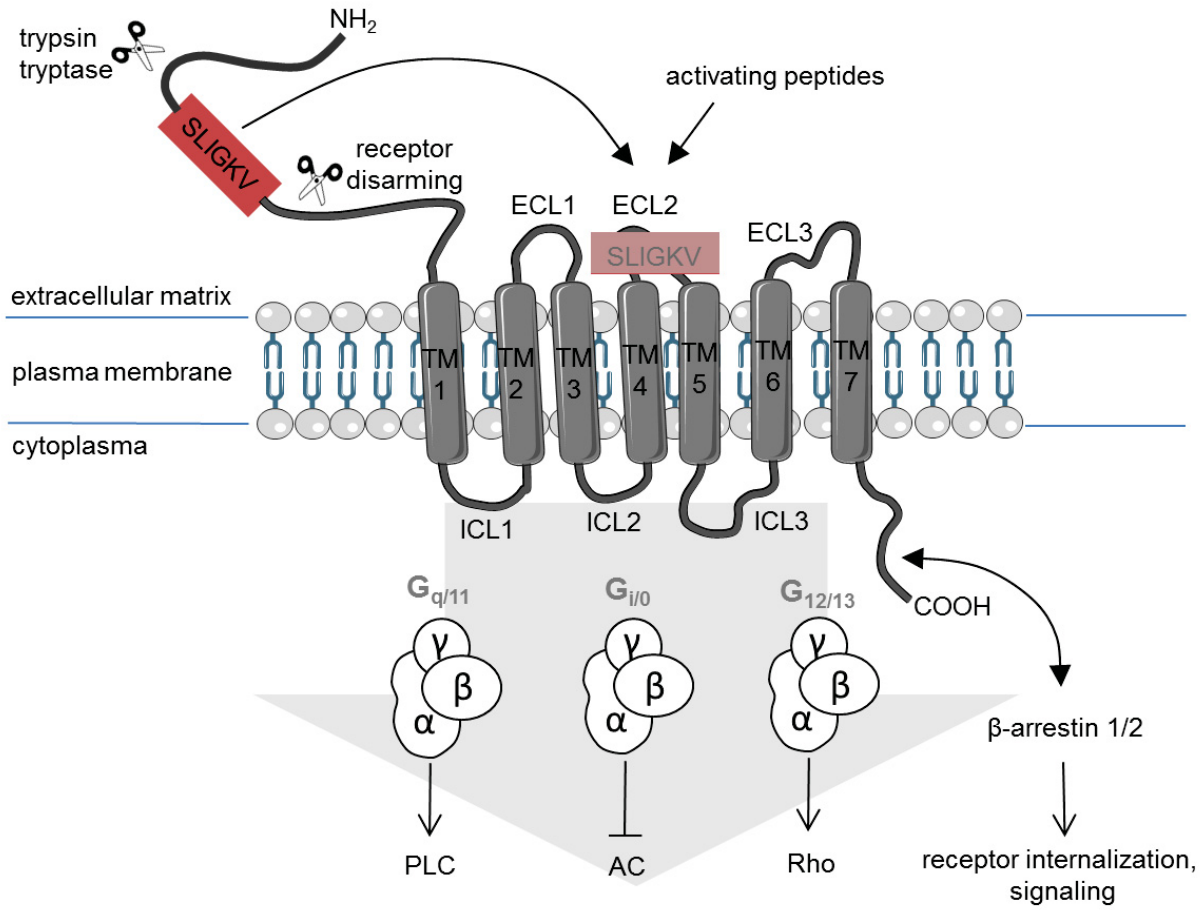

Figure 4. Structure and activating mechanisms of PAR2. PAR2 is a seven transmembrane G-protein coupled receptor. Canonical receptor activation occurs due to cleavage of the extracellular N-terminus by certain proteases and subsequent binding of the tethered ligand to the ECL2. Other proteases, such as cathepsin G, cleave the receptor downstream of its tethered ligand sequence and thereby disarming it. Receptor activation can also be initiated by synthetic agonist peptides which target the binding pocket in the ECL2. Due to intracellular interactions, β-arrestin initiates signaling cascades and receptor internalization. Modified from Kagota et al. Biomed Res Int (2016). Adenylyl cyclase (AC), extracellular loop (ECL), intracellular loop (ICL), phospholipase C (PLC), transmembrane domain(TM).

tissue [131, 148, 149], but not by thrombin, thus PAR2 is functionally different from the other PARs. The proteolytic cleavage of the extracellular N- terminal unmasks the tethered ligand thereby leading to self-activation [150]. Subsequently, activated PAR2 interacts with G-proteins of the four major classes (Gq, Gi, Gs, G12/13) to initiate multiple signaling cascades resulting in a variety of distinct responses [134]. As previously mentioned, proteases cleave the canonical activation site of the receptor, displayed by trypsin, however, there are other proteases splitting distinct sites [151]. Recently, cathepsin S has been demonstrated to activate

PAR2, although weaker, by cleaving the N-terminus thereby exposing a novel tethered ligand (KVDGTS) [152]. Other proteases, such as neutrophil elastase, proteinase-3 or cathepsin G, disarm PAR2 by cleaving downstream of the tethered ligand, it is discussed if they act as receptor antagonists. Anyhow, there is evidence for signaling activities upon PAR2 disarming without use of the tethered ligand [151]. Hence, cleavage per se seems to activate functional PAR2. In addition, PAR2 can be activated independently of N-terminal proteolysis by binding of synthetic peptides corresponding to the amino acid sequence SLIGKV of the tethered ligand [132]. Activating peptides of mouse and human PAR2, namely SLIGRL-NH_2 and SLICKV-NH_2 respectively, are similar in their pharmacological EC_{50} (5-10μM) [153]. These peptides selectively activate PAR2 and are therefore powerful pharmacological targets to study PAR2 function. Degradation of PAR2 is achieved by phosphorylation of the carboxyl terminus, subsequent β-arrestin binding [154] and internalization through clathrin-coated pits [150]. Nonetheless, complex building of PAR2 and β-arrestin promotes pro-migratory signaling pathways, too [155, 156]. Following the irreversible proteolytic cleavage of PAR2, termination of signaling and degradation of the receptor is initialized by ubiquitination of the cytoplasmic domain thereby inducing translocation into endosomes and later lysosomes [157] . Interestingly, activation by activating peptides does not lead to ubiquitination so that the receptor is available at the cell surface for re-stimulation [158].

1.3.3 Functional PAR2 activation

Expression of PAR2 is spread throughout various tissues [132, 159-161]. Thus, analysis of PAR-2 expression by immunoreactivity detected PAR2 in vascular and non-vascular smooth muscle, endothelial and epithelial cells independent of tissues, as well as in numerous cells of the gastrointestinal tract. Moreover, PAR2 was present in astrocytes and neurons of the central nervous system and it was localized in the epidermis and skeletal muscle [162]. The wide, ubiquitous distribution of PAR2 indicates multiple functions in distinct systems which might be tissue-specific and different in receptor activation. However, it is not clear whether PAR2 activation predominates in physiological or upon pathophysiological conditions. In this section we will focus on functional PAR2 activation in EC and SMC.

PAR2 agonism-mediated responses in the vasculature

In the vascular wall, PAR2 is present in human vascular SMC and EC [163-165]. *In situ,* PAR2 is expressed in SMC and endothelium of veins and arteries [162, 166] . Physiological functions of the receptor are the control of vascular tone and coagulation [151, 167]. Several

studies have demonstrated that PAR2 activation due to trypsin or PAR2-AP treatment causes endothelium-dependent vascular relaxation in vessels of different species [168-171]. In this context, NO and prostacyclin were illustrated as mediators of these effect [172, 173]. However, PAR2 has also been linked to vasoconstriction, both in an endothelium-dependent or independent manner [174, 175]. A dual action of PAR2 has been observed also in preparations of rat pulmonary arteries, where low concentrations of PAR2-AP mediated NO-reliant relaxation whereas high contractions end in a contractile response [170]. Thus, one might speculate that high levels of PAR2 activators, such as increased levels of pro-inflammatory cytokines, drive PAR2-mediated hypertension, while low activation of PAR2 might occur in physiological conditions.

Other *in vivo* settings support the role of PAR2 regarding its regulation of vascular tone. Activation of PAR2 triggers hypotension in rats and mice [176, 177]. Accordingly, PAR2 $^{-/-}$ mice are protected against AP-mediated hypotension [178, 179], but display higher blood pressure accompanied by unaffected heart rate compared to WT mice [180]. Contrarily, PAR2 agonists elicit endothelium-dependent relaxation of human coronary arteries after enhanced PAR2 expression due to inflammatory stimuli [181]. Indeed, an important role for the receptor upregulation during acute cardiovascular responses has been earlier proposed [182]. Other functions of PAR2, besides its contribution to vascular tone and blood pressure, might be the control of angiogenesis and wound healing as well as a role in vascular inflammation [132, 183].

In summary, these studies indicate that PAR2 possesses essential tasks throughout the entire body, which may be important in the control of physiological and pathophysiological conditions in a tissue-specific manner.

1.4 Objectives

AT biology gained considerable interest regarding obesity-related diseases such as T2DM and CVD. Being an endocrine organ, AT expresses and secretes a great number of adipokines, through which it influences the function of peripheral organs. Our group has recently identified DPP4 as a novel adipokine playing a substantial role in the regulation of glucose homeostasis. DPP4i have already been extensively studied and are used as oral antidiabetic agents. However, beyond affecting the incretins, local and systemic functions of AT-derived DPP4 during the process of obesity are not yet fully understood.

Therefore the first aim of the thesis was to explore the role of adipose DPP4 during obesity *in vivo*. For this purpose, an adipose-specific DPP4 knockout (AT-DPP4-KO) mouse model was generated and challenged with a HFD. The metabolic phenotype of the AT-DPP4-KO was characterized and both local and systemic impact of circulating adipose DPP4 was analyzed.

Moreover, obesity has been associated with cardiovascular disorders. In adipocytes membrane bound DPP4 is shedded and released into the circulation, where it exerts impact on multiple organs such as skeletal muscle or the vasculature. In obesity circulating DPP4 is upregulated and triggers IR and inflammation, thereby linking obesity and the metabolic syndrome. Furthermore, there is evidence that DPP4 is involved in the pathophysiology of vascular dysfunction and DPP4i have been discussed to act in a protective way. However, up to know, clinical studies rather support a minor role for DPP4i in the treatment of CVD.

The second aim of the thesis was to explore the direct impact and the underlying mechanisms of sDPP4 as an adipokine on murine microvessels *ex vivo*.

CVD is the main cause for mortality worldwide and prevalence increases with the development of obesity. We previously showed that the adipokine DPP4, which links obesity and the metabolic syndrome, signals through PAR2, a G-protein coupled receptor related to vascular function and upregulated in the vascular wall due to inflammatory stimuli. Knowledge about vascular functions of PAR2 during obesity is very limited though.

The third aim of the thesis was the analysis of PAR2 in obesity-associated vascular dysfunction. Therefore, we explored induction of atherogenesis by CM via PAR2 activation and the responsible mechanisms in vascular SMC.

Numerous adipokines have been characterized in the last years in order to find potential therapeutic targets to prevent and treat the development of obesity-associated disturbances. However, the adipocyte secretome is yet not fully identified and other factors besides adipokines are secreted from AT which may play a role in the crosstalk to distant organs. Finally, the role of adipose tissue-derived molecules on skeletal muscle function was determined. Next to adipo-myokines, novel mediators such as inflammasomes, microRNAs or autophagy were also reviewed.

2 PUBLISHED ARTICLES

2.1 DPP4 deletion in adipose tissue improves hepatic insulin resistance in diet-induced obesity

Romacho T, Sell H, **Indrakusuma I**, Roehrborn D, Castañeda T, Jelenik T, Hartwig S, Weiß J, Al-Hasani H, Roden M, Eckel J. Cell reports (2016), under review.

2.2 Soluble dipeptidyl peptidase-4 induces microvascular endothelial dysfunction through proteinase-activated receptor-2 and thromboxane A2 release.

Romacho T, Vallejo S, Villalobos LA, Wronkowitz N, **Indrakusuma I**, Sell H, Eckel J, Sánchez-Ferrer CF, Peiró C. J Hypertension (2016) 34(5):869-76.

2.3 Protease-Activated Receptor 2 Promotes Pro-Atherogenic Effects through Transactivation of the VEGF Receptor 2 in Human Vascular Smooth Muscle Cells.

Indrakusuma I, Romacho T, Eckel J. Front in Pharmacol (2016), in press.

2.4 Novel mediators of adipose tissue and muscle crosstalk

Indrakusuma I, Sell H, Eckel J. Curr Obes Rep (2015) 4(4):411-7.

2.1 DPP4 deletion in adipose tissue improves hepatic insulin resistance in diet-induced obesity

Under review in Cell Reports. Dec 2016

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Abstract— **Besides a therapeutic target for type 2 diabetes, dipeptidyl peptidase 4 (DPP4) is an adipokine potentially linked to human obesity. The role of adipose tissue (AT)-derived DPP4 was explored generating an AT-specific KO mouse (AT-DPP4-KO). Under high fat diet (HFD), AT-DPP4-KO displayed reduced circulating DPP4, proving AT as a relevant source. Independently of glucose-stimulated incretin hormones, glucose tolerance and suppression of endogenous glucose production were improved. AT-DPP4-KO displayed smaller adipocytes, increased M2 macrophages and decreased AT fibrosis. IGF binding protein 3 (IGFBP3) levels were lower in AT and serum, while free IGF1 was increased. In type 2 diabetes patients treated with the DPP4 inhibitor sitagliptin, both free IGF1 and free/total ratio increased. During HFD, lack of adipose DPP4 triggers beneficial remodeling and decreases production of IGFBP3, resulting in increased free IGF1 and improved glucose tolerance and hepatic insulin resistance, supporting a key role of DPP4 in obesity-related metabolic disorders.**

Highlights

- AT-DPP4-KO is a novel model which demonstrates that AT is a major source of soluble DPP4 in obesity.
- Under HFD, AT-DPP4-KO mice display improved glucose tolerance and hepatic insulin resistance, independently of glucose-stimulated incretin hormones.
- Lack of adipose DPP4 triggers favorable AT remodeling during HFD with smaller adipocytes, reduced fibrosis and increased M2 macrophage-markers.
- IGF binding protein 3 (IGFBP3) levels were reduced in AT and serum of AT-DPP4-KO mice, resulting in increased free IGF1 potentially linked to the observed improved hepatic insulin resistance.
- Analogously, type 2 diabetes patients treated with the DPP4 inhibitor sitagliptin, displayed increased serum levels of free IGF1 and free/total IGF ratio.
- Our model underpins a key role of DPP4 in obesity-related metabolic disorders.

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I. INTRODUCTION

Obesity is reaching an epidemic dimension nowadays (Ng et al., 2014) and is intimately related to the development of insulin resistance (Wellen and Hotamisligil, 2003), characterized by decreased insulin-stimulated glucose uptake in muscle (DeFronzo and Tripathy, 2009) and adipose tissue (AT) (Guilherme et al., 2008), as well as by impaired insulin-mediated suppression of endogenous (hepatic) glucose production (EGP) (Perry et al., 2014). Consequently, obesity represents a major risk factor for type 2 diabetes (Hardy et al., 2012). In search for the mechanisms underlying these diseases, AT has been recognized as an important endocrine organ releasing very diverse bioactive factors named adipokines (Romacho et al., 2014). By an in-depth proteomic profiling of the secretome of primary human adipocytes, we identified dipeptidyl peptidase 4 (DPP4) as an adipokine (Lehr et al., 2012). DPP4 is a ubiquitous transmembrane glycoprotein and exoprotease which cleaves N-terminal dipeptides from a wide range of substrates including growth factors, neuropeptides and chemokines (Yazbeck et al., 2009). Of note, DPP4 also cleaves and inactivates the members of the incretin hormone familiy glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), which account for approximately up to 70% of postprandial insulin secretion (Nauck et al., 1986). Thus, DPP4 inhibitors are currently used as glucose-lowering agents to prolong the insulinotropic effect of incretins in type 2 diabetes. Besides its enzymatic activity, DPP4 was originally

identified as the CD26 antigen in lymphocytes involved in T cell activation (Fleischer, 1994). Both, the abundance of the membrane-bound form and the activity of circulating soluble DPP4 are altered in a variety of inflammatory diseases, metabolic syndrome and diabetes mellitus (Rohrborn et al., 2015). Although a fraction of soluble DPP4 most likely originates from immune cells (Cordero et al., 2009), the major source of circulating DPP4 remains unknown.

In vitro, DPP4 knockdown and pharmacological inhibition in human adipocytes improved insulin signaling without affecting adipogenesis (Rohrborn et al., 2016). Studies in animals show that circulating DPP4 activity is higher in C57/BL6 mice under high fat diet (HFD) (Nistala et al., 2014) and in ob/ob mice (Ruter et al., 2004). On the contrary, genetic DPP4 deletion in Fischer rats and congenital DPP4 depletion in Dark Agouti rats resulted in improved glucose tolerance under HFD (Frerker et al., 2009; Yasuda et al., 2002). Furthermore, whole body DPP4 knockout (KO) mice display improved glucose tolerance, increased GLP-1 levels and prevented insulin resistance during HFD (Conarello et al., 2003; Marguet et al., 2000). We previously identified DPP4 as an adipokine that may be a missing link between increased AT mass in obesity and obesity-associated metabolic diseases. Since previous studies suggested that DPP4 may affect AT adipogenesis, inflammation and insulin sensitivity (Rohrborn et al., 2015), we aimed to explore the role of DPP4 within AT in the context of metabolic diseases. However, DPP4 pharmacological inhibition and genetic deletion affect both central regulation of food intake and energy expenditure. Therefore, we have generated an AT specific DPP4 KO mouse (AT-DPP4-KO) in order to characterize the impact of AT-derived DPP4 in obesity.

II. MATERIAL AND METHODS

Materials: Reagents for SDS-PAGE were supplied by GE Healthcare (Freiburg, Germany) and Sigma-Aldrich (Munich, Germany). Antibodies against murine DPP4, CD11b and β-actin were supplied from Abcam (Cambridge, U.K.) and pAkt Ser473 and Thr308 from New England Biolabs (Frankfurt, Germany). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse IgG anti-bodies were supplied by Promega (Mannheim, Germany). Complete protease inhibitor cocktail and PhosStop phosphatase inhibitor cocktail were provided by Roche (Mannheim, Germany). Unless stated elsewhere, all reagents were purchased from Sigma-Aldrich.

Mouse model: The adipose-specific DPP4 knockout (AT-DPP4-KO) mice have been generated by a Cre-lox strategy based on deletion of exons 1 and 2 of the DPP4 gene and crossbreeding with B6.Cg-Tg(Fabp4 cre)1Rev/J mice (Jackson Laboratories), expressing Cre-recombinase under the control of the Fabp4 promoter. Genetic deletion of DPP4 was confirmed at the DNA level by gel electrophoresis and also at the RNA and protein level by real-time PCR and Western blotting.

C57BL/6J floxed mice and adipose-specific DPP4 knockout male mice were housed in mixed groups of maximum 5 animals per cage under a light/dark cycle (12 h/12 h), in a temperature controlled room (22 ºC) with standard food and water ad libitum. At 12 weeks of age animals were randomly allocated to standard chow diet or HFD (60% fat; Research diets D12492) for 24 weeks. All procedures followed the principles outlined in the European Commission Council Directive for the care and use of laboratory animals (86/609/EEC). Animal experiments were approved by the Ethics Committee of the State Agency for Nature, Environment and Consumer Protection (LANUV, North Rhine-Westphalia, Germany) and conducted at the animal facility of the German Diabetes Center.

DPP4 activity in serum, organs and tissues: DPP4 activity was measured with a fluorometric kit from Sigma Aldrich. 10 µl of serum was assayed as detailed by the manufacturer. 10 mg of frozen tissues were homogenized in cold assay buffer. Following homogenization, the samples were centrifuged at 13000 rpm for 15 min at 4°C. The resulting supernatants were collected, diluted 1:10 and were immediately used for DPP4 activity fluorometric assay following manufacturer's instructions. The resulting fluorescent signal was detected with a microplate reader (Infinite M200, Tecan). DPP4 enzymatic activity in each tissue was expressed as the amount of cleaved AMC per minute per total tissue (µU/ tissue) at 37°C.

Western blot: 5 µg of proteins were separated by SDS-PAGE using 10% horizontal gels. The proteins were then transferred to a polyvinylidene difluoride membrane (Millipore) in a semidry blotting system. Membranes were blocked with 5% nonfat dry milk in TBS containing 0.1% Tween for 1 h at room temperature and later incubated with the indicated primary antibodies. After washing with TBST, membranes were incubated with a secondary horseradish peroxidase (HRP)-coupled antibody and processed for chemiluminescence detection using Immobilon HRP substrate (Millipore). Signals were visualized and analyzed on a Bio-Rad VersaDoc 4000 MP work station.

Peritoneal lavage: Mice were sacrificed by cervical dislocation and the peritoneum was flushed with cold PBS with 5% FCS in order to harvest peritoneal cells. The resulting peritoneal lavage was resuspended in 0.4 ml MACS buffer (1× PBS, 2% FCS, 5 mM EDTA). For Western blot analysis about 3 × 106 cells were resuspended in lysis buffer and stained with anti-CD11b (ab53187 Abcam) or a DPP4 antibody.

Body composition: Longitudinal changes in body composition of all animals were assessed using quantitative nuclear magnetic resonance imaging (Whole Body Composition Analyzer; Echo MRI, Texas, USA). Body composition was measured at the beginning of the diet and at every 4 weeks.

Hyperinsulinemic-euglycemic clamp test: At 4-6 days before the clamp, an indwelling silicon catheter (Silastic Laboratory Tubing, Dow Corning, Midland, MI) was placed in the right-side jugular vein of the animals under

isoflurane anesthesia (CP Pharma, Burgdorf, Germany), as previously described (Jelenik et al., 2014). After recovering from surgery, the mice were fasted during the 6 h prior to the clamp, with free access to drinking water. In order to assess basal glucose disposal, D-[6,6-2H2]glucose (98% enriched) (Cambridge Isotope Laboratories, Andover, MA) was infused at a rate of 4 μmol/kg/min for 120 min. The hyperinsulinemic-euglycemic clamp was performed with a primed (40 mU/kg), continuous infusion of insulin (10 mU/kg/min) (Huminsulin; Lilly, Giessen, Germany) for 180 min. Euglycemia was maintained by periodically adjusting a variable 20% glucose infusate. D-[6,6-2H2] glucose was co-infused together with insulin (0.4 μmol/kg/min) and variable glucose infusion to obtain stable tracer concentrations during varying glucose infusion rates (GIR expressed as "mg glucose/ (kg body weight x minute)). Blood samples were taken at 10 min intervals during the basal period and during the last 30 min of the hyperinsulinemic-euglycemic clamps. After the clamps, mice were exsanguinated through cervical incision and killed by cervical dislocation, serum and organs were collected for future analysis.

Glucose tolerance tests: For oral glucose tolerance tests (oGTT), a glucose solution (2 g/kg body weight, 40% solution in 0.9% wt/vol. NaCl) was given by gavage into 16 h fasted animals. Tail blood glucose levels were measured with a glucometer (Free Style Precision Abbot, Wiesbaden, Germany) at 0, 15, 30, 45, 60, and 120 minutes after gavage.

Adipose tissue immunohistochemistry analysis: Pieces of AT were collected from WT and AT- DPP4-KO mice after 24 weeks of HFD or chow diet. The tissues were fixed in 10% formalin and then dehydrated in ethanol followed by paraffin embedding. A total of 200 adipocytes from 3 different levels of the tissue (100 μm distance between levels) were analyzed per animal and depot, respectively.

a) Adipocyte size

Adipocyte area was measured by a blinded observer at a 10-fold magnification with Cellsense software (Olympus SIS). Adipocyte sizes were clustered in corresponding cell size groups and represented as percentage of cells in each size class.

b) Crown-like structures (CLS)

10-μm dewaxed serial sections were incubated with anti-mouse Mac-2/galectin-3 (Cedarlane Laboratories, Canada) followed by incubation with a biotinylated HRP-conjugated secondary antibody goat anti-rat IgG (Mac-2/galectin-3; Vector Laboratories; Burlingame, CA, USA). Histochemical reactions were performed using Vector's Vectastain ABC Kit and diaminobenzidine as substrate. Sections were counterstained with hematoxylin-eosin and mounted in coverslips. CLS structures per 1000 cells were counted by a blinded observer with Image J software in microphotographs obtained at a 40-fold magnification.

Generation of conditioned media from AT explants: Explants from ingWAT and epiWAT from animals fed HFD for 24 weeks were collected in sterile cold PBS. Connective tissue and vessels were removed and fat pads were cut into pieces of 10 mg. After three times washing and centrifugation in fresh PBS supplemented with an antibiotic and antimycotic mix (Life Technologies) explants were weighted and 100 mg of adipose tissue were incubated in in low-glucose DMEM smooth muscle cell growth medium (PromoCell, Heidelberg, Germany) supplemented with 50 mg/ml gentamycin for 24h at 37°C and 5%CO2 to generate 1 ml conditioned medium.

Real-time quantitative PCR (RT-qPCR): Total RNA was extracted from frozen tissue samples using TRIzol Reagent (Ambion Life Technologies, Darmstadt, Germany) and RNeasy purification kit (Qiagen, Hilden, Germany) and cDNA was transcribed using 1 μg of total RNA (Omniscript reverse transcription kit, Qiagen). qPCR was performed using QuantiFast SYBR Green PCR kit (Qiagen). mRNA expression was measured by RT-qPCR with a Step One Plus Real-Time PCR System (AB Applied Biosystems). Values were normalized to the concentration of the housekeeping gene 18S in each sample. mRNA expression was measured in epiWAT and ingWAT from WT and AT-DPP4-KO mice as previously described (Schmittgen and Livak, 2008). The following predesigned primers were used: Mm_Dpp4_1_SG, Mm_Il10_1_SG, Mm_Adipoq_1_SG, Mm_Ccl2_1_SG, Mm_Il6_1_SG, Mm_Col1a1_1_SG, Mm_Col3a1_2_SG Mm_Col6a6_1_SG, Mm_Lgals_3_SG, Mm_Loxl1_2_SG Mm_Mrc_1_SG, Mm_Pparg_1_SG, Mm_Slc2a4_1_SG, Mm_Tnf_1_SG (Applied Biosystems). Customized primers were purchased from Eurofins and had the following sequences Cre fwd: GATTCGACCAGGTTCGTTC Cre rev: GCTAACCAGCGTTTTCGT. UCP-1 rev: CTTTGCCTCACTCAGGAT; UCP-1 fwd: ACTGCCACACCTCCAGTC

Hormone and biochemical assays: Plasma insulin was measured with a mouse insulin ELISA immunoassay (Mercodia, Uppsala, Sweden). Mouse DPP4 (Boster), IGBP3, total IGF1 (R&D systems) and free IGF1 (abcam, United Kingdom) were measured by ELISA. Human IGFBP3, free and total IGF1 were measured using ELISA kits from R&D Systems. Human DPP4 was monitored by ELISA (Abnova).

HepG2 culture and glucose production assay: HepG2 cells were obtained from ATCC to study hepatocytes and checked for mycoplasma contamination. Cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Gibco), 1x Antibiotic-Antimycotic mix (Gibco). Cells were maintained at 37 °C with humidified air and CO2 (5%). At subconfluence, cells were treated with 300 ng/ml human recombinant IGFBP3 (Biotechne) for 18 h. Cells were washed two times with PBS to remove glucose and incubated for 3 h in glucose production medium (glucose- and phenol red-free DMEM, 20 mM sodium lactate, 2 mM sodium pyruvate) in the presence or absence of 100 nM insulin (Sigma) during 10 min. A quantity of 150 μl of medium was sampled for measurement of glucose concentration using a colorimetric glucose assay kit (Sigma). Glucose concentration was normalized with

cellular protein concentration.

Patients: The study was approved by the ethics committee of the Heinrich Heine University Düsseldorf and was conducted in accordance with the Declaration of Helsinki. All participants provided a written informed consent. All patients were participants of the prospective German Diabetes Study (GDS), which evaluates the long-term course of diabetes (Szendroedi et al., 2016). Inclusion criteria for entry into the GDS were type 2 diabetes, known diabetes duration ≤1 year, and 18–69 years of age. Exclusion criteria were: secondary diabetes, pregnancy, severe diseases (cancer), psychiatric disorders, immunosuppressive therapy and limited cooperation ability. Fasting serum samples were obtained from patients treated with sitagliptin in addition to metformin (n=17) and patients treated only with metformin (n=33) matched for age, body max index (BMI) and sex.

Statistics: Statistical analysis was performed with the GraphPad Prism software (La Jolla, CA, USA). p values were calculated using the two-tailed Student's t-test. For statistical comparisons between experimental groups, two-way ANOVA was used, followed by Bonferroni post hoc test. A p value below 0.05 was considered statistically significant. Data are shown as the mean ± S.E.M or mean ± S.D if specified. Normal distribution and equal variances were trested for each analysis. Animals were randomly allocated to the different diets and at least three animals were included in each experimental group. Groups size was calculated by Simple Interactive Statistical Analysis.

III. RESULTS

The AT-DPP4-KO mouse model

Cre expression was only detectable in mature adipocytes from KO animals (Fig 1A), where DPP4 protein levels were significantly reduced by up to 65 % (Fig. 1B) with unaltered DPP4 expression in the stromal vascular fraction (SVF) within AT (Fig. 1C). DPP4 protein levels in immune cells isolated by peritoneal lavage from AT-

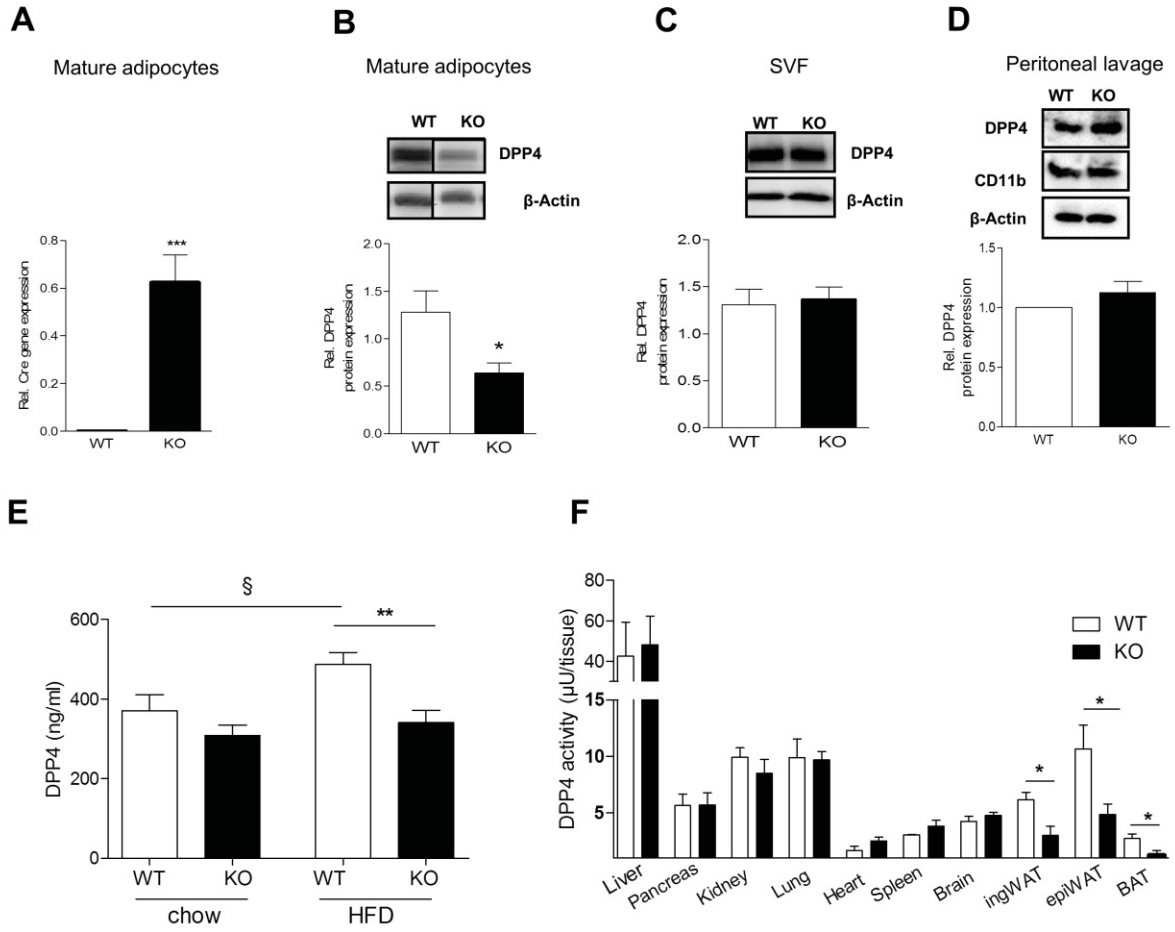


Figure 1: DPP4 deletion is restricted to adipocytes in AT-DPP4-KO mice. (**A**) Cre mRNA levels in mature adipocytes isolated from wild type (WT) or their corresponding DPP4 adipose tissue–specific knockout littermates (KO) (n=12 animals per group). mRNA levels were normalized to 18S levels. (**B**) DPP4 protein levels in mature adipocytes and in stroma vascular fraction (**C**) isolated from the inguinal subcutaneous AT depot, from WT and KO animals (n=4-6 animals per group). Corresponding representative blots are shown on top. (**D**) DPP4 and CD11b expression in resident immune cells isolated from peritoneal lavage (n=3 animals per group). Corresponding representative blot is shown on top. (**E**) Fasting circulating DPP4 levels assessed by ELISA in WT and KO mice after 24 week of chow and HFD (n=11 animals per group in ND and n=22 animals per group in HFD). (**E**) DPP4 enzymatic activity in different tissues and organs (n=4 animals per group). Data expressed as mean ± S.E.M. ***p<0.001; **p<0.01; *p<0.05 vs. WT littermates on the same diet; §p<0.05 vs corresponding genotype on different diet.

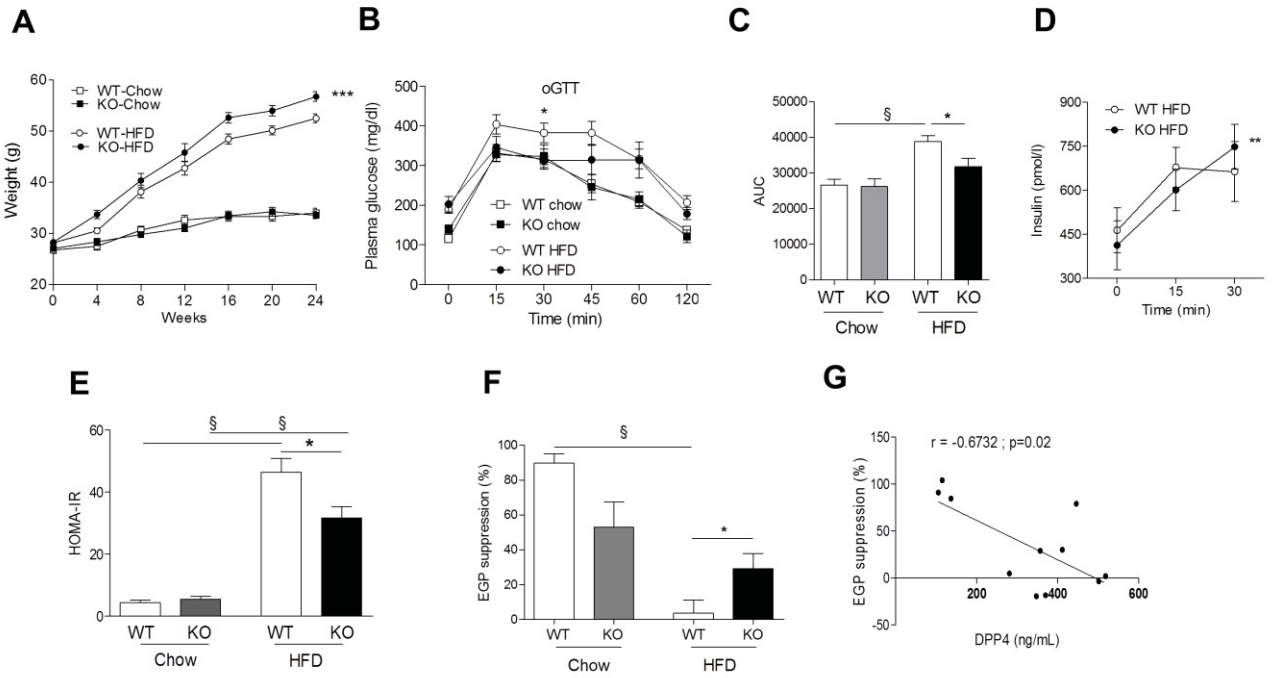


Figure 2: AT-DPP4-KO animals gain more body weight but display improved glucose tolerance and hepatic insulin sensitivity on HFD. (A) Body weight was assessed every 4 weeks during chow and HFD (n=11-16 animals per group on chow, n= 26-31 animals per group on HFD). Data expressed as mean ± S.E.M. ***p<0.01 vs. diet-matched WT littermates. (**B**) oGTTs were performed after 18-20 weeks of chow or HFD (WT chow n=8; KO chow n=6; WT HFD n=9; KO HFD n=6). (**C**) Corresponding AUC of oGTT. (**D**) Plasma insulin levels at 0, 15 and 30 minutes after glucose gavage (2 mg/g body weight) (WT HFD n=9; KO HFD n=7). (**E**) HOMA-IR after 24 weeks of dietary intervention (WT chow n=7; KO chow n=7; WT HFD n=17, KO HFD n=15). (**F**) Percentage of suppression of endogenous glucose production (EGP) during hyperinsulinemic-euglycemic clamps, after 22 weeks of dietary intervention (WT chow n=4; KO chow n=9; WT HFD n=7; KO HFD n=9). (**G**) Correlation between EGP suppression and DPP4 circulating levels (WT chow n=4; WT HFD n=7). Data expressed as mean ± S.E.M. *p<0.05; **p<0.01; ***p<0.001 vs. WT littermates on the same diet, §p<0.05 vs corresponding genotype on different diet.

DPP4-KO mice remained similar to WT mice (Fig. 1D). This specific AT KO resulted in significantly lower serum DPP4 in KO animals on HFD (Fig 1E). As DPP4 KO was restricted to AT, we did not observe differences in incretin levels. Circulating active GLP-1 and GIP were similar between the genotypes both in basal conditions and after 15 min oral glucose load (Suppl. fig. 1). To further prove the specificity of our animal model we explored DPP4 enzymatic activity in different organs and tissues such as liver, pancreas, kidney, lung, heart, spleen, brain, ingWAT, epiWAT and BAT. DPP4 was only decreased in WAT and BAT (Fig. 1F).

Lack of DPP4 in AT improves glucose tolerance in mice under HFD in spite of increased body weight

After 24 weeks of HFD, AT-DPP4-KO mice gained more body weight compared to WT (41.9 ± 3.6 g vs. 56.7 ± 4.3 g; Fig. 2A). This was paralleled by an increase in fat and lean mass (Suppl.Fig. 1A-B). Analogously, the genotype did not affect EE (volume of oxygen consumed (VO2/kg0.75/h), respiratory quotient (RQ) and SPA (Suppl. Fig. 2 A-C). These differences in body weight, fat and lean mass were not due to differences in food intake normalized to body weight (Suppl. Fig. 2d). Body length was similar in both genotypes (Suppl. Fig. 2E). Furthermore, BAT mass and RQ was not affected by the genotype (Suppl. Fig. 2B).

During the oral glucose tolerance test, plasma glucose was lower during the first 60 minutes in KO animals compared to the diet-matched controls (Fig. 2B-C), while plasma insulin levels were significantly increased during the initial 30 minutes (Fig. 2D). HOMA-IR index was significantly lower in KO mice after 24 weeks HFD (Fig. 2E). The hyperinsulinemic-euglycemic clamps revealed increased suppression of endogenous glucose production (EGP) in KO animals after HFD (Fig. 2F). Whole body glucose disposal rate (Rd) and NEFA suppression were not different from WT (Suppl Fig. 3), indicating that AT-DPP4-KO mice exhibit selective improvement of hepatic insulin senstivity upon HFD. Of note, circulating DPP4 levels correlated negatively with hepatic insulin sensitivity, as assessed from suppression of EGP in WT animals (Fig 2G). Under chow diet, insulin sensitivity in AT-DPP4-KO mice was not different from KO.

Liver lipid content as assessed histologically (Supl. Fig 4A) or colorimetrically after extraction (Supl. Fig 4B) did not differ between the WT and KO animals. The KO animals under HFD had similar plasma triglycerides (Suppl. Fig. 4D) but significantly reduced cholesterol plasma levels compared to their matched WT controls (Suppl. Fig. 4C).

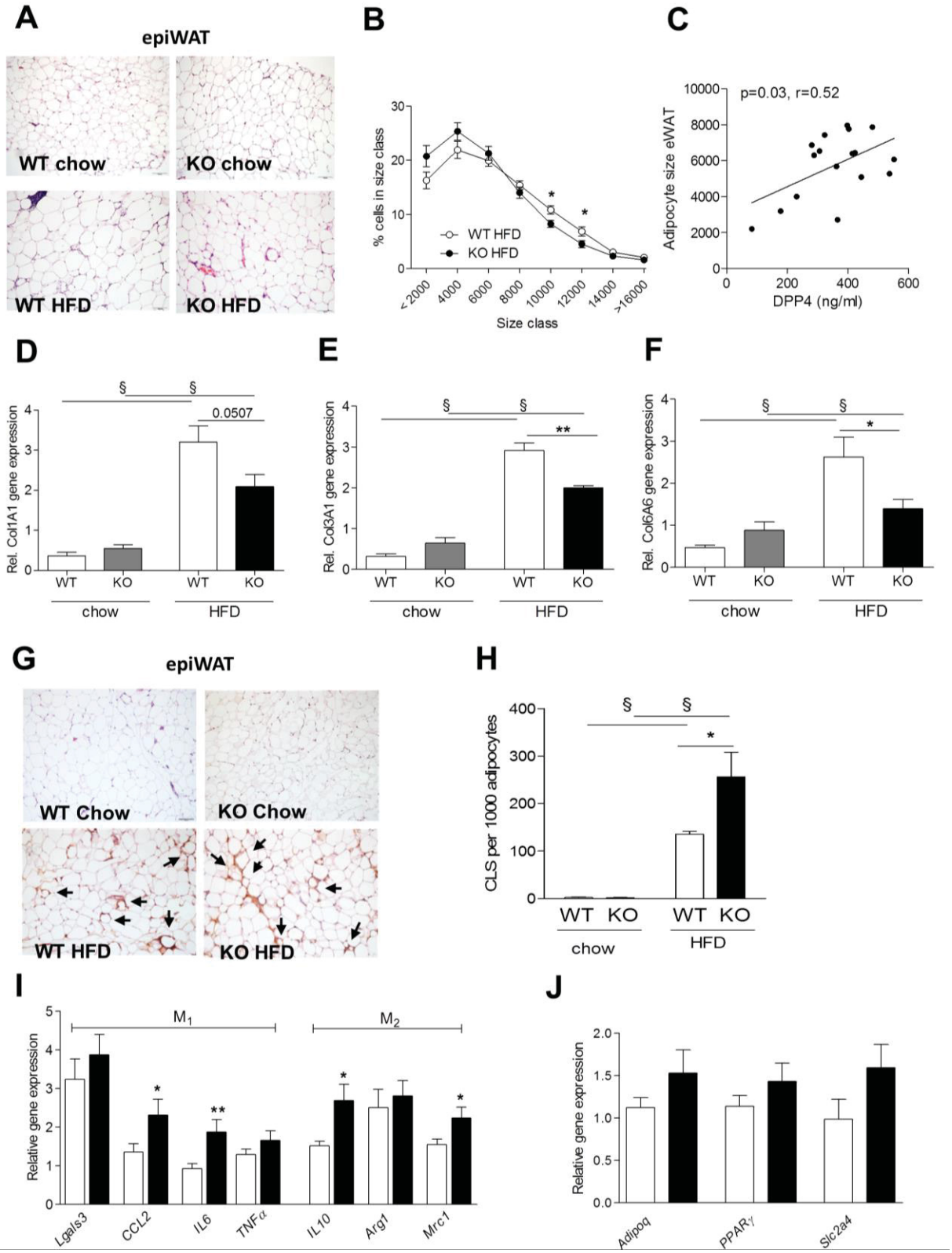


Figure 3: Adipocyte morphology, fibrosis markers and galectin-3 positive macrophage infiltration in epiWAT. (**A**) Representative microphotographs of hematoxylin-eosin stained adipocytes from epiWÁT obtained from WT and KO animals after 24 weeks under chow or HFD. (**B**) Percentage of cells per adipocyte size class (μm2) in epiWAT obtained from WT and KO animals after 24 weeks under chow or HFD (WT HFD n=16; KO HFD n=16). (**C**) Correlation between adipocyte size and DPP4 circulating levels (n=33). mRNA levels of the fibrosis markers collagen 1A1 (**D**), collagen 3A1 (**E**) and collagen 6A6 (**F**), respectively (n=5-8 animals per group). (**G**) Representative microphotographs of immunohistochemistry staining for galectin-3, showing CLS in epiWAT. (**H**) Number of CLS per every 1000 adipocytes calculated after 24 weeks of dietary intervention (WT chow n=4; KO chow n=4; WT HFD n=7; KO HFD n=6). (**I**) mRNA levels of M1 macrophage markers: Lgals3, CCL2, IL-6, TNF-α, and M2 macrophage markers: IL-10, Arg10 and Mrc1 in epiWAT from WT and KO animals after 24 weeks of HFD (WT HFD n= 24; KO HFD n=25). (**J**) mRNA levels of the adipogenic markers: adiponectin, PPARγ (WT HFD n=24; KO HFD n=25) and GLUT4 (WT HFD n=6; KO HFD n=6). Data are expressed as ± S.E.M. *p<0.05; **p<0.01 vs. WT littermates under HFD. All mRNA levels normalized to 18S.

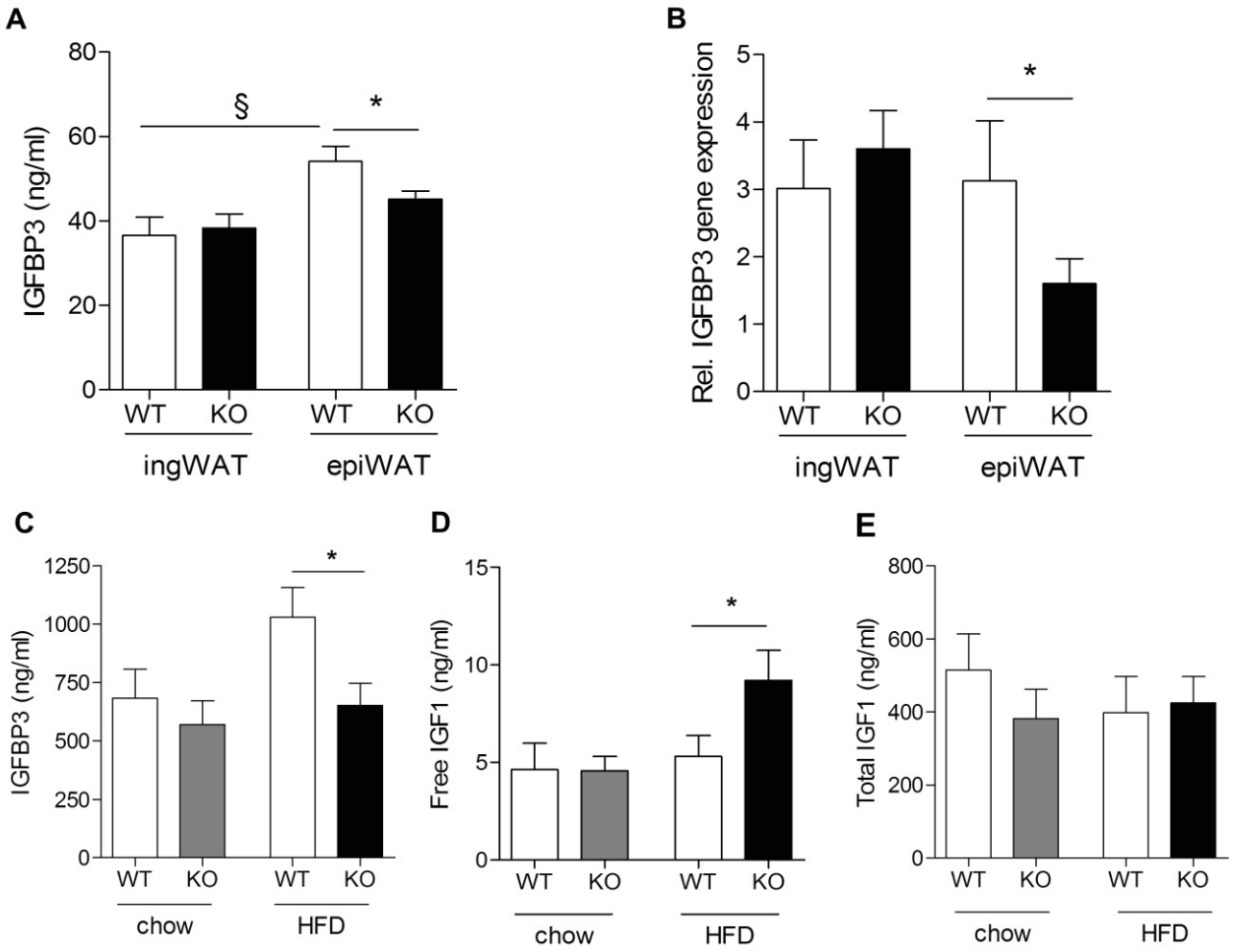


Figure 4: IGFBP3 is downregulated in epiWAT from KO animals under HFD and reduced in serum in parallel to increased concentrations of free IGF1. (**A**) IGFBP3 levels in CM from explants obtained from ingWAT or epiWAT isolated from WT and KO animals after 24 weeks on HFD (ingWAT: WT HFD n=3, KO HFD n=7; epiWAT: WT HFD n=3; KO HFD n=6). (**B**) mRNA levels of IGFBP3 in ingWAT and epiWAT of animals on HFD (ingWAT: WT HFD n=6, KO HFD n=6; epiWAT: WT HFD n=5; KO HFD n=6). (**C**) Plasma IGFBP3 levels in WT and KO animals after 24 weeks dietary intervention (WT chow n=14; KO chow n=13; WT HFD n=8; KO HFD n=18). (**D**) Plasma free IGF1 levels in WT and KO animals after 24 weeks dietary intervention (WT chow n=7; KO chow n=11; WT HFD n=16; KO HFD n=19). (**E**) Plasma total IGF1 levels in WT and KO animals after 24 weeks dietary intervention (WT chow n=8; KO chow n=11; WT HFD n=14; KO HFD n=18). Data are expressed as ± S.E.M. *p<0.05; vs. WT littermates on HFD, §p<0.05 vs different fat depot in corresponding genotype.

The 24-week HFD resulted in alterations of the morphology of epiWAT in both genotypes. Within the HFD group, the KO mice displayed a marked shift in the adipocyte size distribution towards smaller adipocytes in epiWAT (Fig. 3A). A comparison between AT-DPP4-KO animals and WT animals under HFD showed decreased mean adipocyte size in AT-DPP4-KO mice (Fig. 3B). Adipocyte size was positively correlated with circulating DPP4 levels (Fig. 3C), as previously shown for humans (Sell et al., 2013). In order to determine if the changes observed in adipocyte size were related to fibrosis, we investigated the expression of several fibrosis markers, namely Col1A1, Col3A1 and Col6A6. All fibrosis markers were reduced in the epiWAT of KO animals (Fig. 3D-F). Adipocyte size was also substantially reduced in the ingWAT of KO under HFD and circulating DPP4 levels significantly correlated with adipocyte size in this depot (Suppl. Fig. 5C). However, the lack of DPP4 in adipocytes did not affect the expression of fibrosis markers in the subcutaneous fat depot (Suppl. Fig. 5D-F). Since AT inflammation is involved in the development of insulin resistance, we also assessed macrophage infiltration in AT and the expression of the M2 anti-inflammatory macrophage markers vs. M1 pro-inflammatory markers. Crown-like structures (CLS) are formed as a result of macrophages infiltrating into AT to reabsorb dead adipocytes. Macrophage infiltration within AT was analyzed by counting galectin-3 positive CLS. Under HFD, the KO animals displayed significantly increased number of CLS only in the epiWAT (Fig 3G-H). In line with this observation, in the epiWAT, M1 pro-inflammatory markers IL-6 and chemokine (C-C Motif) ligand 2 (CCL2) were significantly increased in KO mice compared to WT animals under HFD (Fig. 3i). On the other hand, the anti-inflammatory M2 macrophage markers mannose receptor 1 (Mrc1) and interleukin (IL)-

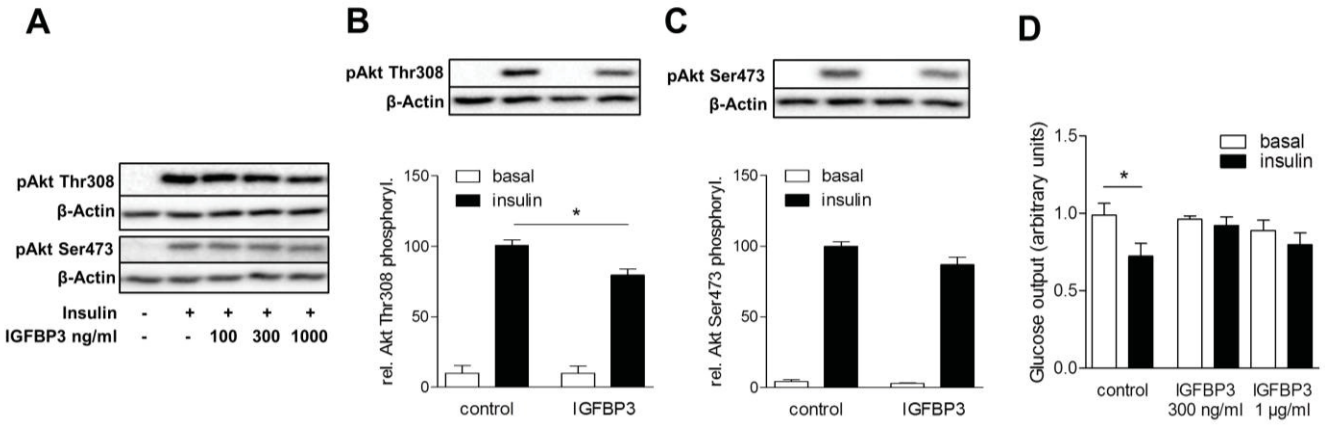


Figure 5: IGFBP3 impairs insulin signaling and insulin-induced suppression of glucose production in HepG2 cells. (**A**) HepG2 cells were treated with 100-1000 ng/ml IGFBP3 for 18 h and stimulated with 100 nM insulin for 10 min. Representative Western Blots are presented. (**B-C**) HepG2 cells were treated with 300 ng/ml IGFBP3 for 18 h and stimulated with 100 nM insulin for 10 min. Data are expressed as ± S.E.M (n=4). *p<0.05; vs. insulin-stimulated control. Representative Western Blots are presented. (**D**) HepG2 cells were treated with IGFBP3 for 18 h and glucose production was measured as detailed in Materials and Methods. Forskolin was used as a positive control and induced glucose production (from 1.0 ± 0.1 to 1.9 ± 0.7, p=0.01). Data are expressed as ± S.E.M (n = 4). *p<0.05; vs. basal control.

10 were also significantly upregulated in KO animals under HFD (Fig. 3J). Regarding the subcutaneous depot an increase of the M1 marker galectin-3 was observed although no differences in CLS were found in this depot (Suppl. Fig. 6A-B). Markers of adipogenesis such as adiponectin, PPARγ and GLUT4 were simultaneously increased in epiWAT (Fig. 3I) but remained unaltered in ingWAT (Suppl. Fig. 6D).

DPP4 deletion results in reduced circulating IGFBP3 and increased free IGF1 levels

In order to characterize how the lack of DPP4 in adipocytes affects adipokine release, we screened the adipokine profile in conditioned medium (CM) obtained from AT explants of animals after 24 weeks of HFD (data not shown) and gained preliminary evidence that IGFBP3 was a factor downregulated in the CM from epiWAT of KO animals. We observed that IGFBP3 release was reduced in the CM from explants of epiWAT of KO animals under HFD in parallel to reduced IGFBP3 gene expression (Fig. 4A-B). This was paralleled by reduced circulating levels in the KO animals which underwent HFD (Fig. 4C). Furthermore, KO animals after HFD displayed increased free IGF1 circulating levels (Fig. 4D), although no changes were observed among total IGF1 levels (Fig. 4E).

IGFBP3 impairs insulin signaling and insulin-stimulated suppression of glucose production in HepG2 cells

In order to further study the effects IGFBP3 on insulin sensitivity in hepatocytes, HepG2 cells were treated with growing concentrations of IGFBP3, which resulted in increasing impairment of insulin-stimulated Akt phosphorylation at Thr308 but not at Ser473 (Fig. 5A). IGFBP3 (300 ng/ml) reduced insulin stimulated Akt phosphorylation at Thr308 by approximately 20% (Fig. 5B) while Akt phosphorylation at Ser473 remained unaltered (Fig. 5C). Finally, IGFBP3 (300 ng/ml and 1 µg/ml) resulted in complete abrogation of insulin-

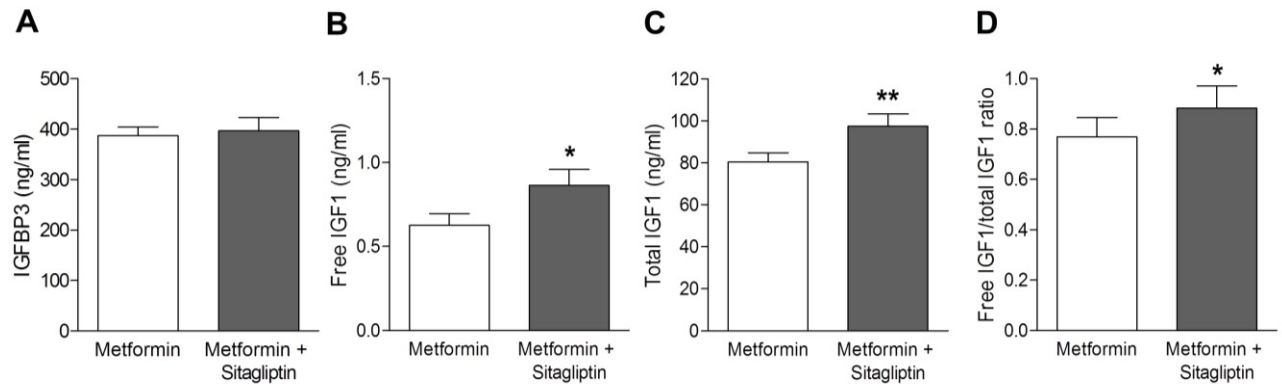


Figure 6: Type 2 diabetic patients treated with metformin alone or in combination with sitagliptin are characterized by increased serum free IGF1, serum total IGF1 and free IGF1/total IGF1 ratio compared to patients with type 2 diabetes treated with metformin alone. IGFBP3 (**A**), free IGF1 (**B**) and total IGF1 (**C**) were measured by ELISA. Free IGF1/total IGF1 ratio was calculated. Data are expressed as ± S.E.M *p<0.05; **p<0.01 vs. patients treated with metformin alone.

stimulated suppression of glucose production by hepatocytes (Fig. 5D).

DPP4 inhibition in patients with type 2 diabetes increases free IGF1 circulating concentrations

IGFBP3, free IGF1, total IGF1, DPP4 protein and DPP4 activity were measured in serum samples obtained from recent onset metformin-treated type 2 diabetes patients with or without treatment with the DPP4 inhibitor sitagliptin. Both groups of patients had comparable anthropometric and and metabolic parameters. However, patients on sitagliptin treatment displayed significantly lower DPP4 activity in serum with comparable amounts of DPP4 protein in circulation (Table 1). Circulating IGFBP3 concentrations were similar in both groups (Fig. 6A). However, patients treated with sitagliptin had significantly higher serum levels of free IGF1 and total IGF1 (Fig. 6B-C). In addition, the free IGF1/total IGF1 ratio was higher in patients receiving sitagliptin (Fig. 6D). Total IGF1 correlated positively with free IGF1 ($r=0.50$, $p=0.0002$) and negatively with hs-CRP ($r=-0.31$, $p=0.02$). The free IGF1/total IGF1 ratio declined with aging ($r=0.30$, $p=0.04$). Of note, IGFBP3 correlated with cholesterol ($r=0.56$, $p<0.0001$), LDL-cholesterol ($r=0.45$, $p=0.001$), serum triglycerides ($r=0.44$, $p=0.002$) and γGT ($r=0.31$, $p=0.03$). DPP4 was positively related to liver transaminases (ALT $r=0.54$, $p<0.0001$; AST $r=0.40$, $p=0.004$; γGT $r=0.40$, $p=0.004$).

Table 1: Antropometric and clinical data obtained from type 2 diabetic patients treated with metformin alone or in combination with sitagliptin. BMI: Body mass Index, WHR: waist to hip ratio. Data expressed as mean ± SD, *$p<0.05$ vs. patients treated with metformin alone

	Metformin	Metformin + Sitagliptin
n (male/female)	33 (25/8)	17 (13/4)
Age (years)	52 ± 11	52 ± 12
BMI (kg/m²)	29.0 ± 4.3	28.3 ± 4.5
Waist circumference (cm)	110 ± 11	96 ± 13
WHR	0.96 ± 0.06	0.93 ± 0.08
M-value (mg/kg*min)	6.18 ± 2.11	6.65 ± 2.53
Fasting blood glucose (mg/dl)	127 ± 25	140 ± 36
Fasting insulin (mU/L)	14.8 ± 6.6	12.8 ± 7.2
C-peptide (ng/ml)	3.07 ± 1.05	2.79 ± 1.02
HbA1c (%)	6.56 ± 1.04	6.42 ± 1.12
hs-CRP (mg/dl)	0.46 ± 0.64	0.25 ± 0.20
Triglyceride (mg/dl)	159 ± 98	131 ± 75
Cholesterol (mg/dl)	208 ± 46	199 ± 42
HDL-cholesterol (mg/dl)	46.6 ± 12.6	54.5 ± 15.8
LDL-cholesterol (mg/dl)	133 ± 39	122 ± 38
AST (U/L)	23.5 ± 9.3	24.0 ± 6.6
ALT (U/L)	32.4 ± 18.3	32.6 ± 19.5
γGT (U/L)	37.1 ± 27.1	33.7 ± 16.4
estimated glomerular filtration rate (ml/min*1.73m²)	87.6 ± 15.2	90.7 ± 14.8
DPP4 activity (µU/ml)	1.03 ± 0.25	0.51 ± 0.16*
DPP4 protein (ng/ml)	314 ± 23	337 ± 31

IV. DISCUSSION

The novel mouse model of adipose-specific knockout of DPP4-KO demonstrates that AT is a major source of circulating DPP4 in obesity neither affecting basal nor glucose-stimulated incretin hormone secretion. When fed a HFD, AT-DPP4-KO mice display improved glucose tolerance and hepatic insulin resistance. DPP4 depletion affects AT itself through a favorable remodeling during HFD with smaller adipocytes, reduced fibrosis and increased M2 macrophage-mediated inflammation.

Our group has previously provided the first evidence for DPP4 being an adipokine (Lamers et al., 2011) and demonstrated that DPP4 is upregulated in human obesity and the metabolic syndrome (Sell et al., 2013). AT-DPP4-KO mice prove that AT is an important source of circulating DPP4 in the obese state. Here, we provide the first comparison of DPP4 enzymatic activity in AT in relation to other organs such as the liver, pancreas or the kidney, which may represent sources of circulating DPP4 (Hildebrandt et al., 1991; Kirino et al., 2009; Shinjo et al., 2015). Of note, except from liver, all other tested tissues are probably only minor contributors to circulating DPP4 due to their relative low mass compared to enlarged AT. The tissue specificity of our model compared to previously described global DPP4 KO models (Conarello et al., 2003; Marguet et al., 2000) allows us to detect the impact of DPP4 deletion within AT and dissect these effects from incretin-mediated actions, since GIP and active GLP-1 did not differ among the genotypes. Therefore, the effects of DPP4 deletion in AT under HFD on body composition, glucose tolerance and hepatic insulin sensitivity occur independent of the incretin system which is not affected by adipose KO of DPP4. In global DPP4 KO mice, active GLP-1 and insulin are significantly increased after an oral glucose load (Marguet et al., 2000). Furthermore, global KO mice display significantly lower food intake resulting in almost complete resistance to diet-induced obesity (Conarello et al., 2003). But not only food intake is responsible for this resistance to HFD, as demonstrated by pair feeding experiments, global KO mice are also characterized by higher oxygen consumption and increased expression of UCP-1 in BAT. Thus, central effects of global DPP4 KO on food intake and increased BAT activity can be attributed to DPP4 depletion in combination with a boosted incretin system. DPP4 ablation only in AT did not affect food intake nor oxygen consumption and occurred without affecting the incretin system which makes our novel model independent from central effects of DPP4 and incretin actions.

AT-DPP4-KO mice display improved glucose tolerance under HFD compared to controls despite higher body weight and fat mass. Furthermore, insulin secretion in the initial phase of oGTTs and HOMA-IR was improved in AT-DPP4 KO mice on HFD compared to WT mice. In fact, deletion of DPP4 in AT seems to play a role specifically for hepatic insulin sensitivity. Compared to WT mice on HFD, AT-DPP4-KO mice displayed significantly increased insulin-mediated suppression of EGP, but no difference in basal EGP, insulin-mediated

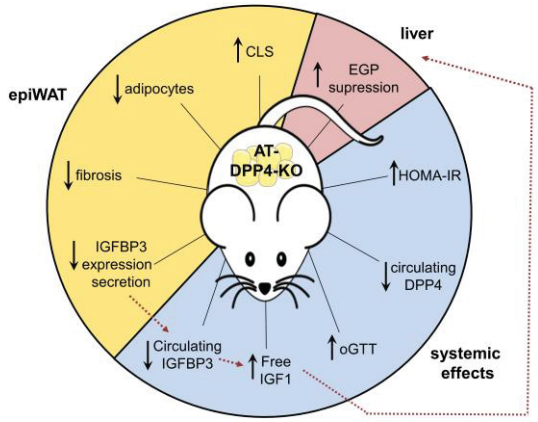
suppression of NEFA and insulin-stimulated whole body glucose disposal. These data indicate that improved insulin action in the liver is the key metabolic feature of AT-DPP4-KO mice on HFD. Impaired insulin-mediated suppression of EGP is an early feature of insulin resistant states and determines the progression to impaired glucose metabolism in obese humans (Ter Horst et al., 2016). Abnormal suppression of EGP further contributes to hyperglycemia in type 2 diabetes mellitus (Mittelman et al., 1997). One may speculate that DPP4 overexpression could contribute to the pathogenesis of hepatic insulin resistance and type 2 diabetes and that its deletion in AT may therefore delay the development of type 2 diabetes. The mechanism by which AT-DPP4-KO affects hepatic insulin sensitivity is still unknown. We can exclude a contribution of NEFA and circulating adiponectin (data not shown) as these parameters were not affected by the KO, while IGFBP3 and IGF1 may be the mediators for improved hepatic insulin sensitivity. Release from AT and circulating levels of IGFBP3 were reduced in KO mice under HFD. In addition to decreased IGFBP3, free IGF1 was specifically increased in serum of KO mice under HFD. IGFBP3 is one of the main circulating IGFBP species found in serum and binds approximately 90% of circulating IGF1 (Jogie-Brahim et al., 2009). Both IGFBP3 and IGF1 are increased in patients with impaired glucose tolerance and type 2 diabetes (Kim and Lee, 2015). Additionally, IGFBP3 overexpression in transgenic mice impairs insulin secretion resulting in fasting hyperglycemia, impaired glucose tolerance, and insulin resistance (Nguyen et al., 2011; Silha et al., 2002). Differently, whole body KO of IGFBP3 results in a complex metabolic phenotype with higher body growth and weight due to increased growth hormone concentrations and decreased resting metabolic rate compared to controls (Yamada et al., 2010). Furthermore, IGFBP3 KO animals display increased basal glucose turnover but preserved insulin sensitivity and reduced WAT mass. However, it should be noted that complete absence of IGFBP3 is not comparable with our model where IGFBP3 is only reduced, total IGF1 unaltered and animal growth normal. In our KO model, reduced IGFBP3 levels occur in parallel to higher free IGF1 which is known to suppress hepatic glucose production (Laager et al., 1993). Alternatively, IGFBP3 could also regulate hepatic glucose production independently from IGF1 as previously shown (Muzumdar et al., 2006). In fact, surface association of IGFBP3 on hepatocytes has been demonstrated by immunohistochemistry (Hallak et al., 2002). Furthermore, treatment of adipocytes with IGFBP3 resulted in impaired insulin signaling and reduced insulin-stimulated glucose uptake (Chan et al., 2005). Here, we also provide evidence that IGFBP3 also impairs insulin signaling and insulin-stimulated suppression of glucose production by hepatocytes. In this study, reduced IGFBP3 levels could therefore contribute to improved hepatic insulin sensitivity in two different ways, first by regulating the bioavailability of IGF1 in the form of increased free IGF1, and second by direct hepatic effects of IGFBP3 independent of IGF1. Taken together, we propose that the reduced release of IGFBP3 from visceral AT might mediate a protective crosstalk between AT and liver in AT-DPP4-KO mice on HFD.

Adipocyte size, a surrogate marker of insulin resistance (Cotillard et al., 2014), was reduced in both subcutaneous and visceral fat of AT-DPP4-KO mice on HFD. Our results are in line with our previous report in humans, where circulating DPP4 positively correlated with adipocyte size (Sell et al., 2013). In diet-induced obesity, DPP4 inhibitors have been proven to reduce adipocyte size (Chae et al., 2015). However, solely in the epiWAT of KO mice under HFD, we observed a trend towards increased markers of adipogenesis potentially suggesting hyperplasia. This is in line with our findings reported in human subcutaneous adipocytes, where DPP4 knockdown did not affect adipogenesis (Rohrborn et al., 2016). We discarded AT fibrosis as the cause of the reduced adipocyte size. Thus, fibrosis was also solely reduced in epiWAT from AT-DPP4-KO mice under HFD. Since AT fibrosis has been linked to insulin resistance (Sun et al., 2013), we hypothesize that reduced fibrosis in visceral fat may represent an additional mechanism for DPP4 deficiency to promote a protective remodeling in AT during diet-induced obesity. Although chronic inflammation in AT links obesity to its complications, there is growing evidence that pro-inflammatory signaling is a prerequisite for AT remodeling and expansion (Wernstedt et al., 2014). We employed galectin-3 to quantify CLS in AT, which besides being a M1 macrophage marker, has been recently proposed as an indicator of phagocytosis of apoptotic cells (MacKinnon et al., 2008). Thus, galectin-3 KO mice exhibit impaired expansion of AT under diet-induced obesity resulting in increased hyperglycemia and glucose intolerance under HFD (Darrow and Shohet, 2015). Therefore, increased galectin-3 positive macrophages in epiWAT from KO mice may contribute to a beneficial AT expansion under HFD. As mentioned, IGFBP3 levels were reduced in both CM from epiWAT and serum from KO animals. IGFBP3 deletion has been shown to improve differentiation in adipocytes via direct interaction with PPARγ, while both administration of exogenous IGFBP3 and overexpression inhibits adipogenesis in 3T3-L1 (Jogie-Brahim et al., 2009). Therefore, we propose that reduced IGFBP3 levels in AT-DPP4 KO mice promotes a beneficial remodeling during HFD, characterized by reduced hypertrophy, fibrosis and inflammation while adipogenesis is increased.

Of note, also in humans with type 2 diabetes DPP4 inhibition increases free and total IGF1 concentrations in serum. Although not directly comparable to our mouse model, where circulating DPP4 is reduced and incretin levels unaltered, these data provide additional evidence for a link between DPP4 and the IGF1/IGFBP3 axis. Recombinant IGF1 and combinations of recombinant IGF1 with IGFBP3 have been previously discussed as treatment options for type 2 diabetes (Ranke, 2005). However, the long term potential of this therapy has not been further developed due to various side effects and the

association of very high IGF1 levels with prostate and breast cancer (Renehan et al., 2004). Nevertheless, IGF1 has been reported to exert vasoprotective effects not only in type 2 diabetes (Ezzat et al., 2008) and lower IGF1 serum concentrations are associated with diabetes (Teppala and Shankar, 2010). Since pharmacological interventions increasing circulating IGF1 have not been reported so far, the observed increase in serum free IGF1 and total IGF1 exerted by the DPP4 inhibitor sitagliptin may represent a novel therapeutic target in type 2 diabetes. It can be speculated that increased IGF1 concentrations could be linked to altered hepatic glucose production in patients treated with DPP4 inhibitors. In fact, sitagliptin improves the suppression of EGP in dogs (Edgerton et al., 2009) and in humans (Muscelli et al., 2012). Also vildagliptin triggers a better suppression of EGP (Balas et al., 2007) or lower basal EGP in patients (Vella et al., 2007).

Future studies will help to clarify how DPP4 deletion in AT can be translated into a therapeutic tool in metabolic diseases. This is of special interest in the case of obese type 2 diabetic patients which are currently treated with DPP4 inhibitors. How exactly DPP4 inhibitors might also affect AT biology and hepatic insulin sensitivity in addition to their incretin-mediated effects remains fully unexplored. Since DPP4 is an adipokine upregulated in adipose tissue in obesity both in human and rodents, AT arises as an important and novel source of DPP4 and as a new therapeutic target for DPP4 inhibitors.



Conflict of interest
There are no conflicts of interest.

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Author contributions
TR wrote the manuscript; TR, HS, MR and JE designed experiments; TR, HS, DR and II performed experiments; TRC collaborated in setting oGTT and performed the analysis of the data of the metabolic cages; TJ performed hyperinsulinemic-euglycemic clamps; TR, HS, DR, II, TRC, TJ, HA-H, JE and MR discussed the manuscript, SH performed Luminex experiments, JW trained for immunohistochemistry.

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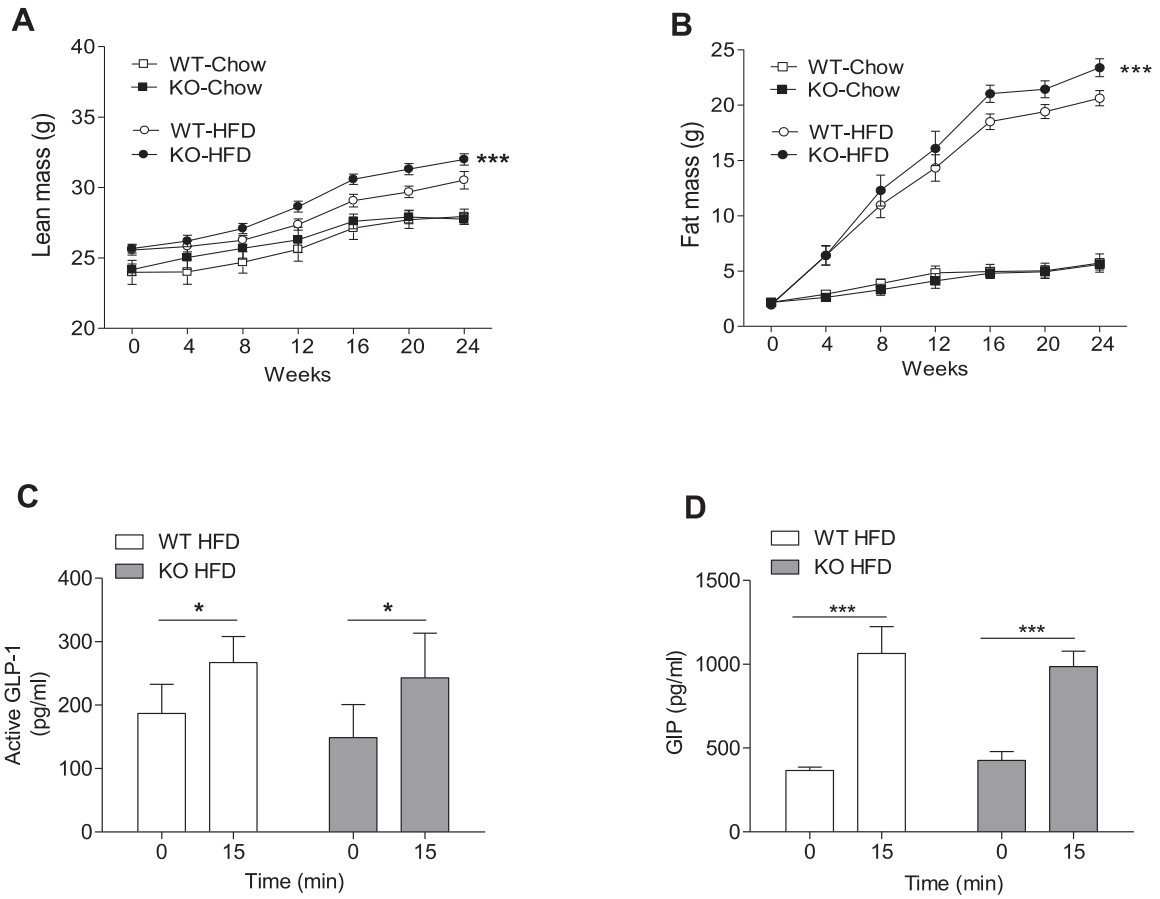
ABBREVIATIONS

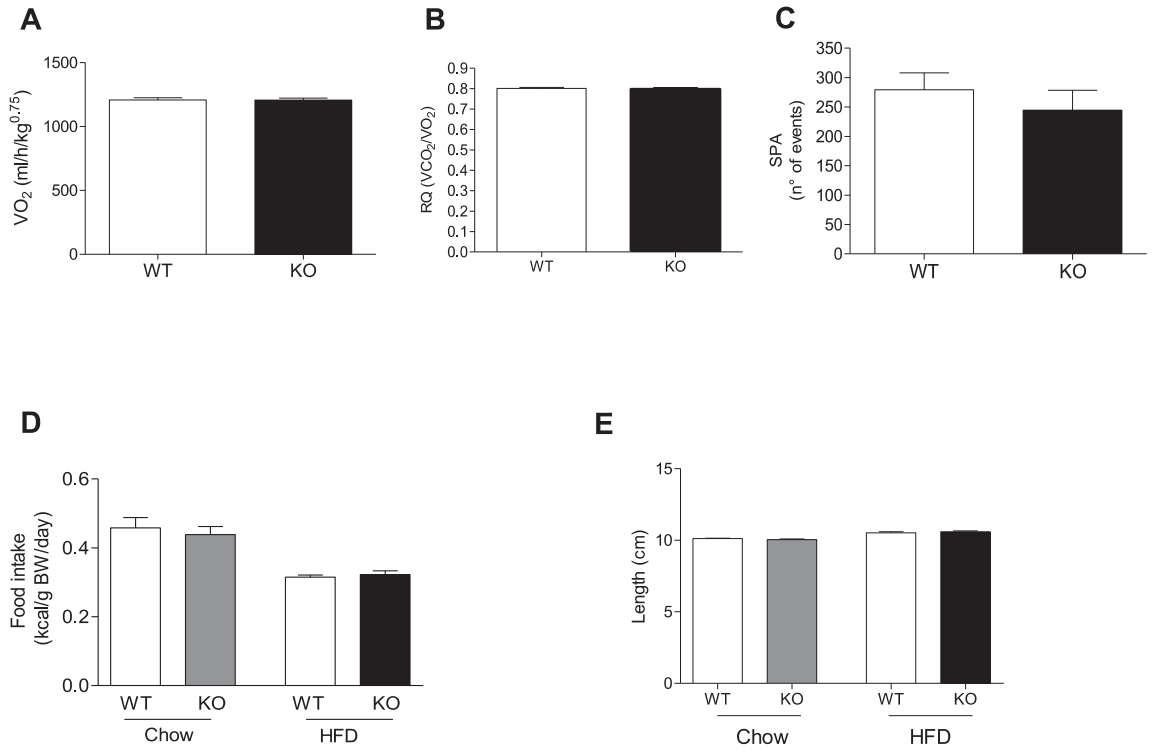
AT, adipose tissue; AT-DPP4-KO , adipose-specific DPP4 knockout; BAT, brown adipose tissue; CCL2 , chemokine (C-C Motif) ligand 2; CLS, crown-like structure; DPP4, dipeptidyl peptidase 4; EE, energy expenditure; EGP, endogenous glucose production; epiWAT, epididymal white adipose tissue; GIP, gastric inhibitory peptide; GLP-1, glucagon-like peptide-1; HFD, high fat diet; HOMA, homeostatic model

assessment; HRP, horseradish peroxidase; IGF1, insulin-like growth factor 1; IGFBP3, IGF binding protein 3; IL, interleukin; ingWAT, inguinal white adipose tissue; Mrc1, macrophage mannose receptor 1; NEFA, non-esterified fatty acids; oGTT, oral glucose tolerance test; PPARγ, peroxisome proliferator-activated receptor γ; RT-qPCR, Real-time quantitative PCR; SPA, spontaneous physical activity; SVF , stroma vascular fraction; WAT, white adipose tissue

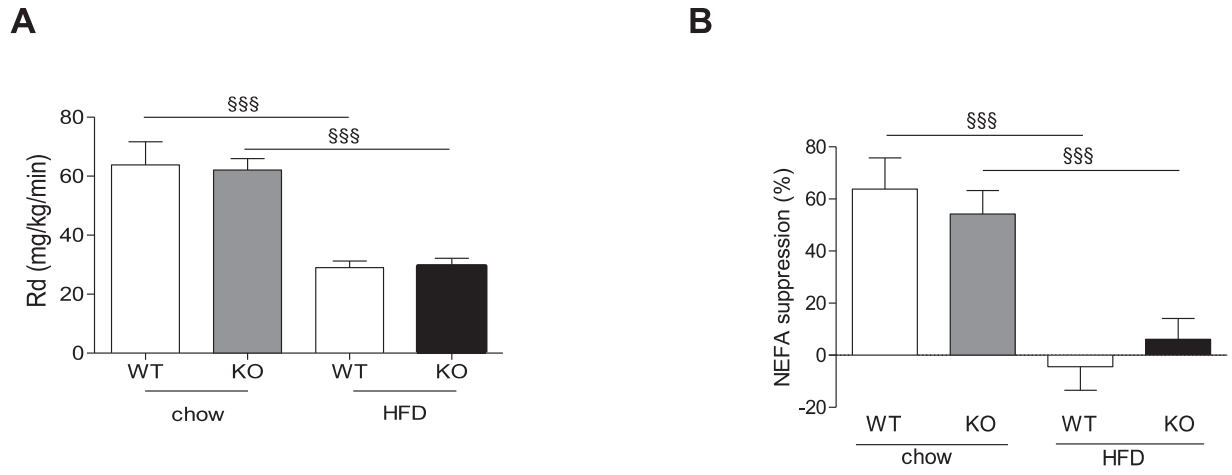
Supplemental Material



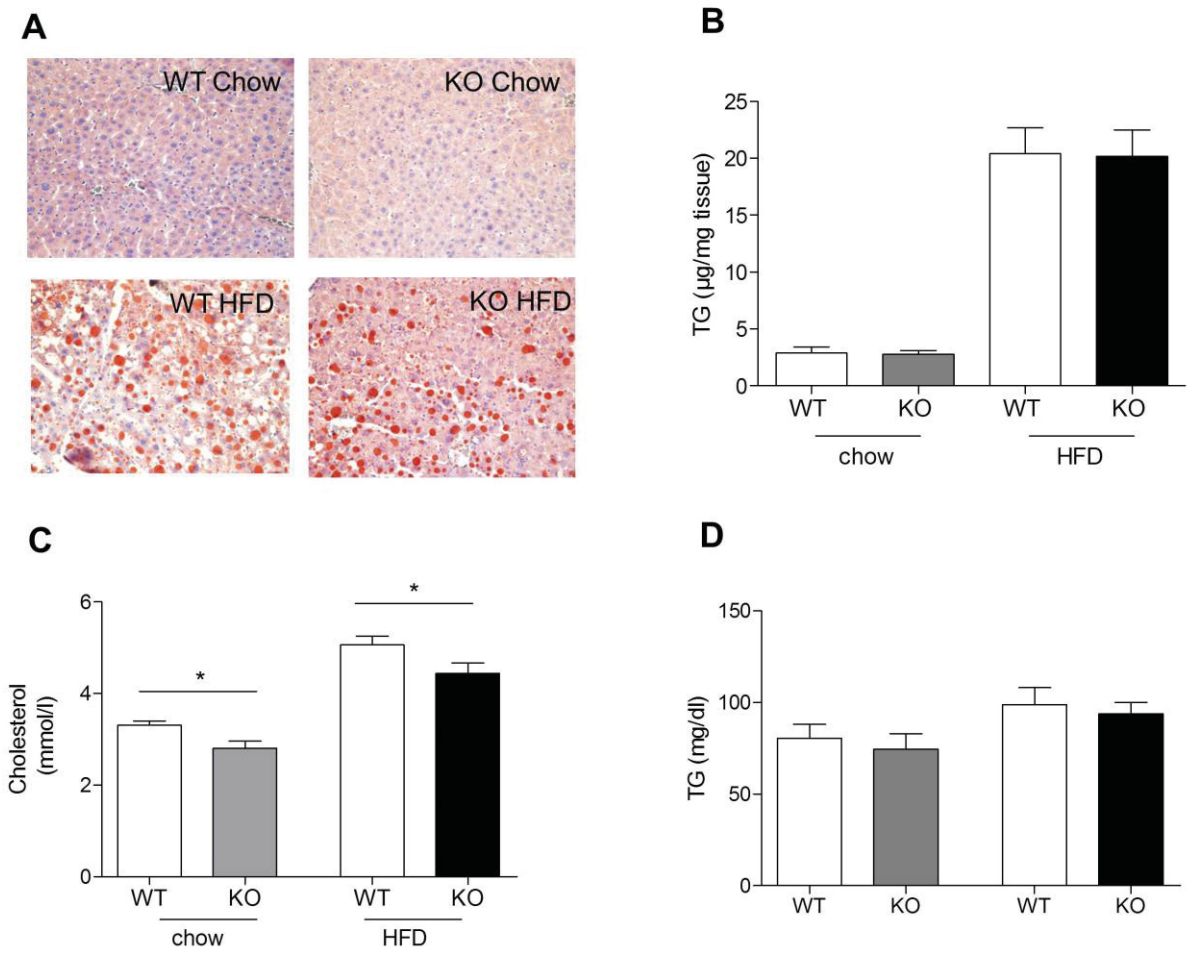
Suppl. Fig. 1: Increased lean and fat mass but unaltered plasma incretins in AT-DPP4-KO animals under HFD. Lean mass (**A**) and fat mass (**B**) was assessed every 4 weeks during dietary intervention (n=11-16 animals per group on chow, n= 26-31 animals per group on HFD). Data expressed as mean ± S.E.M. ***p<0.01 vs. vs. diet-matched WT littermates. Plasma active GLP-1 (**C**) and GIP (**D**) levels at 0 and 15 min after glucose gavage (2mg/g body weight) in animals at week 20 of HFD (WT HFD n=4; KO HFD n=4-5). **p<0.05; ***p<0.001* vs. levels at time 0.



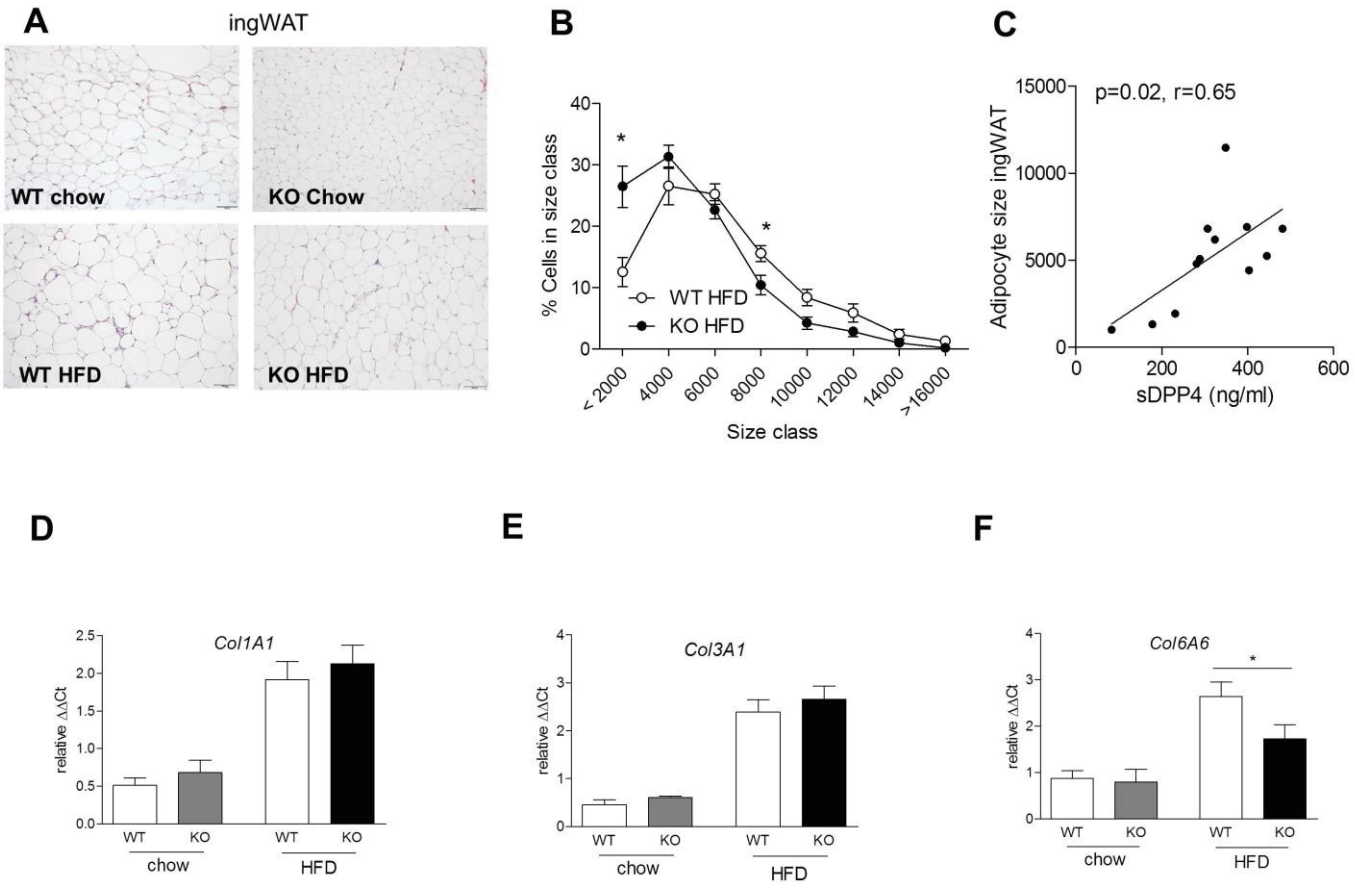
Suppl. Fig. 2: Metabolic phenotyping of AT-DPP4-KO mice. Measurements were carried out for 4 days (48 h adaptation + 48h follow up) at week 20 after dietary intervention. (**A**) Energy expenditure corrected per body surface (WT HFD n=19; KO HFD n=16). (**B**) Respiratory quotient (RQ; WT HFD n=15; KO HFD n=13) (**c**) Spontaneous physical activity (SPA; WT HFD n=20; KO HFD n= 18. (**C**) Daily food intake (WT HFD n=23; KO HFD n=21) (**D**) Nose-tail length was measured in isofluorane-anesthetised animals at week 30 of age (WT chow n=8; KO chow n=8; WT HFD n=14; KO HFD n=14). Data are expressed as mean ± S.E.M.



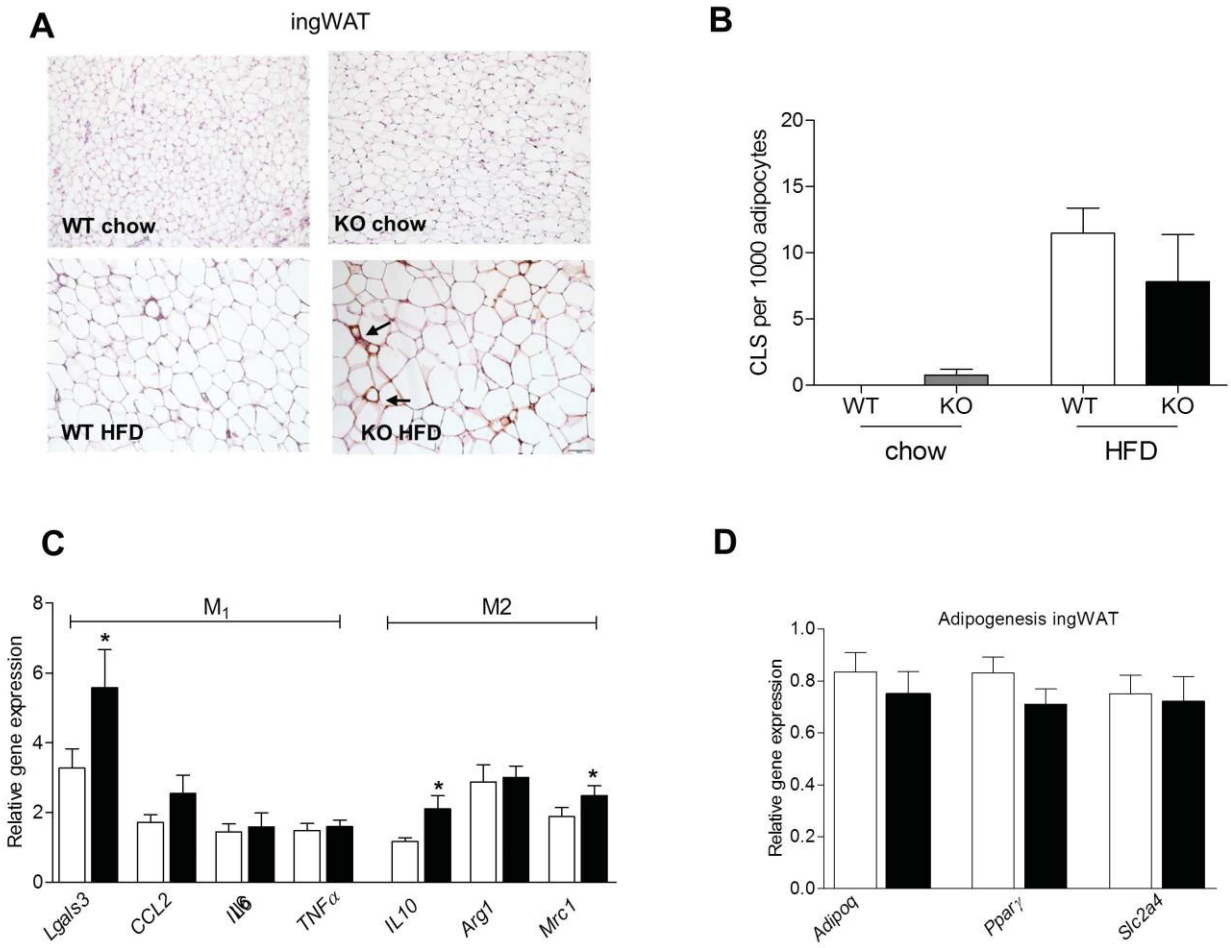
Suppl. Fig. 3: Insulin sensitivity *in vivo*. Hyperinsulinemic-euglycemic clamps were performed after 22 weeks of dietary intervention. (**A**) Glucose disposal rate (Rd) and (**B**) suppression of non-esterified fatty acids (NEFA) were assessed (WT chow n=4; KO chow n= 9; WT HFD n= 7; KO HFD n=9). Data are expressed as mean ± S.E.M. *§§§p<0.001* vs. same genotype under chow diet.



Suppl. Fig. 4: Lipidic profile and hepatic steatosis in AT-DPP4-KO mice. (**A**) Representative microphotographs of Oil Red-stained liver sections adipocytes from WT and KO animals under chow or HFD. (**B**) Hepatic triglyceride content in WT and KO animals under HFD for 24 weeks (WT chow n=7; KO chow n=8; WT HFD n=8; KO HFD n=11). (**C**) Plasma cholesterol and triglycerides (**D**) in WT and KO animals under chow or HFD for 24 weeks (WT chow n=8; KO chow n=8; WT HFD n= 14; KO HFD n=14). *$p<0.05$ vs. matched-WT littermates under same diet.



Suppl. Fig. 5: Improved adipose tissue remodeling in ingWAT. (A) Representative microphotographs of hematoxylin-eosin stained adipocytes from WT and KO animals under chow or HFD. (B) Percentage of cells per adipocyte size class (µm2) in ingWAT obtained from WT and KO animals after 24 weeks under chow or HFD (WT HFD n=11; KO HFD n=11). (C) Correlation between adipocyte size and DPP4 circulating levels in WT animals (n=33). Expression of the fibrosis markers collagen 1A1 (D), collagen 3A1 (E) and collagen 6A6 (F) (WT chow n=5-6; KO chow n=6-8, WT HFD n=6; KO HFD n=6). mRNA levels were normalized to 18S. Data are expressed as mean ± S.E.M. *p<0.05 HFD-matched littermates. *p<0.05 vs. matched-WT littermates under same diet.



Suppl. Fig. 6: Macrophage infiltration in ingWAT. (**A**) Representative microphotographs of immunohistochemistry staining for galectin-3, showing crown-like structures (CLS) in epiWAT. (**B**) Number of CLS per every 1000 adipocytes was calculated by a blind observer in WT and KO under chow or HFD after 24 weeks of dietary intervention. (**C**) mRNA levels of M1 macrophage markers: Lgals3, CCL2, IL-6, TNF-α (WT HFD n=21-24; KO HFD n=15-24) and M2 macrophage markers: IL-10, Arg10 and Mrc1(WT HFD 12-18; KO HFD n=16 animals) in epiWAT from WT and KO animals after 24 weeks of HFD. (**D**) mRNA levels of the adipogenic markers: adiponectin, PPARγ and GLUT4 (WT HFD n=8; KO HFD n=7 animals). mRNA levels were normalized to 18S. Data are expressed as mean ± S.E.M. *$p<0.05$ vs. matched WT littermates under HFD.

EXPERIMENTAL PROCEDURES

Energy balance
Age-matched AT-DPP4-KO and WT control mice were singly housed in an indirect calorimetry system (TSE Phenomaster system, Bad Homburg, Germany) for four light/dark cycles (12 h/12h), at week 20 of dietary intervention. After an adaptation period of 48 h, the data obtained during the last 2 light/dark cycles were used to quantify spontaneous physical activity (SPA), energy expenditure (EE; ml/h/kg $^{0.75}$) and the respiratory quotient (RQ, VCO2/VO2), as previously described (Castaneda et al., 2011). Measurements of food intake were taken manually every day in the morning.

Hormone and biochemical assays
Total GIP and GLP-1 were measured with a DuoPlex assay (BioRad, Hercules, California). Active GLP-1 was measured with a Singleplex kit (Millipore, Billerica, California).
Cholesterol and triglycerides were obtained by standard peroxidase-based clinical assays (ErbaLachema, Czech Republic). Non-esterified fatty acids were quantified using colorimetric assay (NEFA C; Wako, Neuss, Germany).

Liver fat content
Livers were dissected and fixed in 10% formalin, and then dehydrated in ethanol followed by cryopreservation and further sectioning for oil red O stainings. Representative images from at least 5 animals per group were taken with a Leica DM6000 B microscope (Jelenik et al., 2014).
Triglycerides content in liver was assessed by a colorimetric kit (RANDOX) and normalized per protein content as estimated by BCA (Thermo) (Baumeier et al., 2015).

Statistics
Statistical analysis was performed with the GraphPad Prism software (La Jolla, CA, USA). *p* values were calculated using the two-tailed Student's *t*-test. For statistical comparisons between experimental groups, two-way ANOVA was used, followed by Bonferroni *post hoc* test. A *p* value below 0.05 was considered statistically significant. Data are shown as the mean ± S.E.M or mean ± S.D if specified. Normal distribution and equal variances were trested for each analysis. Animals were randomly allocated to the different diets and at least three animals were included in each experimental group. Groups size was calculated by Simple Interactive Statistical Analysis.

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2.2 Soluble dipeptidyl peptidase-4 induces microvascular endothelial dysfunction through proteinase-activated receptor-2 and thromboxaneA2

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Abstract— **Background: Dipeptidyl peptidase-4 (DPP4) is a key protein in glucose homeostasis and a pharmacological target in type 2 diabetes mellitus. This study explored whether the novel adipokine soluble DPP4 (sDPP4) can cause endothelial dysfunction, an early marker of impaired vascular reactivity. Method: Reactivity was studied in mesenteric arteries from 3-month-old female mice, using a small vessel myograph. Thromboxane A2 (TXA2) release was explored in cultured human coronary artery endothelial cells by enzyme immunoassay. Results: Neither the contractility to noradrenaline nor the endothelium-independent relaxations induced by sodium nitroprusside were modified by sDPP4. However, sDPP4 impaired in a concentration-dependent manner the endothelium-dependent relaxation elicited by acetylcholine. The DPP4 inhibitors K579 and linagliptin prevented the defective relaxation induced by sDPP4, as did the proteaseactivated receptor 2 (PAR2) inhibitor GB83. Downstream of PAR2, the cyclooxygenase (COX) inhibitor indomethacin, the COX2 inhibitor celecoxib or the thromboxane receptors blocker SQ29548 prevented the deleterious effects of sDPP4. Accordingly, sDPP4 triggered the release of TXA2 by endothelial cells, whereas TXA2 release was prevented by inhibiting DPP4, PAR2 or COX. Conclusion: In summary, these findings reveal sDPP4 as a direct mediator of endothelial dysfunction, acting through PAR2 activation and the release of vasoconstrictor prostanoids. By interfering with these actions, DPP4 inhibitors might help preserving endothelial function in the context of cardiometabolic diseases.**

Keywords— **Dipeptidyl peptidase-4 inhibitors, endothelial dysfunction, protease-activated receptor 2, soluble dipeptidyl peptidase-4, thromboxane A2, vascular reactivity**

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I. INTRODUCTION

Vascular complications are the main cause of morbidity and mortality associated with type 2 diabetes mellitus (T2DM). An early hallmark of the onset of vascular complications is endothelial dysfunction, which can be defined as a loss of balance among the different factors controlling vascular homeostasis [1]. Endothelial dysfunction is indeed characterized by a shift toward decreased vasodilation, together with pro-oxidant, proinflammatory and prothrombotic properties [1].

Dipeptidyl peptidase-4 (DPP4) is a widely distributed glycoprotein playing a major role in the regulation of glucose homeostasis [2]. Through its enzymatic activity, DPP4 selectively cleaves N-terminal dipeptides from a variety of substrates, including the incretin hormones glucagon- like peptide-1 (GLP1) and gastric inhibitory polypeptide [2]. On this basis, DPP4 inhibitors are currently used as therapeutic tools in T2DM to prolong the insulinotropic effects of incretins and thus ameliorate glycemic control [2].

In a previous study, we identified the soluble form of DPP4 (sDPP4) as a new adipokine whose circulating levels are upregulated in obesity and the metabolic syndrome, wherein it can trigger insulin resistance in human adipocytes and skeletal muscle cells [3]. Beyond these metabolic actions, recent reports suggest that sDPP4 may also participate in the pathophysiology of vascular diseases associated with T2DM [4]. Supporting this hypothesis, DPP4 inhibitors have shown antiatherogenic, anti-inflammatory and antihypertensive properties in a series of experimental assays and clinical trials [5–9]. Nevertheless, a recent clinical study indicates that although the DPP4 inhibitor sitaglipitin is safe, it has no long-term demonstrated cardiovascular benefit [10]. In fact, an effort is being made nowadays to better understand the potential salutary cardiovascular effects of antidiabetic drugs [11].

In addition, we have recently described that sDPP4 can directly favor the proliferation and inflammation of human vascular smooth muscle cells [12]. Despite these observations, the mechanisms through which sDPP4 might negatively affect vascular homeostasis remain largely unknown. In this study, we aimed to explore

whether sDPP4 can induce endothelial dysfunction by causing defective microvascular reactivity, and to identify underlying signaling mechanisms.

II. MATERIAL AND METHODS

Materials: Recombinant human sDPP4 and interleukin-1b (IL-1)b were purchased from R&D Systems (Minneapolis, Minnesota, USA) and Miltenyi Biotec (Bergisch Gladbach, Germany), respectively. Both recombinant proteins had
an endotoxin level below 1.0 EU per mg of protein. The DPP4 inhibitor K579 was from Tocris Bioscience (Bristol, UK), whereas linagliptin was kindly donated by Boehringer- Ingelheim (Ingelheim, Germany). Fungizone and gentamycin were purchased from Gibco (Life Technologies, Lofer, Austria). The protease-activated receptor 2 (PAR2) inhibitor GB83 was obtained from Axon Medchem (Groningen, The Netherlands). Noradrenaline, potassium chloride (KCl), acetylcholine (ACh), sodium nitroprusside (SNP), apocynin, indomethacin, SQ29548, exendin-(9–39) and, unless otherwise stated, all other reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA).
Animals and vascular reactivity: All procedures were in accordance with current institutional guidelines and were approved by the Animal Ethics Committee of Universidad Auto´noma de Madrid (CEI 42-865) in accordance with the European Union Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes. Vascular reactivity experiments were performed in second- branch mesenteric arteries from 3 month-old female C56/BL6 mice using a small vessel myograph to measure isometric tension, as previously described [13]. Prior to endpoint, the animals were anesthetized by a brief exposure to carbon dioxide in a chamber and then immediately killed by cervical dislocation. Mesenteric arteries (mean internal diameter of 212.2 4.5mm, n¼94) were dissected, cleaned free of fat and connective tissue, and mounted in a small vessel wire myograph in Krebs–Henseleit buffer. To determine its viability, each segment was first contracted with 125 mmol/l KCl followed by a washout. Afterward, in the first set of experiments designed to explore the influence of sDPP4 on contractility, some microvascular segments were preincubated for 30 min with sDPP4 (20–500 ng/ml) in parallel to untreated segments and then exposed to increasing concentrations of noradrenaline (3 nmol/l to 3mmol/l). In the second set of experiments aimed to address the influence of sDPP4 on endothelium-dependent vasorelaxation, some microvessels were preincubated for 30 min with sDPP4 (20–500 ng/ml), stimulated with noradrenaline (3mmol/l) and, once the contraction became stable, exposed to cumulative concentrations of the endothelium-dependent vasodilator ACh (1 nmol/l to10mmol/l). In some experiments, segments were preincubated, prior to the addition of sDPP4, with the nonspecific cyclooxygenase (COX) inhibitor indomethacin, the COX-2 inhibitor celecoxib, the specific antagonist of the thromboxane receptor SQ29548, the nicotinamide adenine dinucleotide ph osphate oxidase (NAPDH) oxidase inhibitor apocynin, the PAR2 inhibitor GB83 or the DPP4 inhibitors K579 and linagliptin, at appropriate concentrations. The influence of sDPP4 on endothelium-independent relaxations was assessed using the vasodilator SNP (1 nmol/l to 100mmol/l).
Cell culture: Primary human coronary artery endothelial cells (HCAECs) and endothelial cell growth medium MV were purchased from Promocell (Heidelberg, Germany). HCAECs were grown in 5% fetal calf serum endothelial cell growth medium MV supplemented with antibiotic and antimycotic (50mg/ml gentamycin and 0.05 mg/ml amphotericin B) following the manufacturer's instructions. For the experiments, subconfluent cells of passages 2–5 were used.
Thromboxane A2 release: Thromboxane A2 (TXA2) release was indirectly monitored by assessing the levels of its stable metabolite TXB2, in the cell supernatants with a commercial enzyme immunoassay kit (Cayman Chemical, Ann Arbor, Michigan, USA). For this purpose, HCAECs were stimulated for 1 h with sDPP4 (500 ng/ml). When appropriate, the cells were preincubated for 30 min with selected drugs. IL-1b (100 ng/ml) was used as a positive control for TXA2 release. At the end of the treatment time, the supernatants were collected and frozen (–808C) for further analysis, following manufacturer's instructions.
Statistical analysis: Results are expressed as mean SEM. Statistical analysis was performed using Student's t test, analysis of variance (ANOVA) followed by Bonferroni post-hoc test and twoway ANOVA, as appropriate. A value of $P<0.05$ was considered statistically significant.

III. RESULTS

Soluble dipeptidyl peptidase-4 does not modify microvascular contractility to noradrenaline. Fig. 1a shows that sDPP4 alone (20–500 ng/ml) did not significantly modify the response of murine mesenteric segments to cumulative concentrations of noradrenaline (1 nmol/l to 3mmol/l) (Fig. 1a). Furthermore, sDPP4 alone did not either modify the basal mesenteric vascular tone over the 30-min incubation period prior to the stimulation with noradrenaline (See Table 1 in the online Supplemental material; http://links.lww.com/HJH/A597).

Soluble dipeptidyl peptidase-4 impairs endothelium-dependent vasodilation to acetylcholine. We next tested the influence of sDPP4 on the endotheliumdependent vasodilation evoked by cumulative concentrations of ACh (1 nmol/l to 10mmol/l) in microvessels contracted with noradrenaline (Fig. 1b). The relaxation induced by ACh was dependent on nitric oxide release (Supplementary Figure 1; http://links.lww.com/HJH/A597) and was significantly impaired by sDPP4 (20–500 ng/ml) in a concentration-dependent manner (1 nmol/l to 10mmol/l) (Fig. 1b). For subsequent reactivity experiments with ACh, a submaximal concentration of 200 ng/ml sDPP4 was selected. To explore whether sDPP4 could also interfere with endothelium-independent relaxations, a parallel set

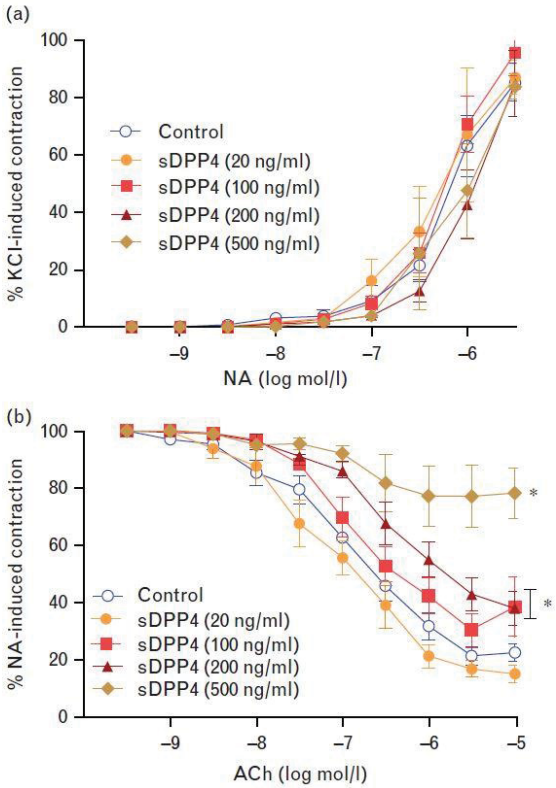

Figure 1: sDPP4 does not modify the contractility to NA, but impairs the endothelium- dependent vasodilation to ACh in mesenteric arteries from C57/BL6 female mice. (a) Concentration-dependent contractile responses induced by NA (3 nmol/l to 3mmol/l) in microvessels exposed to increasing concentrations of sDPP4 (20–500 ng/ml). Results are expressed as mean SEM of 32 segments obtained from six animals. (b) The microvessels precontracted with 3mmol/l noradrenaline prior to analyze the concentration-dependent relaxations induced by ACh in isolated mesenteric microvessels exposed to increasing concentrations of sDPP4 (20–500 ng/ml). Results are expressed as mean SEM of 44 segments obtained from 11 animals. P<0.05 vs. untreated vessels (control). ACh, acetylcholine; NA, noradrenaline; sDPP4, soluble dipeptidyl peptidase-4; SEM, standard

of experiments was performed using SNP, a vasodilator directly acting on vascular smooth muscle. Contrarily to that observed with ACh, sDPP4 did not interfere with the vasodilation evoked by SNP (1 nmol/l to 100 mmol/l), as shown in Table 1.

Dipeptidyl peptidase-4 inhibitors prevent the impaired vasodilatation evoked by soluble dipeptidyl peptidase-4. As DPP4 enzymatic inhibitors have been proposed to exhibit vasculoprotective properties, we tested the capacity of two different DPP4 inhibitors to prevent the direct action of sDPP4 on endothelium-dependent relaxations. The experimental DPP4 inhibitor K579 (100 nmol/l) prevented the impairment of ACh-dependent vasodilation induced by sDPP4, without modifying the responses to ACh itself in the absence of sDPP4 (Fig. 2a). Similarly, the clinically available DPP4 inhibitor linagliptin (1–100 nmol/l) prevented the impact of sDPP4 from a concentration of 10 nmol/l (Fig. 2b). Moreover, in the presence of the GLP-1 receptor antagonist exendin-(9–39) (1mmol/l), linagliptin could still prevent the negative effect of sDPP4 on vasodilation (Fig. 2c). In the absence of sDPP4, exendin alone did not significantly modify the vasorelaxant response to ACh (Fig. 2c) nor did linagliptin, at the highest concentration used (100 nmol/l) (Supplementary Figure 2; http://links.lww.com/HJH/A597).

Protease-activated receptor 2 blockade prevents the impaired vasodilatation evoked by soluble dipeptidyl peptidase-4. We have recently demonstrated that PAR2 pharmacological blockade or silencing prevents the induction of human vascular smooth muscle cells proliferation and inflammation by sDPP4 [11]. To explore whether PAR2 participates in the defective relaxation to ACh induced by sDPP4, we used the specific PAR2 antagonist GB83 (10mmol/l). GB83 blunted the negative effect of sDPP4, therefore permitting a complete relaxant response to ACh (Fig. 3a). It was nevertheless noticed that preincubation with GB83 significantly reduced the contractile effect of noradrenaline prior to the addition of ACh (5.74 0.87 vs. 2.84 0.25 mN for treated segments, respectively; results from eight segments obtained from four animals). In the segments contracted with U46619, GB83 also abrogated the defective relaxant response to ACh induced by sDPP4 (Fig. 3b). In the absence of sDPP4, GB83 did not interfere with the control responses to ACh in microvessels precontracted with noradrenaline or U46619 (Figures. 3a and 3b).

Table 1:. Soluble dipeptidyl peptidase-4 does not modify the endothelium-independent relaxations induced by sodium nitroprusside in murine mesenteric microvessels

Treatment	pEC50	Tone prior to relaxation (mN)	Tone at maximal relaxation (mN)	Maximum relaxation (% of NA)
Control	6.08 ± 0.12	5.13 ± 0.45	0.45 ± 0.12	8.52 ± 2.37
sDPP4 (100 ng/ml)	6.10 ± 0.37	3.43 ± 0.63	0.38 ± 0.12	9.15 ± 2.05
sDPP4 (200 ng/ml)	6.16 ± 0.15	5.23 ± 0.60	0.29 ± 0.04	4.84 ± 0.80
sDPP4 (500 ng/ml)	6.03 ± 0.11	4.60 ± 1.73	0.40 ± 0.16	8.54 ± 4.39

The microvessels precontracted with 3mmol/l noradrenaline were exposed to cumulative concentrations of sodium nitroprusside (1 nmol/l to 100mmol/l), with or without (control) increasing concentrations of sDPP4. Results are expressed as mean_SEM of 4–13 segments from 3 to 8 animals. No significant differences were observed among the different treatments. sDPP4, soluble dipeptidyl peptidase-4. mN, mNewtons; NA, noradrenaline.

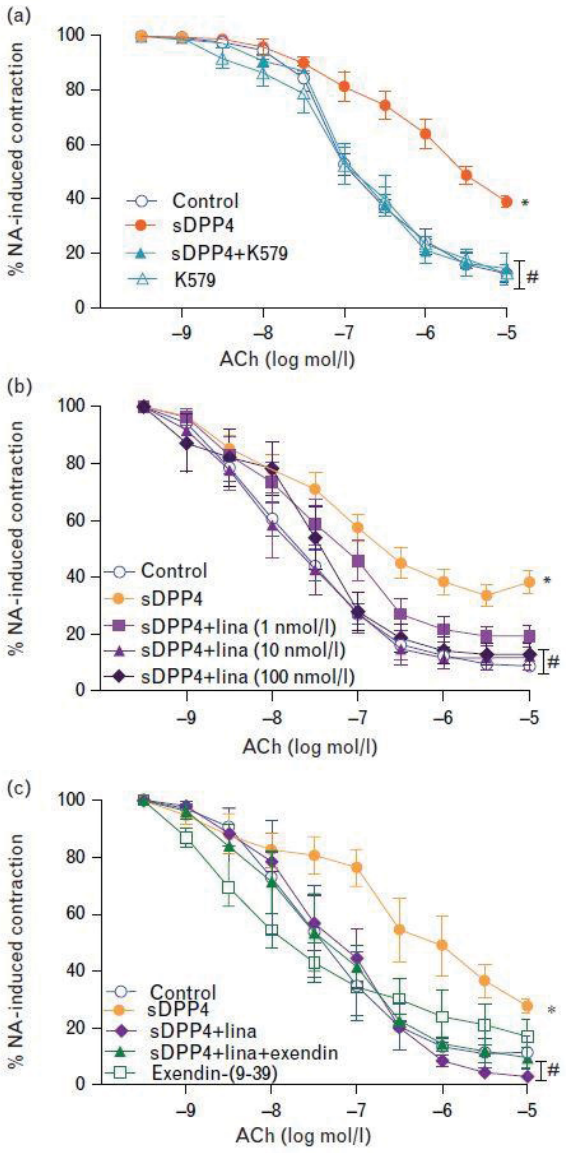

Figure 2: DPP4 inhibitors prevent the defective endothelium-dependent relaxation caused by sDPP4. The microvessels were precontracted with 3mmol/l noradrenaline prior to analyze the effect of (a) K579 (100 nmol/l) and (b) linagliptin (1 nmol/l, 10 nmol/l and 100 nmol/l, respectively) on the defective relaxation to ACh induced by sDPP4 (200 ng/ml). Results are expressed as mean SEM of 25–50 segments obtained from 6 to 12 animals. (c) The GLP-1 receptor antagonist exendin-(9– 39)(10mmol/l) does not significantly modify the effect of linagliptin (10 nmol/l) on the defective relaxation to ACh induced by sDPP4 (200 ng/ml). Results are expressed as mean SEM of 20 segments obtained from four animals. P<0.05 vs. untreated vessels (control); #P<0.05 vs. sDPP4 alone. GLP-1, glucagon-like peptide-1; sDPP4, soluble dipeptidyl peptidase-4; SEM, standard error of the mean.

Apocynin doesn't prevent the impaired vasodilatation evoked by soluble dipeptidyl peptidase-4. Enhanced oxidative stress is regarded a major mechanism mediating endothelial dysfunction and vascular disease [14]. However, the NADPH inhibitor/antioxidant apocynin (10mmol/l) did not modify the defective vasorelaxation elicited by sDPP4 (Fig. 4a). In the absence of sDPP4, apocynin alone did not modify the vasodilation induced by ACh (Fig. 4a).

Cyclooxygenase inhibition and thromboxane receptor blockade prevent the impaired endothelium-dependent vasodilatation evoked by soluble dipeptidyl peptidase-4. The production of COX-derived vasoconstrictor prostanoids is at the basis of endothelial dysfunction in different conditions including aging or diabetes [13,15]. We explored the potential implication of COX-related pathways in mediating the action of sDPP4 downstream of PAR2. The preincubation with the nonspecific COX inhibitor indomethacin (10mmol/l) totally prevented the impaired relaxation to ACh evoked by sDPP4 (Fig. 4b), with similar effects observed using the selective COX2 inhibitor celecoxib (3mmol/l) (Fig. 4c). Moreover, the blockade of thromboxane receptor, to which TXA2 preferentially binds, with the selective antagonist SQ29548 (100 nmol/l) abrogated the effects of sDPP4 (Fig. 4d). Indomethacin, celecoxib or SQ29548 did not modify the relaxation induced by Ach in the absence of sDPP4 (Figures 4b, 4c and 4d).

Soluble dipeptidyl peptidase-4 promotes thromboxane A2 release in human endothelial cells. As the results obtained with SQ29548 pointed at TXA2 as a main mediator of the defective relaxations induced by sDPP4, we explored the ability of cultured endothelial cells to release TXA2 in response to sDPP4. A significant upregulation of TXA2 release was observed after challenging HCAEC with sDPP4 (500 ng/ml; Fig. 5). The preincubation of HCAEC with indomethacin (100 nmol/l) or celecoxib (3mmol/l) abolished the release of TXA2 elicited by sDPP4 (Fig. 5). Similar results were obtained upon DPP4 or PAR2 inhibition with K579 (100 nmol/l) or GB83 (100 nmol/l), respectively (Fig. 5). The proinflammatory cytokine IL-1b (100 ng/ml) was used as a positive control for TXA2 release by endothelial cells (Fig. 5).

IV. DISCUSSION

Beyond its role in regulating glucose homeostasis, DPP4 inhibition has been recently proposed as a promising therapeutic approach to attenuate diabetes-associated vascular risk. In this line, a recent meta-analysis has evidenced that DPP4 inhibitors are safe from a cardiovascular standpoint and may decrease the risk of adverse cardiovascular events in patients with T2DM [8]. However, some controversial results have been reported concerning the cardiovascular benefits of DPP4 inhibitors. Thus, a clinical trial performed inT2DM patients with cardiovascular disease showed no beneficial or detrimental effect by the DPP4 inhibitor saxagliptin on the rate of ischemic events, while the saxagliptin treatment was associated with increased hospitalization for heart failure [16]. Another recent study with sitagliptin did not observe any enhancement of cardiovascular outcomes (cardiovascular death,

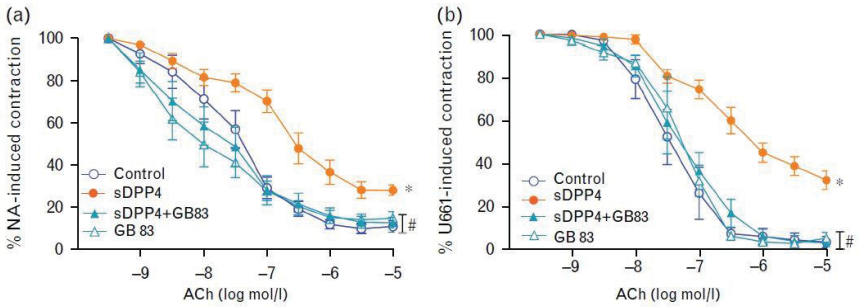

Figure 3: PAR2 inhibition prevents the defective endothelium-dependent relaxation evoked by sDPP4. Effect of the PAR2 inhibitor GB83 (10mmol/l) on the impaired relaxation to ACh induced by sDPP4 (200 ng/ml) in mesenteric arteries contracted with (a) noradrenaline (NA; 3mmol/l) or (b) the prostaglandin H2 analogue U46619 (10 nmol/l). Results are expressed as mean_SEM of 16–33 segments obtained from 4 to 6 animals. _P<0.05 vs. untreated vessels (control); #P<0.05 vs. sDPP4 alone. ACh, acetylcholine; PAR2, protease-activated receptor 2; sDPP4, soluble dipeptidyl peptidase-4; SEM, standard error of the mean.

hospitalization for unstable angina or nonfatal myocardial infarction/ stroke) neither increased hospitalization days because of heart failure [10]. Such discrepancies might reflect that the cardiovascular effects of DPP-4 inhibitors are contextdependent, as suggested by the reported opposite antihypertensive or prohypertensive effects of sitagliptin in different animal models [17]. Therefore, more studies are needed to address in more depth the possible vasculoprotective effects of DPP4 inhibitors, as well as to better characterize both the mechanisms involved and the differences depending on the stage of vascular disease [18].

In the present study, we have demonstrated that sDPP4 can directly impair vascular reactivity by causing defective endothelium-dependent vasodilation, which is considereda main feature of endothelial dysfunction. Both the experimental DPP4 inhibitor K579 and the clinically availableDPP4 inhibitor linagliptin could prevent such detrimental effects of sDPP4 on the vasculature. It is worth noting that effect was in the range of that achieved in the plasma of patients with T2DM treated with this drug [19].

The proposed cardiovascular protective actions of DPP4 inhibitors may rely on incretin stabilization, as GLP1 or its analogues have proved to ameliorate endothelial function in terms of reactivity in both experimental models and patients with T2DM [20,21]. However, the experimentalex-vivo setting used in the present study rather pointed at an incretin-independent action of sDPP4; in fact, we showed that the blockade of

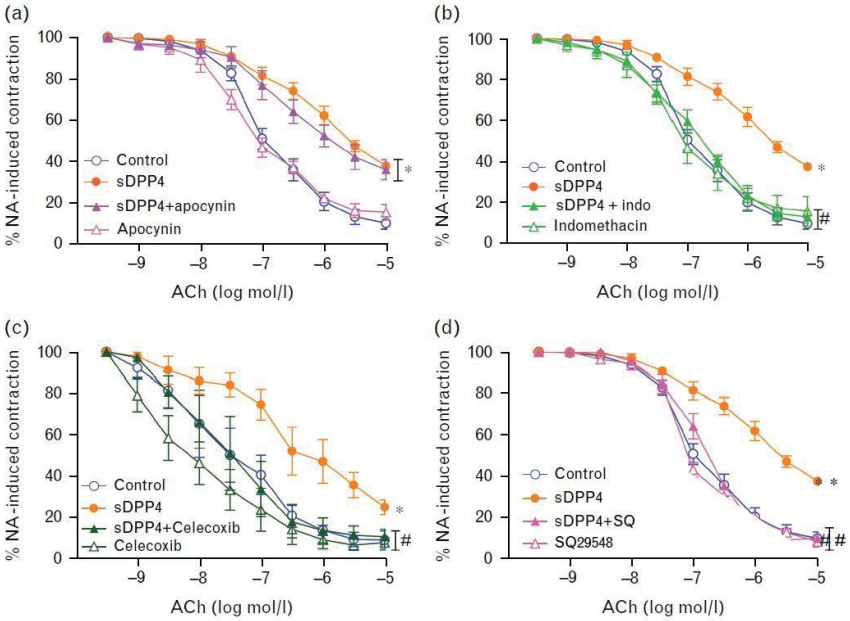

Figure 4: COX inhibition or TP receptors blockade, but not NADPH oxidase inhibition, prevents the impaired endothelium-dependent relaxation evoked by sDPP4. The microvessels were precontracted with 3mmol/l noradrenaline prior to analyze the effect of (a) the NADPH oxidase inhibitor apocynin (10 mmol/l), (b) the nonspecific COX inhibitor indomethacin (10mmol/l), (c) the COX2 inhibitor celecoxib (3mmol/l) and (d) the TP receptors antagonist SQ29548 (100 nmol/l) on the defective relaxation to Ach induced by sDPP4 (200 ng/ml). Results are expressed as mean SEM of 21–28 segments obtained from 4 to 8 animals. P<0.05 vs. untreated vessels (control); #P<0.05 vs. sDPP4 alone. ACh, acetylcholine; COX, cyclooxygenase; NADPH, Nicotinamide adenine dinucleotide phosphate; sDPP4, soluble dipeptidyl peptidase-4; SEM, standard error of the mean.

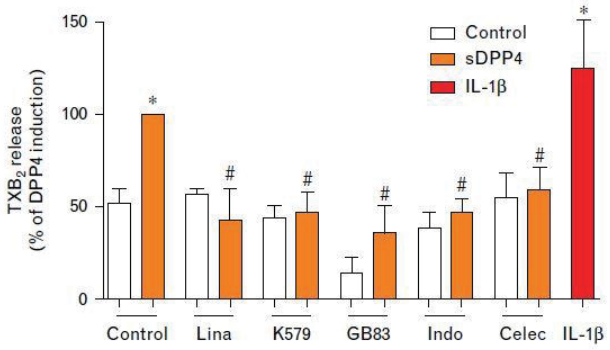

Figure 5: sDPP4 triggers the release of TXA2 in cultured endothelial cells. Human coronary artery endothelial cells were stimulated with sDPP4 (500 ng/ml; 1 h), with or without linagliptin (lina, 10 nmol/l), K579 (100 nmol/l), GB83 (10 mmol/l), indomethacin (10mmol/l) or celecoxib (celec; 3mmol/l), after which TXA2 release was determined in cell supernatants. Interleukin (IL)-1b (100 ng/ml; 1 h) was used as a positive control. Results are expressed as mean SEM of at least four experiments. P<0.05 vs. untreated cells, #P<0.05 vs. sDPP4 alone. sDPP4, soluble dipeptidyl peptidase-4; SEM, standard error of the mean; TXA2, thromboxane A2.

vascular GLP-1 receptors did not suppress the protective action of DPP4 inhibition. This observation is in line with recent evidence suggesting that sDPP4 may act as a modulator of the cardiovascular and immune systems independently of the incretin pathway [6,22]. Thus, beyond inactivating incretin hormones, DPP4 can degrade other substrates, including cytokines, chemokinesand neuropeptides [6,22]. Moreover, DPP4 can alsoexert its actions by directly interacting with membranebound or extracellular matrix proteins relevant for immune and cardiovascular homeostasis [6,22].

In this context, we have recently shown that sDPP4 contains a sequence of four aminoacids (SLIG) that is the key for the activation of the G protein-coupled receptor PAR2 [11]. PAR2 is widely expressed at the surface of endothelial and vascular smooth muscle cells, and plays both a physiological and pathophysiological role in the vasculature [23]. Consistently with sDPP4 being an agonist and activator for PAR2 in vascular cells, we have previously demonstrated that PAR2 silencing or pharmacological blockade prevented the ability of sDPP4 to induce human vascular smooth muscle cell proliferation and inflammation [11]. Here, we have shown that PAR2 blockade abrogates the capacity of sDPP4 to cause defective vasorelaxation and endothelial dysfunction in isolated microvessels. We did not observe any vasoactive effect of GB83 in the absence of the sDPP4, which suggests very little activation of PAR2 receptors in basal conditions of our ex-vivo setting. Taken together, these observations point at PAR2 as a key molecule in mediating some of the direct vascular

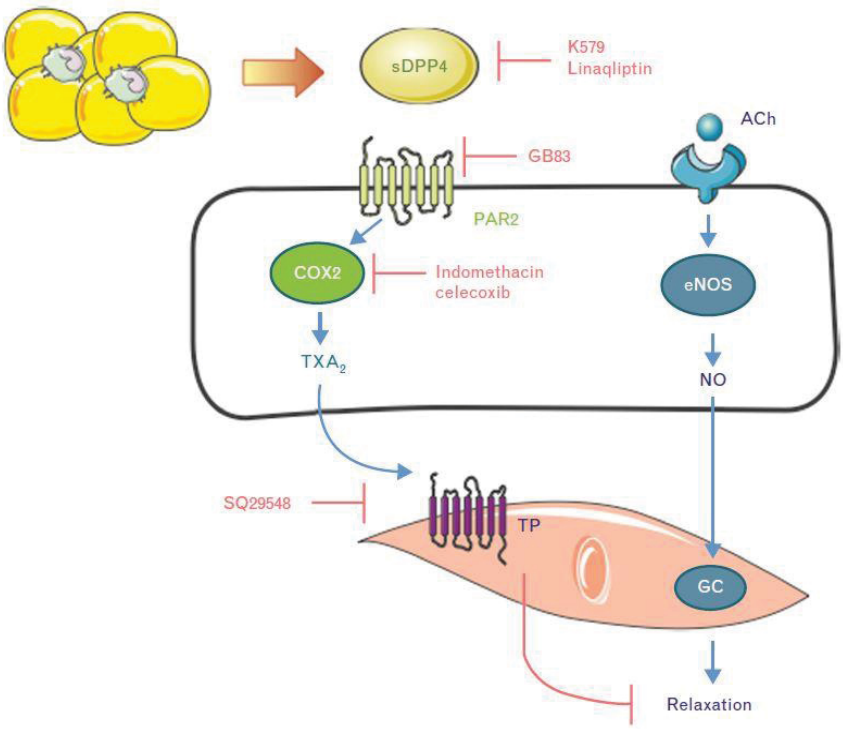

Figure 6: Schematic diagram representing the proposed mechanism by which sDPP4 causes defective vasorelaxation. ACh, acetylcholine; COX2, cyclooxygenase 2; eNOS, endothelial nitric oxide synthase; GC, guanylate cyclase; NO, nitric oxide; PAR2, proteinase-activated receptor 2; TXA2, thromboxane A2. Interference of the different pharmacological inhibitors of the pathway is shown in red.

deleterious actions of sDPP4. Whether and how DPP4 inhibitors couldprevent this interaction between sDPP4 and PAR2 remains to be more accurately defined. In line with our findings, there is recent evidence suggesting that the enzymatic inhibition of DPP4 may affect its direct binding to proteins like fibronectin [24].

To further gain insight into the mechanisms downstream of PAR2 by which sDPP4 caused defective endothelium dependent relaxations, we initially focused on augmented oxidative stress, which has been closely associated with endothelial dysfunction [14]. On the basis of the results obtained with the antioxidant apocynin, reactive oxygen species generation was discarded as major mechanism mediating the negative impact of sDPP4 on vascular reactivity. On the contrary, the activation of COX, and particularly of COX2, together with the activation of thromboxane receptors, seemed to be necessary events for sDPP4 to cause endothelial dysfunction. Accordingly, sDPP4 promoted the endothelial release of the vasoconstrictor prostanoid TXA2, a preferred agonist of thromboxane receptors, through a pathway that was sensitive to DPP4, PAR2 and COX inhibition.

The activation of vascular PAR2 has been described to evoke vasorelaxation [25], as well as vasoconstriction [26]. The activation of PAR2 has further been associated with a rapid cellular release of a series of agonists triggering the immediate autocrine or paracrine transactivation of other non-PAR G protein-coupled receptors, including prostanoid receptors [23]. Further supporting a PAR2-dependent release of prostanoids, a few reports have linked PAR2 activation with enhanced COX2 activity and expression not only in endothelial cells but also in other cell types, including mesangial or mast cells [27–29].

TXA2 is a powerful vasoconstrictor and aggregating factor with proinflammatory properties in the cardiovascular system, whose overproduction is associated with disturbed vascular function in different conditions including aging and diabetes [13,15]. Once activated, thromboxane receptors themselves contribute to amplifying endothelial dysfunction [30]. Overall, the present findings indicate that the release of TXA2 by sDPP4 may be a novel relevant mechanism contributing to impaired reactivity and endothelial dysfunction (Fig. 6).

In conclusion, beyond its role as an adipokine triggering insulin resistance and metabolic complications, the present study identifies sDPP4 as an agent directly causing defective vascular reactivity. Thus, sDPP4 may be an active player in the development of the endothelial dysfunction that characterizes metabolic diseases such as obesity and/or type 2 diabetes, whereas therapeutic tools interfering with sDPP4 might contribute to preserve vascular function in the context of these diseases.

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Conflict of interest
There are no conflicts of interest.

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ABBREVIATIONS

ACh, acetylcholine; COX, cyclooxygenase; DPP4, dipeptidyl peptidase-4; GLP1, glucagon-like peptide- 1; HCAEC, human coronary artery endothelial cells; PAR2, protease-activated receptor 2; sDPP4, soluble DPP4; SNP, sodium nitroprusside; T2DM, type 2 diabetes mellitus; TXA2, thromboxane A2

REVIEWERS' SUMMARY EVALUATIONS

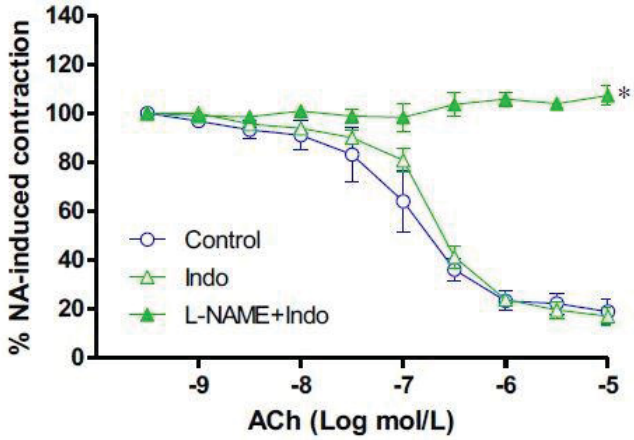
Dipeptidyl peptidase-4 (DPP4) is importantly involved in glucose metabolism, but its role in the pathogenesis of cardiovascular disease is controversial. Although positive effects of inhibiting DPP4 have been demonstrated, these

have not been translated into clinical benefits. This study explores, in a mouse model, one of the possible mechanisms regarding the effect of DPP4 on the endothelium of small arteries. In a careful pharmacological study, the authors demonstrate that DPP4 causes endothelial dysfunction through PAR2 activation and release of vasoconstrictor prostanoids. The relation of these potentially interesting findings to human cardiovascular disease remains to be determined.

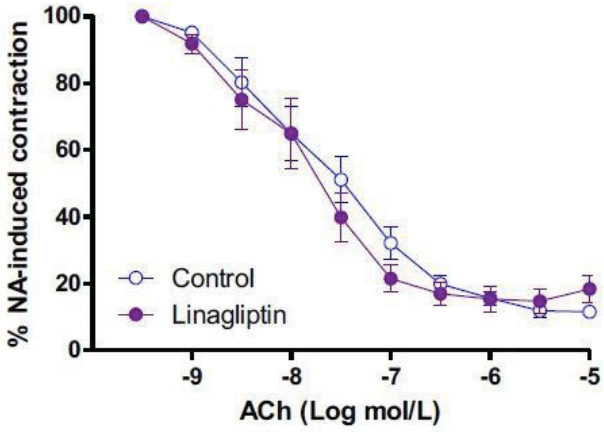
Supplemental Material

sDPP4 concentration (ng/ml)	Vascular tone before sDPP4 addition (mN)	Vascular tone after sDPP4 addition (mN)
0	0.017±0.006	0.035±0.010
20	0.022±0.010	0.016±0.007
100	0.018±0.009	0.065±0.029
200	0.026±0.009	0.044±0.017
500	0.050±0.050	0.032±0.011

Suppl. table 1: sDPP4 did not modify by itself the basal mesenteric vascular tone. The vascular tone was measured before and after 30 min of incubation with sDPP4 alone and prior to NA contraction. Results are expressed as mean ± SEM of 8- 10 segments from 3-5 animals. No significant differences were observed over the 30 min period at any concentration of sDPP4.



Suppl. Fig. 1: The concentration-dependent relaxation induced by ACh in isolated mesenteric microvessels depends on NO release. In some experiments, the vascular segments were pre-incubated with the cyclooxygenase inhibitor indomethacin (indo, 10 μM) alone or supplemented with the endothelial nitric oxide synthase inhibitor L-NAME (10 μM). Results are expressed as mean ± SEM of 6-7 segments obtained from 4 animals. *$p<0.05$ vs control.



Suppl. Fig. 2: The concentration-dependent relaxation induced by ACh in isolated mesenteric microvessels is not modified by linagliptin (100 nM). Results are expressed as mean ± SEM of 7-10 segments obtained from 7 animals.

2.3 Protease-Activated Receptor 2 Promotes Pro-Atherogenic Effects through Transactivation of the VEGF Receptor 2 in Human Vascular Smooth Muscle Cells.

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Abstract – **Background: Obesity is associated with impaired vascular function. In the cardiovascular system, protease-activated receptor 2 (PAR2) exerts multiple functions such as the control of the vascular tone. In pathological conditions, PAR2 is related to vascular inflammation. However, little is known about the impact of obesity on PAR2 in the vasculature. Therefore, we explored the role of PAR2 as a potential link between obesity and cardiovascular diseases.**
Methods: C57BL/6 mice were fed with either a chow or a 60% high fat diet for 24 weeks prior to isolation of aortas. Furthermore, human coronary artery endothelial cells (HCAEC) and human coronary smooth muscle cells (HCSMC) were treated with conditioned medium obtained from in vitro differentiated primary human adipocytes. To investigate receptor interaction vascular endothelial growth factor receptor 2 (VEGFR2) was blocked by exposure to calcium dobesilate and a VEGFR2 neutralization antibody, before treatment with PAR2 activating peptide. Student's t-test or one-way were used to determine statistical significance.
Results: Both, high fat diet and exposure to conditioned medium increased PAR2 expression in aortas and human vascular cells, respectively. In HCSMC, conditioned medium elicited proliferation as well as cyclooxygenase 2 induction, which was suppressed by the PAR2 antagonist GB83. Specific activation of PAR2 by the PAR2 activating peptide induced proliferation and cyclooxygenase 2 expression which were abolished by blocking the VEGFR2. Additionally, treatment of HCSMC with the PAR2 activating peptide triggered VEGFR2 phosphorylation.
Conclusion: Under obesogenic conditions, where circulating levels of pro-inflammatory adipokines are elevated, PAR2 arises as an important player linking obesity-related adipose tissue inflammation to atherogenesis. We show for the first time that the underlying mechanisms of these pro-atherogenic effects involve a potential transactivation of the VEGFR2 by PAR2.
Keywords— **Obesity, PAR2, VEGFR2, smooth muscle cell proliferation, atherosclerosis**

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* Both authors jointly directed this work

I. INTRODUCTION

Obesity is associated with comorbidities such as type 2 diabetes and cardiovascular diseases (CVD) (Despres, 2012). CVD are the main cause for deaths in diabetes mellitus (Morrish et al., 2001) and lead to a significant increase in obesity-associated mortality (Lloyd-Jones et al., 2010). The development of obesity is a consequence of imbalance between energy intake and energy expenditure causing storage of excessive energy in the adipose tissue (AT) (Hill et al., 2012). Besides being a storage organ, it is well established that AT influences systemic metabolism as an endocrine organ secreting a variety of proteins referred to as adipokines (Trayhurn et al., 2011). During the progress of obesity AT undergoes modulation such as the enlargement of adipocytes, or a switch in the secretome towards a prominent release of pro-inflammatory adipokines (Ouchi et al., 2011). In turn, these adipokines are known as inflammatory factors promoting insulin resistance (Hotamisligil et al., 1995). Furthermore, it has been proposed that high levels of circulating pro-inflammatory adipokines found in diseases such as obesity and type 2 diabetes mellitus have an impact on cardiovascular function (Greenberg and Obin, 2006). Thus, adipokines represent a molecular link between metabolic and cardiovascular diseases (Taube et al., 2012). There is evidence that they can directly exert deleterious effects on both endothelial and smooth muscle cells resulting in damage of the vasculature (Karastergiou and Mohamed-Ali, 2010; Lamers et al., 2011; Schlich et al., 2013).

In 1994, protease-activated receptor 2 (PAR2), a seven transmembrane domain, G-protein-coupled receptor, was discovered by Nystedt et al. (Nystedt et al., 1994) and has been found to be expressed in endothelial (Mirza et al., 1996) as well as in smooth muscle cells (Molino et al., 1998). While PAR1, PAR3 and PAR4 activation is driven by thrombin, PAR2 is activated by serine proteases such as tryptase, factor Xa, trypsin and the TF-FVIIa complex (Adams et al., 2011a). The proteolytic cleavage of the extracellular N-terminal domain leads to the unmasking of a tethered ligand which then binds to a binding pocket

in the receptor (Dery et al., 1998). In addition, PAR2 activation can be triggered by synthetic peptides mimicking the sequence of the tethered ligand (Macfarlane et al., 2001b). In the vascular wall, activation of PAR2 initiates multiple signaling pathways exerting distinct responses, among others the control of vascular tone and coagulation (Sriwai et al., 2013; Zhao et al., 2014).

Under pathological conditions, PAR2 is involved in cardiovascular responses such as vasorelaxation and vasoconstriction, as well as inflammatory processes (Hirano and Kanaide, 2003; Aman et al., 2010; Adams et al., 2011a). For instance, administration of PAR2 agonists such as SLIGKV or trypsin induces proliferation in smooth muscle (Bono et al., 1997) and endothelial cells (Mirza et al., 1996). Moreover, smooth muscle cell proliferation via PAR2 can be triggered by pro-inflammatory adipokines such as DPP4 (Wronkowitz et al., 2014). In vivo, PAR2 activation drives the development of hypertension (Emilsson et al., 1997; Cicala et al., 1999) and PAR2 expression is enhanced in aortas of diabetic mice and in human coronary atherosclerotic lesions (McGuire, 2004; Napoli et al., 2004). Furthermore, upregulation of PAR2 content also occurs in endothelial cells and coronary arteries after treatment with inflammatory stimuli such as interleukin (IL)-1α, IL-1β and tumor necrosis factor (TNF)-α (Nystedt et al., 1996; Hamilton et al., 2001; Ritchie et al., 2007). Overall, these studies suggest that PAR2 is an important player in both endothelial and smooth muscle cell function as well as in the overall control of vascular reactivity. However, the role of PAR2 in obesity-related vascular diseases remains unclear. Therefore, we addressed the importance of PAR2 in the vasculature under obesogenic conditions.

II. MATERIAL AND METHODS

Animal model: Animal care and use were approved by the local Animal Ethics Committee according to the principles outlined in the European Commission Council Directive (86/609/EEC). C57BL/6J mice were obtained from Jackson laboratories. 12 week old mice were either fed a high fat diet (HFD, 60% kcal fat; Research diets D12492) or standard chow diet over 24 weeks. Animals were housed in polyacrylamide cages under temperature control (22°C) and a 12 hour light/dark cycle. Mice were weighted every 4 weeks. After 24 weeks on the respective diets mice were sacrificed by cervical dislocation. Aortas were collected, cleaned of fat, snap frozen in liquid nitrogen and stored at -80°C until further analysis.

Preparation of explants: Visceral AT from chow and HFD animals were collected in cold PBS supplemented with an antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA, USA). Connective tissue and vessels were removed and fat pads were cut into small pieces. After washing three times in PBS, liquid was removed by putting explants on a sterile mesh. Relatively dry fat pieces were weighted and 100 mg of AT were incubated in 1 ml of HCSMC starvation medium (fetal calf serum (FCS)-free DMEM low glucose (Invitrogen, Carlsbad, CA, USA) at 37°C and 5%CO2 to generate conditioned medium (CM). After 24 h CM was collected and centrifuged at 1200 rpm for 10 minutes and stored at -20°C until further use.

Isolation of preadipocytes and generation of human adipocyte CM: Preadipocytes were isolated from human abdominal AT obtained from moderately overweight or obese subjects undergoing plastic surgery following the protocol previously described and being approved by the ethical committee of the Heinrich-Heine-University (Düsseldorf, Germany) (Hauner et al., 1995).

Preadipocytes were cultured in DMEM/F12 medium (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 33 µmol/l biotin (Sigma-Aldrich, Schnelldorf, Germany), 17 µmol/l d-panthothenic-acid (Sigma-Aldrich, Schnelldorf, Germany), 14 nmol/l NaHCO3 (Merck, Darmstadt, Germany), human insulin (100 nM, Sigma-Aldrich, Schnelldorf, Germany), dexamethasone (1µM, Sigma-Aldrich, Schnelldorf, Germany) and 10% FCS (Gibco Invitrogen, Carlsbad, CA, USA) and grown until 90% confluence. Then differentiation was induced by addition of 0.25 µM troglitazone (Sigma-Aldrich, Munich, Germany) and 0.2 mM IBMX (Sigma-Aldrich, Munich, Germany). The switch to differentiation medium is indicated as day 0. After 7 days, medium was changed to maintenance medium (differentiation medium deprived of troglitazone and IBMX) until day 14. Medium was changed every two to three days. On day 14, cells were treated with either human coronary artery endothelial cell (HCAEC) starvation medium composed of endothelial cell basal medium MV and 5% FCS or HCSMC starvation medium (DMEM low glucose, 0% FCS) for 48 hours. CM was collected, centrifuged at 1200 rpm for 10 minutes and stored at -20°C until further use.

Cell Culture: HCAEC obtained from 3 different donors were purchased from PromoCell (Heidelberg, Germany) and cultured in endothelial cell basal medium MV (PromoCell, Heidelberg, Germany) supplemented with 20% FCS, 1µg/ml hydrocortisone, 0.004 ml/ml ECGS and 10 ng/ml EGF at 37° C and 5 % CO2. Cells between passages 4-7 were used for experiments. When 90% confluence was reached HCAEC were washed with PBS and treated with CM. HCSMC from 4 different donors were purchased from PromoCell (Heidelberg, Germany), tebu-bio (Offenbach, Germany) and Lonza (Basel, Switzerland) were seeded in HCSMC growth medium (DMEM low glucose, Invitrogen, Carlsbad, CA, USA) containing 10% FCS and cultivated according to manufacturer's protocol at 37° C and 5 % CO2. After reaching 90% confluence, cells were washed with PBS and serum starved for 24 hours. HCSMC were then treated with CM or PAR2 activating peptide (PAR2-AP, SLIGKV-NH_2; 50 µM, Bachem, Bubendorf, Switzerland). All experiments were performed in cells at passage 3.

Immunoblotting: Cells were lysed in a lysis buffer composed of 50 mM HEPES (Promocell, Heidelberg, Germany), 1% TritonX100 (Sigma-Aldrich, Munich, Germany), complete protease inhibitor and PhosStop

phosphatase inhibitor cocktail at pH 7.4 (Roche, Basel Switzerland). Protein lysates were diluted with Laemmli buffer and denatured for 5 min at 94°C. 5 µg of protein were loaded on a 10% gel SDS-PAGE and transferred to a PVDF membrane during blotting process. Reagents for SDS-PAGE were supplied by Amersham Pharmacia Biotech (Braunschweig, Germany) and by Sigma-Aldrich (Munich, Germany). Blots were blocked in a Tris-buffered saline solution containing 0.1% Tween and 5% milk powder and incubated with antibodies against: PAR2 (SAM11: sc-13504, Santa Cruz Biotechnology, Heidelberg, Germany), COX2 (#4842), GAPDH (#2118), phospho-VEGFR2 (Tyr1059) (#3817), VEGFR2 (#2479), NF-κB p65 (#8242) and phospho-NF-κB p65 (Ser536) (#3033). Unless otherwise stated, all antibodies were purchased from Cell signaling Technology (Frankfurt, Germany). We used the corresponding secondary HRP-coupled antibodies against mouse and rabbit (Promega, Mannheim, Germany). After washing, blots were exposed to Immobilon HRP substrate (Millipore, Billerica, MA, USA) and analyzed with a VersaDoc 4000 MP work station (BIO-RAD, Munich, Germany).

qRT-PCR: RNA isolation of human cells was performed in RLT lysis buffer working solution containing 1% β-mercaptoethanol according to manufacturer's protocols. Murine aortas were lysed in 1ml Tripure (Roche, Mannheim, Germany) by a tissuelyser II (Qiagen, Hilden, Germany) for 5 min at 29 s-1 for mRNA isolation. mRNA purification of cells and aortas was done with an RNeasy Kit (Qiagen, Hilden, Germany) and content of mRNA was measured with a NanoDrop2000 (Thermo Scientific, Schwerte, Germany). All samples were reversely transcribed into 1 µg/µl of cDNA using an Omniscript RT Kit (Qiagen, Venlo, Netherlands) and mRNA levels were determined by StepOne Plus real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Primers from Qiagen were used: Hs_F2RL1_1_SG (QT02589377), Hs_ACTB_2_SG (QT01680476) Hs_PTGS2_SG (QF00461055), mM_F2RL1 (QT02255330) and Mm_Rn18S (QT02448075.

Proliferation assay: Prior to determination of proliferation, HCSMC were starved for 24 hours. Cells were pre-incubated with the specific PAR2 antagonist GB83 (10 µM , Axon Medchem, dissolved in DMSO), the vascular endothelial growth factor receptor 2 neutralization antibody (VEGFR2-NA, R&D Systems Wiesbaden-Nordenstadt, Germany, MAB3572) or calcium dobesilate (Sigma-Aldrich, Schnelldorf, Germany) for 1 hour. Subsequently, cells were treated either with CM alone, CM in combination with GB83, the PAR2 activating peptide (PAR2-AP, SLIGKV-NH_2; 50 µM, Bachem, Bubendorf, Switzerland), or PAR-AP in combination with the VEGFR2-NA or dobesilate for 24 hours. 5% FCS was used as a positive control. All treatments contained 10% BrdU. Proliferation was assessed by measuring BrdU incorporation (Proliferation Assay, Roche, Mannheim, Germany) with a microplate reader (Infinite M200, Tecan GmbH, Männersdorf, Switzerland).

Caspase 3/7 activity assay: HCAEC were seeded in a 96-well plate and cultured for 24 hours. Cells were pre-treated with GB83 for 1 hour and treated with CM alone or in combination with GB83 (10 µM) for 18 hours. H2O2 (200 µM, Sigma-Aldrich, Schnelldorf, Germany) was used as a positive control. Caspase 3/7 activity was measured by a Caspase-Glo® 3/7 Assay (Promega, Mannheim, Germany) as described in the manufacturer's protocol. Luminescence was measured in a microplate reader.

VEGF release: In order to monitor vascular endothelial growth factor (VEGF) release from HCSMC, supernatants were collected, centrifuged and stored at -80°C. Supernatants were analyzed with a VEGF ELISA Kit purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany).

Adipokine array: The secretion profile of murine adipose explants was analyzed by usage of a proteome profiler (R&D Systems, Wiesbaden-Nordenstadt, Germany) according to manufacturer's protocol. Membranes were incubated with murine CM from chow and HFD-fed animals.

Statistical analysis: Statistical analysis was performed using the GraphPad Prism software (La Jolla, CA, USA). Unpaired two-tailed Student's t-test or one-way ANOVA (post hoc test: Bonferroni or Dunnett's) were used to determine statistical significance considering a p-value below 0.05 as statistically significant. Data are expressed as mean values ± SEM.

III. RESULTS

PAR2 expression is elevated by HFD in murine aortas and by human adipocyte-derived factors in HCAEC and HCSMC

Animals under HFD gained more weight compared to animals given a chow diet (Fig. 1A). To monitor the impact of obesity on PAR2 expression in the vasculature of these mice, we analyzed PAR2 levels in aortas carefully controlled to be free of adipose tissue. PAR2 mRNA expression in aortas from HFD-fed animals was significantly higher (ΔΔCt of 1.2 ± 0.1) than in chow diet-fed animals (ΔΔCt of 0.8 ± 0.1) (Fig. 1B). Furthermore, PAR2 expression was positively correlated with the body weight of corresponding animals (Fig. 1C). To determine whether the observed changes in aortic PAR2 expression were specifically related to AT, we analyzed the effect of CM from murine adipose tissue explants on HCSMC. Exposure of HCSMC to CM of animals under chow diet had no effect on PAR2 content while treatment with CM obtained from HFD-fed animals provoked a 2-fold increase (Fig. 1D).

Certain cytokines, which are elevated in obesity such as TNF-α or IL-1α are able to induce PAR2 (Nystedt et al., 1996; Hamilton et al., 2001). In order to explore the impact of adipokines on PAR2 induction, we generated CM from differentiated primary human adipocytes obtained from overweight or obese subjects (BMI 30.1 ± 1.9 kg/m²). Human vascular cells were exposed to adipocyte CM. In HCSMC, PAR2 mRNA was significantly elevated up to 1.5 ± 0.2 fold over control

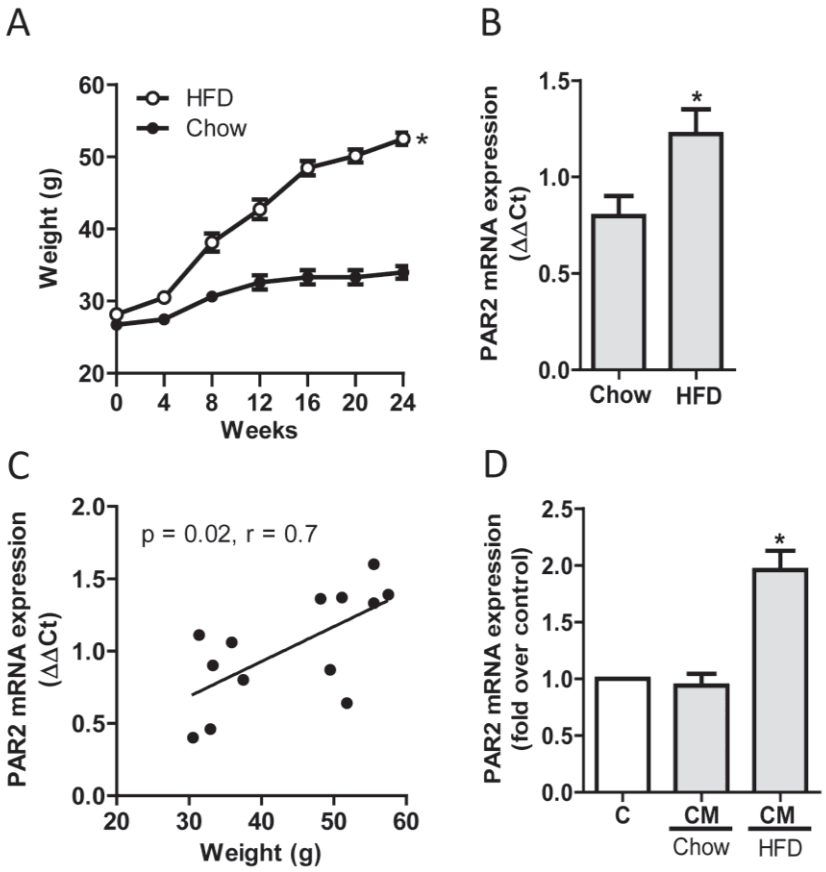


Figure 1: HFD induces PAR2 expression in the vascular wall. (**A**) Weight gain in C57BL/6J wild type mice under HFD or chow diet for 24 weeks; n=11-27. (**B**) PAR2 mRNA expression in murine aortas after 24 weeks. PAR2 expression was normalized to 18S mRNA levels; n=7. (**C**) Correlation of PAR2 mRNA expression in murine aortas and weight of respective animals; n=13. (**D**) CM from murine epididymal AT of chow- and HFD-fed mice were used to determine induction of PAR2 mRNA in HCSMC. Data are normalized to ß-actin mRNA levels (*p<0.05 vs. chow); n= 7. All data represent mean values ± SEM (*p<0.05). Conditioned medium (CM), High fat diet (HFD).

after 1 hour CM treatment (Fig. 2C). At protein level an increase in PAR2 expression occurred after 6 and 24 hours (1.7 ± 0.2 fold over control, respectively) (Fig. 2B). Moreover, PAR2 expression was enhanced in HCSMC exposed to CM of obese subjects. While CM of subjects with a BMI of 25 kg/m² was only capable to induce PAR2 1.2 ± 0.2 fold compared to non-treated cells, CM of subjects showing a BMI of 37 kg/m² could induce PAR2 to a significantly higher extent (Fig. 2C).

PAR2 mediates CM-induced proliferation in HCSMC

A change in intima-media thickness is an important event in the development of vascular remodeling.(Langille, 1993) During this process proliferation of smooth muscle cells results in a thickening of the tunica media (Langille, 1993). Therefore, using CM from human adipocytes we assessed proliferation in HCSMC. Treatment of HCSMC with CM increased proliferation 3.3 ± 0.6 fold over control. Interestingly, we observed that this effect was PAR2-dependent, since it was reduced by 70% with the PAR2 specific antagonist GB83 (Fig. 3A). We further observed a trend towards higher mitogenic activity due to stronger PAR2 expression in HCSMC (Fig. 3B, p=0.06). PAR2 involvement during HCSMC proliferation was supported by the initiation of proliferation with PAR2-AP (Fig. 3C).

CM-induced PAR2 levels and apoptosis in HCAEC

In HCAEC, CM treatment resulted in a biphasic increase of PAR2 expression at mRNA level (Suppl. Fig. 1A), whereas PAR2 was gradually enhanced at protein level with a peak of 2.1 ± 0.4 fold over control after 24 hours (Suppl. Fig. 1B). Since the proliferation of smooth muscle cells during vascular remodeling is accompanied by endothelial cell apoptosis (Langille, 1993), we investigated the impact of adipocyte CM on caspase3/7 activity in HCAEC. Apoptosis of HCAEC was increased by CM and effects of CM were prevented by the PAR2 antagonist GB83 (Suppl. Fig. 1C). GB83 alone had no effect on either cell proliferation or apoptosis.

Induction of cyclooxygenase 2 levels occurs via PAR2

Since cyclooxygenase 2 (COX2) is a key mediator of inflammation and vascular dysfunction (Vane and Botting, 1998; Bagi et al., 2006), we investigated the potential regulation of COX2 by CM. We exposed HCSMC cells to CM for the indicated time points. CM treatment triggered an enhancement of COX2 expression

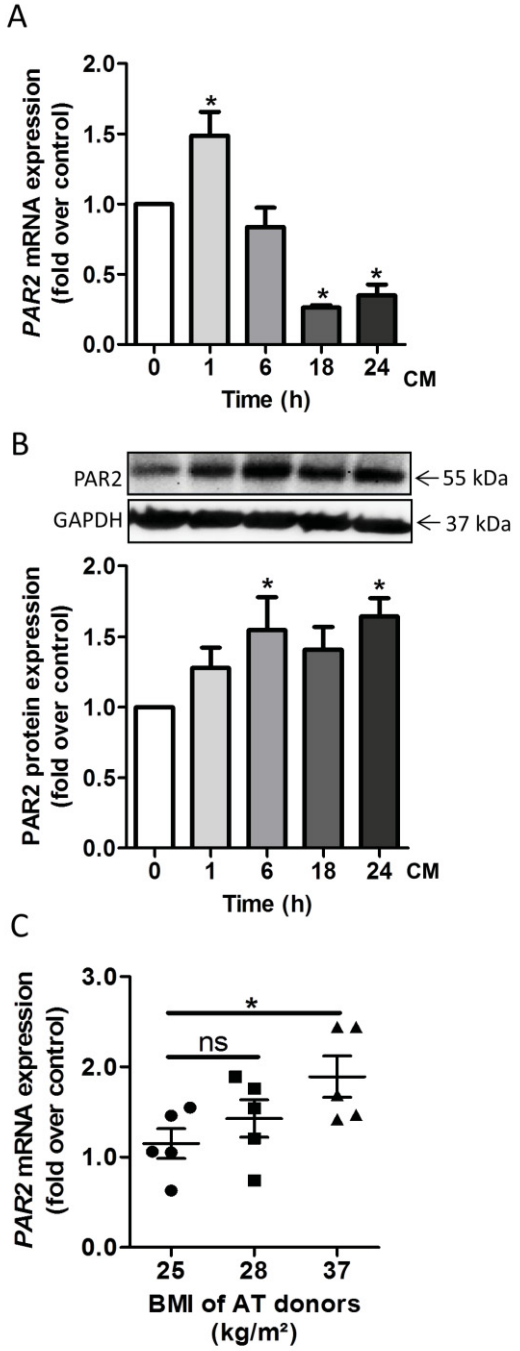


Figure 2: PAR2 induction by adipocyte-derived factors in HCSMC. (**A-B**) Time course of PAR2 mRNA and protein expression after CM treatment for indicated time points was assessed by qRT-PCR and western blot in HCSMC. Data were normalized to β-actin or GAPDH levels respectively; n= 4-6 (*p<0.05 vs. time 0). (**C**) PAR2 expression in HCSMC after challenge to CM for 1 hour and its relation to BMI of AT donors, n=15 (*p<0.05). Data represent mean values ± SEM. Conditioned medium (CM), human coronary smooth muscle cells (HCSMC).

at mRNA level with a peak at 1 hour (2.4 ± 0.4 fold over control) (Fig. 4A) and a peak at protein levels at 24 hours (2 ± 0.3 fold over control) (Fig. 4B). One hour pre-incubation of HCSMC with the specific PAR2 antagonist GB83 downregulated CM-induced COX2 expression to basal levels (Fig.4C). Additionally, we detected a rise in COX2 protein level after challenging cells with the PAR2-AP for 1 hour (1.5 ± 0.2 fold over control) and 2 hours (1.6 ± 0.1 fold over control) (Fig. 4D). As a marker of pro-inflammatory signaling, we examined phosphorylation of the transcription factor NF-κB. After 24 hours CM-induced phosphorylation of NF-κB was prevented by GB83 (Fig. 4E). Accordingly, phosphorylation of NF-κB in HCSMC was induced by the PAR2 agonist PAR2-AP (Fig. 4F).

PAR2 activation leads to proliferation and COX2 induction in a VEGFR2 dependent-manner

As previously described by Schlich et al., VEGF (Schlich et al., 2013) which signals through the vascular endothelial growth factor receptor 2 (VEGFR2) (Ferrara et al., 2003), is a main contributor to smooth muscle cell proliferation. Here, we found increased VEGF levels in CM of adipose explants from mice upon HFD (Suppl. Fig. 2A). In addition, VEGF release from human adipocytes correlated with BMI of adipose tissue explant donors (Suppl. Fig 2B). In order to explore if the VEGFR2 was involved in PAR2-mediated proliferation and COX2 induction, we blocked the VEGFR2 by usage of dobesilate and VEGFR2-NA. Indeed, pre-incubation with either the inhibitor dobesilate or a VEGFR2-NA for 1 hour and subsequent treatment with PAR2-AP resulted in a reduction of proliferation back to basal levels (1.0 ± 0.1 fold and to 0.9 ± 0.1 fold compared to control levels, respectively). VEGFR2 blockade alone had no effect on HCSMC proliferation (Fig.5A). Furthermore, PAR2-AP-mediated COX2 induction of 1.5 ± 0.1 fold over control was diminished to 1.1 ± 0.03 fold over control after VEGFR2-NA and to 1.1 ± 0.1 fold over control after dobesilate addition (Fig. 5B). Next, we explored if PAR2 activation affected VEGFR2 phosphorylation. After 2 hours we observed an enhanced receptor phosphorylation (Fig. 5C), while total VEGFR2 content was unaltered (Data not shown). However, VEGFR2 activation did not occur due to increased VEGF levels since exposure of HCSMC to PAR2-AP did not alter VEGF release compared to control conditions (Fig. 5D).

IV. DISCUSSION

The development of obesity is associated with functional and structural changes in the vasculature (Ouchi et al., 2011). Particularly, during obesity, AT switches to a more pro-inflammatory secretome (Ouchi et al., 2011). These secreted factors, upregulated in obesity, reach the vascular wall through the circulation where they can exert deleterious effects (Goldstein and Scalia, 2007). As a consequence, induction of vascular inflammation, endothelial dysfunction and smooth muscle cell proliferation takes place. These processes are hallmarks of vascular diseases and have been linked to PAR2 (Hirano and Kanaide, 2003; Wronkowitz et al., 2014; Romacho et al., 2016b). To our knowledge, there are

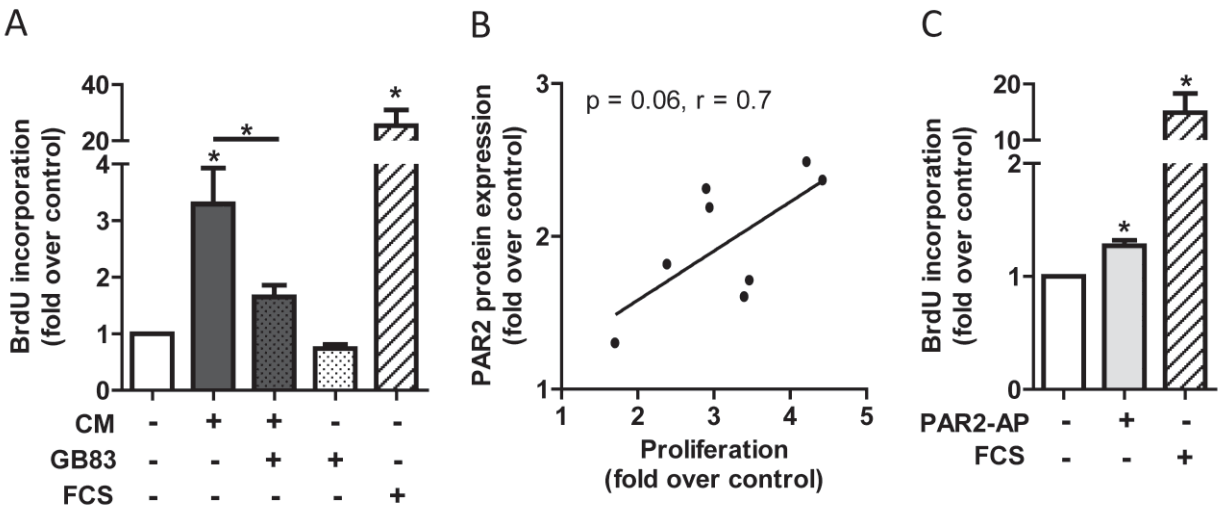


Figure 3: PAR2 mediated proliferation in HCSMC. (A) Proliferation was assessed by BrdU incorporation after 1 hour pre-incubation with GB83 (10 µM) and subsequent treatment with either CM alone or in combination with GB83 for 24 hours in HCSMC. FCS (5%) was included as a positive control. **(B)** Correlation of PAR2 induction after 24 hours and proliferation rate by CM; n=8. **(C)** Effects of PAR2-AP (50 µM) on proliferation was analyzed after 24 hours; n=4-6. Data represent mean values ± SEM (*p<0.05 vs. control). Conditioned medium (CM), fetal calf serum (FCS), human coronary smooth muscle cells (HCSMC), PAR2-activating peptide (PAR2-AP).

scarce reports about a direct connection between obesity and PAR2 in the vasculature. In the present study we aimed to explore the effect of CM as a model of circulating adipokines under obesogenic conditions. We show that obesity caused PAR2 upregulation in murine aortas, and that CM of obese subjects triggered PAR2 induction in HCSMC. Furthermore, CM induced HCSMC proliferation, COX2 and pNF-κB in a PAR2-dependent manner. We demonstrated that PAR2 activation and subsequent effects involved a transactivation of the VEGFR2.

It has been reported that obesity upregulates PAR2 locally in human AT as well as in AT of rats fed a HFD (Lim et al., 2013). Adipokines released from AT are now acknowledged as mediators of inter-organ crosstalk between the AT and the vascular wall (Romacho et al., 2014). Indeed, pro-inflammatory adipokines, which are upregulated in obesity, such as IL-1α, IL-1β and TNF-α have been described as potent PAR2 inducers in the vasculature (Nystedt et al., 1996; Hamilton et al., 2001; Ritchie et al., 2007). Moreover, in human aorta PAR2 expression is enhanced in atherosclerotic lesions (Napoli et al., 2004). Therefore, we aimed to explore if a low-grade chronic inflammatory condition such as obesity had an impact on PAR2 expression not only in AT itself (Lim et al., 2013), but also in the vascular wall. Interestingly, 24 weeks of HFD increased PAR2 mRNA expression in murine aortas. PAR2 expression in aortas was positively correlated with the body weight of mice. Furthermore, solely CM from animals undergoing HFD was able to induce PAR2 in HCSMC. These findings were further corroborated in human vascular cells, namely HCAEC and HCSMC, where human adipocyte-derived CM promoted PAR2 upregulation on mRNA and protein levels. PAR2 mRNA expression was positively correlated to the BMI of AT donors. Altogether these data suggest that an obesogenic environment provokes changes in the AT secretome leading to PAR2 upregulation in the vascular wall.

Vascular remodeling, characterized by proliferation and migration of smooth muscle cells as well as apoptosis of endothelial cells (Langille, 1993), is an early event in the development of atherosclerosis (Lerman et al., 1998). We recently demonstrated that adipocyte-derived CM can initiate HCSMC proliferation (Schlich et al., 2013). In addition we proved the participation of PAR2 in adipokine-induced HCSMC proliferation (Wronkowitz et al., 2014b). Our data suggest that CM-mediated HCSMC proliferation as well as CM-mediated HCAEC apoptosis is PAR2 dependent since it is abolished by the PAR2 specific antagonist GB83 (Adams et al., 2012). The specificity of this antagonist has been previously demonstrated elsewhere (Barry et al., 2010). Accordingly, PAR2-AP induced HCSMC proliferation thereby confirming the involvement of PAR2. This result is in agreement with previous studies where PAR2 agonist induced proliferation of smooth muscle (Bono et al., 1997). Considering that PAR2 activation has been shown to result in migration of smooth muscle cells (Pena et al., 2012), these findings point towards a considerable role of PAR2 in vascular alterations. Further observations showed a trend for correlation between CM-induced HCSMC proliferation and PAR2 expression in these cells. Therefore we assume that PAR2 abundance, upregulated by several CM-derived factors such as TNFα and IL-1β (Nystedt et al., 1996; Ritchie et al., 2007), might facilitate proliferation. However, the precise relevance of PAR2 induction in the context of vascular and metabolic diseases remains poorly understood and has to be further elucidated.

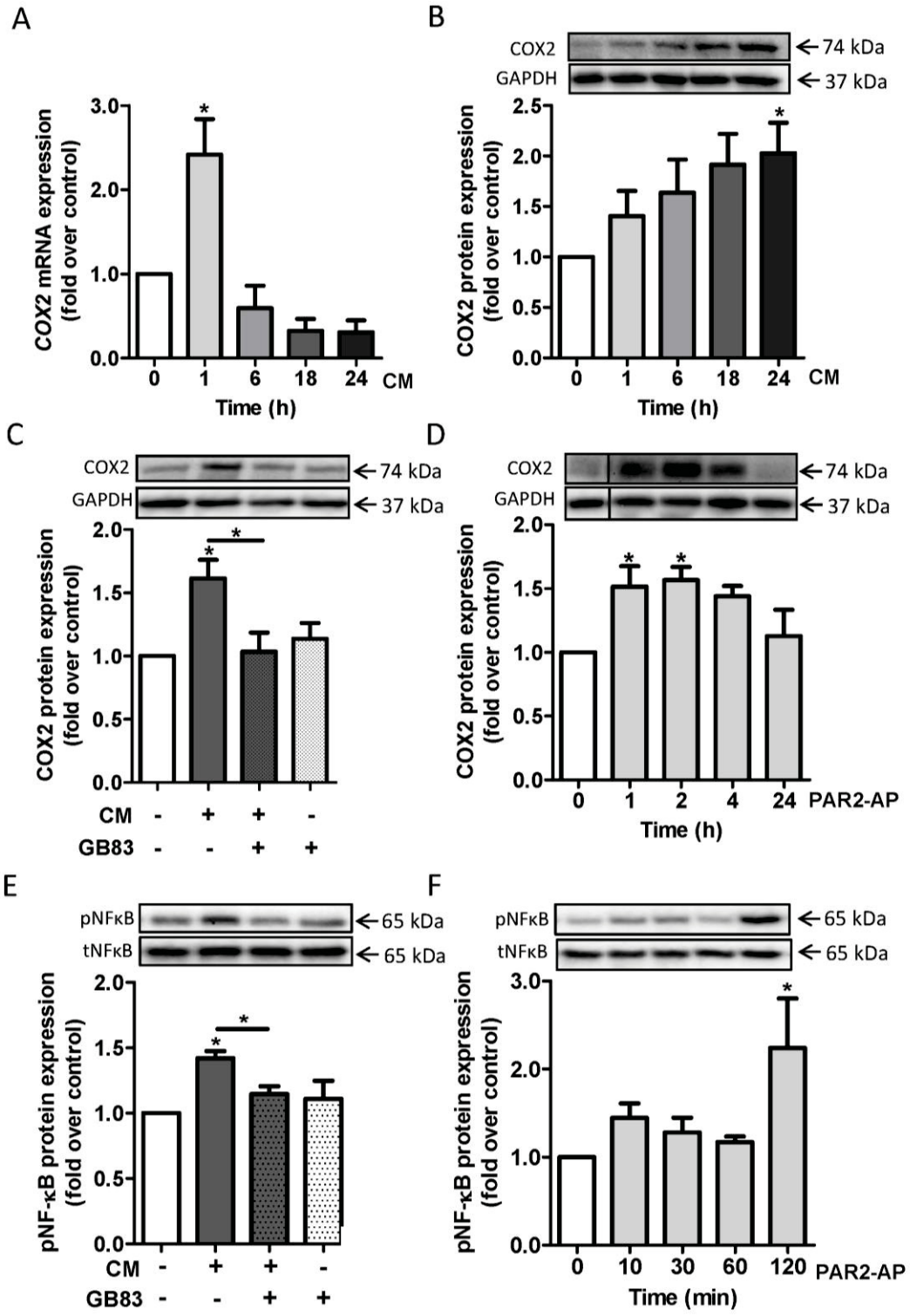


Figure 4: CM induces COX2 expression in HCSMC via PAR2. COX2 expression was determined in HCSMC exposed to CM for the indicated time points and (**A**) mRNA and (**B**) protein levels were quantified. n=4-6. (**C**) CM-induced COX2 expression after 24 hours and pre-incubation with PAR2 antagonist GB83 (10 μM); n=6. (**D**) Time course of COX2 induction by PAR2-AP (50 μM); n=6. (**E**) CM-induced pNF-κB expression after 24 hours and pre-incubation with PAR2 antagonist GB83, n=4. (**F**) Time course of pNF-κB expression by PAR2-AP, n=5. Data of mRNA target gene levels are normalized to β-actin. Protein expression of COX was normalized to GAPDH and pNF-κB was normalized to tNF-κB. Data are mean values ± SEM (*p<0.05 vs. time 0 or control). Conditioned medium (CM), human coronary smooth muscle cells (HCSMC), PAR2-activating peptide (PAR2-AP).

There is evidence for a pivotal role of PAR2 during atherosclerosis (Dery et al., 1998; Wronkowitz et al., 2014). It is known that PAR2 agonism can lead to NF□B activation in human coronary smooth muscle cells (Bretschneider et al., 1999; Wronkowitz et al., 2014).To further elucidate the role of PAR2 in this process, we explored the impact of PAR2 agonism on both NF□B, as a master regulator of inflammatory gene expression

(Macfarlane et al., 2001a) and COX2, another key regulator of vascular inflammatory processes (Bagi et al., 2006). We found that CM activated NF-κB and induced COX2 via PAR2. Analogously, PAR2-AP significantly enhanced COX2 expression and phosphorylation of NF-κB in HCSMC. Consistently, the PAR2 antagonist GB83 prevented these deleterious effects highlighting the importance of this receptor during atherogenesis and inflammation-related diseases. Our results are in line with other studies which showed PAR2-AP-stimulated COX2 expression in HUVECs (Syeda et al., 2006) and activation of NF-κB in smooth muscle cells (Bretschneider et al., 1999). We therefore assume a critical role of PAR2 in controlling COX2- and NF-κB - mediated inflammation. However, one limitation of our study is that we have not dissected the precise interaction between COX2 and NF- κB.

Since it is known that the COX2 promoter region contains putative NFκB binding sites in smooth muscle cells (Tazawa et al., 1994; Nie et al., 2003) we can speculate that PAR2 activation most likely leads to NFκB translocation to the nucleus resulting in COX2 expression. Nontheless, COX2 products such as PGE2 are able to increase the transcription of NFκB, thus positively enhancing the inflammatory signaling (Poligone and Baldwin, 2001). So far there are no data available whether and how exactly PAR2-mediated signaling pathways involve both NFκB and COX2. In the light of our data we cannot specifically dissect whether NFκB leads to COX2 induction, or if COX2 induction leads to NF-κB activity.

In this study we have focused on the effects of CM on inflammation and proliferation as two key processes in atherogenesis, since the role of PAR2 in adipokine-induced impairment of vascular reactivity (Romacho et al., 2016a) and the effect of CM on endothelial migration has already been proven (Hu et al., 2013). CM contains numerous molecules derived from adipocytes, which serve as potential ligands for PAR2, thereby contributing

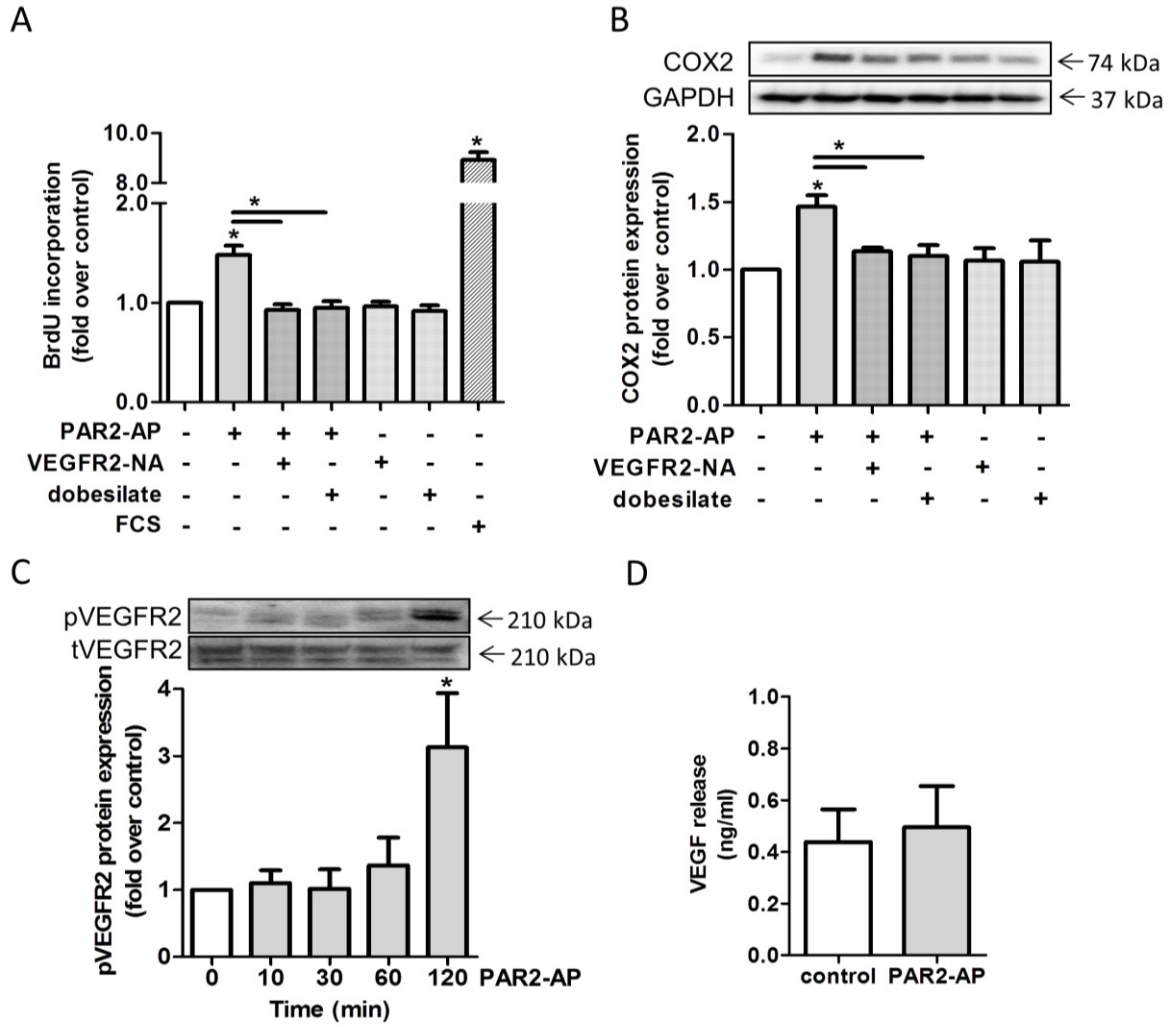
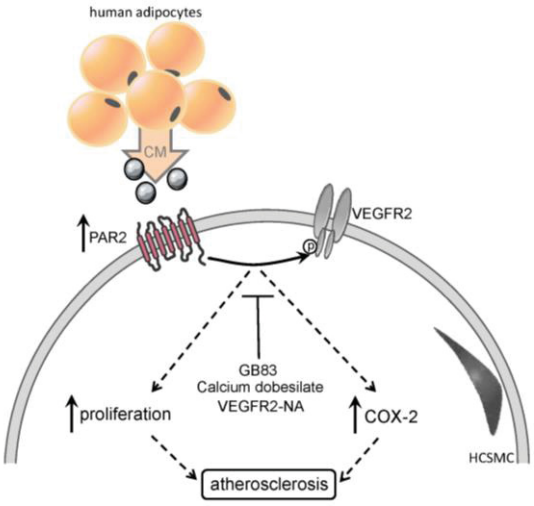


Figure 5: PAR2 mediated proliferation and COX2 upregulation requires intracellular VEGFR2 transactivation. (A) PAR2-AP-induced proliferation. HCSMC were pre-treated with dobesilate (100 µM) or VEGFR2-NA (50 ng/ml) for 1 hour, n=4 **(B)** Effects of dobesilate and VEGFR2-NA on COX2 protein expression after 2 hours; n=3. **(C)** Time course of the PAR2-AP-mediated phosphorylation of the VEGFR2 (Tyr1059); n=4. **(D)** PAR2-AP-triggered VEGF release from HCSMC after 24 hours, n=7. Data are mean values ± SEM (*p<0.05 vs. time 0). Fetal calf serum (FCS), PAR2-activating peptide (PAR2-AP), VEGFR2 neutralizing antibody (VEGFR2-NA).

to promote PAR2-mediated effects, such as proliferation. We have previously demonstrated that the adipokine DPP4 is an activator of PAR2 existing in the CM and driving proliferation through ERK1/2 activation in a PAR2-dependent manner (Wronkowitz et al., 2014). However, our group showed that CM-mediated HCSMC proliferation is mainly due to VEGF abundance (Schlich et al., 2013), which is even higher during obesity (Silha et al., 2005; Loebig et al., 2010; Disanzo and You, 2014). Among the potential factors present in the CM promoting PAR2-mediated proliferation and inflammation we now identified VEGF as an adipokine upregulated in murine CM from AT explants upon HFD (Suppl. Fig. 2A). Moreover, VEGF abundance is positively correlated to BMI of human adipocyte donors (Suppl. Fig. 2B). VEGF signaling occurs via receptor tyrosine kinases (RTKs) particularly VEGF receptor 1 and 2 (VEGFR1 and VEGFR2), of whom the latter is known to be responsible for most of the VEGF-induced actions, including proliferation (Waltenberger et al., 1994; Ferrara et al., 2003; Shibuya and Claesson-Welsh, 2006). In the present study, we prove that PAR2 is involved in HCSMC proliferation. Therefore, we hypothesize that PAR2 and VEGFR2 interaction may lead to COX2 induction resulting in HCSMC proliferation. Nevertheless, there are several factors in the CM, which have to be identified in future studies, potentially leading to PAR2 activation, induction or both via distinct mechanisms such as binding to the binding pocket at the extracellular loop 2 (sDPP4) (Wronkowitz et al., 2014), the cleavage of the N-terminus and unmasking of the tethered ligand (trypsin, cathepsin S) or the disarming of the receptor (cathepsin G) (Kagota et al., 2016). Synthetic PAR2-AP activate PAR2 due to binding to the binding pocket located at the extracellular loop 2 (Adams et al., 2011b) which might result in divergent effects compared with CM-mediated actions. Furthermore we cannot discard the participation of other novel mediators of atherogenesis such as miRNAS (Blumensatt et al., 2014; Novak et al., 2015).

Receptor crosstalk between PARs, other GPCRs and RTKs such as the VEGF receptor has been observed in various cell types for example endothelial or smooth muscle cells (Gschwind et al., 2001; Chandrasekharan et al., 2010; Gieseler et al., 2013; Mazor et al., 2013). To determine if receptor interaction is required in PAR2-mediated proliferation and COX2 upregulation, we assessed the effect of the VEGFR2 inhibitor dobesilate (Angulo et al., 2011) and a VEGFR2-NA. Specific activation of PAR2 by addition of PAR2-AP induced proliferation and COX2 expression which were suppressed by both dobesilate and the VEGFR2-NA. Furthermore, activation of PAR2 resulted in phosphorylation of VEGFR2. Altogether, these results point towards a PAR2-induced transactivation of VEGFR2. Since both receptors are present on the surface of the same cell types, receptor interaction is plausible. Receptor crosstalk occurs via different components such as the release of agonists or transactivation due to intracellular signal transducers (Gieseler et al., 2013). To shed more light onto underlying mechanisms we analyzed whether transactivation of VEGFR2 was ligand dependent or not. The PAR2 agonist did not increase VEGF release from HCSMC indicating that VEGFR2 transactivation is VEGF independent. However, VEGF production in response to PAR2 activation has been described in other cell types (Liu and Mueller, 2006; Dutra-Oliveira et al., 2012; Rasmussen et al., 2012). Nonetheless, other intracellular molecules such as tyrosine kinase Src, ROS and protein tyrosine phosphatases have been discussed to be potential mediators of RTK and PAR interaction (Gieseler et al., 2013). In our case, preliminary data discard the participation of Src (data not shown). Data on receptor transactivation are based mainly on findings focusing on PAR1 and RTKs, others than VEGFR2, thereby demonstrating the novelty of our results. Since precise mechanisms whereby PAR2 promotes activation of VEGFR2 and subsequent proliferation as well as COX2 induction are not yet fully understood, only further investigations will shed light on the specific mechanisms mediating this novel PAR2-VEGFR2 transactivation.



Conclusion

We have demonstrated a pivotal role of PAR2 in obesity-related pro-atherogenic events. Adipocyte-derived CM from obese subjects triggered PAR2 induction in HCAEC and HCSMC. In turn, PAR2 upregulation might facilitate CM-induced proliferation and COX2 induction in HCSMC. We propose that during obesity upregulation and activation of PAR2 by elevated levels of pro-inflammatory adipokines in the circulation, results in NFκB activation, COX-2 induction and proliferation, key processes in the development of atherosclerosis. Importantly, these pro-atherogenic events involved transactivation of the VEGFR2 by PAR2. Therefore, we propose an essential function of PAR2 in vascular cells and present PAR2 as a potentially useful therapeutic target in the treatment of obesity-associated atherogenesis. Therefore, it is of great importance to investigate the exact mechanisms by which PAR2 transactivates the VEGFR2 and to identify certain PAR2 activators triggering the observed effects.

Ethics approval
Animal care and use were approved by the local Animal Ethics Committee according to the principles outlined in the European Commission Council Directive (86/609/EEC).

Conflict of Interest Statement
The authors declare that there is no conflict of interest.

Authors' Contributions
II: data collection and analysis, study design, drafted the manuscript. TR: study design, critical revision of the manuscript. JE: study design, critical revision of the manuscript. All authors read and approved the final manuscript.

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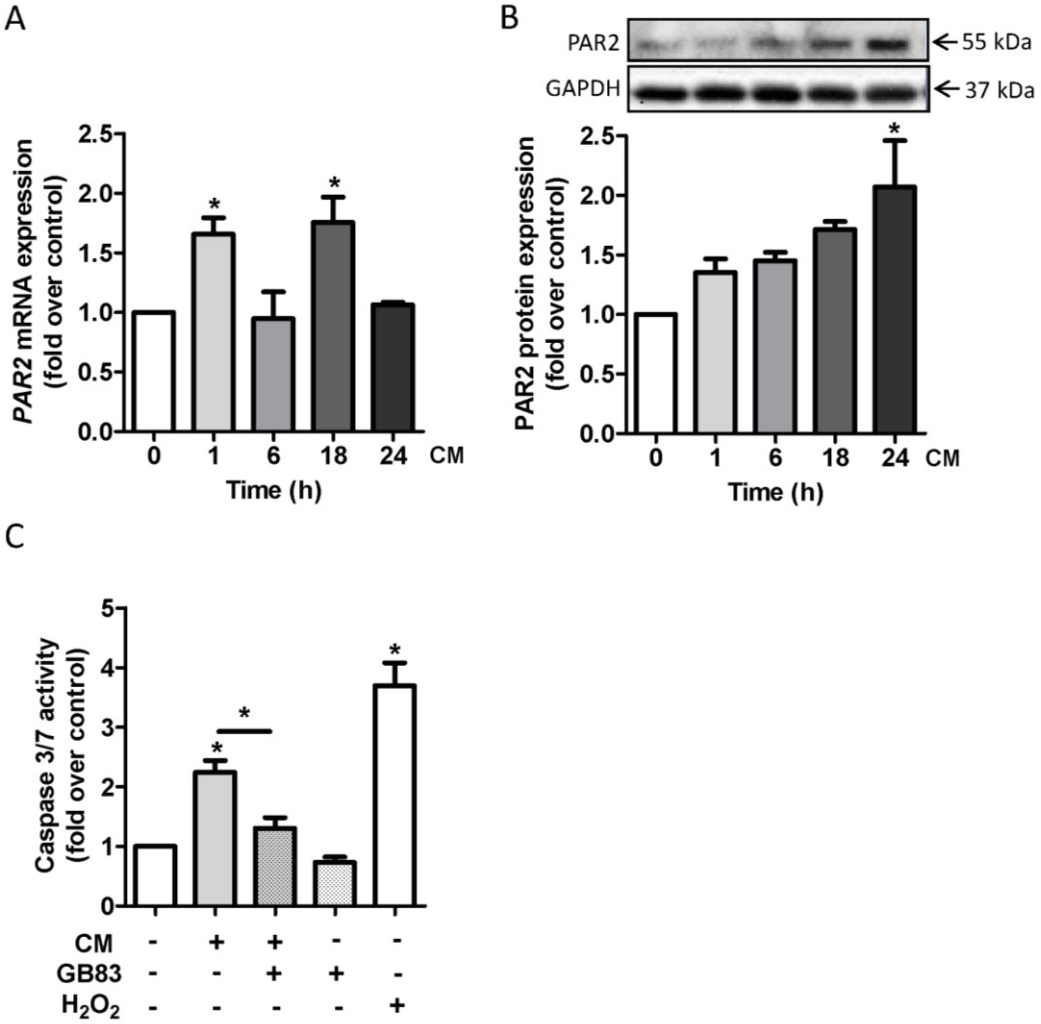
ABBREVIATIONS

AT, adipose tissue; BMI, body mass index; CM , conditioned media; COX2, cyclooxygenase 2; CVD , cardiovascular disease; FCS, fetal calf serum; GAPDH; glyceraldehyde-3-phosphate dehydrogenase; HCAEC, human coronary artery endothelial cells; HCSMC, human coronary smooth muscle cells; HFD, high fat diet; PAR2, protease-activated receptor 2; PAR2-AP , protease-activated receptor 2 activating peptide; SEM , standard error of the mean; VEGF, vascular endothelial growth factor; VEGFR2 , vascular endothelial growth factor receptor 2; VEGFR2-NA, vascular endothelial growth factor receptor 2 neutralizing antibody

Supplemental Material



Suppl. Fig. 1: CM induces PAR2 and enhances apoptosis in HCAEC. (**A-B**) CM induced PAR2 mRNA and protein expression in HCAEC over time. Data were normalized to β-actin or GAPDH, respectively; n=4-6 (*p<0.05 vs. time 0). (**C**) Caspase 3/7 activity in HCAEC exposed to CM for 18 hours and in combination with GB83 (10μM). 200 μM H2O2 was used as a positive control; n=5. Data are mean values ± SEM (*p<0.05 vs. control). Conditioned medium (CM), human coronary artery endothelial cells (HCAEC).

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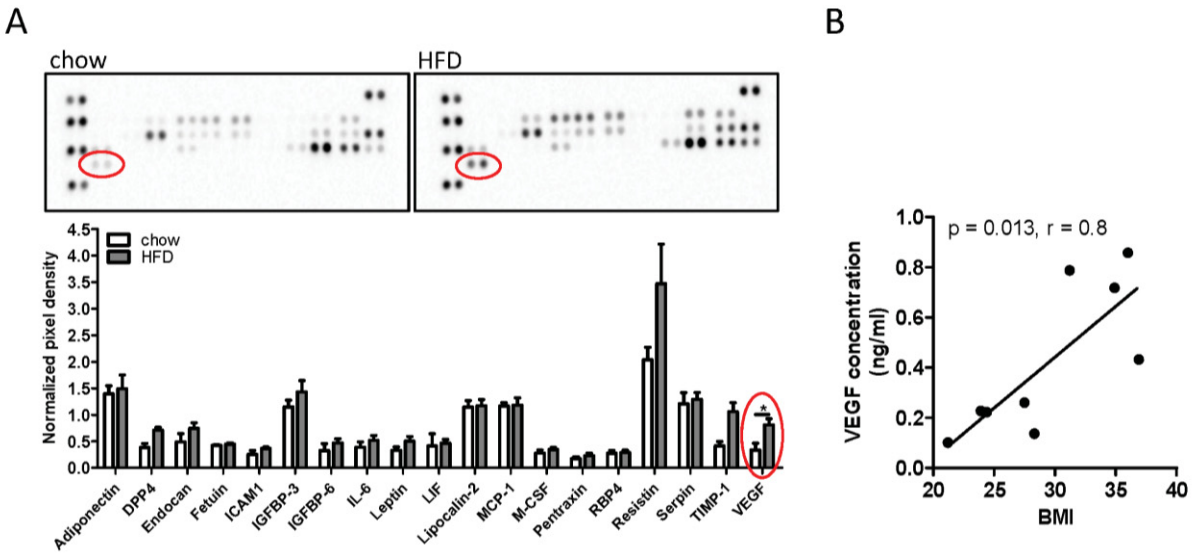

Suppl. Fig. 2: Secretion of adipokines from murine adipose explants or human adipocytes. (A) Secretion profile of murine adipose tissue explants from chow- and HFD-fed animals; n= 3-7 (*p<0.05). **(B)** Correlation of donor BMI and VEGF concentration in CM from human primary adipocytes from respective autopsy donors; n=9 Data are mean values ± SEM (*p<0.05). Body mass index (BMI), Conditioned medium (CM), vascular endothelial growth factor (VEGF).

2.4 Novel Mediators of Adipose Tissue and Muscle Crosstalk

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Abstract— **The crosstalk between adipose tissue and skeletal muscle has gained considerable interest, since this process, specifically in obesity, substantially drives the pathogenesis of muscle insulin resistance. In this review, we discuss novel concepts and targets of this bidirectional organ communication system. This includes adipo-myokines like apelin and FGF21, inflammasomes, autophagy, and miRNAs. Literature analysis shows that the crosstalk between fat and muscle involves both extracellular molecules and intracellular organelles. We conclude that integration of these multiple crosstalk elements into one network will be required to better understand this process.**
Keywords— **Interorgan crosstalk . Adipo-myokines . Inflammasomes . Autophagy . miRNA**

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I. INTRODUCTION

Biological crosstalk occurs between different organs either directly in a paracrine way at tissue interfaces or via biological fluids in an endocrine way. Endocrine organs such as adipose tissue and skeletal muscle express and subsequently release a wide range of proteins and other molecules into the circulation, thereby participating in a multidirectional crosstalk. Secretion of the so-called adipokines or myokines by adipose tissue or skeletal muscle, respectively, plays a crucial role in the regulation of multiple organs or tissues in terms of energy homeostasis [1]. Based on an intensive search of literature on novel concepts and targets for crosstalk between adipose tissue and skeletal muscle from the last 3 years, we selected four innovative topics for this review: 1. While adipokines and myokines are known to be important endocrine mediators in the field of metabolic homeostasis, recent advances have been made to identify common molecules to be released by both tissues, the so-called adipo-myokines. These molecules may play a yet underestimated role in the bidirectional crosstalk between adipose tissue and muscle. 2. Inflammasomes as specialized organelles may contribute to an endocrine crosstalk between adipose tissue and skeletal muscle in a way that is still incompletely understood. 3. Very recently, autophagy has been recognized as an additional mediator of organ crosstalk. 4. In addition to metabolites and proteins, circulating microRNAs have been associated with regulation of metabolic homeostasis, representing a further level of organ communication.

II. ADIPO-MYOKINES – MEDIATORS OF BIDIRECTIONAL CROSSTALK

Skeletal muscle cells and adipocytes secrete a variety of metabolically active proteins called myokines or adipokines, respectively. Besides, a new term referred to as adipo-myokines has been introduced. Adipo-myokines are expressed and released from both skeletal muscle and adipose tissue. Secreted adipo-myokines can affect the organ itself in a paracrine or autocrine manner. Additionally, they have endocrine effects, contributing to a bidirectional crosstalk. Gene expression of adipo-myokines is shifted in obesity or after exercise, thus affecting the circulating pool and the crosstalk between adipose tissue and skeletal muscle.

Besides the well-characterized adipo-myokines such as IL-6 and IL-15, several factors have been identified as adipomyokines in recent years, including follistatin-related protein 1 (Fstl-1), plasminogen activator inhibitor 1 (PAI1), myostatin, and many more [2]. In particular, the adipomyokines apelin and fibroblast growth factor 21 (FGF21) gained considerable interest and will be presented in detail here.

Apelin

Apelin is a peptide binding to the apelin receptor (APJ), a G-protein-coupled receptor [3]. APJ expression is ubiquitously including skeletal muscle and adipose tissue [4], so that both tissues display possible targets. Apelin has been suggested to have cardio-protective impact [5] and is an important player in energy metabolism with anti-obesity and anti-diabetic properties [6]. Until recently, apelin has been described as an adipokine expressed and released from adipocytes [7]. Apelin expression in adipose tissue as well as circulating levels of apelin are elevated in obesity [8]. Furthermore, protein expression of apelin in adipocytes and the circulation have been reported to be increased in mice associated with hyperinsulinemia [8]. In addition, messenger RNA (mRNA) expression of apelin was induced by insulin in murine 3T3-L1 adipocytes [9]. Injection of apelin lowered blood glucose levels in mice [10] and stimulated glucose uptake in adipose tissue [11]. Amplified glucose uptake was also observed in human adipose tissue [12]. Besides, treatment of apelin diminished triglyceride

amounts in adipose tissue in vivo [10] and decreased lipid storage in 3T3-L1 preadipocytes [13]. In summary, these findings disclose the fundamental role of apelin in glucose and lipid metabolism.

Recently, apelin was identified as a myokine, expressed and secreted from human myotubes [14•]. Apelin expression is upregulated due to exercise in obese subjects, and apelin mRNA expression in muscle is positively related to improved insulin sensitivity. In addition, apelin improved glucose uptake in C2C12 myotubes and skeletal muscle insulin sensitivity in mice [11]. These results were supported by observations from apelin-deficient mice, which had reduced insulin sensitivity and were hyperinsulinemic. Indeed, administration of apelin to these mice led to improved insulin sensitivity [15]. Disruption of apelin also had a negative impact on glucose uptake and Akt-phosphorylation in skeletal muscle [15]. Furthermore, apelin is suggested to promote fatty acid oxidation in skeletal muscle [16]. These data indicate that circulating apelin, derived from muscle and adipose tissue, has beneficial effects on lipid and glucose metabolism in both tissues.

FGF21

FGF21 belongs to the fibroblast growth factor (FGF) family which contains 22 members in humans. FGFmembers play an important role in the regulation of whole-body metabolism and development. They are expressed ubiquitously in almost every tissue and act in a paracrine as well as endocrine manner. Endocrine FGFs, like FGF15/19, FGF21, and FGF23, signal through high-affinity FGF receptors (FGFR), depending on α-klotho or β-klotho as a co-receptor [17]. FGF21 in particular requires the co-receptor β-klotho [18] to bind and fully activate FGFR.

FGF21 is mainly expressed and secreted by the liver [19, 20], but also skeletal muscle cells [21–23] and adipocytes [24] have been reported as sources for FGF21. Moreover, these tissues display suitable targets for FGF21 action since they express β-klotho and FGFR [25, 26].

FGF21 serum levels are upregulated in subjects with impaired glucose tolerance, type 2 diabetic patients, and obese nondiabetic subjects [27]. Furthermore, enhanced FGF21 serum levels correlate with high expression of FGF21 in subcutaneous adipose tissue [28], implicating that adipose tissue is a relevant source. FGF21 reduces lipolysis in vivo [29] and in vitro [30], potentially interfering with growth hormone (GH)-mediated lipolysis [29]. Moreover, FGF21 stimulates glucose uptake in human adipocytes [31, 32] in relation to FGF21-induced GLUT1 expression [31]. Thus, based on these data and in line with animal studies [33, 34], FGF21 arises as a potential factor to improve insulin sensitivity in adipose tissue, indicating that elevated levels of FGF21 in obesity could be a compensatory response or resistance to FGF21.

FGF21 has also been described as an exercise-regulated myokine, expressed and secreted by skeletal muscle cells, although at lower levels compared to adipose tissue and liver. In young healthy women, levels of FGF21 in serum were elevated after long-term exercise over 2 weeks, but not enhanced after acute exercise [35]. Controversially, in healthy male subjects, acute exercise raised circulating levels of FGF21 [36]. Another study consisting of nondiabetic obese women showed even reduced FGF21 plasma levels after a 12- month exercise program [37]. Furthermore, Kim et al. observed increased serum FGF21 after a single bout of acute exercise in healthy men and mice. Nevertheless, increase in FGF21 expression could not be detected in WAT or skeletal muscle but was elevated in liver of mice after acute exercise. However, in these studies, the potential source of circulating FGF21 was not determined. It has been suggested that FGF21 plays a special role in mediating the stress response from skeletal muscle to adipose tissue by modulating lipolysis and gene expression in adipose tissue [38]. An adipokine upregulated by FGF21 is adiponectin, potentially mediating the positive metabolic effects of FGF21 on whole-body glucose metabolism and insulin sensitivity [38, 39•]. Functions of FGF21 in skeletal muscle itself might be the induction of insulin-stimulated glucose uptake in a paracrine manner [19] and the protection from insulin resistance [39•, 40].

FGF21 acts in a paracrine fashion by regulating metabolism in tissues of its origin such as liver, adipose tissue, and skeletal muscle. Furthermore, it operates as an endocrine factor released in the circulation and arises as an important player in the regulation of energy homeostasis and whole-body glucose and lipid metabolism. Although its impact on tissues is still not completely understood, this factor might be of interest as a potential therapeutic agent to treat metabolic diseases.

III. INFLAMMASOMES – CRITICAL PLAYERS IN ADIPOSE TISSUE CROSSTALK

Specialized organelles called inflammasomes, especiallyNLR family, pyrin domain-containing 3 (NLRP3) inflammasomes, have gained much attention over the last years in research on metabolic diseases. This organelle is able to sense a wide range of exogenous and endogenous danger signals via pattern recognition receptors in order to translate these signals into activation of caspase 1 and subsequent production of IL-1β from pro-IL-1β. Inflammasomes form on demand by assembly of multiple proteins into a multiprotein complex containing one or more cofactors such as PYD and CARD domain-containing protein (PYCARD, ASC) and caspase 1. Originally, inflammasomes were only described to be present in immune cells as part of the innate immune response. Today, inflammasomes have been described in various cell types including adipocytes, hepatocytes, and pancreatic β-cells. Inflammasomes are major players in regulating adipose tissue inflammation as they act as cellular sensors for obesityassociated activators of caspase-1 such as palmitate and ceramide. Subsequently, inflammasomes act as regulators of inflammation and associated effects on insulin signaling in other organs including skeletal muscle.

Inflammasomes are activated in adipose tissue of obese mouse models [41] as well as in obese humans [42]. In the latter study in humans, induction of inflammasomes in adipose tissue is clearly associated with insulin resistance and impaired glucose metabolism in coincidence with a T cell shift towards T helper cells of the proinflammatory TH1 type. Accordingly, weight loss achieved either by caloric restriction or by exercise reduces inflammasome activity [43]. NLRP3 inflammasome levels also positively correlate with glycemia in type 2 diabetic patients after weight loss induced either by caloric restriction or by exercise [43].

Within adipose tissue, inflammasomes are also important regulators of adipocyte function and insulin sensitivity, which has been demonstrated first in caspase 1 and NLRP3 knockout mice [41, 44]. Lacking inflammasome activity in these models protects from insulin resistance. Inflammasomes are also critical for the induction of obesity [44]. Inflammasomes are crosstalk mediators, a concept that has not been proven directly for the adipose tissue and muscle crosstalk but for a crosstalk between adipose tissue and liver. Activation of inflammasomes, specifically in macrophages, contributes to the development of insulin resistance in hepatocytes in vitro [45]. Similarly, selective activation of inflammasomes in adipocytes also leads to impaired insulin signaling in hepatocytes [46].A functional link between adipose tissue inflammation in obesity and insulin resistance in skeletal muscle can be deduced from activation of inflammasomes specifically in immune cells within adipose tissue which leads to deteriorated insulin tolerance in mice [45]. Indirect evidence comes from inflammasome-deficient mice that have significantly improved insulin signaling in skeletal muscle, adipose tissue and liver in diet-induced obesity demonstrating that all major insulin target tissues are equally involved.

Less is known about the role of inflammasomes in skeletal muscle. Nevertheless, inflammation in skeletal muscle can also result in production of IL-1β [47]. After induction of IL-1β by injury, this cytokine stimulates myoblast proliferation [48]. In myotubes, IL-1β stimulates catabolism [49]. Furthermore, inflammation in skeletal muscle induces amyloid precursor protein (APP) and subsequent intracellular aggregation of β-amyloid in parallel to IL-1β induction [50]. This is an interesting observation, as inflammasome activation is also related to Alzheimer disease where aggregation of β-amyloid causes tissue degeneration, which may be translated to skeletal muscle dysfunction. In addition, IL-1β can also be induced by exercise in vitro [51]. In the light of IL-1β and inflammasomes being also activated in skeletal muscle, therapeutic approaches to modulate inflammasome activation in metabolic diseases have been tested. Blocking of IL-1β signaling by anakinra improves glycemia in type 2 diabetes [52], but does not alter skeletal muscle gene expression [53]. It is currently discussed that IL-1β and inflammasomes are not playing the major role in skeletal muscle in the context of metabolic diseases related to obesity. However, data obtained in animal models clearly reveal a novel metabolic function of the inflammasomes in adipose tissue. Overall, these data suggest that pharmacological modulation of inflammasome activation in obese patients or patients with type 2 diabetes could be a strategy to improve the metabolic function of adipose tissue. This might then contribute to recover insulin sensitivity in skeletal muscle, adding a novel level of organ crosstalk.

IV. AUTOPHAGY – A NEW ELEMENT IN ORGAN CROSSTALK

Autophagy, from Greek “self-eating”, describes a highly conserved catabolic process of degrading and recycling of misfolded or damaged proteins and organelles in lysosomes. Autophagy is an enduring process, regulated for example by amino acid and glucose availability in the basal state [54, 55•], but can also be modulated by diverse factors such as starvation or other kinds of stress [56]. Under these circumstances, the assembly of phagosomes is strongly induced [57, 58] to provide energy and metabolic substrates to the cell. Three different kinds of autophagy, namely chaperone-mediated autophagy, micro-autophagy and macro-autophagy, can be distinguished. However, macro-autophagy is mostly referred to “autophagy” and will be the subject of this section. As a complicated process, autophagy is separated into several steps including the formation of the so-called autophagosome surrounding the target substrate, fusion with the lysosome, degradation, and amino and fatty acid generation. These steps are closely regulated by a group of autophagy-related (Atg) proteins.

Currently, autophagy has gained a lot of interest in the field of energy homeostasis and the pathophysiology of the metabolic syndrome [59]. Impaired autophagy has differential effects as it can on one hand deteriorate metabolic control [45, 60] while it can also lead to improved insulin sensitivity together with beneficial effects in the context of obesity and type 2 diabetes [55•]. In the obese state, autophagy in macrophages is impaired which is accompanied by defective mitophagy and increased IL-1β production by activated NLRP3 inflammasommes [45]. As a result of autophagy-induced IL- 1β release, insulin resistance develops in adipose tissue and skeletal muscle. Furthermore, another study shows that inhibition of autophagy leads to enhanced levels of proinflammatory cytokines such as IL-6, IL-1β, and IL-8 in adipose tissue explants of mice and humans [61]. Thus, autophagy might control the release of pro-inflammatory cytokines and therefore contribute to the prevention of inflammation. Recently published data also show beneficial effects of autophagy, namely adiponectin-stimulating skeletal muscle autophagy to reduce high-fat diet (HFD)-induced insulin resistance [62].

Interesting insight into the role of autophagy in skeletal muscle and adipocytes on whole-body glucose and lipid metabolism has been obtained by tissue-specific depletion of Atgs in mice. Autophagy deficiency due to skeletal muscle-specific deletion of Atg7 contributes to the improvement of diet-induced insulin resistance and obesity [55•]. Interestingly, impaired autophagy is

mechanistically linked to mitochondrial dysfunction and subsequent FGF21 release. Endogenous FGF21 entering the circulation can then act as a mediator of crosstalk between different kinds of tissues by modulating the lipid metabolism. Furthermore, mice with disruption of Atg7 in skeletal muscle show increased β-oxidation and energy expenditure through thermogenic uncoupling. Additionally, the specific knockout of Atg7 in adipose tissue of mice has protective function regarding HFD-induced obesity and glucose tolerance [63]. In detail, impairment of autophagy through Atg7 ablation causes decreased white fat mass, smaller adipocyte size, and higher mitochondrial content potentially coupled to increased energy expenditure in adipose tissue. These animal data are partially contradictory to data obtained in vivo in obese humans and rodents illustrating the need of additional mouse model such as inducible tissue-specific knockouts to clarify the role of autophagy at different disease states.

Taken together, these findings indicate that autophagy deficiency in specific organs affects whole-body metabolism in various ways also involving endocrine mediators. The importance of autophagy is still unclear and has to be further elucidated to benefit from autophagy-related crosstalking mediators, such as FGF21, to prevent and treat obesity and metabolic diseases such as type 2 diabetes.

V. MICRORNAS – AN ADDITIONAL LEVEL OF CROSSTALK

In addition to adipokines and myokines, microRNAs (miRNAs) as a class of small noncoding RNAs increase the complexity of crosstalk between organs in terms of metabolic control [64]. Per definition, miRNAs are 20–22 nucleotides long, single-stranded noncoding RNAs that regulate gene expression at the level of target messenger RNAs (mRNAs). miRNAs bind by base-pairing with complementary sequences within target mRNAs resulting in silencing of target mRNAs by cleavage, destabilization, and less-efficient translation. Today, nearly 2000 different miRNAs are known within the human genome with a constant increase in their number [65]. miRNAs are not only regulated by various stimuli including metabolites, adipokines, and myokines [66], but they also regulate how organs communicate via released factors and thereby modulate metabolic control. Thus, miRNAs are participating in tissue crosstalk in multiple ways, first representing crosstalk molecules themselves altering gene expression in distant organs, second regulating expression of other crosstalk molecules, and third being regulated by other crosstalk molecules.

Both adipose tissue and skeletal muscle release various miRNAs [67]. Although, the overall profile of expressed miRNAs is different between adipose tissue and skeletal muscle, there is also a substantial overlap of common miRNAs for both tissues. Several miRNAs were associated with hypoglycemia in skeletal muscle such as miR-222, miR-27a, miR- 195, miR-103, and miR-10b. For miR-222 and miR-27a, regulation by hypoglycemia is also observed in adipocytes as a response to increased glucose levels, which favors the idea of a crosstalk between skeletal muscle and adipose tissue via miRNA in relation to metabolic dysfunction.

Obesity alters miRNA expression in adipose tissue mostly affecting adipogenesis [68]. Several miRNAs have been described to be regulated in the state of insulin resistance and inflammation in adipose tissue as for example miRNA 143 [69], miRNA 223 [70], and miRNA 93 [71•]. One interesting study revealed that the cluster of miR-126/miR-193b/miR-92a control monocyte chemotactic protein-1 (MCP-1) production by transcription factors, indicating that miRNAs can contribute to adipose tissue inflammation and may be important for the development of insulin resistance and potentially type 2 diabetes. miRNA 378 is increased in obesity and type 2 diabetes in mice and reduces adiponectin expression, which might be related to insulin resistance [72]. Another feature of miRNAs in adipose tissue is the fact that miRNA processing in general and several single miRNAs alter distribution and determination of white and brown adipocytes. Ablation of dicer, the enzyme that catalyzes the final maturation of all miRNAs, in adipose tissue of mice leads to partial dystrophy and a whitening of brown adipose tissue demonstrating the importance of miRNAs in adipose tissue development and determination [73]. As for single miRNAs, miRNA 133 is able to induce browning of skeletal muscle precursors in vitro and to increase browning in vivo [74, 75].

miRNA expression is also altered in skeletal muscle in relation to insulin resistance [76]. Unsaturated fatty acids potentially released from adipose tissue upregulate miRNA 29a which then negatively interferes with insulin signaling [77]. Another miRNA, namely miRNA 106b, induces insulin resistance via mitochondrial dysfunction in skeletal muscle cells [78]. This miRNA is also significantly increased in skeletal muscle of patients with type 2 diabetes [76]. Diminished miRNA expression in general in skeletal muscle is observed in aging [79] and might also be related to age-induced metabolic disturbances, a topic that should be further pursued in the future.

Although many miRNAs are described to be involved in adipose tissue inflammation and insulin resistance as well as in skeletal muscle insulin resistance, so far only single miRs have been identified to be real crosstalk molecules. This is the case for miRNA 130b and miRNA 2 [80, 81•]. miRNA 130b might even be of interest for therapeutic use as its delivery by microvesicle to fat cells decreases lipid deposition via the transcription factor PPAR gamma [82]. As only a few miRNAs are already identified as crosstalk molecules, there is a huge potential of identifying novel miRNAs together with their regulators and targets for diagnostic and therapeutic intervention which is not limited to metabolic disease.

VI. DISCUSSION

Extensive research during the last 20 years has substantially promoted our understanding of how different organs communicate within the body and how

this complex process impacts on metabolic homeostasis in both health and disease. Due to its prominent secretory activity, adipose tissue is now considered as a major player in organ crosstalk. However, skeletal muscle also acts as an endocrine organ, and a highly complex multidirectional crosstalk scenario is operating in humans. Classically, protein molecules (adipokines, myokines) released from these tissues are consider ed to mediate the communication between different organs, but recent evidence points to a much more complex structural hierarchy of organ crosstalk. Thus, intracellular organelles like inflammasomes and autophagosomes have been recognized to regulate organ communication, albeit in an indirect and yet incompletely understood way.Whereas the inflammasome is most important for adipose tissue crosstalk function, autophagy appears to influence the fat/muscle crosstalk in both tissues. Finally, circulating miRNAs constitute an additional element of cellular communication and extend the complexity of this process. The future challenge will be to integrate the different levels of organ crosstalk into one network, to potentially identify new targets of metabolic homeostasis.

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Compliance with ethics guidelines

Conflict of interest
Ira Indrakusuma, Henrike Sell, and Jürgen Eckel declare that they have no conflict of interest.

Human and animal rights and informed consent
This article does not contain any studies with human or animal subjects performed by any of the authors.

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2.5 Contribution statement

DPP4 deletion in adipose tissue improves hepatic insulin resistance in diet-induced obesity

Romacho T, Sell H, **Indrakusuma I**, Roehrborn D, Castañeda T, Jelenik T, Hartwig S, Weiß J, Al-Hasani H, Roden M, Eckel J. Cell reports (2016) under review.

Impact factor: 7.87

Contribution	Total: 32.5 % Conceived / designed experiments: 25 % Performed experiments: 50 % Analysed data: 50 % Contributed to discussion: 30 % Wrote the manuscript: 10 % Reviewed / edited manuscript: 30 % Author: 3rd author

Soluble dipeptidyl peptidase-4 induces microvascular endothelial dysfunction through proteinase-activated receptor-2 and thromboxane A2 release

Romacho T, Vallejo S, Villalobos LA, Wronkowitz N, **Indrakusuma I**, Eckel J, Sánchez-Ferrer CF, Peiró C. J Hypertension (2016) 34(5):869-76

Impact factor: 5.06

Contribution	Total: 8.3 % Conceived / designed experiments: 10 % Performed experiments: 10 % Analysed data: 10 % Contributed to discussion: 10 % Wrote the manuscript: - Reviewed / edited manuscript: 10% Author: 5th author

Protease-Activated Receptor 2 promotes pro-atherogenic effects through transactivation of the VEGF receptor 2 in human vascular smooth muscle cells.

Indrakusuma I, Romacho T, Eckel J. Front Pharmacol (2016), in press.

Impact factor: 4.41

Contribution	Total: 90 % Conceived / designed experiments: 80 % Performed experiments: 90 % Analysed data: 100 % Contributed to discussion: 80 % Wrote the manuscript: 100 % Reviewed / edited manuscript: 90% Author: 1st author

Novel mediators of adipose tissue and muscle crosstalk

Indrakusuma I, Sell H, Eckel J (2015). Curr obes reprots. 118 84-91.

Impact factor: to be released

Contribution	Total: 73.3 % Conceived / designed experiments: - Performed experiments: - Analysed data: - Contributed to discussion: 60 % Wrote the manuscript: 70 % Reviewed / edited manuscript: 90% Author: 1st author

3 DISCUSSION

3.1 Characterization of the adipose tissue specific DPP4 KO mouse model and the role of DPP4 in a local and systemic view

AT is recognized as a true endocrine organ, secreting adipokines, with auto/paracrine and systemic impact. The development of obesity is accompanied by increasing fat mass due to excessive energy intake and a dysfunctional and inflamed adipose tissue. As a consequence, there is a change in the pool of secreted adipokines. High levels of pro-inflammatory adipokines have been reported in T2DM, IR or CVD.

DPP4 has been described as a novel adipokine secreted by human adipocytes and thus potentially affecting function of distant tissues. In pathophysiological conditions such as obesity and insulin resistant states, circulating levels of DPP4 are upregulated [68]. Therefore, our group proposed DPP4 as a candidate linking obesity and the metabolic syndrome. DPP4i are potent glucose lowering agents, due to prolongation of the half-life of the incretins GIP and GLP-1, and are extensively used in the treatment of T2DM. In the vascular system, actions of DPP4 have been reported to impair EC and SMC function. In addition, DPP4i have been discussed to exert cardio-protective properties, too. However, knowledge about the role of DPP4 or direct actions of DPP4 itself is quite limited at the moment. To further advance the therapeutic utility of DPP4i it is of great importance to understand the physiological and pathophysiological role of DPP4. For this purpose, we aimed to elucidate the relevance of AT derived DPP4 locally and on systemic levels. Therefore, a unique AT-DPP4-KO mouse model was generated and analyzed over 24 weeks receiving either a chow or HFD. In a next step our aim was to clarify the role of sDPP4 in the vascular system through *ex vivo* experiments in murine mesenteric arteries.

3.1.1 DPP4 and its role within adipose tissue

3.1.1.1 Adipose tissue inflammation and the role of DPP4

Upon progression of obesity, AT undergoes structural and phenotypic modulation accompanied by the onset of low-grade inflammation. These alterations in AT occur partly due to infiltration of M1 macrophages, preceded by activation of T- and B-cells and a polarization of local anti-inflammatory M2 macrophages towards M1 macrophages [184]. Weisberg et al. proved the overall accumulation of macrophages during the development of obesity [185]. M1 macrophages secrete pro-inflammatory cytokines and chemokines, such as MCP-1 or IL-6 [21]. In this context the dynamics between M1 and M2 macrophages within AT has been assigned an important role in obesity-induced disorders [186, 187] . After 24

weeks of HFD we found higher levels of IL10 and MRC1 in the AT of AT-DPP4-KO mice pointing towards high abundance of M2 macrophages. However, expression of M1 macrophage markers such as MCP-1 and IL-6 were increased too. 90% of adipose tissue infiltrating macrophages are localized around dead adipocytes and form characteristic crown-like structures (CLS) in order to take up and digest cell debris as well as scavenge free lipid droplets [188]. We analyzed the amount of CLS in AT of AT-DPP4-KO and WT mice by the use of galectin-3 immunostaining. Despite being a M1 macrophage marker, galectin-3 might play a role in healthy AT expansion and glucose tolerance under DIO, illustrated by a galectin-3 KO mouse model [189]. Quantification of galectin-3 staining revealed increased appearance of CLS in epididymal AT of KO mice under HFD compared to WT animals. In principle, subcutaneous AT contained a much smaller number of CLS than epididymal AT. However, there were no differences in CLS abundance between genotypes in the subcutaneous AT. A recent paper supports a preferential visceral compared to subcutaneous AT macrophage infiltration also in obese human patients [190].

Inflammation of AT is not only associated with impaired organ function but in a mild form also supports healthy AT expansion [191], as reported for galectin-3. Interestingly, Kostelli et al. observed that acute weight loss or pharmacological induction of adipocyte lipolysis elicits macrophage accumulation illustrated by an increase in macrophage-specific genes, F4/80, CD38 and CSF-R1 [192]. They claim that macrophage infiltration is dependent on lipid metabolism. Due to lipid uptake by macrophages and subsequent decreased adipocyte stress, local adipocyte function is protected [192]. For that reason, accumulation of AT macrophages might be a physiological healthy response. Moreover, recent observations by Schmitz et al. highlight that a subgroup of extremely obese subjects maintains increased numbers of macrophages in AT despite weight loss and improved insulin sensitivity [193]. Another feature of macrophages is helping to remove necrotic adipocytes and their debris, additionally indicating that recruitment of macrophages is beneficial for normal adipocyte function [188]. However, the regulation of macrophage infiltration may vary, thus further investigations are needed to clarify the role of AT macrophages during obesity.

Regarding the data obtained from the AT-DPP4-KO model, we conclude that DPP4 specific deletion in AT might drive local low-grade inflammation illustrated by enhanced macrophage markers and increased CLS, thereby improving beneficial AT expansion. In accordance, Wernstedt et al. demonstrate that pro-inflammatory signaling in AT is essential for healthy AT remodeling and expansion [194]. Studies with DPP4i, however, detect direct beneficial impact on inflammation. Inhibition of DPP4 by des-fluoro-sitagliptin positively

affected AT inflammation independently of GLP-1 [195] . This was confirmed by Dobrian and colleagues who could show that sitagliptin minimized expression of pro-inflammatory cytokines such as MCP-1, IL-6, IL-12 in AT [196]. In vivo, mice challenged with HFD are more insulin sensitive and exhibit lower expression of the macrophage marker F4/80 when treated with linagliptin compared to control mice [197]. However, F4/80 is expressed in both, M1 and M2 macrophages [21], and thus does not serve as a marker for one of the two macrophage types. In addition, Zhong et al. suggest a role for DPP4 in low-grade AT inflammation in obesity or T2DM, because they determined that AT macrophages reveal enhanced DPP4 levels in response to inflammation or obesity, which binds to ADA and subsequently recruits adipose T-cells [107]. Thus, these and our data give evidence for a key role of DPP4 in AT low-grade inflammation during obesity and T2DM. However, the mechanism of action and detailed function of DPP4 remains unclear and has to be further investigated.

3.1.1.2 DPP4 and adipose tissue remodeling

AT remodeling is a crucial determinant of the pathophysiology of obesity [198]. The process comprises not only macrophage infiltration and AT inflammation but also AT expansion due to adipocyte hyperplasia and hypertrophy. Whereas occurrence of hypertrophy in the obese state is well accepted, the likelihood to increase adipocyte number is still under discussion. In this regard, Spalding and colleagues showed by use of ^{14}C incorporation, that adipocyte number is presumably fixed in adulthood [199]. Thus adipocyte turnover, characterized by adipocyte death and generation, is not changed during the onset of obesity [199].

The amount of DPP4 within human AT has been associated with AT inflammation and correlates with adipocyte size [68]. Consistently, under HFD the AT-DPP4-KO revealed reduced adipocyte size compared to WT animals. The abundance of small-sized adipocytes indicates the recruitment of adipocyte precursor cells and differentiated young adipocytes, pointing towards a healthy AT remodeling [187]. Although we observed a significant reduction in adipocyte size in both, visceral and subcutaneous depots, another study could detect parameters of remodeling only in epididymal fat of mice under DIO [200]. However, they did not find increased levels of the preadipocyte marker Pref-1 [201] concluding that smaller adipocytes did not occur due to enhanced classical adipogenesis. Likewise we observed only a small trend towards higher levels of adipogenesis markers such as adiponectin, PPARγ and Slc2a4, in epididymal WAT of AT-DPP4-KO mice. It can be speculated that the reduction in adipocyte size might be based on the loss of DPP4 in

adipocytes, but is independent of adipogenesis. Our findings are consistent with data obtained by Dobrian and colleagues, who discovered reduced number of very large adipocytes accompanied by increased number of small adipocytes after treatment with sitagliptin [196]. Accordingly, DPP4i evogliptin minimzed adipocyte size, but contrarily to our data, also affected weight loss probably due to higher energy expenditure [202]. Moreover, teneligliptin, a DPP4i approved only in Korea and Japan, reduces fat mass and suppresses adipocyte hypertrophy in HFD-fed mice [203].

During AT remodeling the extracellular matrix (ECM), physiologically exhibiting mechanical support for the AT and participating in multiple signaling pathways, accommodates to AT alterations [204]. The flexibility of the ECM to respond to AT expansion counteracts the development of metabolically dysfunctional adipocytes [187]. Obesity leads to upregulation of ECM components such as collagen, thereby promoting AT fibrosis [205], a hallmark of impaired AT function [206]. In detail, enhanced expression of ECM components increases ECM stiffness and reduces AT plasticity and expansion [206]. Since fibrosis has been reported to negatively correlate with adipocyte diameter [204, 206], we wanted to examine if this was the cause for reduced adipocyte size in our AT-DPP4-KO model. Analysis of different fibrosis markers resulted in a rejection of this hypothesis. In contrary, collagens in the AT of the AT-DPP4-KO animals were significantly lower, thereby strengthening the beneficial impact of DPP4 depletion on AT remodeling. A role for DPP4 in AT remodeling has been suggested in omental and subcutaneous AT by other studies, too [207]. However, in the AT-DPP4-KO, AT remodeling is rather restricted to the epididymal AT.

3.1.1.3 DPP4 induced expression and release of adipokines

In addition to incretins, there is a number of potential substrates, such as SDF-1, NPY, PYY, RANTES or substance P, for the enzymatic actions of DPP4. Cleavage of substrates by DPP4 leads to modified proteins which may be inactive or display altered function. Several truncated substances in turn, can affect the production or release of other proteins such as ILs [208], as well as indirectly influence synergistic or suppressive pathways.

Inhibition of DPP4 alters the expression levels and secretion of adipocyte-derived factors, such as adipokines or FFA [209]. Shlomo and colleagues could demonstrate the regulation of numerous adipokines in AT of rats due to global loss of DPP4 [112]. While presence of leptin and adiponectin is increased, TNFα, IL-6, PAI-1 and CAMs are downregulated in AT [104]. Moreover, administration of DPP4i sitagliptin, resulted in

reduced expression of MCP-1, IL-12, IL-6, TNF-a, and IP-10 in murine AT [196]. Owing to analysis of AT explant-derived CM from AT-DPP4-KO and WT mice we identified insulin like growth factor binding protein 3 (IGFBP3) as an adipokine with DPP4-based regulation in AT.

3.1.2 Serum DPP4 and its potential sources

DPP4 expression has been found in multiple tissues and in diverse amounts [210]. Moreover, in rats distribution pattern of DPP4 correspond to measurements of DPP4 activity [211]. In 1968 for the first time DPP4 was detected in serum by Nagatsu et al. [212]. The soluble form lacks the transmembrane and cytoplasmic domain present at the membrane bound form of DPP4. DPP4 serum concentrations are in the range of 400 ng/ml in lean subjects [44], and very similar to DPP4 serum levels of our mice under chow diet. However, sDPP4 protein abundance and serum activity are frequently not associated [101], illustrated by experiments showing that around 10% of DPP4 activity is not related to sDPP4 [210, 213]. A previous study could demonstrate that immunosuppressant therapy drove transient reduction of DPP4 expression on peripheral blood lymphocytes, while serum DPP4 activity was significantly decreased for up to 4 month [214]. Differences in sDPP4 and serum enzymatic activity might be ascribed to the presence of other serum proteins with DPP4 activity, referred to as dipeptidyl-peptidase activity and/or structure homologs [215]. These include DPP7, DPP8, DPP9 and FAP. Moreover, there is evidence for a decrease in serum DPP4 enzymatic activity in an age-dependent manner [210].

In a previous study we could prove expression and release of DPP4 from adipocytes [98]. As mentioned, DPP4 expression in adipose tissue and circulating concentrations of DPP4 are increased during obesity [44], leading to the conclusion that the role of DDP4 might be even more important under obesogenic conditions. Thus, in our AT-DPP4-KO, where depletion of DPP4 resulted in not only diminished DPP4 expression levels in adipocytes but also decreased circulating levels of the enzyme under HFD, AT emerges as an important source of DPP4.

Other organs such as liver, where DPP4 was initially described [94], may additionally contribute to serum DPP4 levels. In vivo, DPP4 expression and activity are notably increased not only in epididymal AT but also in the liver of diabetic rats [113]. Recently it has been reported that in dark agouti rats sDPP4 originates partly from bone-marrow derived cells [216]. Furthermore, Cordero et al. summarized tissues such as spleen and kidney as participants in DPP4 secretion [101]. In our study, organs of chow WT mice such as kidney,

lung and epididymal AT displayed high DPP4 enzymatic activity compared to pancreas. Liver represented the organ with strongest activity of DPP4. However, since AT mass strongly increases within obesity compared to tissue mass of other organs, the latter might be only minor contributors to circulating DPP4 under obesogenic conditions.

Originally DPP4 was independently characterized as CD26, a molecule present on the surface of T-cells [105, 217]. In addition DPP4 has been found in leucocytes as well as lymphocytes [210, 218], pointing towards an important role in immune response. Among other immune cells, macrophages express DPP4. However, the quantity of the membrane bound protein in macrophages seems to be tissue specific, with higher levels of DPP4 in AT macrophages than that in peripheral blood macrophages [107]. Asides from that, the group reports elevated macrophage DPP4 levels as a consequence of obesity and a relation of DPP4 amount to AT IR and T-cell activation [107]. These findings give evidence for a pivotal function of DPP4 during AT low-grade inflammation. However, effects on AT inflammation and modulation in the AT-DPP4-KO are exclusively ascribed to adipocyte derived DPP4 since DPP4 depletion was restricted to these cells.

3.1.3 The systemic impact of circulating DPP4

3.1.3.1 DPP4 and its contribution to systemic insulin sensitivity

Upon shedding, the soluble form of DPP4 is released into the circulation and acts on peripheral tissues [98]. Most investigations on systemic DPP4 functions have been focused on the incretin system. The cleavage of incretins by DPP4 impairs glucose homeostasis. On the basis of this property DPP4 inhibition and following effects have been mostly attributed to modulation of the incretin system. However, differences in the AT-DPP4-KO model versus the WT mice are GLP-1 and GIP independent, since we could not detect any changes in postprandial active GLP-1 and GIP. Therefore, as an advantage of our model, indirect effects of DPP4 KO through GLP-1 and GIP activity can be excluded.

To date, there are scarce reports about the direct effects of sDPP4. Our group was the first to show the harmful effects of sDPP4 on insulin signaling in different cell types such as skeletal muscle cells, adipocytes and SMC [44]. Moreover, there is evidence that DPP4 impairs whole body insulin sensitivity illustrated by correlations with IR [68]. In addition, serum DPP4 activity is associated with IR in T1DM patients [219]. Subjects with T2DM exhibit higher serum DPP4 activity when insulin resistant compared to not insulin resistant individuals [220]. Interestingly, in non-obese healthy T2DM patients, DPP4 serum concentrations and secretion levels are not affected [221]. In vivo, whole body KO of DPP4

enhanced insulin secretion and ameliorated glucose tolerance [115]. Ablation of DPP4 also causes advanced metabolic control due to optimizing insulin sensitivity and protecting against DIO [114]. These alterations were dependent on GLP-1 and might result from lower body weight and less food intake of the DPP4 KO mice [114, 115]. On the contrary, DPP4i have been demonstrated to not modify weight [222]. In our model, DPP4 depletion in AT led to improved glucose tolerance assessed by oral GTT and decreased HOMA-IR, although AT-DPP4-KO animals revealed even higher body weight compared to WT mice. However, energy expenditure, food intake and length were unaltered between genotypes. Therefore, we conclude a direct impact of DPP4 which is not linked to lower body weight per se. Opposing effects by usage of DPP4i corroborate our data.

Thus, administration of vildagliptin in HFD-fed rats significantly enhanced peripheral and brain insulin sensitivity [223]. Despite high safety and efficiency of oral DPP4i in the treatment of T2DM, in respect of their daily administration, they may not exhibit persistent effects. On that account, Pang et al. proposed that immunization may provide an alternative to DPP4i with prolonged effects and avoidance of daily dosage requirements [224]. They established a DPP4 peptide vaccine which facilitated insulin sensitivity and delayed the onset of T2DM by amplifying GLP-1 secretion in a HFD-induced insulin resistant mouse model [224].

3.1.3.2 DPP4 and hepatic insulin resistance

In regard to the latest investigations on DPP4, liver might not only be a primary source of DPP4 but also represents a potential target tissue. Recently, a role of DPP4 in liver disease has emerged. DPP4 depletion in rats attenuated HFD-induced liver dysfunction, lowered levels of triglycerides and reduced cholesterol [112]. In line, Kaji et al. reported that sitagliptin abrogated liver fibrosis and production of hepatic transforming growth factor-β1 indicating that inhibition of DPP4 may facilitate anti-fibrotic effects [225]. Beneficial effects on liver diseases of several DPP4i have been found in mouse models and humans [226-228]. For instance, alogliptin preserves progression of non-alcoholic fatty liver disease (NAFLD) in T2DM [229]. Furthermore, sitagliptin has been suggested as a novel treatment agent for NAFLD in T2DM patients [230].

In obesity and T2DM circulating levels of DPP4 are related to hepatocyte apoptosis and liver fibrosis [231]. Moreover, DPP4 expression is enhanced in subjects suffering NAFLD compared to healthy ones [231], and serum DPP4 is correlated to patients with NAFLD and hepatic steatosis [220, 232]. Intriguingly, KO of DPP4 in AT of mice under HFD

caused an increase in insulin-induced suppression of endogenous glucose production (EGP). In metabolically healthy subjects, there is a tightly insulin and glucagon-controlled balance of glucose disposal from the circulation and EGP by the liver [233]. Insulin directly suppresses EGP in the liver which therefore represents a diagnostic parameter of hepatic insulin sensitivity. Since impaired insulin-induced suppression of EGP is a characteristic of IR in obese subjects [234] and linked to T2DM [235], DPP4 depletion might counteract hepatic IR and the development of diabetes. Indeed, Balas et al. proved that DPP4i vildagliptin augmented insulin secretion thereby increasing EGP suppression in T2DM patients [236]. However, underlying mechanisms whether DPP4 knock down directly or indirectly affects EGP still has to be elucidated. Based on our data, obtained by analysis of CM from explants of AT-DPP4-KO and WT animals, we propose AT-derived IGFBP3 as a potential candidate affecting liver dysfunction.

3.1.4 IGFBP3 as a mediator of adipose tissue and liver crosstalk

IGFBP3 is one of six high affinity binding proteins for insulin like growth factors (IGF) [237]. Circulating IGFBPs bind around 99% of serum IGF and ensure its delivery to target tissues [238]. In the corresponding tissues IGFPBs have different functions, either inhibiting or prolonging IGF actions by sequestering IGF from its receptor or by proteolysis of IGFs from the complex, respectively [237]. IGF, an anabolic peptide hormone, is primarily involved in regulating cell growth, proliferation, differentiation, migration and apoptosis [237]. There are two IGF members, namely IGF-I and IGF-II, which are structurally similar to insulin but divergent in their function [239]. The pronounced homology of IGF-I to insulin indicates a crucial role in glucose metabolism [240]. Indeed, decreased levels of IGF-I is associated with insulin sensitivity in mice [241, 242]. Moreover, recombinant human IGF-I lowers blood glucose and insulin levels [243]. However, these effects might be indirect through regulation of glucagon and growth hormone [244, 245].

The so called IGF axis, consisting of the two ligands (IGF-I and IGF-II), their receptors, IGF-associated proteases and six binding proteins, has been linked to T2DM [237]. In particular, higher IGF-I concentrations have been found to reduce the risk for T2DM. Partly consistent to observations of this group, Drogan and colleagues observed in the EPIC-Potsdam study that levels of IGFBP-3 solely are positively associated with the risk for T2DM although this association might be independent of IGF-I levels [246]. In addition, liver dysfunction, such as liver cirrhosis, is accompanied by lower serum levels of IGFBP3 [247] and IGF-I due to a disturbed synthesis of the polypeptide [248]. In accordance, Wu et al.

postulate that impaired hepatic IGF-I, IGF-II and IGFBP-3 levels might be potential indicators for the prognosis of liver dysfunction [249]. In respect of our data supplied by the AT-DPP4-KO model we assume an intrinsic role of IGFBP3 as a mediator of hepatic IR. We detected diminished concentrations of serum IGFBP3 in KO mice compared to their WT littermates. As expected, since around 75% of circulating IGF-I is bound by IGFBP-3 [250], levels of free IGF-I in the circulation of these mice was increased while total IGF-I was not affected. Since intravenous injection of IGFBP3 was localized mainly (40% of injected dose) in the liver shortly after administration [251], it seems likely that a decrease of IGFBP3 in serum of the AT-DPP4-KO impacts liver function. Indeed, we observed impaired insulin signaling illustrated by abrogated insulin-mediated phosphorylation of AKT (Thr308) *in vitro* and attenuated insulin-mediated suppression of glucose production upon exposure to IGFBP3 in hepatic cells. Alternatively, an indirect impact of IGFBP3 on glucose production, via IGF-I, might be responsible for the observed effects since IGF-I per se is able to suppress glucose production [252]. Furthermore, levels of IGFBP3 gene expression and release from AT explants of KO mice under HFD were reduced. Therefore, we assume that decreased circulating concentrations of IGFBP3 might be ascribed to AT-derived IGFBP3 and might be linked to AT specific DPP4 depletion. Interestingly, IGF-I has putative DPP4 truncation sites [208]. In a recent study, DPP4 was uncovered to proteolytically cleave two amino acids of IGF-I, leading to a truncated inactive form of IGF-I [253]. The shortened form of IGF-I shows amplified affinity to IGFBP3 [253]. In addition to less binding of IGF-I by downregulated IGFBP3, we postulate that reduced levels of circulating DPP4 lead to increased levels of free IGF-I in the HFD-fed AT-DPP4-KO mice. Thus, declined release of IGFBP3 from AT found in AT-DPP4-KO model might facilitate protective crosstalk from AT to liver under HFD. Of note, IGFBP3 has been found to interfere in AT function, too. In 3T3-L1 cells treated with IGFBP3 adipocyte insulin sensitivity was impaired by reducing glucose uptake [254] and adipocyte differentiation was inhibited via PPARγ interactions [255].

IGFBP3 and IGF-I levels are enhanced in type 2 diabetic subjects [256]. To explore the importance of our findings on the IGF axis, we analyzed human serum samples of type 2 diabetic, overweight patients with comparable anthropometric and metabolic parameters. All patients were either solely metformin treated or in combination with DPP4i sitagliptin. The results, although not directly comparable with the mouse model, partly coincide with our previous findings in mice. Free IGF-I levels were upregulated in serum of DPP4i treated subjects, however, also total IGF-I was increased. Moreover, enhanced IGF-I was not dependent on lower circulating IGFBP3 since IGFBP3 levels were similar in all patients. The

divergent outcomes regarding the influence of DPP4 deletion on the IGF axis in mice and human could be ascribed to the AT-specific lack of DPP4 in animals compared to a whole body inhibition through sitagliptin in humans. Furthermore treatment with the DPP4i affects the incretin hormones, which are not affected in our mice. Additionally, patients received treatments after onset of T2DM while mice were DPP4 deficient from birth on.

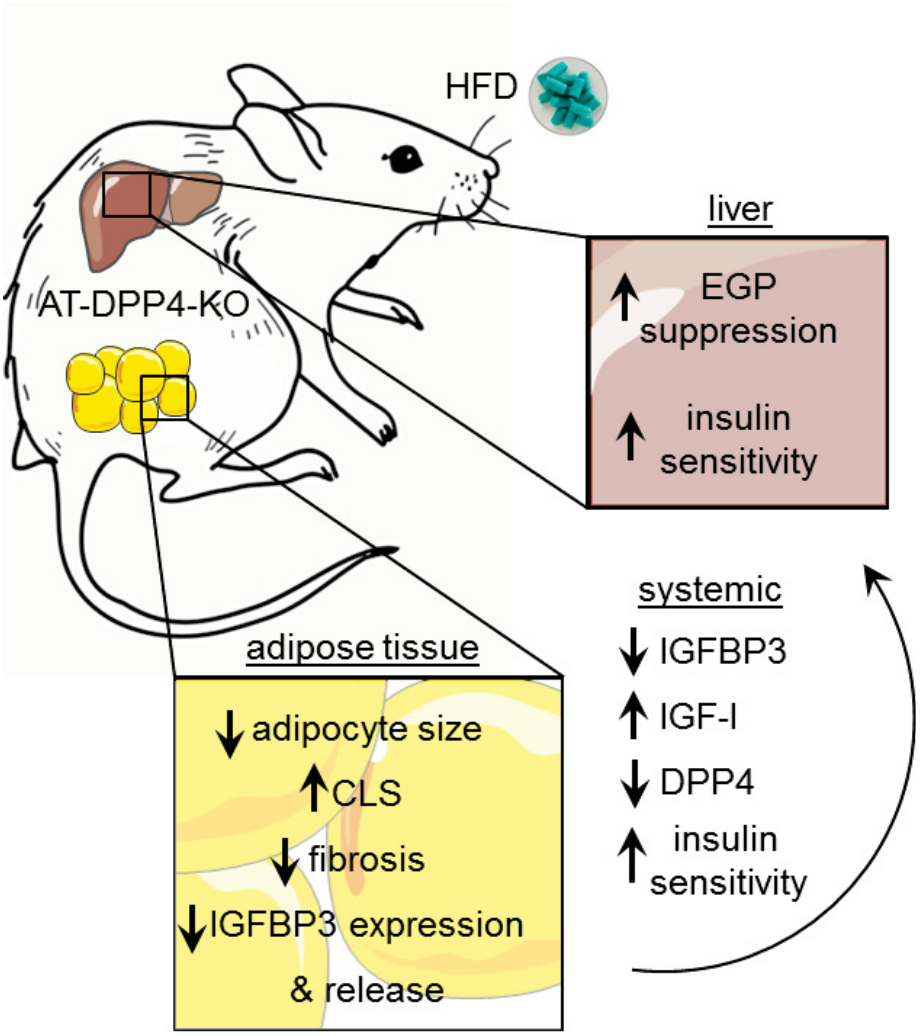

Figure 5. Effects of adipose DPP4 deletion in a mouse model under 24 weeks of HFD. As a consequence of AT-specific deletion of the DPP4 gene in mice receiving a HFD, hepatic insulin sensitivity is improved through the reduced release of IGFBP3 from the AT. Subsequently, circulating free IGF-I levels were increased and might exert additional, beneficial effects on the liver. In AT iself, a healthy AT remodeling process occurred, illustrated by smaller adipocytes, less fibrosis and higher numbers of CLS in KO compared to WT animals. Adipose tissue (AT), Crown-like structures (CLS), endogenous glucose production (EGP), high fat diet (HFD), knock-out (KO), wild type (WT).

Recombinant IGF1 and combinations with IGFBP3 have been discussed in the therapy of 2 diabetes [257]. However, since side effects have been reported the benefit of these treatments is limited. Our hypothesis of improved hepatic insulin sensitivity due to DPP4 deletion or inhibition is supported by findings of improved suppression of endogenous glucose production by sitagliptin and vildaglitpin in human patients [258, 259].

In conclusion, we propose that DPP4 depletion in AT reduces expression and release of IGFBP3 in AT, which in turn leads to higher levels of free IGF-I, thereby stimulating liver insulin sensitivity. In humans, DPP4 inhibition by sitagliptin might entail similar effects but most likely independent of IGFBP3. However, it might provide an additional hint for a relation between DPP4 and the IGF axis. Therefore further clinical investigations should be addressed in the future.

3.1.5 DPP4 and its function in the cardiovascular system

Through release into the circulation, sDPP4 exerts direct effects on the vasculature in an endocrine manner. sDPP4 has been described to participate in the pathophysiology of T2DM-associated vascular diseases [260]. Application of DPP4 increases SMC proliferation through activation of PAR2 [109]. In line, pharmacological inhibition of DPP4 triggers survival pathways in EC and cardiomyocytes [3, 261] as well as improves EC function and attenuates atherosclerotic lesion formation in apolipoprotein E deficient mice [262]. To explore the role of DPP4 in endothelial dysfunction, an early marker of CVD, we exposed murine mesenteric arteries to DPP4 and analyzed vascular reactivity. Treatment with DPP4 resulted in defective acetylcholine-mediated vasodilation, which could be neutralized by DPP4i linagliptin and K579. Administration of DPP4i vildagliptin improved endothelium-dependent vasodilation [263, 264]. Alternatively to a direct effect of DPP4 inhibition, beneficial effects on vascular reactivity might be dependent on prevention of DPP4-mediated GLP-1 degradation. In this context GLP-1 has been found to improve endothelial function in T2DM patients [265] and exert vasorelaxant properties on rat aortas [266]. However, in our study effects of sDPP4 are most likely independent of GLP-1. This is supported by previous studies describing DPP4-induced actions on the cardiovascular system independently of the incretins [267, 268]. Moreover, impairment of endothelium-mediated vascular dilation by DPP4 involved PAR2, cyclooxygenase 2 (COX2) and thromboxane 2 (TXA_2). COX, which comprises a constitutively expressed form COX1 and an inducible form COX2, is an enzyme catalyzing formation of prostanoids such as TXA_2, which is a powerful vasoconstrictor with pro-inflammatory properties in the vascular system. Consistent with our observations an

upregulation of COX2 expression induced by PAR2 has been previously described in other cell types, such as human pancreatic cancer cells, epithelial, and EC [269-271]. Moreover, vasodilatory and vasoconstrictive features of PAR2 are well established. However, DPP4-mediated release of TXA_2 is a novel finding, strengthening the hypothesis that DPP4 plays an essential role in triggering signaling pathways causing defective vascular reactivity. To understand the impact of DPP4 and its inhibitors as therapeutically targets in the treatment of CVD, further investigations are relevant.

3.2 DPP4 and PAR2 as the potential receptor

As mentioned before, we identified PAR2 as a receptor through which DPP4 exerts its detrimental effects on the vasculature. Thus, our group previously demonstrated that sDPP4 induces SMC proliferation in a PAR2-dependent manner [109]. In this study the respective tethered ligand sequence of PAR2 was identified within the cysteine-rich region on the surface of DPP4. Most likely DPP4 activates PAR2 by binding to its binding pocket at the ECL2, rather than cleaving and unmasking the tethered ligand. The fact that DPP4 is an exopeptidase that truncates proteins with proline or alanine in their penultimate position, and that the canonical cleaving domain of PAR2 is located between R36 and S37 excludes this possibility. However, it has to be considered that sDPP4 may cleave PAR2 at a distinct site thereby either exposing a new tethered ligand or disarming the receptor, hence activating it. In addition, we found a link between DPP4 and PAR2 in the AT-DPP4-KO study. PAR2 mRNA levels in aortas of WT but not in aortas of KO mice were upregulated under HFD compared to chow animals, leading to the conclusion that increased levels of DPP4 due to HFD may induce PAR2 mRNA expression (Figure 6A). Whether the underlying mechanism is a direct or indirect effect of DPP4 remains uncertain. Since DPP4 and PAR2 are co-localized in both vascular EC and SMC [98, 164, 165], it seems likely that PAR2 serves as a receptor for DPP4.

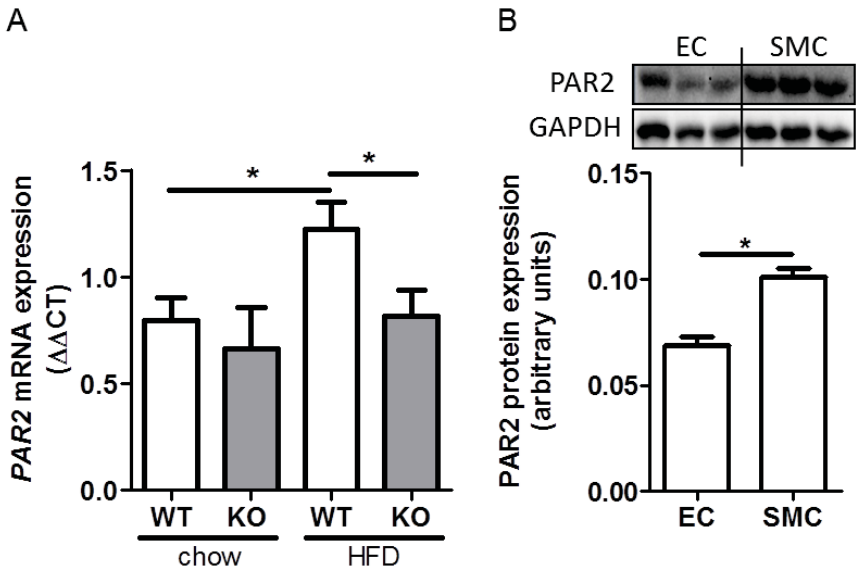

Figure 6. PAR2 expression in murine aorta and human vascular cells. (A) PAR2 mRNA expression, measured in aortas of mice, was elevated in WT animals under 24 weeks of HFD. This effect was absent in KO mice challenging a HFD compared to their chow diet-fed littermates. Data are normalized to 18S mRNA levels (chow WT: n=7, chow KO: n=6, HFD WT: n=7, HFD KO: n=10). (B) Basal expression of PAR2 in human vascular EC and SMC. PAR2 protein abundance was higher in SMC than in EC. Data are normalized to GAPDH and represent mean values ± SEM (*p<0.05); n=3. Endothelial cells (EC), smooth muscle cell (SMC), high fat diet (HFD), knock-out (KO), wild type (WT).

3.3 The relevance of PAR2 in obesity-associated vascular diseases

The control of vascular tone and coagulation are well established features of PAR2 [132]. Up to now other physiological roles of PAR2 in the vascular system remain largely unknown, especially in the context of obesity. As a consequence of obesity, initiation of vascular dysfunction, preceded by impaired endothelial function and SMC proliferation, may lead to the development of atherosclerosis, which is the main cause for mortality in obesity [1]. The above mentioned hallmarks of vascular disturbance have been linked to PAR2. Therefore, we wanted to investigate the role of PAR2 in obesity-related vascular disorders.

3.3.1 PAR2 associated vascular inflammation under obesogenic conditions

3.3.1.1 CM-induced PAR2 levels and smooth muscle cell proliferation

Interestingly, there is emerging evidence for an involvement of PARs under both, acute and chronic inflammatory conditions. Since acute inflammation is rather viewed as a protective state while chronic inflammation is clearly accepted to reflect a harmful, unhealthy state, PAR activation might even mediate opposing effects. Participation of PAR2 in inflammatory

responses has been described in several organs, including the vasculature [150]. For instance, PAR2 deficiency in mice, exhibit decreased leukocyte rolling suggesting a delayed onset of inflammation in the absence of PAR2 [272].

On one hand, the group of Chi et al. found that treatment with TNFα and endotoxin amplified the PAR2-dependent production of IL-6 in EC [273]. Moreover, SMC exposure to PAR2-AP stimulated expression of NFκB [274]. These data could be corroborated in a previous study of our group, showing that sDPP4 treatment of vascular SMC induces IL-6, IL-8, NFκB and ERK1/2 expression in a PAR2-dependent manner. On the other hand, expression of PAR2 is increased through a positive feedback loop by pro-inflammatory cytokines such as IL-1β and TNFα in endothelial cells [181, 182, 275]. Circulating cytokine levels are tremendously upregulated under obesogenic conditions, especially due to release from inflamed AT. Moreover, PAR2 was found to be upregulated in AT of DIO rats [276].

Therefore, we hypothesize that obesity might impact PAR2 expression levels in vascular cells. Since the endothelium is in close contact with circulating factors in the blood and PAR2 is present in these cells [277] modulation of PAR2 expression or activity seems to be very likely. Nonetheless, also SMC express PAR2, even at higher levels compared to EC (Figure 6B). In this study we demonstrated that CM obtained from isolated primary adipocytes of explants of overweight or obese patients, undergoing plastic surgery, were able to induce PAR2 expression at mRNA and protein levels in both EC and SMC. PAR2 mRNA abundance correlated with BMI of adipose biopsy donors. When treated with murine CM from either chow or HFD-fed animals, exclusively CM from mice receiving HFD triggered an upregulation of PAR2 levels in human SMC. In conclusion, vascular PAR2 levels might solely increase under obesogenic conditions. In line, induction of vascular PAR2 appears in animal models of other diseases such as diabetes or atherosclerosis [278, 279]. Nonetheless, the mechanism and purpose of PAR2 induction in the vasculature and other organs remain poorly understood. We suggest that an increase in PAR2 expression might sensitize cell responses to PAR2 ligands. Recently, a study addressed the upregulation of PAR2 in aortas and EC of rats in response to oxidative stress [280]. Aman et al. hypothesize that induction of PAR2 levels serves as a reinforcer for the endothelium-dependent relaxations due to the PAR2 agonist. In our study, CM-induced SMC proliferation increased with CM-induced PAR2 levels thereby confirming our assumption. CM-mediated SMC proliferation was attenuated by pre-incubation with the specific PAR2 antagonist GB83 and initiated by a PAR2 agonist peptide, proving the involvement of PAR2 in this pro-atherogenic process. Consistent, the mitogenic effects of PAR2 proteases such as trypsin or sDPP4 on SMC have

been highlighted previously [109, 164]. However, it might be noted that PAR2-AP promoted proliferation with a much lower potency compared to CM. A possible reason could be the different mechanisms of activation by cleaving or peptide binding. Moreover, CM contains a great number of different factors which are able to not only activate PAR2 but also induce a receptor upregulation. Non-proteolytic activation of PAR2 by agonist peptides causes recycling instead of degradation of the receptor, which makes it sensitive for re-stimulation. These might additionally modulate the activation properties of PAR2 [158].

3.3.1.2 Inflammatory mediators COX2 and NFκB

In the context of vascular inflammation specific key mediators such as COX2 or NFκB play crucial roles. COX2 is an enzyme synthesizing prostanoids from arachidonic acid, thereby regulating for example vascular tone. COX2 is responsible for pro-inflammatory cell processes and its expression is induced by cytokines such as TNFα, NFκB or IL-1 [281, 282]. During acute endothelial cell activation, NO and prostaglandins asides to controlling vascular tone also inhibit the recruitment and activation of immune cells [283]. However, in a chronic inflammatory state, persistent cell activation causes release of cytokines, inducible NO and COX2, which in turn increase activation and accumulation of immune cells, such as leukocytes [284]. We found that CM of adipocytes isolated and cultured from explants of overweight and obese patients induced COX2 and NFκB expression in a sustained, PAR2-dependent manner. Other studies which describe PAR2-AP-induced COX2 and NFκB expression [271, 274] support the hypothesis that PAR2 might control COX2- and NFκB-mediated inflammation. In combination with the mitogenic properties of PAR2, these data lead to the conclusion that PAR2 might accelerate obesity-related vascular inflammation.

3.3.2 Potential activators of PAR2 in the vascular wall

Activation of PAR2 occurs via binding of ligands or cleavage of the N-terminus by proteases, such as trypsin, tryptase, factor Xa and others. In the gastrointestinal tract there is a huge amount of trypsin available, able to activate PAR2. However, trypsin, the most potent activator of PAR2 up to now, is not present in many other tissues so that physiological activators of PAR2 are most likely tissue-specific. Physiological proteases of PAR2 remain to be identified. Trypsinogen, a precursor of trypsin, is secreted by several cell types including EC and epithelial cells and is able to activate PAR2 [285]. Moreover, tryptase, which is released by mast cells, displays a physiological activator of PAR2 [286], concluding a role for this receptor during immune responses. Release of tryptase has also been demonstrated in EC

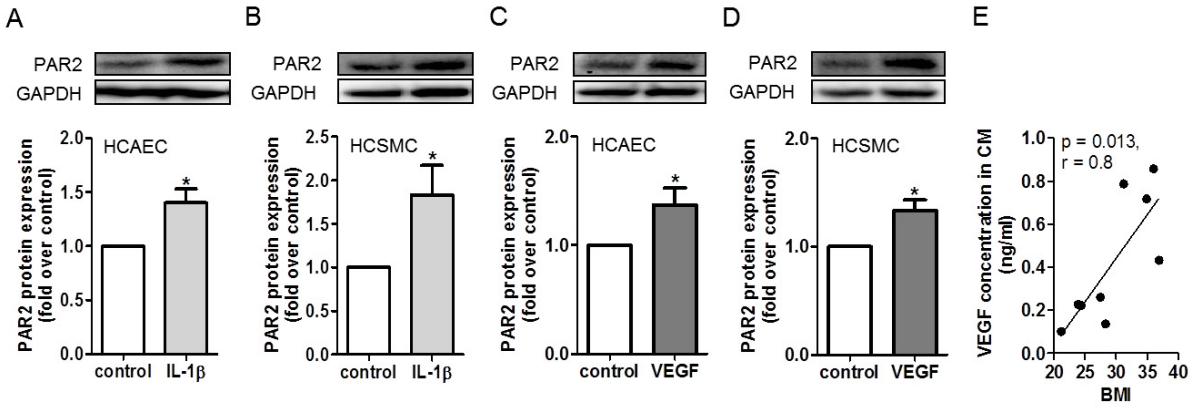

Figure 7: Potential mediators of PAR2 induction in vascular cells. (A and B) Treatment of EC and SMC with IL-1β (10 ng/ml) induced PAR2 protein levels; n=5-6. (C-D) Moreover, exposure of vascular cells to VEGF (250 pg/ml) increased PAR2 expression; n=3-5. Data are normalized to GAPDH. (E) VEGF concentrations in CM of human adipocytes correlate with BMI of AT-donors; n=9. Data represent mean values ± SEM (*p<0.05 vs control). Body mass index (BMI), conditioned medium (CM), endothelial cells (EC), smooth muscle cells (SMC).

[285]. Next to mast cell tryptase, coagulation factor Xa might be a PAR2 activator in the vascular milieu [287]. Nonetheless, trypsin is also a candidate because EC express trypsin in culture and in patients with cancer or intravascular coagulations [284]. During obesity multiple adipokines, such as DPP4, IL-1β, YKL40 or TNFα, are upregulated in the circulation and may therefore serve as potential activators of PAR2 in vascular cells. Furthermore, we observed that concentrations of vascular endothelial growth factor (VEGF) in CM from human adipocytes were positively correlated with BMI (Figure 7E). This is in line with previous data from our group, where VEGF release was increased in obese subjects [288]. Additionally, Disanzo et al. showed in rats that VEGF release from AT was increased under obesity [289]. However, measurements of VEGF serum concentrations in lean and obese subjects are contradictory. While some groups argue that circulating VEGF levels are enhanced in overweight and obese subjects and correlate with BMI [290, 291] other authors report that VEGF serum levels are not altered in obese subjects [292]. Nonetheless, a low concentration of VEGF was sufficient to trigger induction of PAR2 protein expression in both cell types assuming that VEGF represents a potential mediator secreted from adipocytes and able to drive upregulation of PAR2 in the vascular wall. CM is a source of thousands of proteins though, and factors despite VEGF that can be responsible for PAR2 induction and other endogenous ligands responsible remain to be defined.

3.3.3 PAR2 and receptor interactions

VEGF is a powerful inducer of proliferation [288] and our group could previously show that CM-mediated SMC proliferation is mainly driven by VEGF abundance. VEGF binds to receptor tyrosine kinases (RTK), namely VEGF receptor 1 and 2 (VEGFR1, VEGFR2), of whom the latter is responsible for most of the VEGF-mediated actions including SMC proliferation [293-295]. We analyzed whether PAR2 and VEGFR2 interactions might be an underlying mechanism for the inflammatory and mitogenic effects of CM and PAR2-AP. Recently the transactivation of PARs gained considerable interest. It has been shown that due to binding of thrombin the tethered ligand of PAR1 serves as a direct activator for PAR2 [296] In line, other groups found that PAR1 and PAR2 are able to signal together during vascular inflammation [297]. Additionally, receptor crosstalk between PARs and a variety of other receptors, among these the VEGF receptor has been observed [298]. We used potent VEGFR2 inhibitors, namely dobesilate [299] and VEGFR2-neutralizing antibody, and analyzed PAR2-mediated cell proliferation and COX2 induction. These PAR2-AP induced effects were suppressed by both VEGFR2 inhibitors, pointing towards a required transactivation of VEGFR2 due to activation of PAR2. Similarly, transactivation of EGFR was necessary for PAR2-induced COX2 expression in epithelial cells [269].

Transmembrane receptors, such as PARs and RTKs, exist in the same cell types and signaling initiated by diverse ligands occurs not to be necessarily in a distinct manner. They are much more organized in a complex signaling network involving different components such as crosstalk via release of receptor agonists or transactivation by intracellular signal transducers [298]. Several studies could prove that signaling interactions between GPCRs like the PAR family and growth factor receptors occur in various cell types including endothelial cells [300-302] and porcine coronary arteries [303], thereby strengthening our hypothesis. Also, the VEGFR2 and PAR2 are expressed in close proximity on the cell surface so that interaction is easily possible. Interestingly, the PAR2 activation did not increase VEGF release from SMC, indicating that VEGFR2 transactivation in SMC seems likely VEGF independent, although VEGF production in response to PAR2 activation has been previously described [304-306]. Additionally, it has to be taken into account that dobesilate may diminish VEGF production as found in choroidal explants of rats [307], thus possibly counteracting PAR-AP induced VEGF release. Nonetheless, receptor transactivation may occur due to intracellular molecules including the tyrosine kinase Src, ROS or protein tyrosine

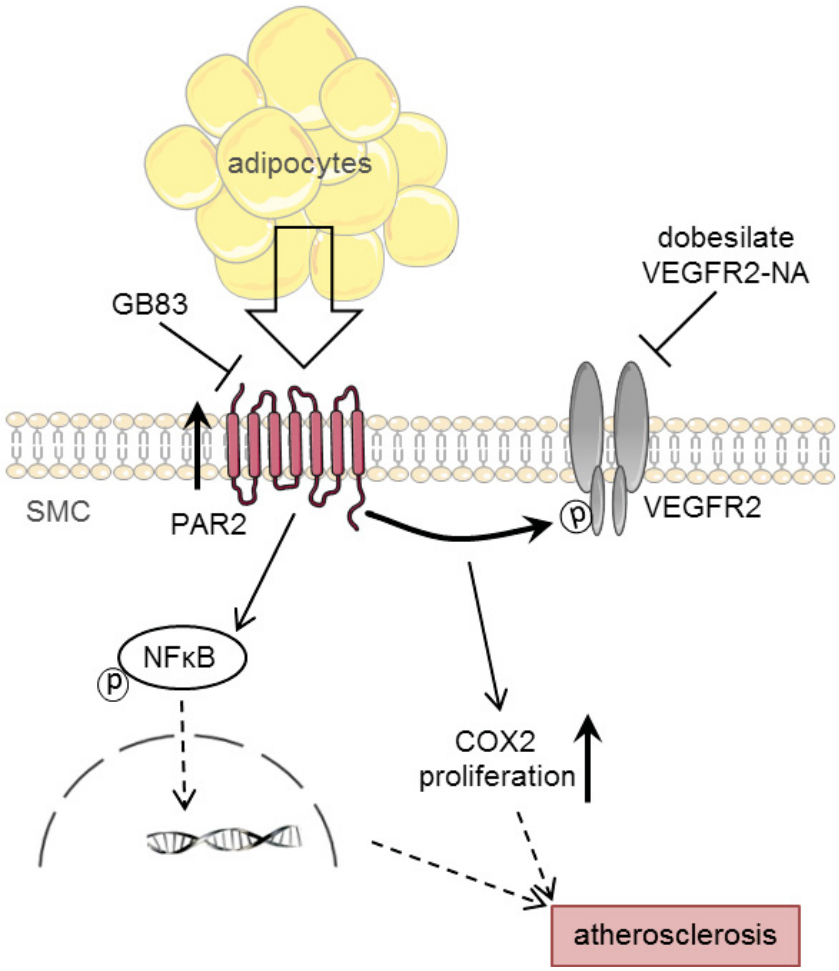

Figure 8. Impact of PAR2 on obesity-induced vascular dysfunction. During obesogenic conditions PAR2 levels are upregulated in vascular SMC. Induction of PAR2 might sensitize PAR2-mediated effects such as proliferation or increase in COX2 and NFκB. Furthermore, the PAR2-induced pro-atherogenic effects require intracellular transactivation of the VEGFR2. Cyclooxygenase 2 (COX2), smooth muscle cell (SMC).

phosphatases [298]. Interestingly, the group of Caruso et al. could show that PAR2 transactivation of epidermal growth factor receptor by Src activation promotes proliferation of gastric cancer cells [308]. Besides its inhibitory properties dobesilate possess antioxidant capacities [309]. The latter represents an alternative mechanism of VEGFR2 transactivation by abrogating PAR2 driven ROS production [298]. Further clarification of PAR2-induced pathways being either upstream or downstream of VEGFR2 and mechanisms of receptor transactivation in SMC in detail is needed to gain deeper insight in the importance of PAR2 signaling during obesity-related vascular dysfunction.

3.3.4 Therapeutic potential of PAR2 targeting antagonists and agonists

Due to its unique activating mechanism the development of antagonists for PARs has been much more difficult than for traditional GPCRs, where specific receptor antagonists block the action of only a restricted number of physiological agonists. For PARs, there exist a great number of different functional proteases and inhibitors, varying between tissues. To date several strategies to interfere in PAR-mediated actions have been evolved. The PAR1 antagonist vorapaxar blocks the tethered ligand binding site [310] while other antagonists such as ATAP2, targeting PAR1, and B5, a PAR2 antagonist, occlude the N-terminal cleavage site for proteases [296]. Alternatively, antagonists that interact with the cytoplasmic ICL3 and the carboxyl-terminus of PARs, which are important for receptor signaling and internalization, have been developed. Through this route for instance p2pal18S inhibit PAR2-mediated and p4pal-10 block PAR1-mediated inflammation [311, 312]. However, except for the PAR1 antagonists vorapaxar and atopaxar which are in phase II and phase III of clinical trials for the prevention of cardiovascular diseases [313-316], other PAR antagonists are still in the discovery stage [317].

Especially for PAR2 there is only limited knowledge about the pharmacology of its anatgonists and function. Based on the tethered ligand sequence PAR2 antagonists such as ENMD-1068 and K-14585 have been developed, which are capable to block PAR2-induced murine joint inflammation and pro-inflammatory signaling, respectively [311]. However for ENMD-1068 another study claimed that the ligand is not PAR2-specific at concentrations used by Kelso and colleagues [131]. The antagonist K-14585 has dual actions, thus besides being an antagonist for PAR2, it partially acts as an agonist, too [318].

Recently, two non-peptidic antagonists, namely GB88 and GB83, have been developed. GB88, a potent PAR2 antagonist, blocks trypsin- and PAR2-AP-mediated PAR2 activation, thereby attenuating inflammation in rat colitis [319]. In line, Suen et al. highlighted the anti-inflammatory properties of GB88, illustrated by the inhibition of PAR2-dependent cytokine release [320]. Analogously, the antagonist GB83 is a powerful inhibitor of trypsin- and PAR2-AP-induced activation of PAR2, already at low (μM) concentrations [321].

Pepducins, another group of PAR2 modulators, have gained attention. These cell-penetrating antagonists, interact with intracellular signal transducers [296]. For instance, P2pal-18S abrogated trypsin-induced neutrophil migration [322].

Moreover, to precisely inhibit certain signaling pathways of PAR2, another option would be to block particular proteases. Alternatively, Kelso et al. suggested the application of small interfering RNA to downregulate PAR2 expression [311]. Therefore the development of

selective compounds to treat PAR2-mediated diseases, such as atherosclerosis, is urgently needed. For this purpose it is of great importance to understand the function of PAR2, which might have beneficial properties under normal conditions while it acts detrimental in certain diseases. Modulations concerning only specific pathways might be of advantage since they preserve physiological actions of PAR2 and in parallel attenuate diseases. This might be applicable to GB88, which has been shown to attenuate cAMP accumulation, Ca^{2+} release and PKC phosphorylation, whereas it stimulates RhoA activity and ERK1/2 phosphorylation [320]. Very recently, the low molecular weight antagonist C391 has been discovered to block both PAR2-triggered Ca^{2+} flux and MAPK signaling pathways activated by peptide agonists or trypsin, but additionally displays weak agonist activity [323].

However, the development of such compounds is most likely very challenging since even single agonists cause diverse signaling cascades due to activation of different G-proteins, β-arrestins and receptor-receptor interactions [320]. Further studies will unravel the role of PAR2 in physiological and pathophysiological conditions and may help to develop potential therapeutic targets for multiple PAR2-associated diseases.

3.4 Perspectives

Circulating DPP4 is associated with obesity-related complications such as CVD. Since we previously described DPP4 as an adipokine upregulated in human obesity and IR, we now aimed to explore the impact of AT-derived DPP4 especially in the context of obesity. In this study, we generated and characterized a new mouse model of specific deletion of DPP4 in AT and could provide evidence for a pivotal role of AT-derived DPP4 under DIO. DPP4 depletion in AT did not only affect the local environment but also had systemic impact on liver function. While loss of DPP4 beneficially modulated AT itself through healthy remodeling, low levels of circulating DPP4 improved liver function probably through decreased release of IGFBP3 from AT and subsequent upregulation of circulating IGF1. To better understand the mechanism by which IGFBP3/IGF-I axis exerts its impact on the liver, further investigations are needed. Moreover, it remains unknown whether other factors regulated by DPP4 influence the crosstalk between AT and other organs and might be worthy to be addressed in future experiments. Although AT could be demonstrated as an important source of DPP4 during obesity, also liver contributes to DPP4 levels in the circulation. Therefore, we suggest to develop a liver-specific DPP4 KO as an approach to analyze and compare the impact of liver derived DPP4 to AT-derived DPP4. Observations regarding the IGFBP3/IGF-I axis from our unique AT-DPP4 KO model could be partially confirmed in human samples obtained from subjects achieving sitagliptin treatment. However, it is difficult to compare AT specific knockdown in mice with an entire inhibition of DPP4 activity in humans. Therefore, further investigations in that direction might be helpful to get better insight in potential advantages of DPP4i beyond the incretin level on hepatic insulin sensitivity.

Since DPP4 inhibition is discussed to have protective cardiovascular impact, this issue has been addressed in the second study. Here, we report that sDPP4 impairs vascular reactivity in murine mesenteric vessels via COX2 and TXA_2. Inhibition of DPP4 by linagliptin and K579 reversed the DPP4-mediated defective relaxation. Once more we could demonstrate that DPP4 exerts its actions on the vasculature via the receptor PAR2. It would be interesting to further elucidate the relevance of DPP4-mediated activation of PAR2 in vascular diseases. Intervention in the synergistic actions of DPP4 and PAR2 by inhibitors or antagonists might maintain EC and SMC function and thus represents a potential therapy counteracting obesity-related CVD.

PAR2 is poorly studied as a therapeutic target, although there is increasing evidence that the receptor plays an essential role for vascular function in health and disease. Therefore we investigated the impact of vascular PAR2 on obesity-associated diseases. In the third study we show that PAR2 is an important mediator of a pro-atherogenic state under obesogenic conditions, such as SMC proliferation and inflammation, involving induction of COX2 and NFκB. We could further prove the transactivation of the VEGFR2 as an underlying mechanism for PAR2 actions. Data about receptor transactivation by PARs are based primarily on PAR1 and RTKs, others than VEGFR2, thus emphasizing the novelty of our results. However, it remains to be elucidated which ligands activate PAR2 and which intracellular signal transducers are involved in receptor transactivation. This would be of interest regarding biased signaling of PAR2, so that only specific, harmful pathways can be modulated. We suggest PAR2 as a mediator of atherogenesis under obesogenic conditions. For the development of therapeutic agents targeting PAR2 or its agonists and thereby preventing PAR2-related disorders, it is important to gain detailed knowledge about the PAR2 specific ligands. Furthermore, PAR2 ligands are often only evaluated concerning Ca^{2+} and solely in a few cell types. There is need to more widely investigate PAR2 functions in different cell types and more deeply.

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

(Ira Indrakusuma)

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