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Dipeptidyl peptidase-4 promotes human endothelial cell senescence and dysfunction via PAR2-COX-2-TP axis and NLRP3 inflammasome activation

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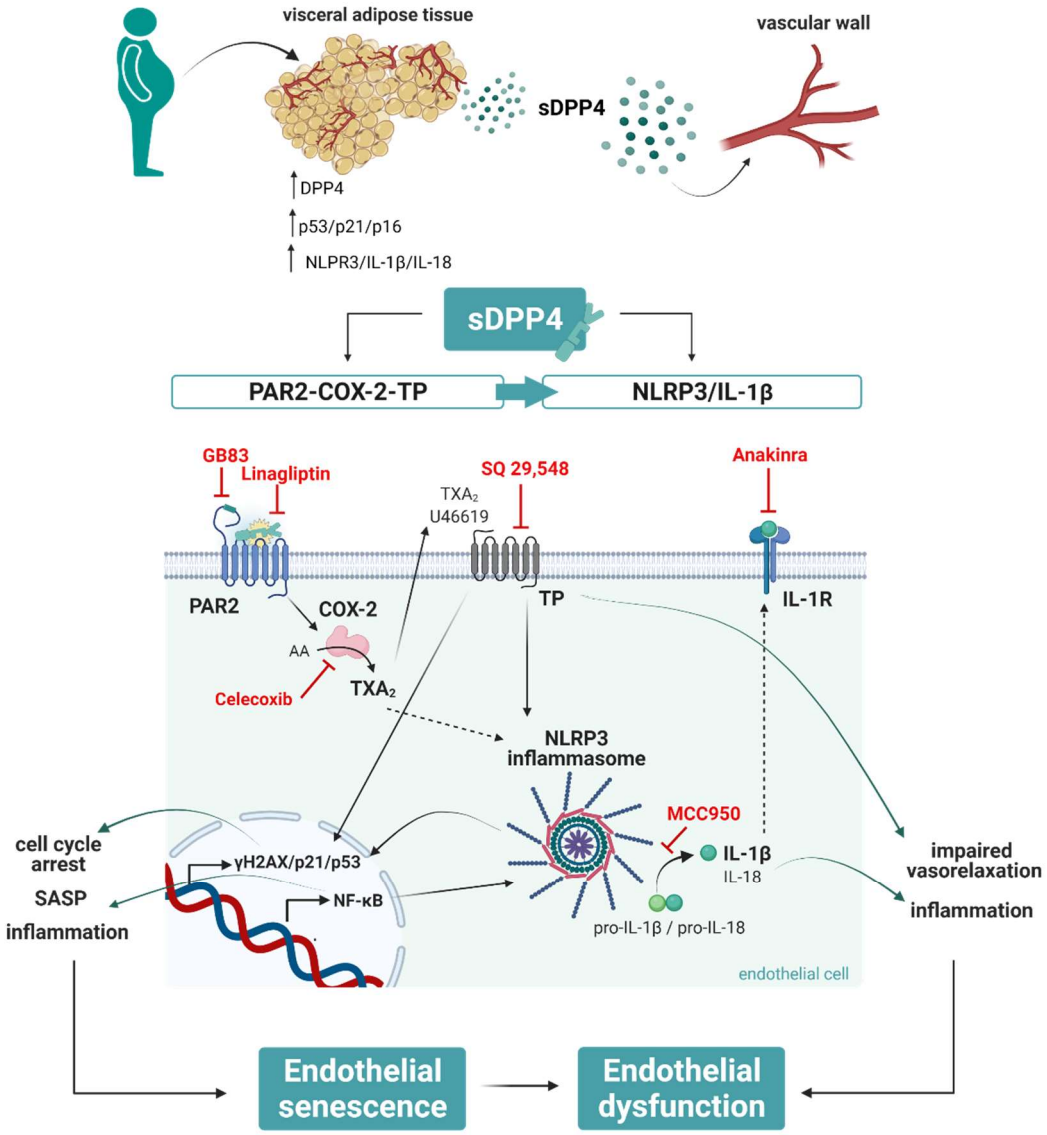
Abstract

Vascular aging is a multifaceted process characterized by structural modifications in blood vessels, like the abnormal accumulation of senescent cells leading to a compromised vascular function. Obesity and type 2 diabetes mellitus (T2DM) are considered progeric conditions leading to premature vascular aging. Soluble dipeptidyl peptidase 4 (sDPP4) secretion from adipose tissue (AT) is enhanced in obesity and T2DM, where it was proven to mediate deleterious effects, albeit its contribution to vascular aging is unknown. We aimed to explore sDPP4 involvement in vascular aging, unravelling the molecular pathway by which sDPP4 acts on the endothelium. The present study demonstrates that, by a common mechanism, sDPP4 triggers senescence in cultured human endothelial cells and endothelial dysfunction in isolated human resistance arteries. sDPP4 activates the metabotropic receptor PAR2, cyclooxygenase 2 (COX-2) activity and the production of TXA_2 acting over TP receptors (PAR2-COX-2-TP axis), leading to NLRP3 inflammasome activation. Additionally, in the pathological context of human obesity, we explored some related parameters *in vitro* and *ex vivo*. Obese patients exhibited impaired microarterial functionality in comparison to control non-obese counterparts. Importantly, endothelial dysfunction in obese patients positively correlated with greater expression of DPP4, pro-senescent, and pro-inflammatory markers in the visceral AT nearby the resistance arteries. Moreover, when DPP4 activity or sDPP4-induced pro-senescent mechanism were blocked, endothelial dysfunction was restored back to levels of healthy subjects. These results reveal sDPP4 as a relevant mediator in early vascular aging and highlight its capacity activating main pro-inflammatory mediators in the endothelium that might be tackled with pharmacological tools.

Keywords: *dipeptidyl peptidase-4, endothelial senescence, endothelial dysfunction, vascular aging, obesity. inflammaging, NLRP3 inflammasome.*

Graphical Abstract



Graphical abstract. Dipeptidyl peptidase-4 promotes human endothelial cell senescence and dysfunction via PAR2-COX-2-TP axis and NLRP3 inflammasome activation. Increased DPP4 release and expression in the context of obesity and other cardiometabolic diseases contributes to the inflammaging-prone microenvironment in which DPP4 can trigger endothelial cell senescence and endothelial dysfunction, contributing to further vascular disease. While DPP4 activates PAR2-COX-2-TP axis and NLRP3 inflammasome machinery, pharmacological inhibition of its enzymatic activity and downstream signalling prevents endothelial senescence and reverses endothelial dysfunction in a human model of obesity. DPP4 is an inducer of endothelial senescence and dysfunction and antidiabetic DPP4 inhibitors and other anti-inflammatory tools may entail therapeutic potential to delay vascular inflammaging and disease in cardiometabolic diseases.

List of abbreviations

ANOVA	Analysis of variance
ASC	Apoptosis-associated speck-like protein containing carboxyl-terminal CARD
AT	Adipose tissue
BK	Bradykinin
BMI	Body mass index
CMD	Cardiometabolic disease
COX-2	Cyclooxygenase-2
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide-1
HUVEC	Human umbilical vein endothelial cells
IL	Interleukin
MCP-1	Monocyte chemoattractant protein 1
NLRP3	Nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing-3
PAR2	Protease-activated receptor 2
SASP	Senescence-associated secretory phenotype
SA-β-gal	Senescence-associated β-galactosidase
SEM	Standard error of the mean
SNP	Sodium nitroprusside
TIFs	Telomere dysfunction-induced foci
TNF-α	Tumour necrosis factor
TP	Thromboxane receptor
TRF-1	Telomere repeat binding factor-1
TXA_2	Thromboxane A_2

Introduction

Vascular aging is a complex and multifaceted process that profoundly alters vascular structure and functionality, predisposing to vascular diseases such as atherosclerosis and endothelial dysfunction (1,2). Importantly, over the last years, cardiometabolic diseases (CMD) such as obesity and type 2 diabetes mellitus, have been acknowledged as progeric diseases exhibiting signs of premature vascular aging (3).

Endothelial senescence is the major process contributing to vascular aging (4). Cell senescence is a cell irreversible state where a core of damage-sensing signalling pathways is activated and cells lose their ability to divide while remaining metabolically active (5). A causative relation between cellular senescence and endothelial dysfunction has been demonstrated, as the progressive accumulation of endothelial senescent cells can induce the dysregulation of vascular tone and arterial stiffening (4). Endothelial senescent cells acquire a senescence-associated secretory phenotype (SASP) with pro-inflammatory, pro-thrombotic and pro-atherogenic effects (6). The SASP profile in vascular endothelial cells involves an enhanced release of mediators like tumour necrosis factor (TNF-α) and chemokines and cytokines as monocyte chemoattractant protein 1 (MCP-1), interleukin-(IL)-6 and IL-1β, among others (7). This microenvironment contributes to an unresolved and uncontrolled low-grade chronic inflammation that propagates the senescence phenotype and exacerbates even more the aging process, named after the term 'inflammaging' (8). Therefore, a better understanding of the molecular and physiological basis of endothelial senescence is decisive to identify new therapeutic approaches to tackle vascular aging.

Obesity is acknowledged as a chronic non-infectious disease that has reached epidemic proportions globally, with about 2.5 million people dying annually as consequence of being overweight or obese (9). The main characteristic of obesity is the enlargement and

113 inflammation of the adipose tissue (AT), currently recognized as a true endocrine organ.
114 The imbalanced secretion of active factors from AT, adipokines, towards a more pro-
115 atherogenic and pro-inflammatory phenotype, influences the local and systemic vascular
116 responses and contributes to vascular disease (10). In this pro-inflammatory context, the
117 innate immune response mediator nucleotide binding oligomerization domain, leucine-
118 rich repeat and pyrin domain-containing-3 (NLRP3) inflammasome have been proven as
119 a relevant contributor to the low-grade chronic inflammation underlying vascular disease
120 in obesity (11). AT is engaged in paracrine crosstalk with adjacent vascular trees (12)
121 and, therefore, not only perivascular AT but also visceral AT inflammation can modulate
122 the function of blood vessels in close proximity or even within the tissue.

123 Dipeptidyl peptidase 4 (DPP4) is a surface serine protease that can be shed from the
124 plasma membrane as a soluble form (sDPP4), recently identified among the substrates
125 released by the AT (13). Both the soluble and membrane-bound DPP4 cleaves N-terminal
126 dipeptides from diverse substrates like the incretin hormones glucagon-like peptide-1
127 (GLP-1) and gastric inhibitory polypeptide (GIP). The gliptin family of antidiabetic drugs
128 inhibit DPP4 activity, thus prolonging the half-life of incretins and glucose homeostasis
129 (14). Interestingly, sDPP4 plasma levels and activity are increased in type 2 diabetes
130 mellitus and obesity and they positively correlate with insulin resistance and an increased
131 risk score for the metabolic syndrome (13,15). Moreover, sDPP4 directly induces
132 inflammation in vascular cells and impair vasorelaxation in isolated murine microvessels
133 (16,17) by activating G-protein-coupled proteinase-activated receptors 2 (PAR2), which
134 are upregulated in animal models of obesity (18), and by triggering the activation of the
135 cyclooxygenase 2 (COX-2) and thromboxane receptor (TP) axis (16,17), namely the
136 PAR2-COX-2-TP axis. However, whether sDPP4 itself induces endothelial cell

137 senescence and thus contributes to premature vascular aging and endothelial dysfunction
138 is yet to be established.

139 In the search of new therapeutic targets to tackle accelerated vascular aging, we first
140 studied whether sDPP4 can directly induce cell senescence in cultured human endothelial
141 cells and endothelial dysfunction in human isolated mesenteric microvessels, while
142 exploring the underlying molecular pathway with focus on the PAR2-COX-2-TP axis and
143 NLRP3 inflammasome. We next determined DPP4 and pro-senescent markers expression
144 in visceral AT from control and obese patients and assessed the endothelial function of
145 the close microvasculature. Finally, we assessed whether gliptins or the pharmacological
146 blockade of sDPP4 downstream signalling pathways may improve the impaired reactivity
147 of microvessels from obese patients.

148

149 **Materials and Methods**

150 ***Human umbilical vein endothelial cells (HUVEC) isolation and cell culture***

151 HUVEC were isolated from donated umbilical cords obtained in Hospital Universitario
152 La Paz (Madrid, Spain), with informed consent, following the Spanish legislation and
153 under approval of the appropriate Ethical Committee. Umbilical cord donors with CMD
154 were rejected. HUVEC were extracted by chemical digestion with 2 mmol/l type II
155 collagenase (Sigma, Saint Louis, Missouri, USA) and characterized by von-Willebrand
156 factor detection. Cells were cultured in supplemented M199 medium as previously
157 described (19).

158 ***Collection of human omentum biopsies and health-associated parameters***

159 Omentum biopsies and basic blood analysis registers were obtained from a group of
160 patients (mean age 50±13 years old, 57% male), classified as non-obese controls without

CMD (body mass index, BMI<30 kg·m^{-2}) and obese patients (BMI≥30 kg·m^{-2}). All procedures were approved by the Ethical Committee of Hospital Fundación Jiménez Díaz (Madrid, Spain) and following the principles outlines in the Declaration of Helsinki. The patients participating in the study were submitted to a non-urgent, non-septic abdominal surgery consisting in cholecystectomies and bariatric surgery for control and obese patients, respectively. After signature of an appropriate written consent, a 3 cm piece of omentum was dissected during surgery and kept at 4ºC until experimental use. Two donors were dismissed from the experiments due to inappropriate manipulation of tissue sample. Mesenteric arteries were obtained, cleaned, and mounted on a small vessel myograph, while surrounding visceral AT was frozen immediately until total RNA extraction was performed, as indicated below.

Senescence Associated-β-galactosidase (SA-β-gal) assay

SA-β-gal staining was performed following the prescriptions of a commercial kit from Sigma, as previously described (20). The percentage of SA-β-gal positive cells over total cells was determined by blind manual scoring of at least 2,000 cells per sample in 12 randomized fields, under an inverted microscope Nikon Eclipse TE300 (Tokyo, Japan) connected to a Nikon DS-U3 camera and using the NIS-Element 4.50 imaging software, in phase contrast mode with a 20X objective.

Detection of γH2AX foci and telomere dysfunction-induced foci (TIFs) by double-indirect immunofluorescence

γH2AX *foci* representing DNA damage and TIFs, where γH2AX co-localizes with the telomere protein telomere repeat binding factor-1 (TRF-1), were examined by indirect double-immunofluorescence as described before (20). Samples were viewed under a Nikon Eclipse 801 microscope connected to a Hamamatsu Orca 285 digital camera. Fluorescence images were captured using the Volocity 3D image analysis software (Pekin

Elmers Inc., version 5.5). Z-stacks were converted to voxels (volume pixels) and further analysed with the Volocity co-localization module after image projection. The average fluorescence corresponding to each detected protein (expressed as voxels/cell) and the percentage of TIFs-positive cells (considering cells with ≥5 γH2AX-TRF-1 co-localization sites), were determined by analysis of at least 200 nuclei in 10 random fields per sample.

Western Blotting

HUVEC were treated as indicated and lysed, while mechanically detached from the plate. Protein content was quantified by a colorimetric protocol using the bicinchoninic acid (BCA) method (Thermo Fisher Scientific, Illinois, USA). Thereafter, 20 μg of protein lysates were separated by SDS-PAGE electrophoresis and later transferred to polyvinyl membranes (Merck, Darmstadt, Germany). Proteins were detected with primary antibodies followed by corresponding horseradish peroxidase-labelled secondary antibodies (please see http://hyper.ahajournals.org). Protein levels were normalized to β-actin signal (Sigma). Immunoreactive bands were detected using an enhanced chemiluminescence ECL detection kit (Bio-Rad, California, USA) and quantified by densitometry using ImageJ 1.51w free software.

Determination of NLRP3 inflammasome activation by indirect immunofluorescence

Apoptosis-associated speck-like protein containing carboxyl-terminal CARD (ASC) specks were detected by incubation with primary anti-ASC antibody (Enzo Life Sciences, New York, USA), followed by the secondary antibody Alexa Fluor 647-conjugated goat anti-rabbit IgG (Jackson Immuno Research, Cambridge, UK). Nuclei were counterstained with 1 μmol/l 4'-6'-diamidino-2-phenylindole (DAPI). ASC specks per field were quantified by manual blind scoring of 17 radial distributed fields in each sample, under

an inverted microscope Eclipse TE300 (Nikon). Representative images were acquired with a TCS SPE confocal microscope (Leica, Wetzlar, Germany).

Microvascular reactivity

Isolated microvessels from fresh omentum biopsies were cleaned free of fat and connective tissue under a light microscope and later mounted as ring preparations on a small vessel myograph (DMT, Denmark), as previously described (17). The passive tension and internal diameter were determined with MyoNorm-4 software (Cibertec), for later subject the arteries to a tension equivalent to 90% of 100 mmHg intramural pressure. Arteries were contracted with 35 mmol/l KCl, and endothelium-dependent and independent relaxation responses were assessed by addition of cumulative concentrations of bradykinin (BK, 0.01 nmol/l to 10 µmol/l) or sodium nitroprusside (SNP, 1 nmol/l to 100 µmol/l), respectively. In some experiments, mesenteric microvessels were treated with *ex vivo* addition of sDPP4 (200 ng/ml) alone or with pre-treatment with the drugs: linagliptin (100 nmol/l), GB83 (10 µmol/l), SQ 29,548 (10 µmol/l), MCC 950 (1 µmol/l) or anakinra (1 µg/ml).

Total RNA isolation and quantitative real-time (qRT)-PCR

For gene expression analysis, total RNA was extracted from frozen visceral AT pieces (30 mg) with NZYol (NZYTech, Lisbon, Portugal). RNA integrity was tested by a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific) and cDNA synthesis was performed using the First-Strand cDNA Synthesis kit (NZYTech), with 500 ng of RNA as template. qRT-PCR reactions were performed with iTaq Universal SYBR Green Supermix (Bio-Rad) on a 7500 Fast Real-Time PCR System (ThermoFisher Scientific) and specific primers against DPP4, p53, p21, p16, NLRP3, IL-1β and IL1-18 (please see http://hyper.ahajournals.org). The relative quantification of gene expression was determined by $2^{-\Delta\Delta Ct}$ method and rRNA *18S* housekeeping gene was used for normalizing.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) for the indicated number of experiments. Statistical analysis was performed using GraphPad, Prism 8.0.2 software (California, USA). Normality and homoscedasticity were checked for each variable by Shapiro-Wilk and Brown-Forsythe test, respectively. Two-tailed unpaired Student's t-test (parametric variables) or Mann Whitney test (non-parametric variables) was used to determine differences between two groups. One-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test (parametric variables) or Kruskal-Wallis followed by Dunn's multiple comparison test (non-parametric variables) was used to determine differences among more than two groups. Two-way ANOVA was employed to compare two variables when appropriate. For correlations, the Spearman coefficient was determined. A p value$\leq$0.05 was considered statistically significant.

Results

sDPP4 induces endothelial cell senescence in vitro

In HUVEC primary cultures, human recombinant sDPP4 caused a concentration-dependent increase in the percentage of SA-β-gal positive stained cells (Figures 1A and 1B), almost doubling over 24 hours. From 4.39±0.29% of positive senescent cells in non-stimulated cultures, submaximal stimulation with 200 ng/ml sDPP4 reached 8.54±0.12% SA-β-Gal^{+} cells, and it was the concentration chosen for all subsequent experiments. It should be noted that circulating sDPP4 levels in healthy patients varies in the rage of 200 to 600 ng/ml (15).

sDPP4 also induced DNA damage in HUVEC, as depicted by both an increased expression of the DNA-damage marker γH2AX (Figure 1D) and the accumulation of

DNA damage spots assessed by immunofluorescence. We detected in the sDPP4-treated cultures a higher number of γH2AX *foci* and TIFs, indicating DNA injury both at non-telomeric and telomeric position (Supplementary figures S1A, S1B, S1C, and S1D).

DNA damage is indeed a major stimulus for the activation of cellular alarm mechanisms leading to cessation of the cell cycle and senescence (21). Consistently, sDPP4 induced an increase in the expression of the effector protein for cell cycle arrest p21 (Figures 1E). Moreover, sDPP4 also induced the phosphorylation of the p-65 (Pp65) NF-κB subunit and the secretion of the pro-inflammatory cytokine IL-6 (Supplementary figures S1E and S1F), which is consistent with the establishment of an active SASP.

sDPP4 induces endothelial dysfunction ex vivo

Endothelial senescence is itself a promoter of endothelial dysfunction, another hallmark of vascular aging and one of the earliest age-related vascular modifications (22). In this context, we next examined whether sDPP4 affected the functionality and vasodilator properties of human resistance vessels. We used isolated mesenteric microvessels from human omentum biopsies of control non-obese subjects, whose health-related parameters are indicated in Supplementary table S1A.

Figure 1F shows the vasorelaxant responses of mesenteric microvessels in response to increasing concentrations of BK (0.01 nmol/l to 10 μmol/l). We observed that the incubation with sDPP4 (200 ng/ml) impaired the endothelium-dependent relaxation in response to BK (Figure 1F). Indeed, the mean half-maximal effective concentration value (pEC50) significantly decreased in presence of sDPP4 (5.88±0.19) in comparison to control conditions (7.52±0.17), as shown in Supplemental table S1B.

The PAR2-COX-2-TP axis mediates sDPP4-induced endothelial senescence and dysfunction

Since sDPP4 can bind PAR2 leading to downstream COX-2 activation (17,23), we assessed whether this pathway was involved in sDPP4-induced senescence and endothelial dysfunction. First, we observed that either the PAR2 antagonist GB83 (10 μmol/l), the COX-2 blocker celecoxib (3 μmol/l), or the TP antagonist SQ 29,548 (10 μmol/l) were able to attenuate the percentage of SA-β-gal positive stained cells (Figure 2A) and γH2AX expression (Figure 2B) in HUVEC treated with sDPP4. In line with these results, the blockade of PAR2 and TP prior to sDPP4 exposure in human isolated microvessels, also prevented the sDPP4-induced endothelial dysfunction (Figure 2C and 2D).

sDPP4 activates NLRP3 inflammasome via the PAR2-COX-2-TP axis

Since the overactivation of NLRP3 inflammasome has been involved in the sterile chronic inflammation and inflammaging associated to vascular disease (24), we addressed the capacity of sDPP4 to activate this innate immune response system. In HUVEC, sDPP4 enhanced the levels of the inflammasome components NLRP3, ASC and pro-IL-1β (Figures 3A, 3B, and 3C).

Upon activation, NLRP3 protein oligomerizes and interacts with ASC to assemble into a multiprotein scaffold (ASC-speck) wherein caspase-1 is activated to process pro-IL-1β and pro-IL-18 into mature forms. Thus, the formation of ASC-speck structures becomes a hallmark of inflammasome activation (25). In our experiments, sDPP4 enhanced ASC-speck formation by 2-fold over the basal speck levels, raising from 2.65±0.46% of ASC-specks positive cells in non-treated cultures to 5.38±0.71% after sDPP4 (200 ng/ml) challenge (Figures 3H and 3I). The latter was shown to be abrogated by MCC 950 (1 μmol/l), a specific inhibitor of NLRP3 inflammasome assembly (Figures 3H and 3I). In addition, sDPP4 significantly increased the ratio of the cleaved form of

active caspase-1 versus pro-caspase-1 (Figure 3D), as well as the expression of IL-1β, the final product underlying NLRP3 inflammasome activation (Figure 3E).

Blocking the PAR2-COX-2-TP axis with GB83 (10 μmol/l), celecoxib (3 μmol/l), or SQ 29,548 (10 μmol/l), significantly reduced the capability of sDPP4 to induce NLRP3 and IL-1β expression (Figure 4A and 4B) and ASC-speck formation (Figures 4C and 4D).

The NLRP3 inflammasome mediates endothelial cell senescence induced by sDPP4

The fact that PAR2-COX-2-TP axis activation by sDPP4 was mediating both cell senescence and the NLRP3 inflammasome activation, suggested a connexion between these latter cellular events. Indeed, the thromboxane (TXA_2) stable analogue U46619 (1 μmol/l) enhanced the expression and the activation of NLRP3 inflammasome (Supplementary figures S2A, S2B, and S2C), as well as the percentage of SA-β-gal^+ cells by 1.5-fold, and γH2AX levels (Supplementary figures S2D and S2E), in a TP-dependent manner.

Moreover, blocking the NLRP3 inflammasome activation with MCC 950 (1 μmol/l) or the IL-1 receptors with anakinra (1 μg/ml) significantly reduced the number of SA-β-gal^+ cells induced by sDPP4 (200 ng/ml) or by U46619 (1 μmol/l) (Figures 5A). Similarly, both drugs abolished sDPP4 or U46619-induced γH2AX expression (Figures 5B).

Additionally, both MCC 950 and anakinra prevented the endothelial dysfunction induced by sDPP4 *ex vivo* in isolated human microvessels (Figure 5C and 5D), thus confirming that human endothelial senescence and endothelial dysfunction elicited by sDPP4 were both mediated by a common pathway involving the NLRP3 inflammasome.

DPP4 inhibitors abolish the detrimental vascular effects of sDPP4 in vitro and ex vivo

We next aimed to identify pharmacological interventions to prevent the detrimental actions of sDPP4 on human endothelial cells or microvessels. To determine

the potential therapeutical benefit of DPP4 inhibition, we evaluated the effects of linagliptin, a non-peptidomimetic DPP4 inhibitor within the top-three most used gliptins for the management of type 2 diabetes mellitus. Linagliptin (10 nmol/l) reduced the increase in SA-β-gal positive cells (Figure 1C), γH2AX and p21 expression (Figures 1D and 1E) induced by sDPP4. We next tested whether the action of gliptins might be mediated by the relative increase of incretins like GLP-1 due to DPP4 inhibition. However, in the presence of the GLP-1 receptor antagonist exendin-(9-39) (1 μmol/l), linagliptin could still prevent the pro-senescence capacity of sDPP4, while exendin-(9-39) alone did not significantly modify the basal SA-β-Gal^{+} cells in absence of sDPP4 (Supplementary figure S1G).

In line with a role for the NLRP3 inflammasome in HUVEC senescence, linagliptin abolished the expression of NLRP3 (Figure 3F) and the functional activation of the complex, as evaluated by IL-1β expression (Figure 3G) and ASC-speck formation (Figure 3H and 3I). In another set of experiments, we address DPP4 inhibition with the experimental peptidomimetic inhibitor K759 (100 nmol/l), observing similar effects than linagliptin (Figures 1C-1E and 3F-3I). Moreover, linagliptin prevented the defective relaxation of human arteries to BK induced by sDPP4 (Figure 1G).

AT content in DPP4 is associated to endothelial dysfunction and inflammaging in human obesity

We next aimed to assess the role of sDPP4 and its inhibition in the context of a human disease. Obesity has been acknowledged as a progeric condition in which patients exhibit features of vascular aging such as endothelial dysfunction (26). We studied a cohort of patients (mean age 50±13 years old, 57% male), classified as non-obese controls without CMD (BMI<30 kg·m^{-2}) and obese patients (BMI≥30 kg·m^{-2}), whose health-related parameters are indicated in Supplementary table S1A.

We first observed that the vascular segments from obese patients exhibited a significant impaired endothelium-dependent relaxation as compared to the control non-obese group, with pEC_{50} values for BK-relaxation of 7.52 ± 0.17 and 6.10 ± 0.16 for non-obese and obese group of patients, respectively (Figure 6A and supplementary tables S1B and S1C). Interestingly, pEC50 values inversely correlated with BMI (Supplementary table S1B). On the contrary, the endothelium-independent relaxations evoked by SNP (1 nmol/l to 100 μmol/l) were similar in both groups (Figure 6B).

In the close proximity of the dissected mesenteric microarteries, we determined in the visceral AT the local expression of DPP4, which was significantly higher in the visceral AT explants from obese patients as compared with control non-obese subjects (Figure 6C). We also quantified the visceral AT mRNA expression of the senescence markers p53, p21, and p16, as well as those of the inflammasome-related genes NLRP3, IL-1β, and IL-18. The heatmap depicted in Figure 6D shows that all these values were higher in obese patients, validating a pro-inflammatory and pro-senescence neighbouring microenvironment around their mesenteric vasculature. Detailed graphs for each marker are supplied in Supplementary figures S3C-S3H.

Interestingly, the pEC_{50} values for the BK responses inversely correlated with DPP4 levels of expression in visceral AT from both non-obese and obese patients (Figure 6E), indicating a close association of local DPP4 expression with the loss of functionality in the near vasculature. We thus explored whether the pharmacological blockade of DPP4 and its signalling pathways could ameliorate the impaired vascular functionality in the microvasculature from obese patients. Indeed, the pre-incubation with linagliptin (10 nmol/l), GB83 (10 μmol/l), or SQ 29,548 (10 μmol/l) significantly improved the BK-induced relaxation in microvessels from obese patients bringing it back to values similar to those observed in control non-obese patients (Figures 6F, 6G, and 6H). These

treatments had not affected the vascular reactivity responses in control non-obese microarteries (Figures 1G, 2C-D and Supplementary table S1B).

Discussion

Accelerated vascular aging is a common feature of most age-associated diseases (2) and an important driver of frailty and disability (22). The progression of obesity and type 2 diabetes mellitus shares common hallmarks with aging, so these diseases are now acknowledged as progeric conditions (3,26-28). Although adipokines have been identified as pivotal effectors in several deleterious actions in the vasculature (29,30), evidence about adipokines playing a role in the progression of inflammaging is still very limited. The adipokine sDPP4 was found to be upregulated in type 2 diabetes and obesity patients (13,15), where it triggers detrimental actions in the vasculature and contributes to insulin resistance and atherosclerosis (17,23), yet its contribution to vascular aging has not been stablished.

In the present study, we have demonstrated that sDPP4 directly induces cell senescence in primary cultured human endothelial cells. The endothelium is a main cell layer in the vascular wall as it tightly controls vascular homeostasis (31). The accumulation of endothelial senescent cells disrupts endothelial functionality and impairs its regenerative capacity (7), and also boosts the pro-thrombotic, pro-atherogenic and pro-inflammatory microenvironment that contributes to inflammaging (32). Therefore, understanding the molecular mechanism underneath endothelial senescence is essential to delay vascular disease in CMD and aging.

sDPP4 triggered endothelial cell senescence by activation of PAR2, a metabotropic receptor whose activity in the context of CMD has been related to vascular inflammation

405 (33). PAR2 controls vascular tone and coagulation (34,35) and its expression is
406 upregulated in human coronary atherosclerotic lesions (36), and in animal models of
407 obesity (18) and type 2 diabetes (37). Although the mechanism by which sDPP4 may
408 activate PAR2 is not fully dilucidated, the fact is that DPP4 contains a high homologous
409 sequence (SLIG region) in its structure that may directly activate PAR2 independently of
410 its protease activity (16,17,38).

411 In vascular smooth muscle cells and endothelial coronary cells, PAR2 stimulation
412 can directly activate COX-2 (18,23). These results are in line with the molecular pathway
413 we hereby describe in HUVEC. In our hands, the blockade of COX-2 or TP receptors,
414 which are activated by COX-2-derived TXA_2, prevented cell senescence induced by
415 sDPP4 via PAR2, thus pointing at TXA_2 behaves as a final effector of sDPP4 pro-
416 senescence effects. In fact, we observed that TXA_2 could induce endothelial senescence
417 by itself, as previously reported in fibroblasts (39). TXA_2 is a potent vasoconstrictor
418 released by activated endothelial cells that may contribute to the pro-thrombotic status in
419 the senescent endothelium. Interestingly, an enhanced vasoconstrictor prostanoid activity
420 has been associated to obesity-associated complications (40). At the same time, aged
421 blood vessels produce increased amounts of COX-derived contractile factors promoting
422 the early onset of endothelial dysfunction (41,42). In this regard, we have also
423 demonstrated here that sDPP4 induces endothelial dysfunction in human mesenteric
424 microarteries, and that PAR2 or TP blockade prevented the impaired endothelium-
425 dependent relaxation induced by sDPP4.

426 We further hypothesized that sDPP4 might serve as a signal to activate the innate
427 immune response NLRP3 inflammasome machinery and the subsequent production of
428 IL-1β as mediator of sDPP4-induced endothelial senescence and dysfunction. It has been
429 reported that NLRP3 inflammasome over-activation contributes to vascular disease in

metabolic syndromes (11,43,44), while its self-activation reinforces endothelial senescence propagation and vascular aging progression (45). In our *in vitro* setting, the PAR2-COX-2-TP sequence would act upstream NLRP3 inflammasome activation triggered by sDPP4. In fact, we revealed TXA_2 as being able to fire NLRP3 inflammasome expression and assembly. A former study observed that TP activation in rat pulmonary arteries leads to activation of voltage-gated K^+ channels causing cellular potassium efflux (46), one well-established mechanism leading to NLRP3 inflammasome canonical activation. However, the detailed mechanism connecting TP and NLRP3 inflammasome activation needs further investigation. In any case, both the pharmacological inhibitor of NLRP3 inflammasome assembly MCC 950 and the direct antagonism of IL-1 receptors by anakinra were able to prevent the endothelial dysfunction induced by sDPP4 in human isolated microarteries. Therefore, we conclude that the same molecular pathway, namely the PAR2-COX-2-TP axis followed by NLRP3 inflammasome activation, is mediating either endothelial senescence and dysfunction triggered by sDPP4.

The adipokine sDPP4 thus appears as a relevant effector of vascular senescence and dysfunction. The relationship between endothelial senescence and endothelial dysfunction has been previously reviewed (47). While mice models of accelerated aging present impaired reactivity responses (48), the pro-inflammatory microenvironment and decreased nitric oxide bioavailability of endothelial senescent cells seems to be compromise vascular function (49).

To evaluate the relevance of our hypothesis in a pathological context of disease, we worked with a cohort of obese patients from whom human omentum samples were obtained. The obese patients (mean IMC 39.7±1.74) exhibited higher basal glucose levels and diastolic arterial pressure in comparison to the control non-obese participants,

455 together with an impaired microarterial endothelium-dependent relaxation to BK in
456 comparison to the control group. Importantly, when DPP4 activity or DPP4 downstream
457 signalling was pharmacologically inhibited *ex vivo*, the endothelial dysfunction of the
458 microvessels from obese participants was restored back to the levels of the control group.
459 At this point, we searched for a potential mechanistical explanation in the context of the
460 AT from which the microarteries were isolated. DPP4 was found overexpressed in the
461 visceral AT from obese over non-obese patients, as previously observed (13,15).
462 Furthermore, the visceral AT from obese patients drew a pro-senescent and pro-
463 inflammatory microenvironment, in which DPP4 expression positively correlated with
464 that of senescence-related genes.

465 Thus, DPP4 might emerge as a novel biomarker of AT senescence. In this line, it
466 was previously found upregulated in senescent fibroblasts and lymphocytes (50) and in
467 the aorta of aged versus young rats (51). Here we must consider a limitation of our study
468 due to the scarce human tissue availability, which makes it difficult to establish a cause-
469 effect relationship between DPP4 content in the AT and endothelial dysfunction in the
470 microarteries. Although we could not establish a direct relation between sDPP4-induced
471 endothelial senescence and dysfunction *in vivo,* we do demonstrate that the
472 pharmacological inhibition of DPP4 activity and its downstream pro-senescence pathway
473 reverted the impaired vasorelaxant response in the microarteries from obese patients,
474 without affecting those from control subjects. Moreover, we show that the tissular context
475 is different in the pathological obesity scenario and that a worse endothelial function
476 indicated by pEC50 values is positively associated with BMI, DPP4 expression and the
477 pro-senescence and pro-inflammatory picture in the visceral AT.

478 In line with our *in vitro* results, we propose that sDPP4 coming from visceral AT
479 might act as a direct inducer of local tissue senescence later propagated to the underlying

vascular wall. Previous studies indicated that the visceral AT explants from obese patients express and release higher amounts of sDPP4 in comparison to non-obese subjects (15). In addition, circulating DPP4 activity was formerly associated with an impaired skin microcirculation and flow mediated dilation in type 2 diabetes patients (52,53). Further supporting our results, recent *in vitro* studies have revealed the induction of senescence in the bidirectional crosstalk between AT and the vasculature (54,55). Therefore, the accumulation of senescent adipocytes has been related to an obesity-associated low-grade chronic inflammation (56) and insulin resistance (3), while senescent cells ablation and senolytic therapies alleviated the obese-related metabolic alterations (57,58).

At this point, we assessed whether DPP4 inhibitors might entail a therapeutic potential against vascular aging. Linagliptin prevented sDPP4-induced endothelial senescence, improving drastically the defective reactivity responses of obese patients' microvessels. Indeed, previous preclinical data have shown that DPP4 inhibitors exert anti-inflammatory, anti-proliferative (23), and anti-senescence effects *in vitro* (59), yet there are few indirect evidences about their *in vivo* implication in the context of aging. Based on our results, the *in vitro* anti-senescence effect of sDPP4 did not rely on an increased bioavailability of incretins. Interestingly, however, gliptins can bind to DPP4 in a region in the close proximity with the SLIG sequence and may thus hamper the direct interaction between sDPP4 and PAR2 in endothelial cells (60).

Interestingly, it has been reported that linagliptin treatment ameliorates the progression of premature aging phenotype in klotho KO mice, a model of premature aging (61). Besides, obese mice treated with DPP4 inhibitors exhibited greater survival and lifespan (62). Additionally, in elderly type 2 diabetes patients, gliptin treatment improved mild cognitive impairment in subjects with or without Alzheimer's disease (63). In this context, the SAVORO clinical trial (NCT02576288) will evaluate the effect of saxagliptin

preserving arterial safety and dysfunction in obese patients without cardiovascular disease. At present, nevertheless, there is not yet evidence of the ability of DPP4 inhibitors to reduce signs of accelerated vascular aging in humans although, based in the present results, gliptins might appear as a promising strategy to preserve and even improve endothelial function and delay vascular inflammaging.

Perspectives

sDPP4 is an AT-derived product whose levels are enhanced in the context of obesity and type 2 diabetes mellitus, where cardiovascular diseases are the main cause of death and disability. In this study, we identified sDPP4 as a relevant contributor both *in vitro* and *ex vivo* to the progression of two major aspects of vascular aging in the context of human obesity, namely endothelial senescence and endothelial dysfunction. It is accepted that preservation of vascular function is essential for a healthy aging and delay of vascular disease. In this line, we have demonstrated that the pharmacological inhibition of DPP4 itself, with approved gliptins, or the downstream signalling was able to reverse DPP4 effects in the vasculature, recovering normal vascular function in obese patients. Our results suggest that DPP4 and other AT-derived products are new therapeutic targets to tackle vascular inflammaging.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

CP, CFSF and TR conceived the manuscript. CP, CFSF, TR and IV designed the experiments. CP, CFSF, IV and JDE wrote the manuscript. IV performed the senescence, and WB experiments and statistically analysed all the data. SV and AR performed and analysed the vascular reactivity experiments. MP, RC and DGO recluted the patients and collected the omentum biopsies. PD and ASHL performed and analysed the qRT-PCR experiments. All authors approved the final version of the manuscript.

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Novelty and Significance

What is new?

- The adipokine sDPP4 induces human endothelial cell senescence *in vitro* and human microvascular endothelial dysfunction *ex vivo* by activation of PAR2-COX-2-TP axis and NLRP3 inflammasome.
- Over-expression of DPP4 is associated with a pro-senescence and pro-inflammatory microenvironment in the adipose tissue and a worse endothelial function in obese patients.

What is Relevant?

- Pharmacological blockade of DPP4 and its related signalling drastically improves the *ex vivo* vascular function of obese patients exhibiting hyperglycaemia and hypertension.

Summary

- DPP4 is a mediator of accelerated vascular aging.
- Approved antidiabetic DPP4 inhibitors (gliptins) arise as a promising strategy to preserve endothelial function and delay vascular inflammaging.

Figure Legends

Figure 1. **sDPP4 induces cell senescence *in vitro* and endothelial dysfunction *ex vivo*.** HUVEC were treated with human recombinant sDPP4 (10-500 ng/ml) for 24h. **(A)** Representative phase contrast microphotographs (200X magnification) showing SA-β-gal staining. Images were captured with a Nikon DS-U3 camera using the NIS-Element 4.50 imaging software. Black arrows indicate SA-β-gal^{+} cells, which **(B)** were quantified by blind manual scoring after the indicated treatments. (n=3-4, *p<0.05 vs. untreated cells by one-way ANOVA). DPP4 inhibitors linagliptin (10 nmol/l) and K579 (100 nmol/l) were used in presence of the submaximal concentration of sDPP4 (200 ng/ml) selected for further experiments, and **(C)** SA-β-gal staining **(D)** γH2AX and **(E)** p21 protein levels were determined. Data are expressed as fold over sDPP4-induced levels. Representative blots are shown on top of the corresponding graphs (n=3-13, *p<0.05 vs. untreated cells, #p<0.05 vs. sDPP4 (200 ng/ml)-induced response by one-way ANOVA). In other set of experiments, mesenteric microvessels were isolated from omentum biopsies from control non-obese patients that were exposed to **(F)** sDPP4 (200 ng/ml) alone or **(G)** in combination with linagliptin (10 nmol/l) and later submitted to BK (10^{-11}-10^{-5} mol/l) to evaluate endothelium-dependent relaxation. Values (mean ± SEM) in contraction curves were calculated as average percentage of KCl 35 mmol/l contraction for all segments (n) coming from pooled patients (p) in each group. The curves were expressed as the percentage of the previous potassium-evoked contraction. (*p<0.05 vs. control untreated

segments response, #p<0.05 vs. sDPP4 (200 ng/ml)-induced response by two-way ANOVA). L: linagliptin, K: K579.

Figure 2. PAR2-COX-2-TP axis mediates the sDPP4-induced vascular effects. HUVEC were exposed to sDPP4 (200 ng/ml) alone or in presence of GB83 (10 μmol/l), celecoxib (3 μmol/l), or SQ 29,548 (10 μmol/l), for later assessment of **(A)** SA-β-gal staining and **(B)** γH2AX protein level expression. In panel A data are presented as fold over the number SA-β-Gal^{+} cells after sDPP4 challenge; in panel B data are expressed as fold over sDPP4-induced levels with representative blots shown on top of the corresponding graph (n=3-11, *p<0.05 vs. untreated cells. #p<0.05 vs. sDPP4 (200 ng/ml)-treated cultures levels by one-way ANOVA). Results are expressed as mean ± SEM (error bars). Isolated human mesenteric microvessels were exposed to sDPP4 (200 ng/ml) alone or in combination with **(C)** GB83 or **(D)** SQ 29,548, and their relaxing capacity was evaluated by myography. Values (mean ± SEM) in contraction curves were calculated as average percentage of KCl 35 mmol/l contraction for all segments (n) coming from pooled patients (p) in each group. The curves were expressed as the percentage of the previous potassium-evoked contraction. (*p<0.05 vs. control untreated segments response. #p<0.05 vs. sDPP4 (200 ng/ml)-induced response by two-way ANOVA). G: GB83; C: celecoxib; S: SQ 29,548.

Figure 3. sDPP4 triggers NLRP3 inflammasome activation in HUVEC. The expression of NLRP3 inflammasome components **(A)** NLRP3 **(B)** ASC and **(C)** pro-IL-1β were determined by western blot in total cell lysates after sDPP4 (200 ng/ml) challenge for 24h. As indicative of NLRP3 inflammasome activation, **(D)** caspase-1 activation (represented as pro-caspase-1 (p45) versus cle-caspase-1 (p10) fragment expression) and **(E)** mature IL-1β expression werevalidated by western blot. In some experiments, HUVEC were treated with linagliptin (10 nmol/l) or K579 (100 nmol/l) in presence of

784 sDPP4 and **(F)** NLRP3 and **(G)** IL-1β protein expression were evaluated. For panels A-
785 G, representative blots are shown on top of corresponding graphs (n=3-12, *p<0.05 vs.
786 untreated cells by unpaired t-test or Mann Whitney test). **(H)** The percentage of ASC-
787 specks^{+} cells was quantified under a fluorescence microscope (n=5-10, *p<0.05 vs.
788 untreated cells, #p<0.05 vs. sDPP4 (200 ng/ml)-treated cultures levels by one-way
789 ANOVA). **(I)** ASC-speck visualization by indirect immunofluorescence after 24h
790 challenge with sDPP4 (200 ng/ml) alone or in presence of MCC950 (1 μmol/l), linagliptin
791 (10 nmol/l) or K579 (100 nmol/l). Confocal representative photographs (630X
792 magnification) where white arrowheads indicate the location of toroidal-shaped ASC
793 specks. Specific antibody against ASC (red) was used, while cell nuclei were
794 counterstained with DAPI (blue). Images were captured with a TCS SPE confocal
795 microscope. Results are shown as mean ± SEM (error bars). L: linagliptin, K: K579.

796 **Figure 4. sDPP4-induced NLRP3 inflammasome activation is mediated by PAR2-**
797 **COX-2-TP axis. (A)** NLRP3 and **(B)** mature IL-1β protein levels expression were
798 detected by western blot in total cell lysates of HUVEC exposed to sDPP4 (200 ng/ml)
799 alone or in combination with GB83 (10 μmol/l), celecoxib (3 μmol/l) or SQ 29,548 (10
800 μmol/l). Data are represented as fold increase of each protein expression levels in sDPP4-
801 treated cells. Representative blots are shown on top of the corresponding graphs (n=4-8,
802 p<0.05 vs. untreated cells, #p<0.05 vs. sDPP4 (200 ng/ml)-treated cultures levels by one-
803 way ANOVA). ASC-specks^{+} cells were detected by indirect immunofluorescence in
804 presence of the aforementioned treatments. **(C)** Percentage of ASC-specks positive cells
805 was quantified by scoring under a fluorescence microscope (n=3-13, *p<0.05 vs.
806 untreated cells. #p<0.05 vs. sDPP4 (200 ng/ml)-treated cultures levels by one-way
807 ANOVA) **(D)** Representative confocal microscopy images (630X magnification) show
808 specific antibody against ASC (red) and cell nuclei counterstained with DAPI (blue).

White arrowheads depict the location of toroidal-shaped ASC specks. Images were captured with a TCS SPE confocal microscope. Results are shown as mean ± SEM (error bars). G: GB83; C: celecoxib; S: SQ 29,548.

Figure 5. NLRP3 inflammasome and its end-product IL-1β are the final effector of sDPP4-induced vascular effects. HUVEC were exposed to sDPP4 (200 ng/ml) or the TXA_2 stable analogue U46619 (1 μmol/l) for 24h alone or in combination with MCC 950 (1 μmol/l) or anakinra (1 μg/ml). **(A)** SA-β-gal$^+$ cells were quantified by manual scoring after indicated treatments. Data are represented as fold over the number of senescent cells in non-treated cultures. **(B)** γH2AX protein levels were determined by western blot in total cell lysates exposed to the aforementioned treatments. A representative blot is shown on top of the graph. Data are expressed as fold over protein levels in non-treated cultures (n=3-7, *p<0.05 vs. untreated cells, #p<0.05 vs. sDPP4 (200 ng/ml), $p<0.05 vs. U46619 (1 μmol/l) by one-way ANOVA). Isolated human mesenteric microvessels were exposed to sDPP4 (200 ng/ml) alone or in combination with **(C)** MCC 950 or **(D)** anakinra, and their relaxing capacity was evaluated by myography. Values (mean ± SEM) in contraction curves were calculated as average percentage of KCl 35 mmol/l contraction for all segments (n) coming from pooled patients (p) in each group. The curves were expressed as the percentage of the previous potassium-evoked contraction. *p<0.05 vs. control untreated segments response. #p<0.05 vs. sDPP4 (200 ng/ml)-induced response by two-way ANOVA. Results are shown as mean ± SEM (error bars). MCC: MCC 950; AK: anakinra.

Figure 6. AT content in DPP4, pro-senescence and pro-inflammatory genes is associated to endothelial dysfunction in human obesity. Mesenteric microvessels were isolated from omentum biopsies from a group of control non-obese (n=13) and obese (n=12) human donors were submitted to crescent concentration of **(A)** BK (10^{-11}-10^{-5}

834 mol/l) or **(B)** SNP (10^{-9}-10^{-4} mol/l) to evaluate their endothelium-dependent and
835 independent relaxation responses, respectively. **(C)** The local expression of DPP4 in the
836 visceral AT in the close proximity of the microvessels from control vs. obese patients was
837 analysed by qRT-PCR. **(D)** Heatmap summarizing the levels of visceral AT mRNA
838 expression detected by qRT-PCR of pro-senescence markers p53, p21 and p16 and the
839 NLRP3-inflammasome components and products NLRP3, IL-1β and IL-18, relative to
840 each patient represented by each one of the columns in the graph. Red intensity depicts
841 the level of expression of each gene as fold increase over the expression of pooled non-
842 obese levels and normalized to 18S rRNA levels. **(E)** Linear correlation analysis was
843 performed between pEC50 values for BK relaxation responses and DPP4 mRNA
844 expression in visceral AT. p-value and Spearman coefficient (r_S), are indicated on top of
845 the graph. Obese patients' dissected microvessels were submitted to cumulative
846 concentration of BK in presence or not of the drugs: **(F)** linagliptin, **(G)** GB83 or **(H)** SQ
847 29,548. In panels A, B, E, F and G, the values (mean ± SEM) in contraction curves were
848 calculated as average percentage of KCl 35 mmol/l contraction for all segments (n)
849 coming from pooled patients (p) in each group. The curves were expressed as the
850 percentage of the previous potassium-evoked contraction, which is indicated in the
851 Supplemental figure S4C. *$p<0.05$ vs. control (non-obese or obese) untreated segments
852 response. (#$p<0.05$ vs. sDPP4 (200 ng/ml)-induced response by two-way ANOVA).