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Soluble DPP4 induces inflammation and proliferation of human smooth muscle cells via protease-activated receptor 2

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ABSTRACT

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

1. Introduction

Type 2 diabetes and obesity are frequently associated with the development of cardiovascular disease (CVD) [44]. CVD remains the major cause of death worldwide and represents a key global health problem due to the epidemic increase of obesity-induced insulin resistance and type 2 diabetes [21]. Adipose tissue was long seen as a passive lipid storage depot but it is now considered as an endo- and paracrine organ that produces a large number of mediators that affect metabolism, inflammation and coagulation. Expansion of adipose tissue in obesity is highly associated with an enhanced secretion of pro-inflammatory and pro-atherogenic factors [33,38]. Thus, alterations in adipocyte derived factors, the so called adipokines, may link obesity to cardiovascular dysfunction.

Dipeptidyl peptidase 4 (DPP4) is an ubiquitously expressed cell-surface protease, which selectively cleaves N-terminal dipeptides from a variety of substrates including growth factors, hormones, neuropeptides and chemokines. Substantial DPP4 activity is also found in plasma and other body fluids because of a soluble form of DPP4 lacking the cytoplasmic tail and the transmembrane region [28]. In a previous study we could identify sDPP4 as a novel adipokine [29], which is widely known for its role in the regulation of glycemia through catabolism of incretin peptides, and we could show that sDPP4 serum concentrations positively correlate with various parameters of the metabolic syndrome like BMI, adipocyte surface, leptin and insulin levels [29,37]. Interestingly, comparison of different fat depots showed the highest DPP4 expression in visceral adipose tissue of obese patients [37]. Furthermore, we were the first in investigating direct effects of sDPP4 on human adipocytes, human skeletal muscle cells and hVSMC showing a sDPP4-induced insulin resistance and an increased hVSMC proliferation [29]. Therefore sDPP4 is a promising candidate linking obesity to CVD. Up to now DPP4 has gained most interest as a therapeutic target for type 2 diabetes treatment due to the degradation of the incretin hormone glucagon-like peptide (GLP)-1 by this enzyme. Thus, DPP4-inhibitors are able to prolong the insulinotropic effect of GLP-1 and are now in

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clinical use as anti-diabetic drugs [2]. Additionally, preclinical and preliminary clinical data suggest that DPP4-inhibitors hold interesting promise for cardiovascular protection. Since GLP-1 itself has favorable cardiovascular effects [3,32] most of these protective effects of DPP4 inhibition are ascribed to the increased bioavailability of GLP-1. Nevertheless, there are accumulating data suggesting that DPP4-inhibitors mediate vascular protection independent of GLP-1, involving endothelial repair [19], anti-inflammatory effects [15,19,42] and blunting of ischemic injury [48,49].

Vascular endothelial cells and smooth muscle cells represent the major cell types of the artery wall preserving vessel wall homeostasis. Migration of hVSMC from the media to intima and their concomitant proliferation occurring in the synthetic state are critical causes of arterial wall thickening and the development of arteriosclerosis [27]. Moreover, hVSMCs are involved in the ongoing low-grade inflammation within arteriosclerotic lesions by recruitment of immune cells [9]. However, the underlying mechanism of hVSMC dysfunction taking place in type 2 diabetes and obesity is not fully understood.

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

2. Material and methods

2.1. Cell culture of hVSMC

Primary human coronary artery SMCs were obtained from PromoCell (Heidelberg, Germany), tebu-bio (Offenbach, Germany) and Lonza (Basel, Switzerland). hVSMCs from three different donors (Caucasian, male, 58 years old; two female, both 55 years old) were supplied as proliferating cells and kept in culture according to the manufacturer's protocol. For all experiments, subconfluent cells of passage 5 were used. Cells were characterized as hVSMCs by morphologic criteria and by immunostaining with smooth muscle α-actin. Cells were seeded in smooth muscle cell growth medium (PromoCell, Heidelberg, Germany) with 5% fetal calf serum (FCS) (Gibco Invitrogen, Carlsbad, CA, USA) and allowed to attach for 24 h. Afterwards cells were serum-starved for 24 h and then exposed to sDPP4 (R&D Systems, Wiesbaden-Nordenstadt, Germany) alone or in combination with the specific DPP4-inhibitor K579 (Tocris Bioscience, Bristol, UK), the ERK1/2-inhibitor PD98059 (Calbiochem, Merck Biosciences, Schwalbach, Germany), the specific PAR2 antagonist GB83 or the highly specific IκB-Kinase (IKK)-inhibitor I229 [27]. K579 is a slow binding but long acting DPP4-inhibitor, which was successfully tested as a hypoglycemic agent in Zucker fatty rats [43]. The IKK-inhibitor was from Sanofi-Aventis (Frankfurt, Germany). It has submicromolar activity on the isolated IKK complex and is highly specific on IKK. The general structure of I229 is described in PCT/EP00/05340. The DPP4- and IKK-inhibitor were dissolved in DMSO as a 100 mM stock solution, and were further diluted in sterile serum-free SMC medium up to 100 nM and 100 μM, respectively. The ERK1/2-inhibitor was already dissolved in DMSO as a 5 mg/ml stock solution, and was further diluted in sterile serum-free SMC medium up to 10 μM. Cells were 1 h preincubated with the ERK1/2- and the IKK-inhibitor and subsequently treated with sDPP4 with and without concomitant administration of the specific inhibitors. The specific PAR2 antagonist GB83 was dissolved in DMSO as a 32.8 mM stock solution and was further diluted in sterile serum-free SMC medium up to 1 μM. All controls of experiments involving the DPP4-, the IKK-inhibitor or the PAR2 antagonist were treated with DMSO alone. Furthermore, hVSMCs were treated with the PAR2-activating peptide (AP) (Bachem, Bubendorf, Switzerland) alone or in combination with PAR2 antagonist GB83 for 10 min.

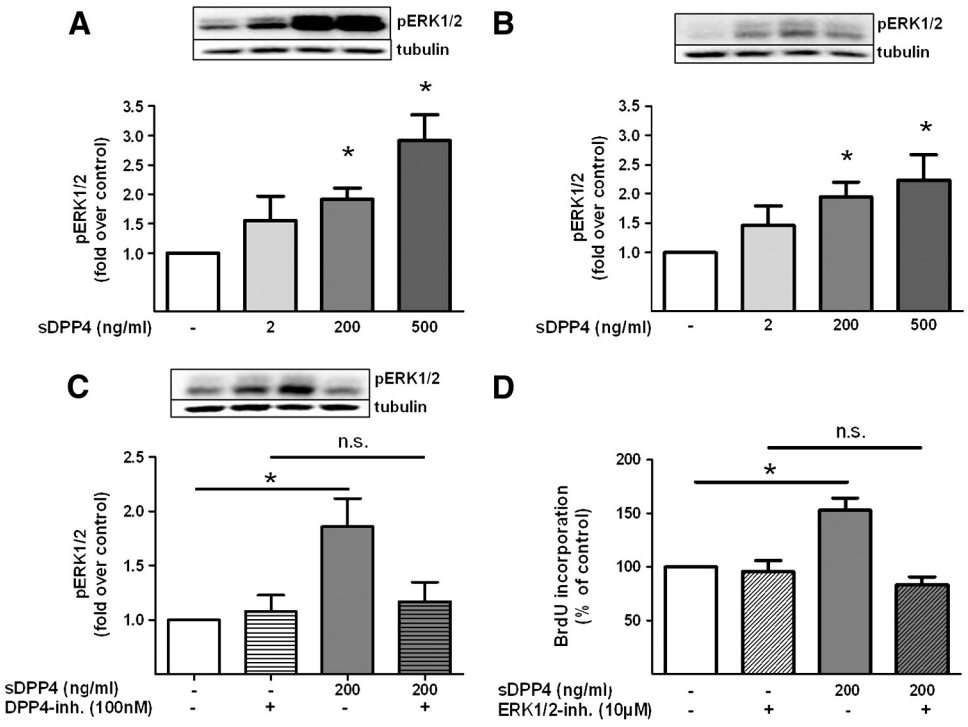


Fig. 1. Effect of sDPP4 on the ERK1/2 signaling pathway in hVSMCs. Cells were serum-starved for 24 h and subsequently treated with the indicated amounts of sDPP4 with and without concomitant administration of the specific DPP4-inhibitor K579 for 6 (A and C) and 24 h (B). (A–C) Total cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody to the phosphorylated form of ERK1/2. Data are tubulin normalized mean values ± SEM of four independent experiments. *$p < 0.05$ compared as indicated. Representative Western Blots are presented. Two-way ANOVA: sDPP4 $p < 0.05$, Inhibitor n.s. and sDPP4xInhibitor $p < 0.05$. (D) hVSMCs were treated with sDPP4 alone or in combination with the ERK1/2 Inhibitor PD98059 for 24 h. Proliferation of hVSMCs was determined by measuring the incorporation of BrdU into DNA. Data are expressed relative to the basal control value, taken as 100%. Data are mean values ± SEM of three independent experiments. *$p < 0.05$ compared as indicated. Two-way ANOVA: sDPP4 $p < 0.01$, Inhibitor $p < 0.05$ and sDPP4xInhibitor $p < 0.01$.

2.2. In vitro analysis of hVSMC proliferation

To monitor DNA synthesis, 10.000 hVSMC per 15 mm^2 well were seeded in 96 well culture dishes and allowed to attach for 24 h, followed by serum starvation for an additional 24 h period. hVSMC were exposed to sDPP4 alone or in combination with the ERK1/2-inhibitor or the PAR2 antagonist in the presence of BrdU (10 μM) for 24 h. All controls of experiments involving ERK1/2-inhibitor or PAR2 antagonist were treated with DMSO alone. 5% FCS was used as a positive control. The BrdU ELISA (chemiluminescent) (Roche, Mannheim, Germany) was used to determine the proliferation according to the manufacturer's protocol. Signals were visualized and evaluated on a LUMI Imager work station (Boehringer, Mannheim, Germany).

2.3. Transfection of hVSMC with PAR2 siRNA

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

2.4. Analysis of protein expression and phosphorylation

hVSMCs were treated as indicated and lysed in a buffer containing 50 mM HEPES, pH 7.4, 1% TritonX100, complete protease inhibitor and PhosStop phosphatase inhibitor cocktail. After incubation for 2 h at 4 °C on a rotation-shaker, the suspension was centrifuged at 10,000 *g* for 15 min. Thereafter, 5 μg protein of lysates were separated by SDS-PAGE using 10% horizontal gels and transferred to polyvenylidene fluoride filters in a semi-dry blotting apparatus [45]. Filters were blocked with Tris-buffered saline containing 0.1% Tween and 5% non-fat dry milk and subsequently incubated overnight with primary antibodies for anti-phospho-NF-κB (P65) (Ser536) (Cell Signalling Technology, Frankfurt, Germany), anti-phospho-p44/42 MAPK (ERK1/2, Thr202/Tyr204) (Cell Signalling Technology, Frankfurt, Germany), anti-iNOS (BD Transduction Laboratories, Heidelberg, Germany), PAR2 (Santa Cruz Biotechnology, Heidelberg, Germany) and alpha-tubulin (Calbiochem Merck Biosciences, Schwalbach, Germany). After washing, filters were incubated with corresponding secondary HRP-coupled antibody (Promega, Mannheim, Germany) and processed for enhanced chemiluminescence detection using Immobilon HRP substrate (Millipore, Billerica, MA, USA). Signals were visualized and evaluated on a LUMI Imager work station.

2.5. Determination of cytokine secretion

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

2.6. RNA isolation, cDNA synthesis and qRT-PCR

To assess the effects on mRNA expression, hVSMCs were harvested 24 h after the addition of sDPP4 with and without the different inhibitors. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden,

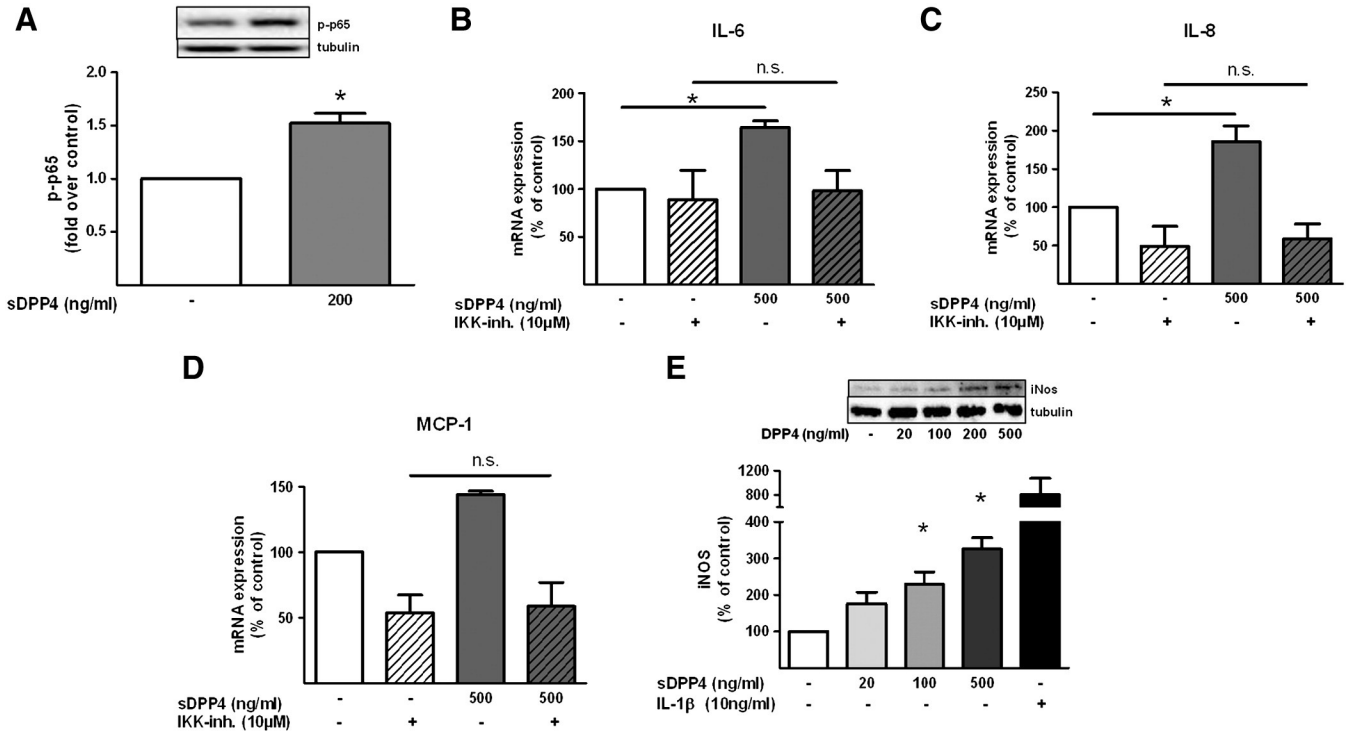


Fig. 2. Impact of sDPP4 on the NF-κB signaling pathway and the expression of pro-inflammatory cytokines in hVSMCs. (A) After 24 h serum-starvation, cells were treated with the indicated amounts of sDPP4 for 6 h. Phosphorylation of the NF-κB subunit p65 was assessed by Western Blot analysis. Data are tubulin normalized mean values ± SEM of four independent experiments. *$p < 0.05$ compared to control hVSMCs. Representative Western Blots are presented. (B–D) Cells were serum-starved for 24 h and then treated with indicated concentrations of sDPP4 alone or in combination with the IKK-inhibitor I229 for 24 h. mRNA level of IL-6 (B), IL-8 (C) and MCP-1 (D) were quantified by Real-time PCR and normalized to the level of β-actin. Data are expressed relative to the basal control value and are means ± SEM of three independent experiments. *$p < 0.05$ compared as indicated. Two-way ANOVA: for B sDPP4 $p < 0.05$, Inhibitor $p < 0.05$ and sDPP4xInhibitor n.s.; for C sDPP4 n.s., Inhibitor $p < 0.05$ and sDPP4xInhibitor $p < 0.05$; for D sDPP4 $p < 0.05$, Inhibitor n.s. and sDPP4xInhibitor n.s. (E) After 24 h serum-starvation, hVSMCs were exposed to the indicated amounts of sDPP4 for 18 h. The expression of iNOS was assessed by Western Blot analysis and normalized to tubulin. Representative Western Blot and the respective quantification are presented. IL-1β was used as a positive control. Data are expressed relative to the basal control value and are means ± SEM of four independent experiments. *$p < 0.05$ compared to control hVSMCs.

Germany) according to the manufacturer's instructions. RNA concentration and purity was measured with a NanoDrop 2000 (Thermo Scientific, Schwerte, Germany). One microgram of RNA was reversely transcribed using an Omniscript RT Kit (Qiagen, Venlo, Netherlands). mRNA expression levels were determined with predesigned Quantitect Primer Assays for IL-6 (QT00083720), IL-8 (QT00000322), MCP-1 (QT00212730) and β-Actin (QT01680476) and GoTaq® qPCR Master Mix (Promega) on a StepOne Plus real-time PCR system (Applied Biosystems, Carlsbad, CA, USA).

2.7. Presentation of data and statistics

Data are expressed as mean ± S.E.M. Unpaired two-tailed Student's *t*-test or one-way and two-way ANOVA (post hoc test: Bonferroni) were used to determine statistical significance. All statistical analyses were done using Prism (GraphPad, La Jolla, CA, USA) considering a p-value < 0.05 as statistically significant.

3. Results

3.1. sDPP4-induced ERK1/2 activation leads to increased proliferation of hVSMCs

In a previous study, we showed that sDPP4 induces proliferation in hVSMC in a concentration-dependent manner which can be prevented by the inhibition of sDPP4 activity [29]. Therefore we investigated the effects of sDPP4 on the MAPK signaling pathway and its involvement in hVSMC proliferation. Using increasing concentrations of sDPP4 (2–500 ng/ml), we could observe a concentration-dependent activation of ERK1/2 (3-fold) after 6 h (Fig. 1A), which remained stable for up to 24 h (Fig. 1B). To demonstrate the specificity of this effect, 100 nM of the DPP4-inhibitor K579 was used. The DPP4-inhibitor alone had no effect, but in combination with sDPP4 the ERK1/2 activation could be completely abrogated after 6 h (Fig. 1C). Furthermore, we could show that the sDPP4-induced hVSMC proliferation could totally be prevented by 10 μM of the ERK1/2-inhibitior PD98059 (Fig. 1D).

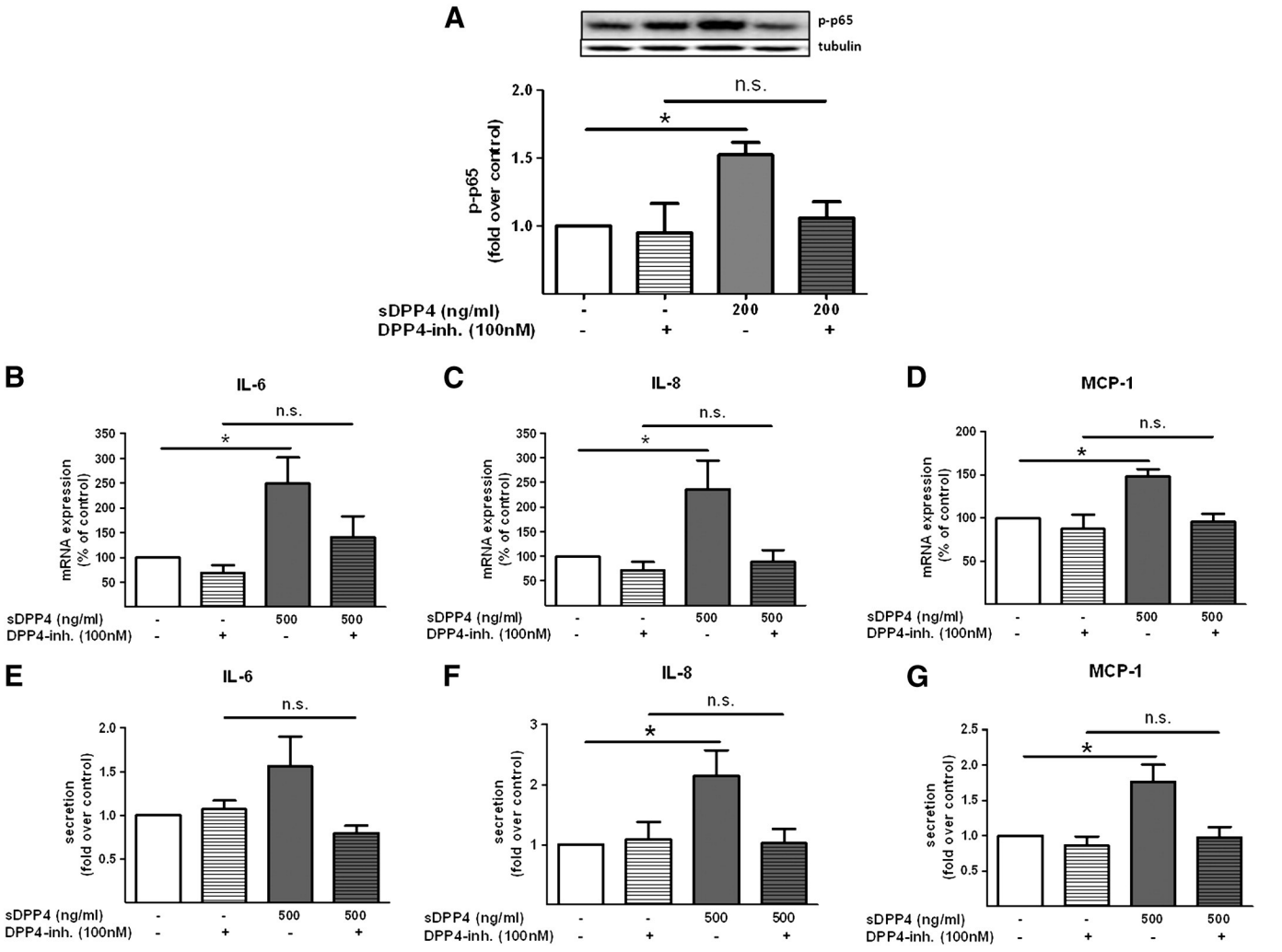


Fig. 3. Effect of the DPP4-inhibitor on the sDPP4-induced cytokine expression and secretion. (A) After 24 h serum-starvation, cells were treated with indicated amounts of sDPP4 alone or in combination with the DPP4-inhibitor K579 for 6 h. Phosphorylation of the NF-κB subunit p65 was assessed by Western Blot analysis. Data are tubulin normalized mean values ± SEM of four independent experiments. *$p < 0.05$ compared to control hVSMCs. Representative Western Blots are presented. Two-way ANOVA: sDPP4 $p < 0.05$, Inhibitor $p < 0.05$ and sDPP4xInhibitor n.s. (B–D) hVSMC were serum-starved for 24 h and then treated with the indicated amounts of sDPP4 alone or in combination with the DPP4-inhibitor K579 for 24 h. mRNA level of IL-6 (B), IL-8 (C) and MCP-1 (D) were quantified by Real-time PCR and normalized to the level of β-actin. Data are expressed relative to the basal control value and are means ± SEM of three independent experiments. *$p < 0.05$ compared as indicated. Two-way ANOVA: for B sDPP4 $p < 0.01$, Inhibitor n.s. and sDPP4xInhibitor n.s.; for C sDPP4 $p < 0.05$, Inhibitor $p < 0.05$ and sDPP4xInhibitor = n.s.; for D sDPP4 $p < 0.05$, Inhibitor $p < 0.05$ and sDPP4xInhibitor $p < 0.05$. (E–G) After 24 h exposure to the respective treatments, supernatants were collected and IL-6 (E), IL-8 (F) and MCP-1 (G) concentration was measured by ELISA assay. Data are expressed relative to the basal control value and are means ± SEM of four independent experiments. *$p < 0.05$ compared as indicated. Two-way ANOVA: for E sDPP4 n.s., Inhibitor n.s. and sDPP4xInhibitor $p < 0.05$; for F sDPP4 n.s., Inhibitor n.s. and sDPP4xInhibitor $p < 0.05$; for G sDPP4 $p < 0.05$, Inhibitor $p < 0.05$ and sDPP4xInhibitor n.s.

3.2. sDPP4-mediated NF-κB activation results in elevated cytokine and iNOS expression in hVSMCs

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

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

Consistent with our findings on the ERK signaling pathway, we were able to prevent the sDPP4-induced NF-κB activation after 6 h by the inhibition of DPP4 (Fig. 3A), whereas the inhibitor itself showed no effect. Accordingly, we were also able to block the sDPP4-induced expression (Fig. 3B–D) and secretion (Fig. 3E–G) of the pro-inflammatory cytokines IL-6, IL-8 and MCP-1 by DPP4 inhibition. These data underpin the notion of incretin-independent effects of DPP4-inhibitors [18].

3.4. DPP4 contains a potential PAR2 activating sequence

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

3.5. PAR2 silencing prevents sDPP4-induced proliferation and inflammation

It could be shown that PAR2 is highly expressed in epithelial cells and smooth muscle of vascular and nonvascular origins [14]. Here we assessed PAR2 expression in three different primary human cell types and observed a 2-time higher PAR2 expression in hVSMC compared to

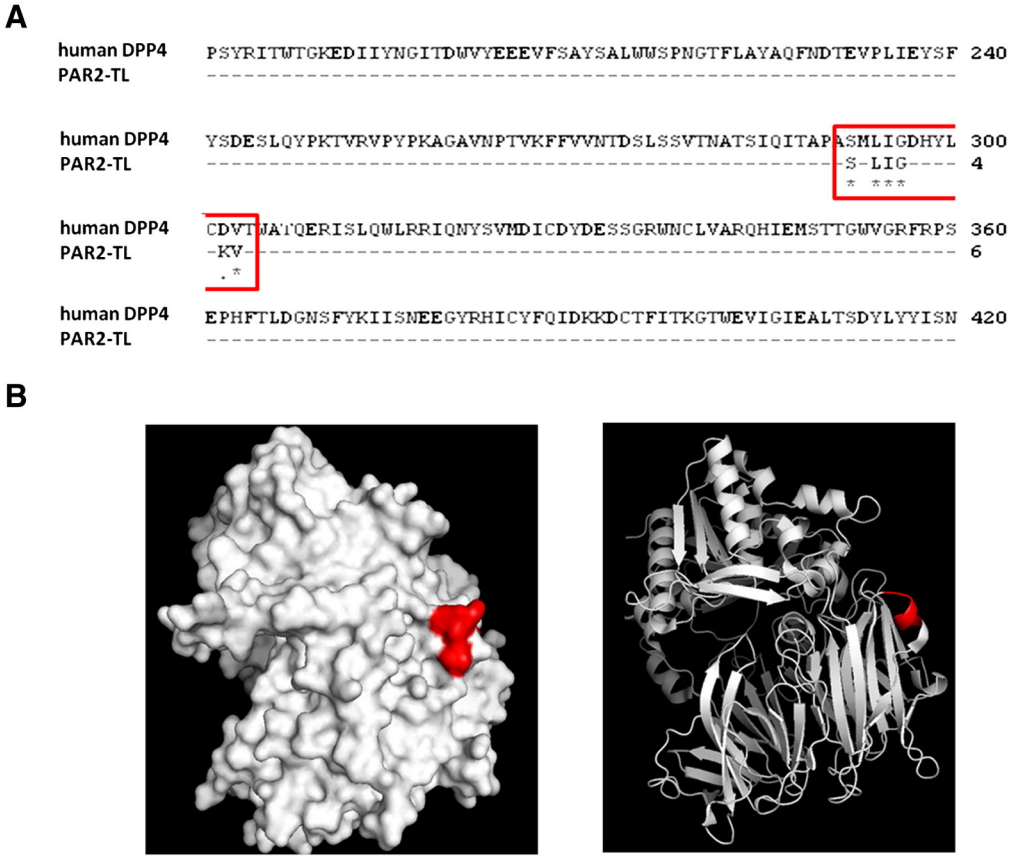
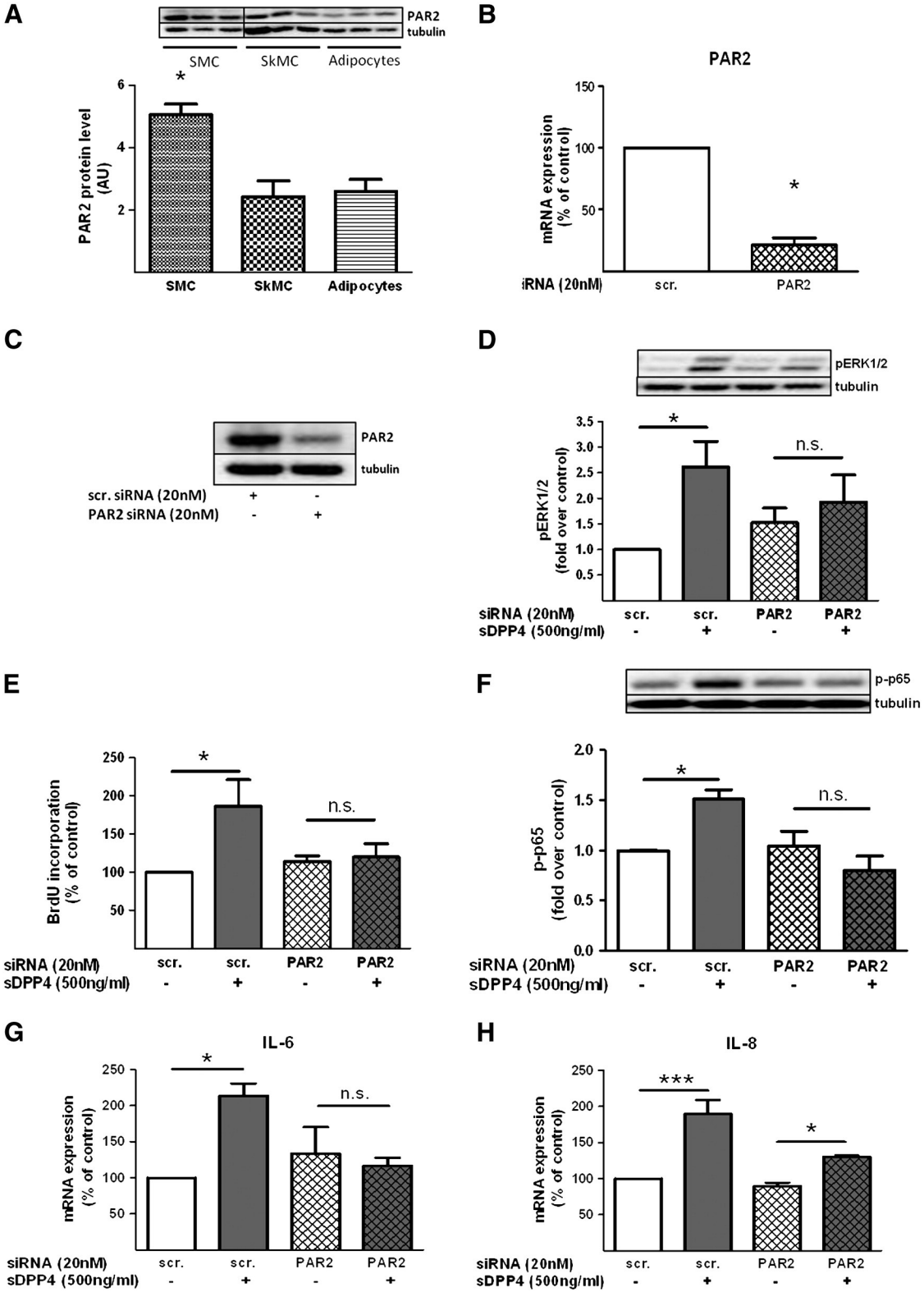


Fig. 4. Homology between DPP4 and the tethered ligand (TL) sequence of PAR2. (A) CLUSTALW was used to align the sequence of the human DPP4 (P27487 UniProtKB) and the TL sequence of PAR2 (SLIGKV). The TL sequence is also aligned to the sequence of the crystallized DPP4 (PDB ID 4A5S). (B) Surface representation (left) and ribbon diagram (right) of the crystal structure human DPP4. The potential TL sequence (SLIG) is highlighted in red. Images were created with PyMol (PyMOL v1.6). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



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

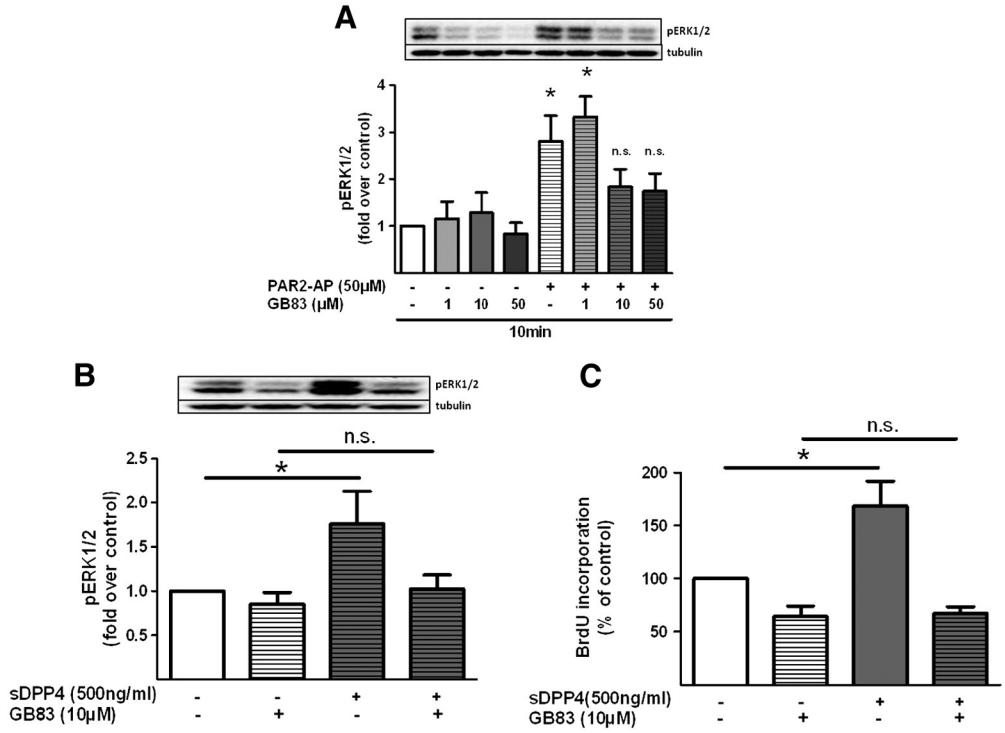


Fig. 6. Impact of the PAR2 antagonist GB83 on sDPP4-induced ERK signaling and proliferation. (A) Cells were pretreated for 1 h with the indicated amounts of the PAR2 antagonist and then stimulated with the PAR2-activating peptide (AP) (SLIGKV) for 10 min. Phosphorylation of ERK1/2 was assessed by Western Blot analysis. Data are tubulin normalized mean values ± SEM of three independent experiments. *$p < 0.05$ compared to control hVSMC. Representative Western Blot is presented. (B) After 1 h pre-incubation with the PAR2 antagonist GB83, hVSMCs were treated with sDPP4 for 6 h. Phosphorylation of ERK1/2 was assessed by Western Blot analysis. Data are tubulin normalized mean values ± SEM of three independent experiments. *$p < 0.05$ compared as indicated. Representative Western Blot is presented. Two-way ANOVA: sDPP4 $p < 0.05$, Antagonist n.s. and sDPP4xAntagonist n.s. (C) Cells were treated for 24 h with sDPP4 alone or in combination with the PAR2 antagonist GB83. Proliferation of hVSMCs was determined as described before. Data are expressed relative to the negative control value, taken as 100%. Data are mean values ± SEM of four independent experiments. *$p < 0.05$ compared as indicated. Two-way ANOVA: sDPP4 $p < 0.05$, Antagonist $p < 0.001$ and sDPP4xAntagonist $p < 0.05$.

IL-6 could be abolished by PAR2 silencing (Fig. 5F,G). Additionally, sDPP4-induced upregulation of IL-8 could be partially prevented by PAR2 silencing (Fig. 5H).

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

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

4. Discussion

Since central obesity is characterized by an enhanced cardiovascular risk, it has been speculated that adipocyte derived factors might directly contribute to the pathogenesis of atherosclerosis [4,11]. In a previous study, we identified sDPP4 as a novel adipokine, which was markedly higher expressed in and released from visceral compared to subcutaneous adipose tissue of obese patients [29,37]. Additionally, we could demonstrate that sDPP4 directly impairs insulin signaling in three different primary cell types, namely, adipocytes, skeletal muscle, and smooth muscle cells and increases hVSMC proliferation [29]. Here we further assessed direct effects of sDPP4 on hVSMC in vitro and aimed to identify the underlying signaling pathway for the observed inflammation and proliferation. For our study, we used sDPP4 concentrations reflecting circulating levels that were measured in both lean and obese subjects [29]. Interestingly, high serum levels of sDPP4 have

Fig. 5. Impact of PAR2 silencing on the sDPP4-induced proliferation and inflammation. (A) Expression of PAR2 in three different primary human cell types, namely SMC, in vitro differentiated skeletal muscle cells (SkMC) and adipocytes was assessed by Western Blot. For each cell type three different donors were analyzed. Data are tubulin normalized and expressed as mean values ± SEM. *$p < 0.05$ ($n = 3$). Representative Western Blots are presented. Cells were transfected either with 20 nM scramble or PAR2 siRNA. (B) 24 h after transfection PAR2 mRNA levels were quantified by Real-time PCR. PAR2 mRNA was normalized to the level of β-actin. Data are expressed relative to the negative control value and are means ± SEM of three independent experiments. *$p < 0.05$ compared to control hVSMC. (C) 30 h after transfection PAR2 protein level was assesed by Western Blot analysis. Representative Western Blot is presented. (D) 24 h after transfection hVSMCs were exposed to sDPP4 for 6 h. Phosphorylation of ERK1/2 was detected by Western Blot analysis. Data are tubulin normalized mean values ± SEM of six independent experiments. *$p < 0.05$ compared as indicated. Representative Western Blots are presented. Two-way ANOVA: sDPP4 $p < 0.05$, Silencing n.s. and sDPP4xSilencing n.s. (E) 24 h after transfection hVSMCs were exposed to sDPP4 for further 24 h. Proliferation of hVSMC was determined as described before. Data are expressed relative to the negative control value, taken as 100%. Data are mean values ± SEM of three independent experiments. *$p < 0.05$ compared as indicated. Two-way ANOVA: sDPP4 $p < 0.05$, Silencing n.s. and sDPP4xSilencing n.s. (F) 24 h after transfection hVSMCs were exposed to sDPP4 for 6 h. Phosphorylation of p65 was assessed by Western Blot analysis. Data are tubulin normalized mean values ± SEM of three independent experiments. *$p < 0.05$ compared as indicated. Representative Western Blots are presented. Two-way ANOVA: sDPP4 n.s., Silencing $p < 0.05$ and sDPP4xSilencing $p < 0.01$. (G and H) 24 h after transfection hVSMCs were exposed to sDPP4 for further 24 h. IL-6 (G) and IL-8 (H) mRNA levels were quantified by Real-time PCR and normalized to the level of β-actin. Data are expressed relative to the negative control value and are means ± SEM of three independent experiments. *$p < 0.05$, ***$p < 0,001$ compared as indicated. Two-way ANOVA: for G sDPP4 n.s., Silencing n.s. and sDPP4xSilencing $p < 0.05$; for H sDPP4 $p < 0.001$, Silencing $p < 0.01$ and sDPP4xSilencing $p < 0.05$.

been described in various conditions [20] including atherosclerosis [17]. In the present study, we could show that sDPP4 activates the MAPK and NF-κB signaling cascade resulting in pro-atherogenic changes in hVSMC illustrated by an increased proliferation, the induction of iNOS and elevated expression and secretion of pro-inflammatory cytokines.

DPP4-inhibitors, a class of oral anti-hyperglycemic agents that prolong the bioavailability of the endogenously secreted incretin hormone GLP-1 and the glucose-dependent insulinotropic polypeptide (GIP), are effective in the treatment of type 2 diabetes. DPP4-inhibitors have been validated and approved as drugs that can lower both fasting and postprandial glucose levels and improve islet β-cell function in patients with diabetes [16]. In addition to their anti-diabetic properties, accumulating data indicate that DPP4-inhibitors also have important protective effects on the cardiovascular system. Several meta-analyses mainly using phase 3 trials repeatedly demonstrated beneficial cardiovascular effects of DPP4-inhibitors [25,31,34]. However, a very recently published clinical trial using the DPP4-inhibitor saxagliptin in type 2 diabetic patients with prior cardiovascular diseases revealed the safety of the compound without additional cardiovascular benefits [36]. Most cardiovascular endpoints studied in the trial were not modified by saxagliptin but hospitalization due to heart failure was significantly higher in the group treated with the DPP4-inhibitor. As sDPP4 is an adipokine upregulated in obesity and type 2 diabetes that triggers insulin resistance and metabolic complications [29,37], it might be speculated that the beneficial effects of DPP4-inhibitors would be higher in those early phases of the metabolic disorders previous to the development of established cardiovascular disease. This would be a possible explanation for the lack of positive results in the study by Scirca and collaborators [29,37]. However, several clinical trials with different DPP4-inhibitors and defined cardiovascular endpoints (Carolina, TECOS, Prediab) are still ongoing and will help to clarify safety and cardioprotective effects in type 2 diabetes patients with and without prior cardiovascular events. Studies in animals have already shown that DPP4-inhibitors exert cardiovascular protective effects. In mice and pigs, DPP4-inhibitors decrease myocardial infarct size, stabilize the cardiac electrophysiological state during myocardial ischemia, reduce ischemia/reperfusion injury, and prevent left ventricular remodeling following myocardial infarction [13,46]. Furthermore, in ApoE$^{-/-}$ mice fed with a high fat diet the DPP4-inhibitor sitagliptin reduces atherosclerosis lesion formation and improves endothelial dysfunction [30]. Additionally, in LDLR$^{-/-}$ mice DPP4 inhibition prevents monocyte activation and chemotaxis, thereby reducing aortic plaque formation [39]. Most of these effects could be ascribed to the increased bioavailability of DPP4 substrates like GLP-1 [5,6], stromal derived factor (SDF)-1α [19,47] or brain natriuretic peptide (BNP) BNP [7,8]. But despite of the classical view of DPP4 acting only as an exopeptidase degrading numerous peptide substrates accumulating data suggest that DPP4-inhibitors exert vascular protective effects also independent of DPP4 substrates. Ta et al. [42] could demonstrate that the specific DPP4-inhibitor alogliptin blocked LPS-induced ERK phosphorylation in U937 histiocytes, representing a model of cells involved in the progression of atherosclerosis, such as foam cells. Downstream of ERK, the inhibition of DPP4 prevented the activation of matrix metalloproteinases (MMP) via the Toll-like receptor pathway, which contributes to the destabilization of atherosclerotic plaques leading to acute vascular events. Whether the observed effects could be ascribed to direct effects of sDPP4 or to inhibition of enzymatic degradation of DPP4 substrates remained unknown. Furthermore, it could be demonstrated that sitagliptin prevents TNFα-induced PAI-1, ICAM and VCAM gene and protein expression in vascular endothelial cells independent of GLP-1 [23]. This effect could partially be explained by a reduction of the TNFα-induced NF-κB mRNA expression by sitagliptin. Additionally, incubation of human umbilical vein endothelial cells with the DPP4-inhibitor alogliptin resulted in endothelial nitric oxide synthase (eNOS) and Akt phosphorylation (Ser1177 and Ser473, respectively) paralleled by a rapid increase in nitric oxide [40]. These data suggest that DPP4 inhibition might play a pivotal role in prevention of endothelial dysfunction. Nevertheless, direct effects of sDPP4 were not taken into consideration in most of these studies. In a recently published study, a sDPP4-induced short-term activation of ERK1/2 in rat VSMC was observed, which could partially be blocked by DPP4 inhibition [17]. In the same study, the authors also demonstrate decreased SMC proliferation in vessels of ApoE knockout mice treated with anagliptin for 16 weeks. However, here we observed a very robust and long-lasting ERK1/2 activation, which can completely be prevented by DPP4 inhibition. Moreover, by using an ERK1/2-inhibitor, we could confirm that the sDPP4-induced proliferation is ERK-dependent. Thus, our data strongly support a direct role for sDPP4 in regulating vascular function independently of GLP-1.

Furthermore, our data on the direct effects of sDPP4 suggest that PAR2 is involved in signaling induced by sDPP4. In the literature, it is currently discussed that fibronectin [12], TLR4 [42] and mannose-6-phosphate receptor/IGFII receptor [24] may bind DPP4. Bioinformatic analysis and the signaling signature induced by sDPP4 suggest that sDPP4 might be an agonist for PAR2. PAR2 is activated by proteolytic cleavage of its N-terminus exposing a tethered ligand (TL) that then auto-activates the receptor. In addition, a synthetic peptide corresponding to the TL (SLIGKV) for PAR2 specifically activates PAR2 in the absence of proteases [22]. This activating sequence of PAR2 could be found in the cystein-rich region of DPP4 responsible for partner binding. We observed an almost perfect match for the first four amino acids, which are most important for the activation of PAR2 [1]. Within the tertiary structure of DPP4, the potential PAR2 TL sequence SLIG could be located on the protein surface. It might be speculated, that sDPP4 activates the PAR2 receptor with its TL sequence and that DPP4-inhibitors affect the binding properties of sDPP4 possibly by a conformational change. A similar phenomenon was observed by Piazza and colleagues, who showed that enzymatic inhibition of DPP4 affects its well known binding to fibronectin [35]. Interestingly, the binding site of fibronectin and the potential PAR2 binding side are both located in the cystein-rich region of DPP4. Additionally, the sDPP4 induced signaling in our cell model is consistent with the known PAR2 signaling signature. In the literature it has already been shown that stimulation with the PAR2-AP leads to NF-κB [10] and ERK1/2 [26] activation resulting in SMC mitogenesis. Regarding the sDPP4 effects on cytokine secretion, in human neutrophils, it could be shown that the PAR2-AP induces IL-6 and IL-8 secretion [41]. Thus, our in vitro data indicate that PAR2 is involved in the sDPP4-induced effects in hVSMC. Whether sDPP4 directly binds to PAR2 and how the DPP4-inhibitor might interfere with this potential binding requires further investigations.

5. Conclusion

In conclusion, in this study we characterized a novel sDPP4-induced signaling cascade in hVSMC. sDPP4 directly and markedly activates the MAPK- and NF-κB signaling pathway in a PAR2 dependent manner leading to pro-atherogenic changes in hVSMC like increased proliferation and inflammation. The observed in vitro effects might also play a role in vivo but would certainly coincide with the classical effects of DPP4 to cleave and inactivate incretin hormones and other substrates. Considering that sDPP4 is an adipokine with increased circulating levels in obesity, it can be speculated that sDPP4 may act in a para- or endocrine fashion on the vascular wall which could potentially contribute to inflammation in this setting.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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