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Involvement of furin-like proprotein convertases in the arterial response to injury

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Abstract

Background: Furin-like proprotein convertases (PCs) are proteolytic activators of proproteins, like membrane type 1-matrix metalloproteinase (MT1-MMP) and transforming growth factor β (TGF-β), that are described in the arterial response to injury. However, the involvement of furin-like PCs in the arterial response to injury has not been studied yet. We studied furin, MT1-MMP, MMP levels and TGF-β signaling after arterial injury. We also investigated the effect of an inhibitor of furin-like PCs, α1-antitrypsin Portland (α1-PDX), on arterial injury following balloon dilation.

Methods and results: NZW rabbit femoral and iliac arteries ($N=42$) were balloon dilated unilaterally and harvested after 2, 7, 14, 28 or 42 days. Furin mRNA levels were increased after 2 and 7 days. MMP-2 and MT1-MMP levels were increased after day 7 and TGF-β signaling, by phosphorylating Smad 1/5 and 2/3, was increased at all time points. Inhibition of furin-like PCs, by adenoviral over-expression of α1-PDX, blocked proTGF-β activation and Smad phosphorylation, and reduced MT1-MMP and MMP-2 activation ($N=5$). In vivo adventitial inhibition of furin-like PCs ($N=9$) resulted in a reduction of 13.1±5.2% in advential and 23.6±7.9% in intimal areas ($P<0.05$), but had no effect on lumen size due to decreased vessel areas.

Conclusions: This study demonstrates that furin-like PCs are involved in the arterial response to injury possibly through activation of the TGF-β–Smad signaling pathway and identifies furin-like PCs as a possible target to inhibit intimal hyperplasia.
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Keywords: Gene therapy; Remodeling; Balloon dilation; Transforming growth factor β (TGF-β); Matrix metalloproteinases

Furin-like proprotein convertases (PCs) are calcium dependent proteases and function mainly as proteolytic activators of proproteins [1] and have been described to play a potential role in the pathogenesis of disease [2].

Among these PCs, furin, PC5 and PC7 are widely distributed in tissues and found to be present in rodent arteries [3]. An upregulation of PC5 mRNA was reported in the neointima upon balloon injury in rats [3], and increased furin expression has been reported in flow-induced arterial remodeling [4]. However, it is not known if furin-like PCs are involved in the arterial response to injury, characterized by neointimal formation and arterial remodeling and if inhibition of furin-like PCs can modulate these processes.

Several substrates that are activated by furin have been described in the arterial response to injury, like pro-membrane type 1-matrix metalloproteinase precursor (proMT1-MMP), [5] and the pro-transforming growth factor β (proTGF-β) [6]. Although these furin substrates are described to be present in the artery after injury and the role

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of furin is described in in vitro systems, it is still unclear when proMT1-MMP is activated and if the activated TGF-β is able to induce downstream signaling pathways through phosphorylation of Smad 1/5 and Smad 2/3 after arterial injury.

Inhibition of furin-like PCs has been studied using a potent furin-like PC inhibitor, the α1-antitrypsin Portland (α1-PDX). This a mutant form of α1-antitrypsin, and is more than 3000-fold more effective than α1-antitrypsin at inhibiting furin-like PCs in vivo, moreover, it does not inhibit either elastase or thrombin [7].

In the present study, we studied furin expression, the MT1-MMP–MMP2 activation pathway and TGF-β–Smad signaling pathway in time following balloon dilation. This revealed that furin, MT1-MMP, MMP-2 and phosphorylated Smad 1/5 and 2/3 levels increased after balloon dilation. We locally administered an adenovirus, expressing α1-PDX, on the adventitia of the balloon dilated segment. Inhibition of furin-like PCs after balloon dilation resulted in a reduction in advential and intimal areas. This demonstrates the involvement of furin-like PCs in the arterial response to injury and suggests that furin-like PCs are a potential interesting target to intervene in neointima formation.

1. Materials and methods

1.1. Animals

Animals were housed conforming to the Guide for the Care and Use of Laboratory Animals (NIH publication No.85-23, 1985) and all experiments were approved by the ethical committee on animal experiments of the University Medical Center, Utrecht. New Zealand white rabbits (Broekman Charles River, 3–3.5 kg) were anesthetized by methadone (0.15 ml) and vetranquil (0.15 ml) followed by etomidate (1 mg/kg) and ventilation with N2O:O2/0.6% halothane.

Femoral and external iliac arteries of forty-two rabbits were balloon dilated as described before [8]. Arterial lumen diameter was determined using angiography after balloon dilation and at termination. Geometrical remodeling and intimal formation were analyzed as previously described [8]. The balloon dilated segments and the contralateral control arteries were harvested after 2, 7, 14, 28 and 42 days and immediately frozen at −80 °C for RNA and protein isolation [8] ($N=6–7$ rabbits per time point).

1.2. Quantitative RT-PCR

Rabbit furin (Forward: 5′-ccatccaggctggttttgta-3′; Reverse: 5′-gtccattaaatagaaccaacaatgc-3′) and ribosomal 18S (Forward:5′-tcaacacgggaaacctcac-3′; Reverse:5′-acaaatcgctccagcaac-3′) primers were designed using the Prime program at CMBI (Nijmegen).

Quantitative RT-PCR, using cybergreen and the I-cycler iQ™ RT-PCR (Biorad), was performed as previously described [8]. The PCR reactions started with 2 min at 94 °C followed by 40 cycles of: 30 s at 94 °C, 30 s at 60 °C and 60 s at 72 °C. Data are presented as the ratio of the dilated and control arteries.

1.3. Western blotting and zymography

For Western blotting, samples (12 μg) were separated on 10% SDS–PAGE gel and transferred onto a Hybond-ECL membrane (Amersham). MT1-MMP was detected with a monoclonal antibody for MT1-MMP (diluted 1:50, clone113-5B7, Oncogene) and a goat-α-mouse-HRP (diluted 1:1000; DAKO). Only activated MT1-MMP is recognized by this antibody. Phosphorylated Smad 1/5 and Smad 2/3 with a polyclonal antibody for pSmad 1/5 (diluted 1:500; clone sc-12353, Santa Cruz) or for pSmad 2/3 (diluted 1:250; clone sc-11769, Santa Cruz) and a rabbit-α-goat-biotin (diluted 1:1500; DAKO) and streptavidin-HRP, followed by chemiluminescence substrate (Sigma) and exposed to the ChemiDoc XRS system (Biorad). Negative controls were performed using an isotype control antibody and by omitting the first antibody.

Zymography was performed as described before [8]. In short, protein samples (8 μg) were separated on a 10% SDS–PAGE gel containing 1 mg/ml gelatin (Sigma). After incubation overnight at 37 °C in Brij solution (0.05 M Tris–HCl pH 7.4, 0.01 M CaCl2, 0.05% Brij 35 (Sigma)), the gel was stained (25% methanol, 15% acetic acid, 0.1% Coomassie blue) and proMMP-2 and active MMP-2 bands were assayed by densitometry on the ChemiDoc XRS system (Biorad) and presented as the ratio between dilated and contralateral control arteries. Bands were characterized by size and co-migration of recombinant MMP-2.

1.4. α1-PDX adenoviral construct

The α1-PDX construct, containing the furin-like PC inhibitor (modified α1-antitrypsin), was kindly provided by Dr. G. Thomas (Portland, USA). The α1-PDX was cloned into the Ad-Easy Xl vector (Stratagene) (Ad.CMV.α1-PDX), according to the manufacturers' protocol. We also created two control viruses, an empty virus (Ad. CMV.Empty) and a virus expressing β-galactosidase (Ad.CMV.LacZ). Cesium chloride purification was performed on amplificated virus stocks and titers (plaque forming units (pfu) per milliliter) were determined by repeated plaque assays [9].

1.5. Ex vivo adventitial gene delivery and protein expression

Normal rabbit aortas were harvested and transfected periadventitially, with the α1-PDX construct or the empty virus (50 μl of 2.10^{10} pfu/ml), by slight pressing with a brush [10]. The segments were cut into aortic rings for culture

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(3+7 days) as described before [8] and total protein was isolated. Equal amounts of total protein were loaded. Western blotting was performed for MT1-MMP and for phosphorylated Smad, and for TGF-β by using rabbit-α-TGF-β (diluted 1:500; pan-specific, R&D systems) and goat-α-rabbit-HRP (diluted 1:1000; DAKO) antibodies. Zymography was performed as described above. Three independent experiments were performed and analyzed, and representative blots are depicted.

1.6. In vivo adventitial gene delivery

The Ad.CMV.LacZ virus was applied on the artery to explore transfection efficiency of the used peri-adventitial brushing method. β-galactosidase expression was stained as described by the manufacturer of the Ad-Easy Xl vector.

Both femoral arteries of rabbits ($N=15$) were carefully explored and embedded in a saline solution containing 5 mg/ml papaverin. Balloon dilation was performed bilateral as described before [8] and, either the Ad.CMV.α1-PDX virus or an empty virus (Ad.CMV.Empty) was applied locally (50 μl of 2.10^{10} pfu/ml) on the 2 cm long dilated arterial segment. Each rabbit received the α1-PDX construct at one side and the control construct at the contralateral artery. For applying the virus peri-adventitially, the arteries were lifted and the viral constructs were spread by slight pressing with a brush as described before [10]. After a minute the arteries were put back in position and tissues were closed. Angiographic images were taken before, during, and after balloon dilation and at termination and arterial diameters were digitally analyzed. Rabbits completely recovered after surgery and were terminated after 1 ($N=1$), 3 ($N=5$), and 14 days ($N=9$).

1.7. Biochemical and histological analysis

Transfected balloon dilated arteries ($N=5$) were harvested after 3 days, immediately frozen for protein isolation, and MMP activation and pSmad signaling is studied as described above.

Transfected balloon dilated arteries ($N=9$) were harvested after 14 days fixated in 4% formaldehyde and embedded in paraffin. Adventitial, medial, intimal and lumen areas were analyzed by cross sectional analysis (10 sections per artery, 500 μm in between) on Elastin van Giesson stained sections and median values were compared. The observer that analyzed the sections was blinded for the different treatment groups. Regions of interest were drawn on digital images, and computerized analysis was performed to analyze the different areas (Analysis 3.2). Regions of interest were drawn at the outside of the adventitia (1), at the external elastic lamina (2) and internal elastic lamina (3), and the luminal border (4) (Fig. 5). The adventitial area is defined as the area between 1 and 2, the medial area between 2 and 3, and the intimal area between 3 and 4.

1.8. Statistical analysis

Statistical analysis of the data was performed using a Wilcoxon matched pairs signed rank sum test. Expression data are presented as ratio operated versus control mean ± the standard error of the mean. The α1-PDX and empty virus treated arteries were compared by the median of each artery. p-values of <0.05 were considered statistical significant.

2. Results

2.1. Remodeling and neointima formation after balloon injury

Upon balloon dilation, a decrease in internal elastic lamina diameter was assessed from day 7, which became statistically significant at day 14 (−0.27 mm, $p=0.05$) (Table 1). At day 28, the internal elastic lamina diameter had decreased by −0.43 mm ($p=0.01$) and at day 42 by −0.59 mm ($p<0.001$). The onset of intimal formation was also detectable at day 7 (0.096 mm^2) and this increased till day 28 (0.305 mm^2), when it reached a plateau till day 42 (Table 1). As expected no neointima was detected in contralateral uninjured arteries.

2.2. Arterial furin, MT1-MMP and MMP-2 levels after injury

After balloon dilation, we found increased furin mRNA levels at 2 and 7 days ($p=0.01$ and $p=0.004$, respectively) (Fig. 1A) compared to contralateral control arteries. After 2 days, furin mRNA levels declined in time and returned to basal levels. Active MT1-MMP protein levels were significantly increased at day 7 ($p=0.003$), reached a maximum at 28 days ($p=0.038$), but remained elevated until 42 days ($p=0.016$) (Fig. 1B). Active MMP-2 levels were increased after balloon dilation compared to control levels at 2 days ($p=0.004$), reached its maximum at 14 days ($p=0.002$), and remained elevated throughout the period studied (Fig. 1C). Also the proMMP-2 levels were increased at day 2

Table 1
Remodeling of the artery and intimal hyperplasia 2, 7, 14, 28 and 42 days after balloon dilation

Survival	IEL change (mm)	S.E.M.	p-value	IH (mm^2)	S.E.M.	p-value
2	0.1	0.07	0.17	0		
7	−0.04	0.09	0.69	0.096	0.304	0.3
14	−0.27	0.12	0.05	0.049	0.040	0.04
28	−0.43	0.11	0.01	0.305	0.003	0.003
42	−0.59	0.07	<0.001	0.262	0.017	0.018

Remodeling is calculated as mean relative change ± S.E.M. (millimeter) in internal elastic laminae (IEL) diameter between post dilation and at termination. Intimal hyperplasia (IH) is presented as mean absolute area (mm^2) ± S.E.M. ($N=6-7$ rabbits per time point, $p<0.05$ = significant).

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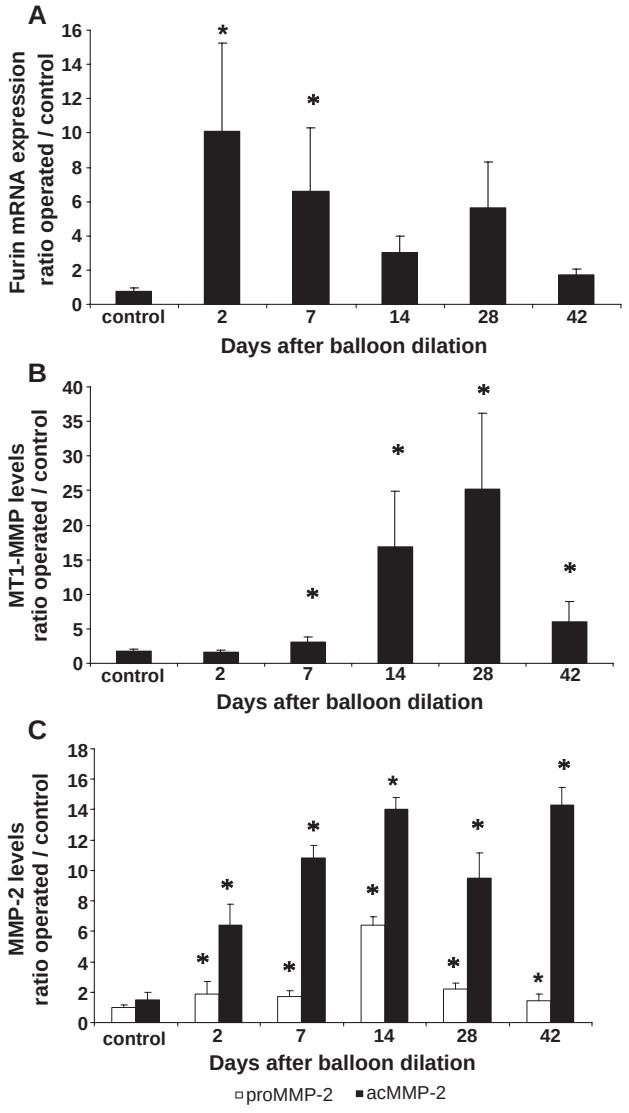

Fig. 1. (A) Furin mRNA expression 2, 7, 14, 28 and 42 days after balloon dilation/contralateral control artery. Furin expression is significantly increased at days 2 and 7. Expression levels of active membrane type 1-matrix metalloproteinase (MT1-MMP) (B) pro- and activated MMP-2 (C) were calculated relative to levels detected from contralateral control arteries. Injury increases levels of activated MT1-MMP and MMP-2 protein levels. (Ratio mean number of pixels ± S.E.M.) ($N=6-7$ rabbits per time point, *$p<0.05$).

($p=0.005$) and reached their maximum at day 14 ($p=0.002$) (Fig. 2C). We did not observe an increase in MMP-9 activity levels after injury at all time points.

2.3. Arterial phosphorylated Smad 1/5 and 2/3 levels after balloon injury

After balloon dilation, we observed an increase in phosphorylated Smad 1/5 (pSmad 1/5) at day 2, 7, and 28 ($p=0.03$, $p=0.04$, $p=0.009$ respectively) (Fig. 2A).

Phosphorylated Smad-2 (pSmad-2, Fig. 2B) was also increased from day 2 onwards ($p=0.009$) and slowly reached a maximum at day 28 ($p=0.009$). The same pattern was seen with pSmad-3, but with a maximum at day 14 ($p=0.008$) (Fig. 2C).

2.4. Ex vivo inhibition of furin-like PCs and MT1-MMP, MMP-2, and TGF-β1 levels

We transduced aortic rings ex vivo with α1-PDX (Ad.CMV.α1PDX) to study the effect of furin inhibition on MT1-MMP and TGF-β activation. α1-antitrypsin is expressed 3 and 7 days after α1-PDX transfection, and is not present in the control aortic rings (Fig. 3A). After transduction with α1-PDX, MT1-MMP activation was inhibited after 3 and 7 days of culture while a reduction in MMP-2 activation was found after 7 days of culture (Fig. 3B and C, respectively). Interestingly, 3 and 7 days after transduction with α1-PDX, we found no TGF-β activation (Fig. 3D) in contrast to the aortic rings transduced with the

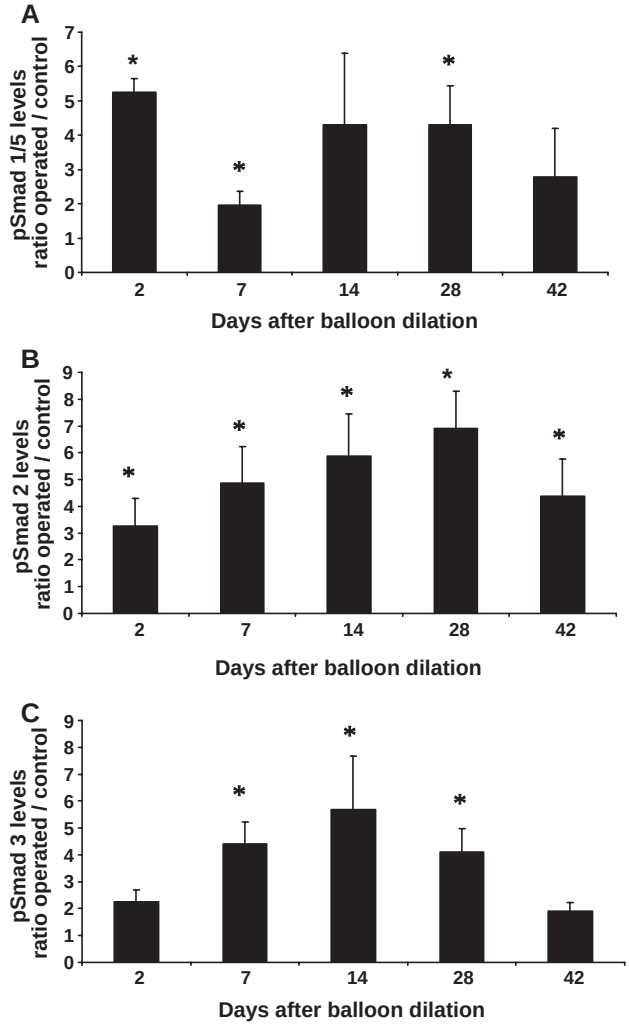

Fig. 2. Western blot analysis of phosphorylated Smad 1/5 (A: pSmad 1/5), phosphorylated Smad-2 (B: pSmad-2), and phosphorylated Smad-3 (C: pSmad-3). Expression levels were calculated relative to levels from contralateral control arteries. pSmad expression levels are increased in time after balloon dilation. (Data are presented as the measured mean number of pixels ± S.E.M.) ($N=6-7$ rabbits per time point, *$p<0.05$).

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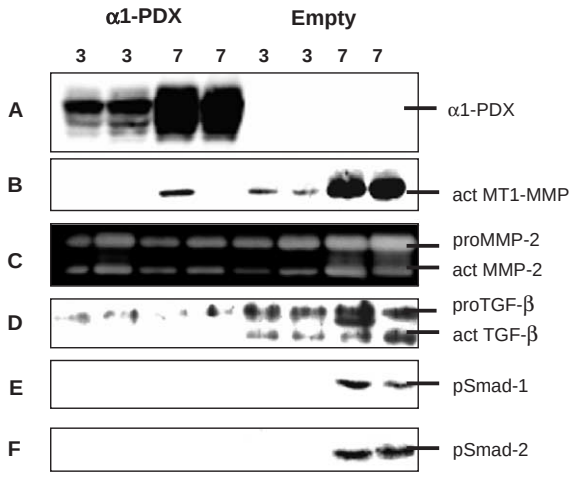

Fig. 3. Western blot and zymography analysis of α1-antitrypsin (α1-PDX) (A), MT1-MMP (B), MMP-2 (C), transforming growth factor β (TGF-β) (D), phosphorylated Smad-1 (pSmad-1) (E), and phosphorylated Smad-2 (pSmad-2)(F) expression levels. Aortic rings were cultured 3 and 7 days after transfection with Ad.CMV.α1-PDX or Ad.CMV.Empty. The expression of the modified α1-antitrypsin (α1-PDX) reduced MT1-MMP and MMP-2 activity, and inhibited active TGF-β levels and phosphorylation of Smad-1 and 2 (representative for $N=3$ independent experiments).

empty virus. TGF-β downstream signaling was also affected by α1-PDX transduction. Both pSmad-1 and pSmad-2 were present 7 days after culture in the aorta rings after transduction with the empty virus. In contrast, no phosphorylated Smad was detected in the α1-PDX infected rings (Fig. 3E and F).

Since equal amount of protein were loaded, Fig. 3D suggests that TGF-β synthesis is also regulated by furin-like PCs because proTGF-β levels were decreased after α1-PDX transduction.

2.5. In vivo inhibition of furin-like PCs

Transduction efficiency in vivo was tested by locally administering a β-galactosidase producing adenovirus. The peri-adventitial gene delivery was able to transduce the adventitial layer but did not transduce medial smooth muscle cells (SMCs) (Fig. 4A). The α1-PDX expression could be detected by Western blotting in vivo, 1 and 3 days after transfection (Fig. 4B). Three days after balloon injury and transduction with Ad.CMV.α1-PDX or Ad.CMV.Empty ($N=4$), we observed a reduction in MMP-2 activation in the

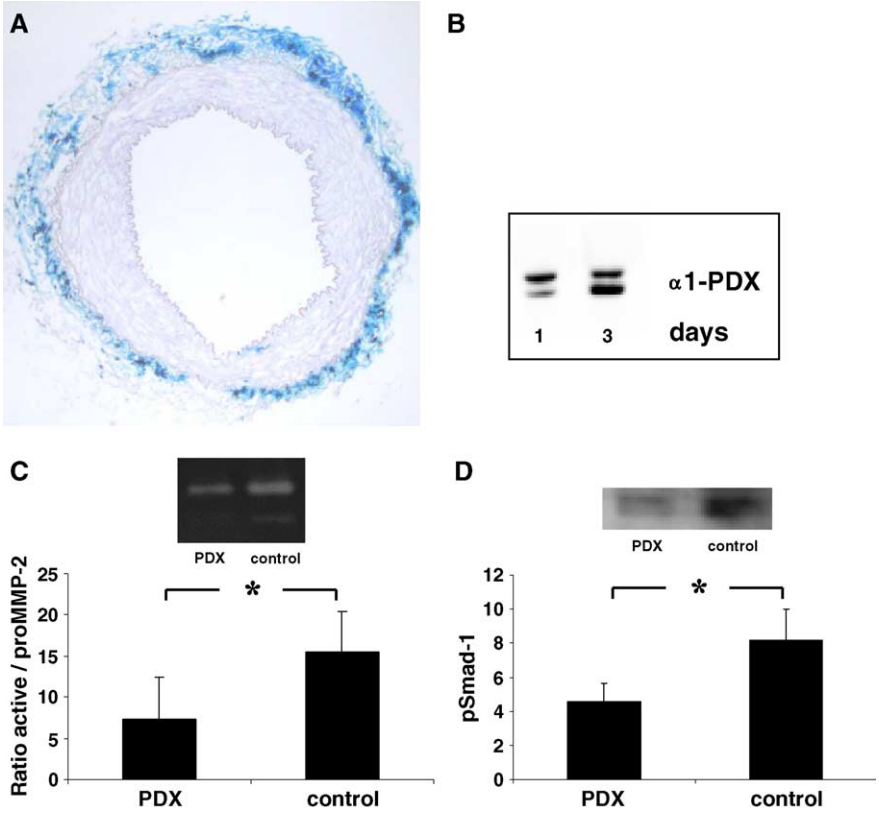

Fig. 4. (A) Staining for β-galactosidase 3 days after peri-adventitial gene delivery using 1.10^9 pfu Ad.CMV.lacZ was able to transfect the adventitial area. (B) By Western blotting α1-antitrypsin, produced by α1-PDX, could be detected at days 1 and 3. (C) Ratio active/proMMP-2 for α1-PDX and empty virus treated arteries (Ratio × 100%, $N=5$ rabbits, $*p<0.05$). After treatment with α1-PDX, MMP-2 activation is reduced at 3 days. (D) Western blot analyses of phosphorylated Smad-1 levels for α1-PDX and empty virus treated arteries ($N=4$ rabbits, $*p<0.05$). After treatment with α1-PDX, phosphorylated Smad-1 levels are reduced at 3 days (data are presented as the measured mean number of pixels ± S.E.M.).

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α1-PDX treated arteries (PDX: 7.4±0.94 vs. Empty: 15.5±4.1, arbitrary units; $p<0.05$) (Fig. 4C). Moreover, we observed also a reduction in pSmad-1 signaling in the α1-PDX treated arteries (PDX: 4.6±1.1 vs. Empty: 8.2±1.8, arbitrary units; $p<0.05$) (Fig. 4D), confirming results obtained ex vivo.

2.6. Angiographic and morphometric analysis after α1-PDX transduction

Nine rabbits were balloon dilated in both femoral arteries and transduced with Ad.CMV.PDX or Ad.CMV.Empty. The angiographic diameters measured before dilation, during balloon dilation and post-dilation, did not differ between the α1-PDX and empty virus transduced arteries. Also at termination no significant differences were found in lumen diameter, and in late lumen loss (LLL=diameter post-dilation − diameter termination) (Table 2).

Morphometry confirmed the angiographic data and revealed no difference in mean luminal area between the α1-PDX and control treated arteries (α1-PDX: 0.43±0.07 mm^2 vs. Empty: 0.44±0.06 mm^2) (Table 2). Reductions in areas measured at the outside of the adventitia, external elastic lamina, and internal elastic lamina, were observed in the α1-PDX arteries compared to the control treated artery, but were not significant (Table 2). The medial areas (α1-PDX: 0.42±0.03 mm^2 vs. Empty: 0.42±0.03 mm^2) did not differ significantly. Comparison of the adventitial and intimal areas, however, revealed a 13.1±5.2% reduction in adventitial area (α1-PDX: 0.44±0.02mm^2 vs. Empty: 0.53±0.04 mm^2, $p=0.03$) and a 23.6±7.9% reduction in intimal area (α1-PDX: 0.40±0.05mm^2 vs. Empty: 0.49±0.02mm^2, $p=0.05$) in the α1-PDX treated arteries (Fig. 5).

Table 2
Angiographic diameter (micrometer) and morphometric area parameters (micrometer square) of the empty and α1-PDX virus treated arteries

Angiographic	Empty virus		Alpha1-PDX		p
	Mean	S.E.M.	Mean	S.E.M.	
Pre-BD	1494	100	1439	96	0.6
BD	2875	134	2761	58	0.26
Post-BD	2324	71	2246	65	0.31
Termination	1778	137	1666	79	0.54
LLL	−546	167	−580	84	0.87
Morphometric parameters					
Outside adventitia	1886468	90858	1696488	103816	0.12
EEL	1362963	67273	1246639	89903	0.23
IEL	937285	70491	833116	94356	0.29
lumen	447554	61998	430134	73844	0.82

The angiographic diameters are measured pre-dilation (pre-BD), during balloon dilation (BD) and post-dilation (post-BD), as well as at termination. Late lumen loss (LLL) is calculated by the diameter post-dilation minus the diameter at termination. The morphometric area parameters are measured at sections from each artery ($N=9$ rabbits). Angiographic diameters and morphometric areas did not differ significantly between the two groups. (IEL=internal elastic laminae, EEL=external elastic laminae).

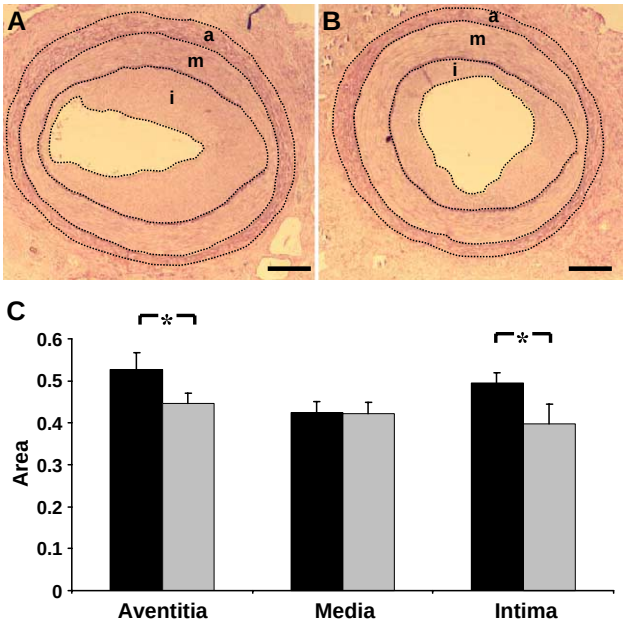

Fig. 5. Balloon dilated arteries were transfected with Ad.CMV.Empty (A) or Ad.CMV.PDX (B) and rabbits were terminated after 14 days. (a=adventitia, m=media, i=intima; bar=250 μm). (C) Histomorphometric adventitial, medial, and intimal layer areas (millimeter square) in the empty (black) and α1-PDX (grey) treated arteries. (Data are mean±standard error of the mean; $N=9$ rabbits, $*p<0.05$). A significant reduction in adventitial and intimal area is observed after inhibition of furin-like PCs.

3. Discussion

Proprotein convertases (PCs) are proteolytic activators of proproteins and potential targets to modulate the arterial response to injury. We studied furin, MT1-MMP, MMP levels and TGF-β signaling and investigated the effect of an inhibitor of furin-like PCs on the arterial response to injury following balloon dilation.

We found that furin mRNA levels increased at 1 and 2 days after balloon dilation. Rabbit specific furin could not be detected by Western blotting, however, strong correlations are found between furin mRNA and transforming growth factor (TGF) β1 activation [11]. This implies that furin activity is transcriptionally regulated and that furin mRNA levels are good indicators for furin activity. This finding suggests that furin, next to PC5 [12], is an important PC during neointima formation in vivo.

Since the role of substrates of furin-like PCs after arterial injury was unclear, we studied both the MT1-MMP–MMP-2 activation pathway and the TGF-β signaling pathways after balloon injury. Active MT1-MMP levels were increased from day 7, with a maximum at 28 days and pro- and active MMP-2 levels increased at day 2, with a maximum at 14 days, confirming previous

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reports on MMP-2 expression [13,14]. The shift between the onset of MT1-MMP and MMP-2 activation points to an initial role of a different activation mechanism, probably by $\alpha_v\beta_3$ [14].

Analyzing the TGF-β signaling cascade showed that both pSmad 1/5 and pSmad 2/3 were increased from day 2 following balloon dilation, suggesting that TGF-β is able to regulate downstream targets. This is in agreement with previous reports showing that active TGF-β1 levels are increased between 2 h and 7 days, with a maximum at 3 days [15], after PTCA injury in porcine arteries. Early after injury, TGF-β1 expression was localized within the adventitia and from day 7 also in the developing neointima [16,17], suggesting that an adventitial adenoviral approach to inhibit TGF-β1 signaling might be effective.

Adventitial ex vivo transduction of aortic rings with α1-PDX reduced both MT1-MMP activation and MMP-2 activity and inhibited the activation of TGF-β and subsequent phosphorylation of Smad-1 and 2. This was confirmed in vivo by a reduced activation of MMP-2 and reduced pSmad-1 levels. Activation of proTGF-β is complex and includes the intracellular removal of the prodomain performed by furin. However, TGF-β activity and binding to its receptor is also dependent of the release of the latency binding peptide (LBP) which is executed by e.g. different MMPs.

Adventitial in vivo transduction showed no differences in arterial lumen size and in late lumen loss between the α1-PDX treated arteries and the control arteries. We observed a significant reduction, however, in intimal and adventitial areas in the α1-PDX treated group, showing that furin-like PCs are involved in the arterial response to injury.

The earlier increase in furin expression, compared to PC5 [12] makes furin activity more susceptible for adenoviral inhibition. For this, adenoviral inhibition of furin-like PCs will mostly affect early furin-like PCs dependent processes like TGF-β1 expression and activation [15,17] and subsequent Smad phosphorylation. MT1-MMP activation occurred later in time after balloon dilation and is therefore less susceptible for inhibition by an adenoviral approach. Next to this, furin knockout cells showed no TGF-β maturation, however, active MT1-MMP was still present. This suggests the existence of a furin-independent activation of MT1-MMP [18,19], and might reduce the efficiency of inhibition of MT1-MMP activation even more.

The reduced intimal and adventitial areas, after furin-like PC inhibition, are in accordance with the previous studies on TGF-β1 inhibition [17,20,21]. However, the previous observations on TGF-β1 inhibition showed less luminal narrowing and less arterial shrinkage. We found no changes in lumen areas, because the reduction in neointima was accompanied with a tendency to increased shrinkage of the artery (decreased vessel areas).

Although involvement of TGF-β in arterial remodeling has been reported, these results are confusing. Inward arterial remodeling can be inhibited using a TGF-β signaling inhibitor [17] while adenoviral overexpression of TGF-β (stimulating TGF-β signaling) also inhibits inward arterial remodeling [22]. This might be explained by the recent observation, that in endothelial cells TGF-β can activate distinct pathways. The activin receptor-like kinase 5 (ALK5)–Smad 2/3 pathway stimulates collagen production but inhibits cell proliferation, migration and MMP production while the ALK1–Smad 1/5 pathway stimulates cell proliferation, migration and MMP production but inhibits collagen production [23]. This might also apply to the non-endothelial cells of the arteries. We found that both pathways are activated after balloon dilation. Since both pathways have different kinetics and threshold levels for TGF-β, and both pathways can influence each other, local levels will determine which pathway is dominant. This might also explain why other researchers found an increase in neointima/plaque formation after TGF-β inhibition [24] in contrast to this and other studies [17,20,21].

Active TGF-β levels were observed early after injury, whereas the increases in pSmad pathways last longer. This might be explained by the family of bone morphogenic proteins (BMP) or activin. Next to TGF-β, BMP can activate the Smad 1/5 pathway [25], whereas activin can activate Smad 2/3 [26]. The increased expression of activin was observed at 2, 4, 7 and 14 days after arterial injury [27], whereas BMP can influence Smad phosphorylation of SMCs [28]. Moreover, activin overexpression inhibits neointima formation in mice and in cultured human saphenous vein segments [29].

Thus, based on our observation, we cannot conclude if one TGF-β/Smad pathway is responsible for geometric remodeling or neointima formation and is locally dominant. Specific TGF-β pathway deletion in adult mice using an inducible Cre transgenic background might help to answer these questions.

In conclusion, our data show that furin, the MT1-MMP–MMP2 activation cascade and the TGF-β–Smad signaling pathway are activated after balloon injury. Advential inhibition of furin-like PCs shows that these PCs, probably via the TGF-β–Smad signaling pathway, are involved in the arterial response to injury and identifies furin-like PCs as a possible target to inhibit intimal hyperplasia.

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