FEBS Letters 588 (2014) 3870–3877

journal homepage: www.FEBSLetters.org

Shedding of dipeptidyl peptidase 4 is mediated by metalloproteases and up-regulated by hypoxia in human adipocytes and smooth muscle cells

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ARTICLE INFO

Article history:
Received 20 May 2014
Revised 13 August 2014
Accepted 27 August 2014
Available online 12 September 2014

Edited by Laszlo Nagy

Keywords:
CD26/DPP4
Hypoxia
Non-classical secretion
Protease

ABSTRACT

Dipeptidyl peptidase 4 is an important drug target for diabetes and a novel adipokine. However, it is unknown how soluble DPP4 (sDPP4) is cleaved from the cell membrane and released into the circulation. We show here that MMP1, MMP2 and MMP14 are involved in DPP4 shedding from human vascular smooth muscle cells (SMC) and MMP9 from adipocytes. Hypoxia increased DPP4 shedding from SMC which is associated with increased mRNA expression of MMP1. Our data suggest that constitutive as well as hypoxia-induced DPP4 shedding occurs due to a complex interplay between different MMPs in cell type-specific manner.

1. Introduction

Dipeptidyl peptidase 4 (DPP4) is a glycoprotein of 110 kDa, which is ubiquitously expressed on different cell types. The extracellular part of this type II cell surface protein is substantially glycosylated, which plays an important role in the interaction with different proteins [1]. As an exopeptidase of the serine protease type, DPP4 cleaves numerous substrates at the penultimate position and thereby mostly inactivates them. Among these are peptides (e.g. stromal cell-derived factor 1 alpha (SDF1α), eotaxin) and cytokines (*monocyte chemotactic protein-1* (MCP-1), interleukin 2 (IL-2)) as well as the incretin hormones [1]. The incretin hormones glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are major regulators of the postprandial insulin release [2]. Therefore gliptins, a class of specific DPP4 inhibitors, are now widely used as a monotherapy or combination therapy for type 2 diabetes. By inhibition of DPP4 activity, these drugs prolong the half-life of GLP-1 and GIP which then stimulate pancreatic insulin secretion, suppress glucagon production and thereby contribute to an improved glycemic control [2,3].

DPP4 is not only present on the surface of cells, but can also be found in the circulation [4]. By comprehensive proteomic profiling of the adipocyte secretome, we could identify soluble DPP4 (sDPP4) as a novel adipokine, with an upregulated release throughout the differentiation of adipocytes [5,6]. We confirmed that mature adipocytes in comparison to macrophages and preadipocytes are a major source of DPP4 [5]. Elevated serum levels of DPP4 were found in obese patients and correlate with the size of adipocytes and risk factors for the metabolic syndrome.

It has been reported, that DPP4 as a type II transmembrane protein is cleaved of the cell membrane in a process called shedding [7]. The nature of enzymes contributing to the shedding of DPP4 and the regulation of this process is largely unknown. The aims of our study were to elucidate the underlying shedding mechanism and to explore the regulation of sDPP4 release in vitro. We show here for the first time that members of the metalloprotease family are involved in the release of sDPP4 from different primary human cells.

Abbreviations: ADAM, a disintegrin and metalloprotease; bref A, brefeldin A; DPP4, Dipeptidyl peptidase 4; sDPP4, soluble dipeptidyl peptidase 4; MMP, matrix metalloprotease; SMC, human vascular smooth muscle cell; TACE, tumor necrosis factor α converting enzyme

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http://dx.doi.org/10.1016/j.febslet.2014.08.029
0014-5793/

2. Materials and methods

2.1. Materials

Complete protease inhibitor (04693116001), and PhosStop phosphatase (04906837001) inhibitor cocktail were provided by Roche. Reagents for SDS–PAGE were supplied by Amersham Pharmacia Biotech and by Sigma. DPP4 rabbit polyclonal antibody (H00001803-D01P) was obtained from Abnova. Beta actin mouse antibody (ab6276) was supplied by Abcam. HRP-conjugated goat anti-rabbit (W4011) and goat anti-mouse (W4021) IgG antibodies where purchased from Promega.

Collagenase NB4 (17465.02) was obtained from Serva. FCS (10270-106), Dulbecco's modified Eagles/HAM F12 (DMEM/F12) medium (42400-010), α-modified Eagle's (α MEM) medium (11900-016) and Ham's F-12 medium (21700-026) was supplied by Gibco (Invitrogen). Troglitazone was obtained from Sigma Aldrich.

The Protease inhibitors AEBSF (ALX-270-022), E64 (ALX-260-007) were dissolved in water. BB-94 (196440), MMP9 Inhibitor I (444278) and MMP2 Inhibitor III (444288) were purchased from Calbiochem and dissolved in sterile DMSO. Human Protease Array Kit (ARY021) was obtained from R&D Systems.

2.2. Adipocyte isolation and culture

Preadipocytes of human subcutaneous adipose tissue were obtained from lean or moderately overweight subjects undergoing plastic surgery. Isolation was performed as previously described [8]. Cells from up to passage 4 were grown to confluence in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12) supplemented with 10% FCS with medium change every 2-3 days. Passaging of the preadipocytes was performed according to Skurk et al. [9]. Differentiation was started by adding 5 μmol/l troglitazone for 3 days to adipocyte differentiation medium (DMEM/F12, 33 μmol/l biotin, 17 μmol/l D-panthothenic-acid, 66 nM insulin, 1 nM triiodo-L-thyronine, 100 nM cortisol, 10 μg/ml apo-transferrin, 50 μg/μl gentamycin, 15 mmol/l HEPES, 14 nmol/l $NaHCO_3$, pH 7.4) as described previously [5]. After 14 days cells where treated with the indicated substances diluted in α-modified DMEM and incubated for the indicated periods.

2.3. Smooth muscle cell culture

Primary human smooth muscle cells (SMC) from three different donors (Caucasian, one male, two females) were supplied as proliferating cells from Lonza, TebuBio and PromoCell and kept in culture according to the manufacturer's protocol. For all experiments subconfluent cells of passage three were used. SMC were characterized by morphologic criteria and by immunostaining with smooth muscle α-actin. For the experiments 100.000 cells/ml were seeded and grown for 24 hours in Growth medium (Promocell) with appropriate supplements. After washing with PBS and serum starvation for 24 hours cells were treated as indicated.

2.4. Hypoxia

For hypoxic experiments, cells were exposed to 1% O_2 supplemented with 5% CO_2 and respective concentrations of nitrogen in an Xvivo hypoxia chamber system (Biospherix) for 24 or 48 h.

2.5. ELISA

DPP4 release to the cell culture medium was measured by human DPP4 DuoSet ELISA (R&D Systems, DY1180) according to the manufacturer's instructions.

2.6. Silencing of target genes

SMC were seeded in 6-well plates and grown until 60–80% confluence. Silencing experiments were performed by using 40 nM FlexiTube siRNA (Qiagen, MMP1 (SI03021802), MMP14 (SI03648841)) and 12 μl HiPerfect (Qiagen, 301705) according to the manufacturers' instructions. At day 10-12 of differentiation adipocytes were treated with 40 nM of the respective siRNA and 9 μl of HiPerfect. Optimal transfection conditions were tested by separate titration experiments. To control for unspecific effects, control cells were treated with AllStars Negative Control siRNA (QIAGEN, 1027280). After 24 h mRNA was isolated to check for silencing efficiency. Supernatants were collected after 24 or 48 h respectively.

2.7. qRT-PCR

Total RNA was isolated and reverse transcribed using the RNeasy Mini Kit (Qiagen, 74106) and Omniscript Reverse Transcription kit (Qiagen, 205113) according to the manufacturer's instructions. Gene expression was determined by quantitative real-time PCR (qRT PCR) using QuantiTect primer assays (Qiagen, ACTB, QT00057428, QT00001533, QT00040040, QT00088396, QT00014581, QT00055580) and GoTaq qPCR Master Mix (Promega, A6002) with 0.04-0.4 ng of generated cDNA on a Step One Plus Cycler (Applied Biosystems). Beta-actin was used as a reference gene and expression levels of investigated genes were normalized to beta-actin. Gene expression was analyzed via the ΔΔCt method and compared with the designated control.

2.8. Statistical analysis

Data are expressed as mean ± SEM. Unpaired two-tailed Student's *t* test or one-way ANOVA (post hoc test: Bonferroni's multiple comparison test) were used to determine statistical significance. All statistical analysis was done using Prism (GraphPad, La Jolla, CA, USA) considering a *P* value of less than 0.05 as statistically significant. Corresponding significance levels are indicated in the figures.

3. Results

3.1. sDPP4 release is insensitive to brefeldin A treatment

In accordance with previously published data on sDPP4 release from skeletal muscle cells [10], sDPP4 release did not follow the classical ER/Golgi-dependant pathway in SMC and adipocytes, because it was insensitive to brefeldin A (bref A) treatment (Fig. 1A and B). The applied concentrations of bref A were effective in blocking IL-6 secretion from SMC (Fig. 1A) and adiponectin secretion from adipocytes (Fig. 1B).

3.2. Protease profile differs between SMC and adipocytes

To find out what types of proteases are released from different cell types and if there is a difference in the release profile of proteases, we used supernatants of adipocytes and SMC in a Protease Profiler Array. This array enables us to assess the release of 34 different proteases of the four main types of proteases, namely matrix metalloproteases (MMP), serine proteases, cysteine proteases and aspartyl proteases. A huge panel of proteases spotted on this membrane belongs to the Cathepsins, which are serine, cysteine or aspartyl proteases. The comparison of different members of the Cathepsin family showed, that the detectable subtypes were more or less identical in the different cell types (Data not shown). In

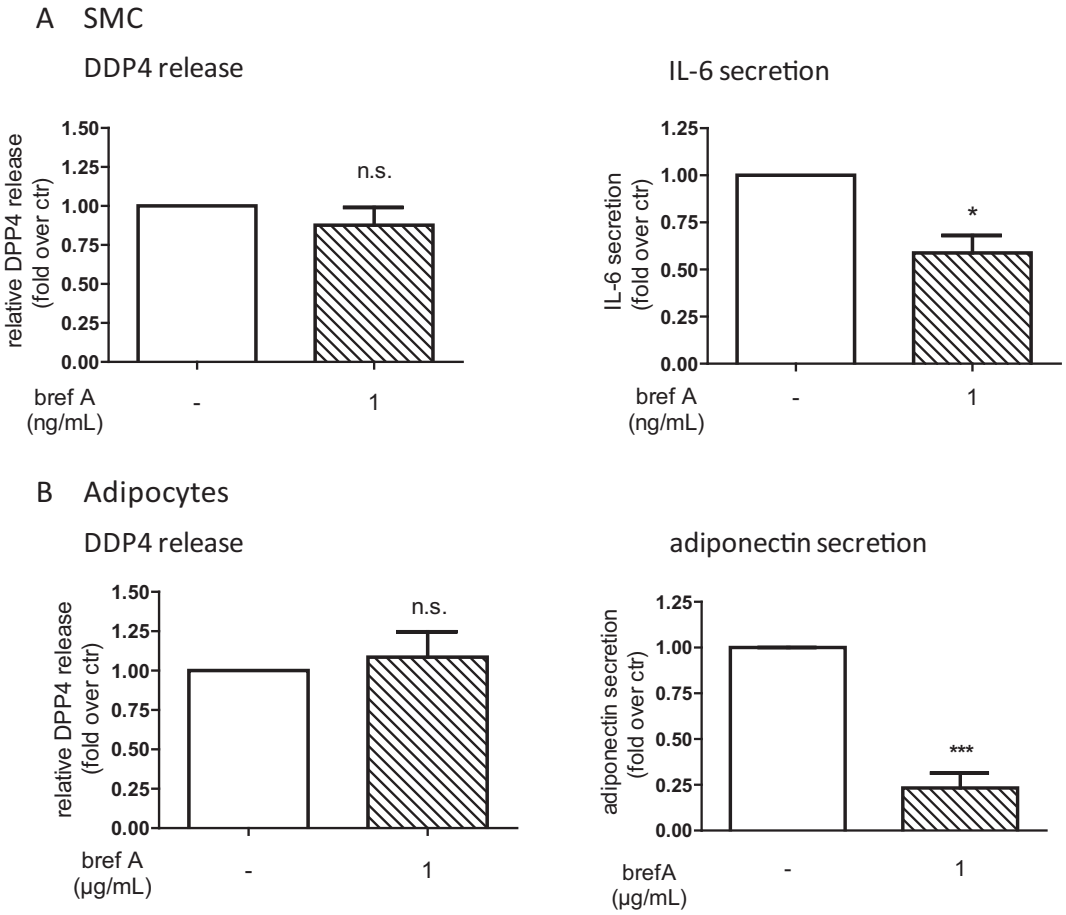


Fig. 1. Influence of bref A treatment on sDPP4 release in SMC (A) and adipocytes (B). Cells were treated for 24 h with indicated concentrations of bref A. sDPP4 release and IL-6 or adiponectin secretion respectively to the culture medium was measured by ELISA. Data are mean values ± SEM, $n = 3$-4; $^{*}P < 0.05$, $^{***}P < 0.001$ vs. non-treated control; n.s. not significant; bref A, brefeldin A.

SMC, MMP1 and MMP3 signals were very prominent (Fig. 2A), whereas in adipocytes MMP2 and MMP9 signals were the strongest for all MMPs tested in the array (Fig. 2 B).

3.3. sDPP4 release can be reduced by general broad spectrum protease inhibitors

To identify the proteolytic enzymes involved in the DPP4 shedding, different classes of protease inhibitors were screened for their ability to block sDPP4 release. In SMC, sDPP4 release was impaired by the general broad spectrum MMP inhibitor BB-94, the general cysteine protease inhibitor E64 and the general serine protease inhibitor AEBSF to the same extent (Fig. 3A). To elucidate if a combination of these inhibitors could further diminish sDPP4 release, E64 and AEBSF, E64 and BB-94 as well as AEBSF and BB-94 were combined. None of these combinations showed additive effects on sDPP4 release (Fig. 3B). Furthermore, none of the treatments affected DPP4 protein expression (data not shown).

In adipocytes, only BB-94 showed a significant effect on sDPP4 release (Fig. 3A). In the combination of the different classes of inhibitors no additive effects were observed (Fig. 3B). The protein expression of DPP4 remained unchanged after treatment with the different classes of protease inhibitors (data not shown).

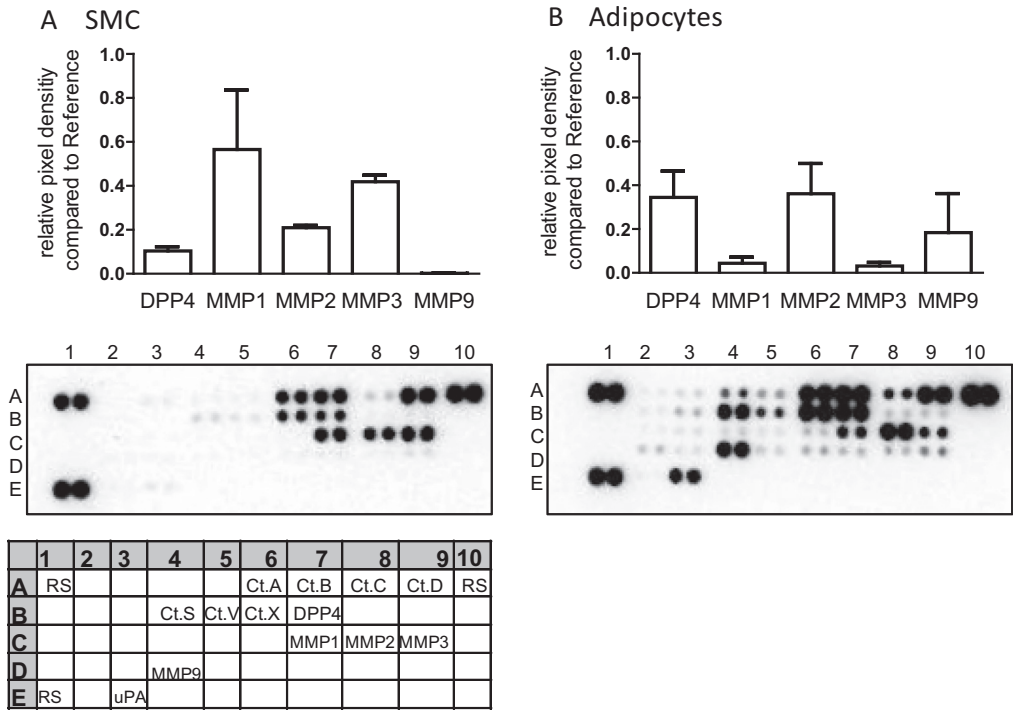
3.4. sDPP4 release is mediated by metalloproteases

To correlate the shown effects of the general MMP inhibitor to a subset of MMPs, specific inhibitors or gene silencing were used. The IC_{50} values of BB-94 are lowest for MMP14 (2 nM), MMP1 (3 nM), MMP2 and MMP9 (4 nM) and some of these MMPs are elevated in mouse models of obesity [11,12]. To deduce our findings with BB-94 to a specific MMP, we used specific inhibitors if available or gene silencing if no specific inhibitors could be used. Expression of the respective MMP could be reduced by about 70–80% using specific siRNAs (Suppl. Fig. 1). In SMC, inhibition of MMP2 and silencing of MMP1and MMP14 showed the most prominent effects on sDPP4 release, which was blocked by 20-30%, respectively (Fig. 4A). Inhibition of MMP9 in SMC showed only a slight effect on sDPP4 release (Fig. 4A). In contrast to SMC, MMP2 inhibition did not affect sDPP4 release in adipocytes, but MMP9 inhibition significantly reduced DPP4 shedding (Fig. 4B). Silencing of MMP1 or MMP14 in adipocytes had no effect on DPP4 shedding (Fig. 4B).

3.5. Hypoxia increases DPP4 shedding from SMC

To test whether lower oxygen levels can influence DPP4 shedding, SMC were incubated in an Xvivo hypoxia chamber system at 1% and 21% O_2 for 24 or 48 h. After 24 h as well as after 48 h sDPP4 release was upregulated between 40% and 50%, respectively (Fig. 5A). However, DPP4 mRNA levels were not significantly elevated compared to normoxic conditions (Fig. 5B).

In addition to DPP4 shedding, mRNA expression of selected MMPs was tested after challenging of the SMC with 1% O_2. The most prominent effect of hypoxia could be shown for the expression of MMP1, which was upregulated more than 3-times by hypoxia-treatment after 48 h in SMC. Even after 24 h, mRNA levels increased 2-fold in the hypoxic situation (Fig. 5C). MMP9 mRNA



	1	2	3	4	5	6	7	8	9	10
A	RS					Ct.A	Ct.B	Ct.C	Ct.D	RS
B				Ct.S	Ct.V	Ct.X	DPP4			
C							MMP1	MMP2	MMP3	
D				MMP9						
E	RS		uPA							

Fig. 2. Supernatants from SMC (A) and adipocytes (B) under control conditions were used to perform human Protease Profiler array. Data are depicted as relative pixel density compared to the reference spots. Representative arrays are presented and labeled with a grid pattern to identify corresponding spots. Data are mean values ± SEM, $n = 2$; RS reference spot; Ct, cathepsin; uPA, urokinase-type plasminogen activator.

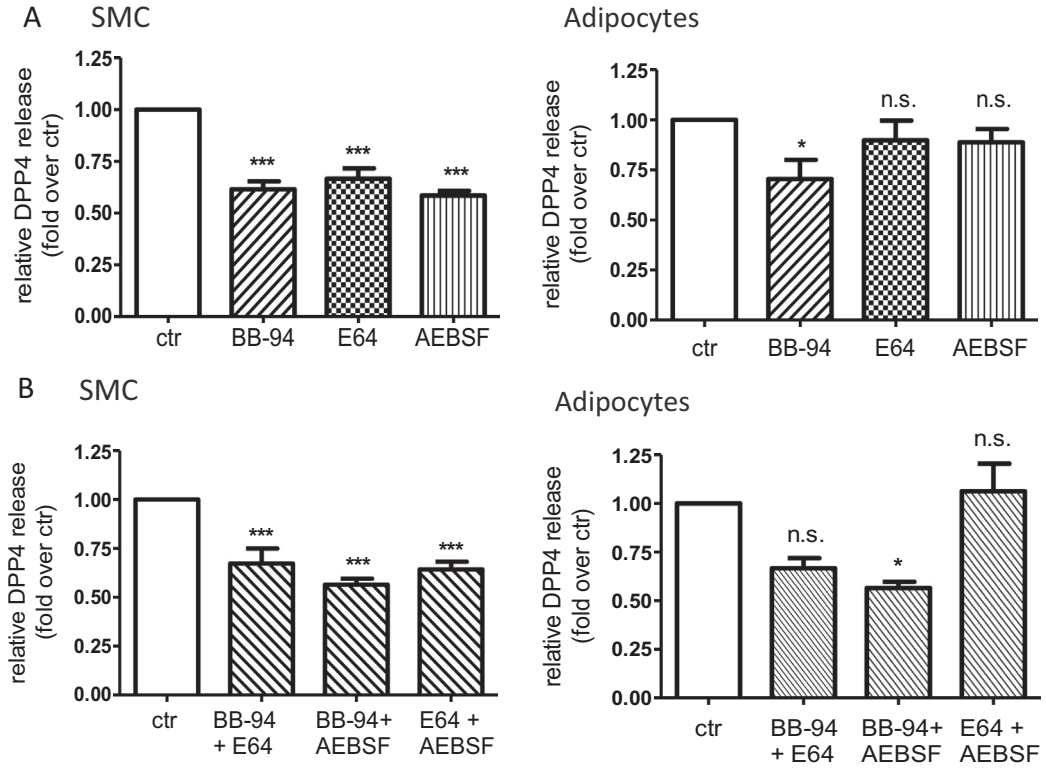


Fig. 3. sDPP4 release after stimulation with different classes of Protease inhibitors in SMC and adipocytes. (A) sDPP4 after treatment with 50 μM BB-94, 20 μM E64 and 20 μM AEBSF for 24 h. sDPP4 release was measured by ELISA. Data were normalized to the respective control and are shown as fold over control. Data are mean values ± SEM, $n > 3$, $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. respective control. (B) sDPP4 after combined treatment with 20 μM E64, 20 μM AEBSF and 20 μM BB-94 as depicted for 24 h. Data are mean values ± SEM, $n > 5$, $^{*}P < 0.05$ and $^{***}P < 0.001$ vs. respective control; n.s., not significant; ctr, control.

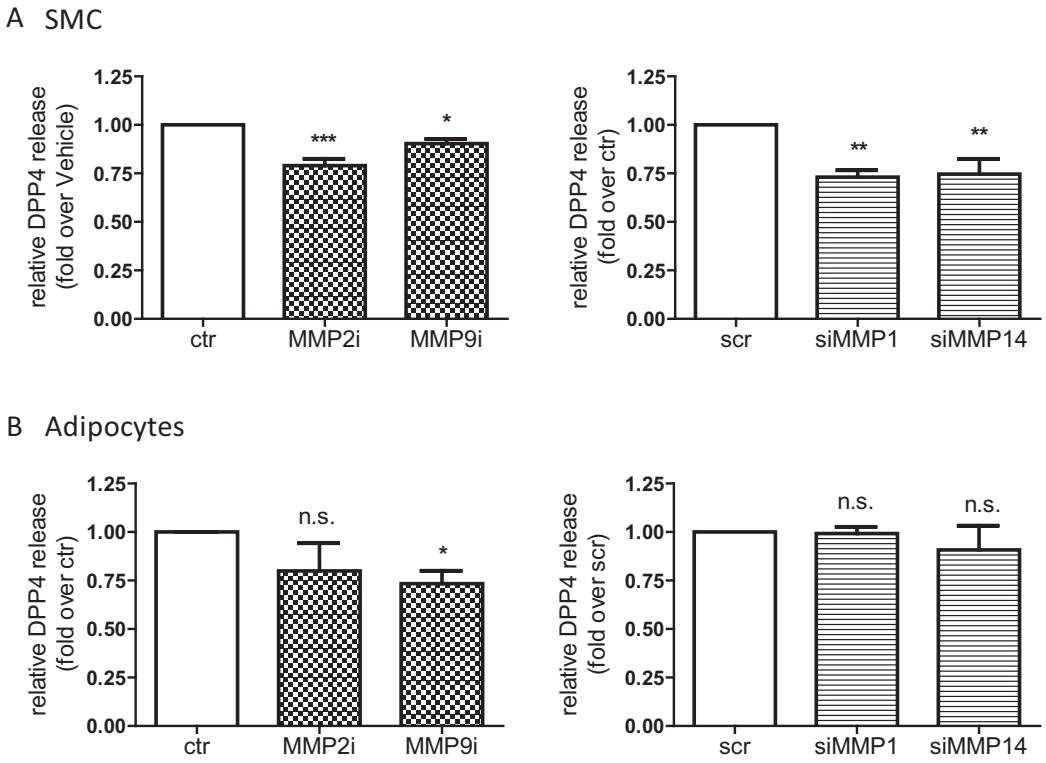


Fig. 4. Inhibition and silencing of specific metalloproteases in SMC and adipocytes partially prevented sDPP4 release. Treatment of SMC (A) and adipocytes (B) with specific MMP2 and MMP9 inhibitors in the indicated concentrations for 24 h or with 40 nM MMP1 siRNA and 40 nM MMP14 siRNA respectively or with 40 nM scrambled siRNA for 48 h. sDPP4 release is measured by ELISA. Data are presented as fold over respective control. Data are mean values ± SEM, $n = 4$-5, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. respective control; n.s., not significant; ctr, control; scr, scrambled.

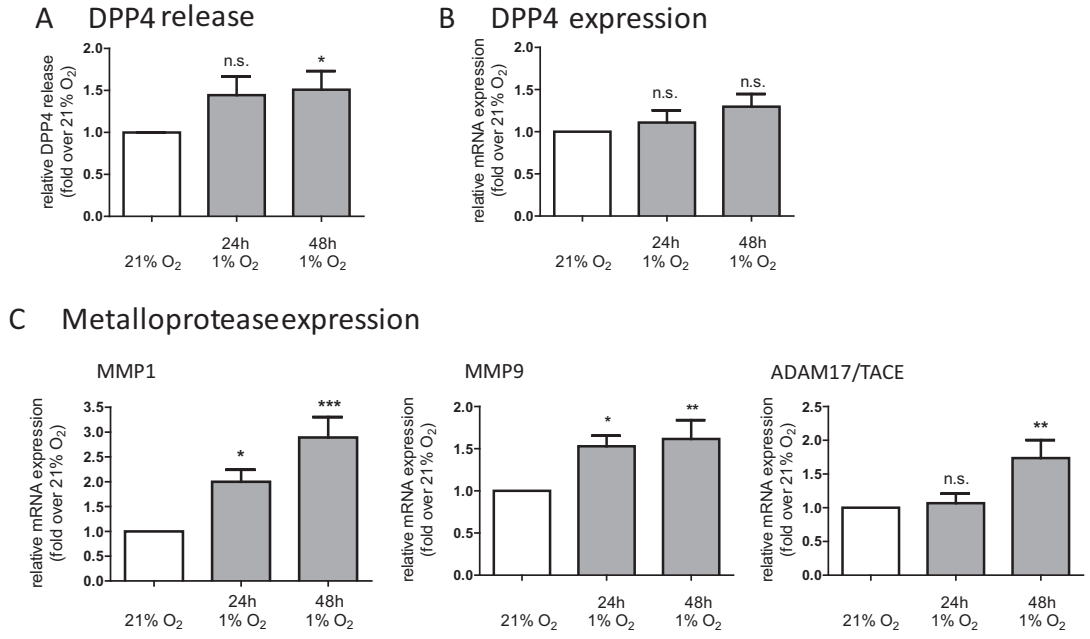


Fig. 5. Influence of hypoxia on sDPP4 release and mRNA expression of selected genes in SMC. (A) SMC were cultivated in parallel under normoxic (21% O_2) and under hypoxic (1% O_2) conditions for 24 and 48 h DPP4 release to the culture medium was measured by ELISA. Data are depicted as fold over normoxic control. Data are mean values ± SEM, $n = 7$, $^{*}P < 0.05$ vs. normoxic control. (B) DPP4 mRNA expression from the above mentioned treatment was measured by qRT PCR. Data were normalized to the mRNA expression level of actin and expressed relative to the normoxic control. Data are mean values ± SEM, $n = 7$. (C) MMP1, MMP9 and ADAM17/TACE mRNA expression from the above mentioned treatment was measured by qRT PCR using specific primer sets for the indicated targets. Data were normalized to the mRNA expression level of actin and expressed relative to the normoxic control. Data are mean values ± SEM, $n > 4$, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. normoxic control. Grey bars indicate treatment with 1% O_2; n.s., not significant.

expression showed at least a 1.5-fold increase in this setting (Fig. 5C). A slight but significant increase was also detected after 48 h for the expression of a disintegrin and metalloprotease (ADAM)17/tumor necrosis factor α converting enzyme (TACE) (Fig. 5C). In contrast, expression of MMP2 and MMP14 remained comparable to normoxic conditions (Data not shown).

Additional protease arrays were performed using supernatants from SMC cultured under hypoxic and normoxic conditions. Protease array results confirmed that hypoxia increased relative sDPP4 release and indicate that the increased expression of MMP1 mRNA is paralleled by increased MMP1 release (Data not shown).

4. Discussion

DPP4 is a type II transmembrane glycoprotein that is released from the membrane in a non-classical secretion mechanism. This is evidenced by insensitivity to brefeldin A treatment and the lack of a cleavable signal sequence at the N-terminus [13,14]. Type II single-pass transmembrane proteins possess a signal anchor sequence which is positioned within the membrane-spanning domain and which targets these proteins to the rough endoplasmatic reticulum. But in contrast to a classical secreted protein, the signal for the peptidase which cleaves off the signal sequence is missing [15]. We were able to confirm, that DPP4 is insensitive to brefeldin A treatment not only in skeletal muscle cells [10], but also in SMC and adipocytes. Endogenous proteolytic release of transmembrane proteins is limited to type I or type II transmembrane proteins. This occurs by post-translational hydrolysis, the so called shedding [7]. Bioinformatic analysis with Secretome 2.0 predicted DPP4 as non-classically secreted protein with a score of 0.719 (exceeding the threshold of 0.5). The cleavage of sDPP4 most likely occurs in the spacer region spanning amino acid 29–39 of the full length DPP4 according to the protein annotation at UniProt. However, the responsible enzymes are unknown so far.

Our data suggest that various types of proteases are able to induce sDPP4 release, because DPP4 shedding was significantly reduced by general serine-(AEBSF), cysteine-(E64) and metalloprotease (BB-94) inhibitors in SMC. Because combined treatment with the effective broad spectrum protease inhibitors lead to no additive effects we speculate, that in case of sDPP4 release a proteolytic cascade which involves cathepsins and MMPs could be supposed. This goes in line with the rather complex activation of MMPs. MMPs belong to the family of zinc-dependent enzymes which are synthesized as an inactive proenzyme released into the extracellular space. Propeptides of MMPs are covalently bound to the zinc ion in the active center of these enzymes and thereby suppress the enzymatic activity until the propeptide is cleaved off [16]. Different factors are able to activate MMPs like urokinase-type plasminogen activator (uPA), coagulation factors, phorbol esters or cytokines [16]. But even MMPs can activate each other, which is known in case of MMP2 activation by MMP14 [17] or MMP9 activation by MMP2 and MMP3 [18]. Also serine proteases like plasmin and kallikreins or cysteine proteases like Cathepsin G were shown to directly activate MMP1, MMP2 and MMP9 in vitro or in vivo [19].

For MMP2, MMP9 and MMP14 it has already been reported, that their expression levels are elevated in obese mice [11,12]. Furthermore MMP1, MMP2, MMP9 and MMP14 play a role in the shedding of a large variety of substrates [20–24]. These studies also emphasize that one MMP can shed several substrates and shedding of a specific target might not be limited to a specific MMP, but is a rather complex interplay between different enzymes.

Our results suggest that MMP1, MMP2 and MMP14 play a role in constitutive DPP4 shedding in SMC. One may keep in mind that MMP14 is able to activate MMP2 [17] and therefore might not only directly contribute to DPP4 shedding in vitro. Proteome profiler array data confirmed that MMP2 and MMP1 are released by SMC, whereas MMP9 was below the detection level in the supernatants of unstimulated SMC. Although also MMP3 was released from SMC, we did not further investigate this MMP due to the following reasons. First, MMP3 is described as a protective MMP in the context of atherosclerosis and it is not clear if this MMP is involved in shedding processes [25,26]. Furthermore the IC_{50} of BB-94 for MMP3 is significantly higher in comparison to the other candidates. In adipocytes only the specific inhibition of MMP9 could decrease DPP4 shedding, whereas MMP2 inhibition, MMP1 or MMP14 silencing respectively showed no effect. MMP1, MMP2 and MMP9 were detectable in the unstimulated supernatants of adipocytes. This result suggests, that constitutive DPP4 shedding is cell-type specific. None of the investigated mechanisms to block MMPs where able to reduce DDP4 release below 50%, which indicates, that DPP4 shedding is not only regulated by a single MMP.

We were able to demonstrate that low oxygen levels of 1% O_2 increase DPP4 shedding in SMC. In developing lesions during atherogenesis the oxygen supply is often exhausted, which is partly due to the highly oxygen-consuming foam cells and the relatively low diffusion rate of oxygen through the cell environment [27]. This can result in severe hypoxia (< 1% O_2) in some areas. Hypoxia affects transcription of genes, which are for example responsible for matrix remodeling [28]. For some MMPs like MMP1 and MMP9, it is already known that hypoxia stimulates their release and expression [29,30]. In our setting, we were able to show an increased expression of MMP1, MMP9 and ADAM17/TACE under low oxygen supply. MMP1 release was relatively high in SMC compared to other MMPs, whereas it was relatively low in adipocytes. Therefore, MMP1 might be an important player in DPP4 shedding both under normoxic and particularly under hypoxic conditions in SMC. Though MMP9 mRNA expression is also upregulated due to hypoxia, this MMP could only play a minor role in DPP4 shedding in SMC, because it is barely detectable in supernatants of these cells compared to supernatants from adipocytes as shown by the Protease Array Analysis. Furthermore, MMP9 inhibition had only a very slight effect on DPP4 shedding. MMP2, which showed an impact on sDPP4 release after treatment with a specific inhibitor, was not influenced by hypoxia. ADAM17/TACE, cannot be ruled out to be involved in DPP4 shedding. From the literature it is known that it is involved in the processing of different substrates despite its eponymous target TNFα [31]. Although we were able to silence ADAM17/TACE in SMC using different siRNAs, we always observed a concomitant upregulation of MMP1 thus preventing us from clarifying the role of ADAM17/TACE in DPP4 shedding. As no specific ADAM17/TACE inhibitor is available, it is unfortunately impossible to further study ADAM17/TACE at the moment. In the end, DPP4 expression itself is not influenced by hypoxia which shows, that the increased release of sDPP4 is not due to a higher DPP4 expression, but seems to be mediated at the level of DPP4 processing. In adipocytes, we could already show that culture at 1% O_2 for 24 h had no effect on DPP4 release [5].

Adipose tissue and immune cells are recognized sources of DPP4. Circulating DPP4 concentrations are not only higher in obese patients but also in various inflammatory diseases [4,32]. In a previous study, we were already able to show a significantly decreased DPP4 release from adipose tissue explants and in serum from obese patients who underwent bariatric surgery [5]. MMP2 and MMP9 are key elements of extracellular matrix modulation in adipose tissue and both MMPs are significantly higher in serum of obese patients [33] and MMP2 is significantly downregulated in serum of patients after bariatric surgery [34]. High serum and mRNA expression levels of MMP1 and MMP9 are also associated with carotid atherosclerosis and plaque stability in patients [35–37]. Accordingly, patients with a higher risk for cardiovascular

disease are characterized by both higher circulating DPP4 and increased serum levels of MMP1 [38,39]. In vitro, we have previously shown that sDPP4 in concentrations similar to circulating levels induces SMC proliferation [5]. In parallel, sDPP4 induces inflammatory and stress pathways in vascular cells [40,41]. Accordingly, sDPP4 generated in ischemic tissue or derived from perivascular and visceral adipose tissue might have similar effects in vivo [32,42]. The multiple targets and functions of DPP4 and its inhibition by gliptins suggest that it could also play an important role beyond its effects on the incretin axis [43]. As for the relation of DPP4 and potential shedding enzymes of DPP4 to obesity and atherosclerosis, it should be noted that only correlative data is available at the moment and no causal relationship can be established based on our current knowledge. In the future, research should focus on the molecular mechanisms of DPP4 shedding in the context of atherosclerosis and obesity which might finally help to better understand the role of sDPP4 in physiological and pathophysiological conditions.

In conclusion, we could show that several MMPs are involved in the constitutive DPP4 shedding as well as under hypoxic conditions in vitro. Our data suggest, that not a single MMP is involved in sDPP4 release, but it is rather an interplay between different shedding enzymes in a cell type-specific manner.

Acknowledgements

This work was supported by the Ministry of Science and Research of the State of North Rhine-Westphalia (MIWF NRW) and the Federal Ministry of Health (BMG). This study was supported in part by a Grant from the Deutsche Forschungsgemeinschaft (SE-1922 2-2), a grant from the German Diabetes Association (DDG) and a fellowship to HS from the European Foundation for the Study of Diabetes (EFSD).

We wish to thank the Dept. of Plastic Surgery, Florence-Nightingale-Hospital Düsseldorf, for support in obtaining adipose tissue samples. The technical assistance of Andrea Cramer, the scientific support from Dr. Nina Wronkowitz and the secretarial assistance of Birgit Hurow is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.08.029.

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