A novel role of endothelium in activation of latent pro-membrane type 1 MMP and pro-MMP-2 in rat aorta

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Aims Aortic stiffness is an independent risk factor for progression of cardiovascular diseases. Degradation of elastic fibres in aorta due to angiotensin II (ANGII)-stimulated overactivation of latent membrane type 1 matrix metalloproteinase (MT1MMP) and matrix metalloproteinase-2 (MMP2) is regarded to represent an important cause of aortic stiffness. Therefore, clarification of the causal mechanisms triggering the overactivation of these MMPs is of utmost importance. This study addresses the endothelium as a novel key activator of latent pro-MT1MMP and pro-MMP2 in rat aorta.

Methods and results Using a co-culture model of rat aortic endothelial cells (ECs) and smooth muscle cells (SMCs), we found that ANGII stimulation resulted in activation of latent pro-MT1MMP and pro-MMP2 in SMCs exclusively when co-cultured with ECs (assessed with western blot and gelatin zymography, respectively). EC-specific AT1 receptor stimulation triggered endothelin-1 release and paracrine action on SMCs. Endothelin-1 increased expression and activity of pro-protein convertase furin in SMCs via endothelin receptor type A (assessed with qPCR and furin activity assay, respectively). Consequently, furin acted in two ways. First, it increased the activation of latent pro-MT1MMP and, second, it activated pro-protein convertase furin in SMCs via endothelin receptor type A (assessed with qPCR and furin activity assay, respectively). Consequently, furin acted in two ways. First, it increased the activation of latent pro-MT1MMP and, second, it activated pro-protein convertase furin in SMCs via endothelin receptor type A (assessed with qPCR and furin activity assay, respectively). Consequently, furin acted in two ways. First, it increased the activation of latent pro-MT1MMP and, second, it activated pro-protein convertase furin in SMCs via endothelin receptor type A (assessed with qPCR and furin activity assay, respectively). Consequently, furin acted in two ways. First, it increased the activation of latent pro-MT1MMP and, second, it activated pro-protein convertase furin in SMCs via endothelin receptor type A (assessed with qPCR and furin activity assay, respectively). Consequently, furin acted in two ways. First, it increased the activation of latent pro-MT1MMP and, second, it activated pro-protein convertase furin in SMCs via endothelin receptor type A (assessed with qPCR and furin activity assay, respectively). Consequently, furin acted in two ways. First, it increased the activation of latent pro-MT1MMP and, second, it activated pro-protein convertase furin in SMCs via endothelin receptor type A (assessed with qPCR and furin activity assay, respectively).

Conclusions We propose that the endothelium under ANGII stimulation acts as a novel and key activator of latent pro-MT1MMP and pro-MMP2 in SMCs of rat aorta. Therefore, endothelium may critically contribute to pathophysiology of aortic stiffness.

Keywords Angiotensin II • Endothelium • Endothelin-1 • Smooth muscle cell • MMPs

1. Introduction Aortic stiffness is a hallmark of aging and hypertension. Higher resistance caused by stiffer aorta increases the workload of the left ventricle and impairs coronary blood flow which leads to cardiac dysfunction. Aortic stiffness is attributed to adverse remodelling of extracellular matrix (ECM) in the aortic wall, such as degradation of elastin and fibrosis. Pathophysiology of aortic stiffness is not fully understood. However, given the fact that it substantially accelerates during hypertension and involves complex remodelling of aortic wall ECM, the role of angiotensin II (ANGII) and matrix metalloproteinases (MMPs) as drivers of hypertension and adverse ECM remodelling, respectively, are of great importance. Both the ANGII and MMPs can contribute to aortic stiffness by degrading elastin and by that facilitating fibrosis. MMPs are proteolytic enzymes mediating physiological and pathological remodelling of ECM. MMP2 is the major enzyme found in cardiovascular tissues including aorta. MMP2 is secreted into ECM as a latent pro-enzyme (pro-MMP2). Unlike other MMPs, pro-MMP2 is hardly activated by general proteases, but mainly by membrane type 1 MMP (MT1MMP). MT1MMP is also synthesized as a latent pro-enzyme (pro-MT1MMP) within cells followed by intracellular proteolytic activation via the pro-protein convertase (PC) furin (to MT1MMP). This mechanism of activation is common for cancer cells. In vascular SMCs, however, at least partial furin-independent activation of pro-MT1MMP may occur, yet via other furin-like PCs. In addition, furin and furin-like PCs may directly contribute to pro-MMP2 activation. In order to ensure the...
proteolytic activation of latent pro-MMP2 with MT1MMP, pro-MMP2 has to bind MT1MMP on the cell membrane. This step is mediated via integrin αvβ3 in vascular SMCs.16,17 Similar to other α-integrins, αvβ3 is activated from its precursor pro-αvβ3 and this is mediated by furin and furin-like PCs.14,23 Therefore, furin and furin-like PCs remain essential in activation of latent pro-MT1MMP and pro-MMP2. Besides its role as a main activator of pro-MMP2, MT1MMP too has been strongly implicated in degradation of elastin in aorta.5,10 Therefore, both MT1MMP and MMP2 might contribute to aortic stiffness.

Loss of balanced regulation of expression and activation of pro-MMP-2 may induce adverse ECM remodelling in aorta.3,4 Increased formation of ANGII and increased expression and activation of pro-MMP2 strongly coincide with development of aortic stiffness.4 Finally, inhibition of MMP2 activity can prevent degradation of elastin.19 Previously, we reported that ANGII is a strong regulator of latent pro-MMP2 expression in cultured endothelial cells (ECs) and smooth muscle cells (SMCs) of rat aorta.18 We provided comprehensive evidence that regulatory mechanisms employed by ANGII in rat aortic ECs and SMCs are cell specific, yet commonly activated by angiotensin II type 1 receptor (AT1R). Unlike latent pro-MT1MMP, however, neither SMCs nor ECs exhibited active MMP2.18 Most interestingly, we showed that active MMP2 cannot be detected in isolated rat aorta when the endothelium is removed. Even though there is a better understanding of the regulation of pro-MMP2 expression in aorta, understanding of mechanisms controlling the activation of pro-MMP2 remain incomplete. Because latent pro-MMP2 in ECM does not exert enzymatic activity in vivo,6,7 the activation of the latent pro-MMP2 is a limiting step in its overall catalytic efficacy and thus potential contribution to aortic stiffness. Therefore, unravelling the mechanisms regulating the activation of latent pro-MMP2 is of great importance to improve our causal understanding of aortic stiffness. In the present work, we propose a conceptually novel role of the endothelium as a critical regulator of activation of latent pro-MMP2 in aorta in the presence of ANGII stimulation. We tested the hypothesis that the ANGII-stimulated endothelium essentially regulates the activation of latent pro-MMP2 by initial activation of latent pro-MT1MMP. Moreover, we identified the underlying activating pathways.

2. Methods
A detailed description of the methods and further information about materials is provided in Supplementary material online.

2.1. Co-culture and validation
The model was established based on a previous report.20 Second and third passages of rat aortic ECs and SMCs were used (Cell Applications, Inc., San Diego, CA, USA). Five different vials of cells were used to perform five independent experiments. The cells were co-cultured on Transwell polyester membrane inserts (Corning Inc., Corning, NY, USA). As a control of the co-culture model, pure culture of SMCs, cultured on one side of the same insert, was used. For experimentation, stimulation of the cells with ANGII (0.1, 0.5, 1 μmol/L, 8 h) and treatments with various inhibitors were performed in non-supplemented, serum-free DMEM medium with glucose. Before performing experiments on the co-culture, the model was validated. Results of the validation are demonstrated in Supplementary material online, Figure S1 with a detailed description of the procedure.

2.2. Western blot
Western blot was used to quantify the expression of pro-MT1MMP (64 kDa) and MT1MMP (54 kDa) protein in cell lysates and extracts from aorta, as previously described.18

2.3. Gelatin zymography
Gelatin zymography enables us to detect active 62 kDa MMP2, but also uniquely latent 72 kDa pro-MMP2 through its ability to exert in vivo gelatinolytic activity. Therefore, the method was used to quantify the active MMP2 (62 kDa) in EC and SMC supernatants, as well as in extracts from rat aorta, as previously described.18

2.4. Experiments with aortic rings
Thoracic aorta for ex vivo experiments was isolated from 10-week-old male Wistar rats (total n = 20, Charles River Laboratories, Germany). The use of animals was approved by the institutional committee and the local authorities (permission: 24-9168.24-1/2012-16). Animal experiments conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Animals were anaesthetized with intraperitoneal injection of ethyl carbamazepine (1.3 g/kg body weight). Anaesthesia was monitored by assessment of pain and corneal reflexes. Isolation of aorta was started after both reflexes completely disappeared. Endothelium from aortic rings was removed as previously described.18 Successful removal of the endothelium from aorta was validated using a Mullvany myograph as previously reported.21 In addition, the removal of the endothelium from aorta was confirmed by histological sections as previously described.22 For experimentation, stimulation of aorta with ANGII (1 μmol/L, 8 h) and treatments with various inhibitors were performed in the same media used for the co-cultured cells.

2.5. Furin activity assay
Furin activity assay was performed in lysates of SMCs and extracts of aortic rings using furin-specific fluorescent substrate Pyr-Arg-Thr-Lys-Arg-AMC (Calbiochem-Novabiochem, Bad Soden, Germany) as previously reported.14

2.6. RNA isolation and reverse transcription
Total mRNA from SMCs and aortic rings for reverse transcription qPCR (RT-qPCR) was isolated using TRIzol Reagent (Life Technologies, Eggenstein, Germany) according to the user manual. RT-qPCR was performed as previously described.23 The detailed experimental procedure is described in Supplementary material online.

2.7. Statistical analysis
Results from independent experiments (n = 5) were analysed using one-way ANOVA followed by Bonferroni post hoc test. In case which compared two groups (n = 5), unpaired Student’s t-test was used. A P-value < 0.05 was considered statistically significant. Values are shown as mean ± SD (standard deviation).

3. Results
3.1 ANGII-stimulated ECs activate latent pro-MT1MMP and pro-MMP2 in SMCs
In order to address our hypothesis, we employed a validated co-culture model of rat aortic ECs and SMCs. Results of the validation are demonstrated in Supplementary material online (Figure S1) and described in the figure legend. ECs and SMCs in the co-culture were stimulated simultaneously with different concentrations of ANGII (0.1, 0.5, and 1 μmol/L) for 8 h. As a control of the co-culture, SMCs were cultured alone (pure SMCs culture) and stimulated similarly. Results show that ANGII dose-dependently increased latent pro-MT1MMP protein in SMCs both in pure and co-culture models (Figure 1A). However, ANGII increased activation of latent 64 kDa pro-MT1MMP to active 54 kDa MT1MMP in SMCs only in the co-culture, demonstrated by the dose-dependent increase in active 54 kDa MT1MMP. In pure SMC culture, active MT1MMP was below the level of detection. ANGII also
Endothelium: a novel activator of MMPs

were not detected in ECs, whereas both latent pro-MT1MMP and MMP2. In contrast to SMCs, active MT1MMP and MMP2 was detected in the EC compartment, even with ANGII stimulation, excludes the transfer of active MMP2 from EC to SMC compartment.

Results described above showed that the increase in latent and active forms of MT1MMP and MMP2 was highest at a concentration of 1 µmol/L of ANGII. Therefore, this concentration was used in all consecutive experiments. To investigate the mechanism of EC-dependent activation of latent forms of pro-MT1MMP and pro-MMP2 in SMCs, the co-culture of ECs and SMCs was used in all consecutive experiments.

To provide evidence for the specific involvement of the AT1R in the effects of ANGII, we used the selective AT1R blocker losartan (50 µmol/L), which was applied 30 min prior to ANGII stimulation. Effective concentration of losartan was chosen based on our previous report. Results show that selective pre-treatment of only ECs with losartan significantly (P < 0.01) inhibited ANGII-stimulated activation of the latent pro-MT1MMP (Figure 2A) and pro-MMP2 (Figure 2B) in SMCs. This is demonstrated by a decrease in active 54 kDa MT1MMP protein and gelatinolytic activity of 62 kDa MMP2. However, treatment of ECs with losartan did not affect the increase in latent pro-MT1MMP protein or gelatinolytic activity of latent pro-MMP2 in SMCs. The latter finding serves as an evidence that losartan when added to the EC compartment did not reach the SMC compartment, at least in effective concentration. Similarly, selective stimulation of EC compartment with ANGII did not increase MMP2 and MT1MMP in supernatant of SMC compartment (see Supplementary material online, Figure S5), further excluding the compound diffusion from the EC to the SMC compartment.

Selective pre-treatment of only SMCs with losartan, however, significantly (P < 0.01) inhibited the increase in latent pro-MT1MMP protein (Figure 2A) and the gelatinolytic activity of latent pro-MMP2 (Figure 2B) and, hence, also the active forms of these MMPs. Simultaneous pre-treatment of ECs and SMCs with losartan inhibited the increase of both latent and active forms of MT1MMP and MMP2 in SMCs. These findings suggest that whereas expression of latent pro-MT1MMP and pro-MMP2 in SMCs depends on AT1R stimulation in SMCs, their activation depends on AT1R stimulation specifically in ECs. In order to elucidate the signal by which ECs trigger the activation of latent pro-MT1MMP and pro-MMP2 in SMCs, we addressed the role of endothelin-1 by using selective endothelin-1 receptor inhibitors for type A (ET\textsubscript{A}, BQ-123, Tocris) and type B (ET\textsubscript{B}, BQ-788, Tocris). Our results show that pre-treatment of SMCs with 1 µmol/L ET\textsubscript{B}, but not 1 µmol/L ET\textsubscript{A} inhibitor significantly (P < 0.05) decreased ANGII-stimulated activation of latent pro-MT1MMP and pro-MMP2 (Figure 2D and E). Dual ET\textsubscript{A}/ET\textsubscript{B} inhibition showed the same effect as ET\textsubscript{A} alone. The ET\textsubscript{A} receptor inhibitor worked in a dose-dependent manner (0.1, 0.5, and 1 µmol/L, Supplementary material online, Figure S4). We also detected an increase in endothelin-1 protein release in co-cultured EC and SMC supernatants after ANGII stimulation (Figure 2C). In cell lysates, however, increase in endothelin-1 protein was detected only in EC lysates, while no endothelin-1 protein was detected in SMC lysates even after ANGII stimulation. These results suggest that, in co-culture, endothelin-1 was released by ECs subsequently diffusing to SMCs and acting in a paracrine manner.

We further performed complementary experiments where we stimulated pure culture of SMCs together with ANGII and endothelin-1. Dual stimulation with ANGII and endothelin-1 (0.01, 0.1, and 1 µmol/L, Sigma)

Figure 1 ANGII-stimulated ECs activate latent pro-MT1MMP and pro-MMP2 in co-cultured SMCs. (A) Representative graph and western blots with latent and active forms of MT1MMP in pure and co-culture of SMCs after stimulation with ANGII (0.1, 0.5, and 1 µmol/L, 8 h). While ANGII dose-dependently increased latent pro-MT1MMP protein in SMCs both in pure and co-cultures, increase of active MT1MMP was detected only in the co-culture. (B) Representative graph and zymogram with latent and active forms of MMP2 in SMCs in pure and co-culture of SMCs after stimulation with ANGII (0.1, 0.5, and 1 µmol/L, 8 h). Similar to MT1MMP, ANGII dose-dependently increased latent pro-MMP2 in SMCs of both pure and co-cultures, but increase of active MMP2 was detected only in the co-culture. (C) Representative western blot (upper) and zymogram (lower) with latent and active forms of MMP2 in SMCs in pure and co-culture of SMCs after stimulation with ANGII. Latent pro-MMP2 was detected in EC lysates, while no endothelin-1 protein was detected only in the co-culture. (*) and (**, ***): ANGII dose-dependently increased latent pro-MT1MMP protein expression. ANGII dose-dependently increased latent pro-MT1MMP protein in SMCs only in the co-culture, demonstrating by the increase in gelatinolytic activity of active 62 kDa MMP2. Again, an active MMP2 was not detected in pure cultures of SMCs. Consistent with our previous report, ANGII did not influence MMP9 neither in ECs nor in SMCs (see Supplementary material online, Figure S2).

In order to exclude that the active MMP2 detected in the SMC compartment was transferred from EC compartment, we also examined ECs in co-culture to assess latent pro- and active forms of MT1MMP and MMP2. In contrast to SMCs, active MT1MMP and MMP2 were not detected in ECs, whereas both latent pro-MT1MMP and pro-MMP2 was detected (Figure 1C). Consistent with our previous report, ANGII dose-dependently increased pro-MMP2 protein expression. However, no effect on latent pro-MT1MMP protein expression was detected (Figure 1C). The fact that neither active MMP2 nor active MT1MMP was detected in the EC compartment, even with ANGII stimulation, excludes the transfer of active MMP2 from EC to SMC compartment.

We further performed complementary experiments where we stimulated pure culture of SMCs together with ANGII and endothelin-1. Dual stimulation with ANGII and endothelin-1 (0.01, 0.1, and 1 µmol/L, Sigma)
dose-dependently increased the activation of both latent pro-MT1MMP and pro-MMP2 (see Supplementary material online, Figure S5A).

3.2. ANGII-stimulated ECs activate latent pro-MT1MMP and pro-MMP2 in SMCs via gap junctions and furin

In co-culture, paracrine action of ECs on SMCs would require cell–cell contacts—most likely gap junctions. Consistent with previous report, gap junctions were also detected in our model (see Supplementary material online, Figure S1C and D). We investigated whether these gap junctions are essential for ECs to ensure the activation of latent pro-MT1MMP (A) and pro-MMP2 (B) in SMCs, without affecting the increase in latent forms per se. Selective pre-treatment of SMCs (Los. SMC), as well as of both ECs and SMCs (Los. EC and SMC) inhibited ANGII-mediated increase of both latent and active forms of MT1MMP (A) and MMP2 (B). (C) Representative western blot with endothelin-1 protein. ANGII increased endothelin-1 protein in supernatants of co-cultured ECs and SMCs, as well as lysates of ECs, but not lysates of SMCs. (D) Representative graph showing inhibition of ANGII-stimulated activation of latent pro-MT1MMP and pro-MMP2 with ETα (ETα-i, BQ-123, 1 μmol/L), but not with ETβ (ETβ-i, BQ-788, 1 μmol/L) receptor blocker in SMCs. Ctrl, untreated control; Alb, albumin; (A) ***p < 0.01 vs. control; **p < 0.01 vs. 64 kDa pro-MT1MMP; (B) ***p < 0.01 vs. control; **p < 0.01 vs. 72 kDa pro-MP2; (C) ***p < 0.01 vs. control; **p < 0.01 vs. 64 kDa pro-MT1MMP; (E) ***p < 0.01 vs. control; *p < 0.05 vs. 72 kDa pro-MMP2; n = 5.

**Figure 2** Role of EC-specific AT1R and SMC-specific ETα receptor in activation of latent pro-MT1MMP and pro-MMP2 in co-cultured SMCs. (A) Representative graph and western blot with latent (64 kDa) and active (54 kDa) forms of MT1MMP and (B) representative graph and zymogram with latent (72 kDa) and active (62 kDa) forms of MMP2. Prior to stimulation with ANGII (1 μmol/L, 8 h) selective pre-treatment of ECs, SMCs, or both with AT1R blocker losartan (50 μmol/L, 30 min) was performed. Selective pre-treatment of ECs (Los. EC) with losartan inhibited the ANGII-mediated activation of latent pro-MT1MMP (A) and pro-MMP2 (B) in SMCs, without affecting the increase in latent forms per se. Selective pre-treatment of SMCs (Los. SMC), as well as of both ECs and SMCs (Los. EC and SMC) inhibited ANGII-mediated increase of both latent and active forms of MT1MMP (A) and MMP2 (B). (C) Representative western blot with endothelin-1 protein. ANGII increased endothelin-1 protein in supernatants of co-cultured ECs and SMCs, as well as lysates of ECs, but not lysates of SMCs. (D) Representative graph showing inhibition of ANGII-stimulated activation of latent pro-MT1MMP and pro-MMP2 with ETα (ETα-i, BQ-123, 1 μmol/L), but not with ETβ (ETβ-i, BQ-788, 1 μmol/L) receptor blocker in SMCs. Ctrl, untreated control; Alb, albumin; (A) ***p < 0.01 vs. control; **p < 0.01 vs. 64 kDa pro-MT1MMP; (B) ***p < 0.01 vs. control; **p < 0.01 vs. 72 kDa pro-MP2; (C) ***p < 0.01 vs. control; **p < 0.01 vs. 64 kDa pro-MT1MMP; (E) ***p < 0.01 vs. control; *p < 0.05 vs. 72 kDa pro-MMP2; n = 5.

In co-culture, paracrine action of ECs on SMCs would require cell–cell contacts—most likely gap junctions. Consistent with previous report, 20 gap junctions were also detected in our model (see Supplementary material online, Figure S1C and D). We investigated whether these gap junctions are essential for ECs to ensure the activation of latent pro-MT1MMP and pro-MMP2 in SMCs. For this, we used two different gap-junction inhibitors (18-alpha GA and FFA) at various concentrations. The cells were treated with these inhibitors 30 min prior to ANGII stimulation. Results show that ANGII-mediated increase in activation of latent pro-MT1MMP and pro-MMP2 (Figure 3A and B) was dose-dependently inhibited with both 18-alpha GA and FFA. This is
used the synthetic peptide inhibitor of furin (Decanoyl-Arg-Val-Lys-Arg-CMK, 10, 50, 100 μmol/L, Calbiochem). The peptide also inhibits other furin-like PCs, which may be involved in latent pro-MT1MMP activation. Before simultaneous stimulation of ECs and SMCs with ANGII, SMCs were treated 24 h with furin antisense oligonucleotide and 1 h with synthetic furin inhibitor before the stimulation with ANGII. Results show that furin antisense oligonucleotide inhibited ANGII-mediated increase in activation of latent pro-MT1MMP to active MT1MMP significantly (P < 0.05, Figure 4A), yet maintaining ~50% of the activation. However, the oligonucleotide abolished the activation of latent pro-MMP2 to active MMP2 (Figure 4B). Interestingly, the less specific synthetic furin inhibitor almost abolished activation of both latent pro-MT1MMP and pro-MMP2 (Figure 4A and B). Moreover, combining furin antisense oligonucleotide with the synthetic furin inhibitor abolished activation of both latent pro-MT1MMP and pro-MMP2 (Figure 4A and B). Neither, latent pro-MT1MMP protein nor pro-MMP2 was affected by furin antisense oligonucleotide or furin inhibitor treatment. A control with scrambled furin oligo is shown in Supplementary material online, Figure S7. These results suggest that latent pro-MT1MMP may be activated via other furin-like PCs, whereas furin seems to be critical in activation of latent pro-MMP2.

In order to obtain more insights into the underlying mechanism, using the same samples, we tested a potential predominant role of furin in the activation of pro-αvβ3, an integrin necessary for binding of latent pro-MMP2 to active MT1MMP on a cell membrane. Indeed, ANGII increased the activation of precursor 150 kDa pro-αvβ3 to its active 125 kDa αvβ3 form, which was abolished by pre-treatment with furin antisense oligonucleotide (Figure 4C). Combining furin antisense oligonucleotide with the synthetic furin inhibitor showed a similar inhibitory efficacy to the furin antisense oligonucleotide or the synthetic furin inhibitor alone. This strongly suggests that predominantly furin activated pro-αvβ3. ANGII did not change expression of the pro-αvβ3 protein (Figure 4C).

### 3.3. Endothelium is pivotal in ANGII-mediated activation of latent pro-MT1MMP and pro-MMP2 in rat aorta

We further investigated the applicability of the findings obtained in the co-culture model of rat aortic ECs and SMCs using isolated rat aorta ex vivo. For this, we prepared intact and denuded aortic rings and stimulated them with ANGII (1 μmol/L) for 8 h. Latent and active forms of MT1MMP protein and MMP2 were assessed in aortic extracts. Consistent with in vitro findings, ANGII significantly (P < 0.01) increased both latent pro- and active MT1MMP in the intact rat aorta. Removal of the endothelium from aorta abolished the ANGII-mediated increase in activation of latent pro-MT1MMP to active MT1MMP. This is demonstrated by a significantly (P < 0.01) lower protein fraction of active MT1MMP. However, increase in latent pro-MT1MMP protein was preserved (Figure 5A). Consistent to our results reported previously, removal of the endothelium abolished the ANGII-mediated increase in activation of pro-MMP2 to active MMP2 without affecting the increased latent pro-MMP2 (Figure 5B). Interestingly, immunostaining of ANGII stimulated aortic sections for latent and active forms of MT1MMP and MMP2 of aortic sections, stimulated with ANGII, showed that the signal was mainly localized to the media, while it was not detectable in the endothelium (see Supplementary material online, Figure S8).

We further investigated the involvement of furin in endothelium-dependent activation of latent pro-MT1MMP and pro-MMP2 in response to ANGII stimulation. For this, aortic rings were pre-treated...
We further examined an involvement of endothelin-1 in the activation of latent pro-MT1MMP and pro-MMP2 in rat aorta. Similar to the co-culture, pre-treatment of the isolated intact aorta with 1 μmol/L ET₄ blocker significantly \((P < 0.05)\) decreased the activation of latent pro-MT1MMP (Figure 5E) and pro-MMP2 (Figure 5F) stimulated by ANGII (1 μmol/L). Furthermore, in contrast to single ANGII stimulation, dual treatment of denuded rat aorta with ANGII and endothelin-1 (1 μmol/L) increased the activation of both latent pro-MT1MMP and pro-MMP2 (see Supplementary material online, Figure S5B).

3.4. Endothelium regulates activity and expression of Furin in SMCs and thereby critically contributes to ANGII-stimulated activation of latent pro-MT1MMP and pro-MMP2 in rat aorta

The findings in co-culture and isolated rat aorta suggest that the endothelium-dependent activation of latent pro-MMP2 in SMCs in response to ANGII stimulation is mediated predominantly via furin. We further investigated whether the activity and expression of furin in SMCs is regulated by ECs. For this, we used a furin activity assay and RT-qPCR, respectively. Results show that ANGII significantly \((P < 0.01)\) increased furin activity (Figure 6A) and mRNA expression (Figure 6B) in SMCs co-cultured with ECs, as well as in intact aorta. However, neither activity nor expression of furin was increased in pure SMC culture and denuded aorta. Therefore, furin activity and mRNA expression was significantly \((P < 0.01)\) lower in pure SMC culture and denuded aorta (Figure 6A and B) compared with SMCs co-cultured with ECs and intact aorta. Both losartan and ET₄ receptor blocker significantly \((P < 0.01\) for losartan and \(P < 0.05\) for ET₄ blocker) inhibited ANGII-mediated increase in furin activity in SMCs and isolated intact aorta. We further assessed activation of pro-αvβ3 integrin. An increase in activation of pro-αvβ3 integrin was evident in SMCs co-cultured with ECs and in intact aorta (Figure 6C). However, processing of pro-αvβ3 did not occur in pure culture of SMCs or in denuded aorta.

Finally, specificity of the furin substrate was controlled by incubating SMC lysates from the co-culture, pre-treated with furin antisense oligonucleotide, with the furin substrate. Compared with untreated SMCs, furin activity was abolished in SMCs treated with furin antisense oligonucleotide (Figure 6D).

4. Discussion

Our study demonstrates a novel pivotal role of the endothelium in ANGII-triggered activation of latent pro-MT1MMP and pro-MMP2 in SMCs of rat aortic media. Endothelium activates these latent MMPs without affecting their expression, which supports the specific role of endothelium in the activation step of these MMPs. We further demonstrate that the endothelium regulates expression and activity of furin and possibly other furin-like PCs in SMCs via paracrine action of endothelin-1. This appears to be central in endothelial control of latent pro-MT1MMP and pro-MMP2 activation. Previous studies were able to show active MT1MMP or MMP2 using pure cultures of SMCs only while applying additional stimuli.\(^{16,24,25,31}\) Interestingly, these stimuli activate the MT1MMP/MMP2 axis. In studies where SMCs were not exposed to external stimuli only latent pro-MT1MMP and pro-MMP2 prevailed.\(^{26,30}\) Active MT1MMP or MMP2, however, are typically observed in animal studies assessing extracts from intact aortic tissues.\(^{3,4,5,8}\)
These studies clearly indicate that SMCs are unable to activate latent pro-MT1MMP and pro-MMP2 in a pure culture without additional stimuli. In the present study, we used a co-culture model of ECs and SMCs in comparison to pure culture of SMCs. Stimulation of pure culture of SMCs with ANGII resulted in an increase of latent pro-MT1MMP and pro-MMP2, but not the activation of these latent MMPs (Figure 1A and B). In co-culture model, however, ANGII increased the activation of latent pro-MT1MMP and pro-MMP2 in SMCs indicating a critical role of ANGII-stimulated endothelium activates latent pro-MT1MMP and pro-MMP2 in isolated rat aorta in a furin-dependent manner. (A) Representative graph with activation of latent pro-MT1MMP and (B) pro-MMP2 in rat aorta. Aortic rings, with or without endothelium, were stimulated with ANGII (1 μmol/L, 8 h). Removal of endothelium abolishes the ANGII-stimulated activation of latent pro-MT1MMP and pro-MMP2, without affecting the increase in latent pro-MT1MMP and pro-MMP2. (C) Representative graph with activation of latent pro-MT1MMP and (D) pro-MMP2 in aortas with endothelium (intact) stimulated with ANGII. Endothelium-dependent activation of latent pro-MT1MMP and pro-MMP2 in response to ANGII stimulation is significantly inhibited with synthetic Furin inhibitor I (Fur-i, 100 μmol/L). (E) inhibition of activation of latent pro-MT1MMP and (F) pro-MMP2 with ETα receptor blocker (ETα-i, BQ-123, 1 μmol/L). Ctrl, untreated control; STD, recombinant 72 kDa pro-MMP2 and 62 kDa MMP2; **P < 0.01 vs. control +/endothelium; △△P < 0.01 vs. ANGII stimulation +/endothelium; n = 5 independent experiments on aortic rings isolated from five rats.
showing that there was no active MMP2 and MT1MMP in supernatants of EC compartment even after ANGII stimulation (see Supplementary material online, Figure S1C).

AT1R is critical in ANGII signalling. We demonstrate that activation of latent pro-MT1MMP and pro-MMP2 in SMCs in co-culture requires stimulation of AT1R in ECs. Application of the AT1R blocker losartan selectively to the EC compartment abolished the activation of latent pro-MT1MMP and pro-MMP2 in SMCs. However, selective application of losartan to the EC compartment did not affect the increase in protein expression of the latent pro-MT1MMP and pro-MMP2 in SMCs. Instead, application of losartan to the SMC compartment reduced the expression of latent pro-MT1MMP and pro-MMP2 in SMCs. In- stead, application of losartan to the SMC compartment reduced the expression of latent pro-MT1MMP and pro-MMP2 in SMCs. Thus, while SMC stimulation with ANGII may result in enhanced expression of latent pro-MT1MMP and pro-MMP2, stimulation of AT1R in ECs is required to activate these latent pro-MMPs. In vivo ET\textsubscript{A} receptor blockade was reported to decrease pro-MMP2 activation in rat aorta.\textsuperscript{23} Our findings further demonstrate that inhibition of ET\textsubscript{A} receptor in SMCs in co-culture decreased the activation of latent pro-MT1MMP and pro-MMP2 (Figure 2D and E). Furthermore, in co-culture, ANGII-stimulated increase in endothelin-1 protein was detected in both lysates and supernatants of ECs, whereas only SMC supernatants, but not lysates contained endothelin-1 protein (Figure 2C). This suggests that ECs, via endothelin-1, acted in a paracrine manner to activate latent pro-MT1MMP and pro-MMP2 in SMCs. In support, stimulation of SMCs in pure culture only with ANGII did not trigger activation of latent pro-MT1MMP and pro-MMP2, but it did by dual stimulation with endothelin-1. Furthermore, dual stimulation of denuded aorta with ANGII and endothelin-1 showed similar effect as seen in pure SMCs culture (see Supplementary material online, Figure S5).

In our co-culture model, SMCs and ECs were in contact through the cell extensions, most likely gap junctions (see Supplementary material online, Figure S1C and D). This is in accordance with a previous study using the same co-culture model of ECs and SMCs.\textsuperscript{20} Our findings suggest that the formation of cell-cell contacts in the co-culture was a prerequisite to ensure the activation of latent pro-MT1MMP and pro-MMP2 in SMCs. In support of this, pharmacological gap-junction inhibitors abolished the activation of these MMPs (Figure 3). Furthermore, SMCs cultured distantly (4 mm) from ECs, most likely preventing formation of cell-cell contacts, where unable to activate latent pro-MT1MMP and pro-MMP2 (see Supplementary material online, Figure S6). In this aspect, however, the in vivo situation may differ. It is described in the literature that myoendothelial gap junctions are absent in adult rat aorta.\textsuperscript{28} In accordance with this notion is that application of gap-junction inhibitors on isolated rat aorta was ineffective in preventing activation of pro-MT1MMP and pro-MMP2 (see Supplementary material online, Figure S9). This indicates that in contrast to the co-culture, gap junctions do not represent a critical path of communication in adult rat aorta to ensure the endothelium-dependent activation of latent pro-MT1MMP and MMP2 in SMCs in response to ANGII.

Our findings strongly suggest that ECs regulate expression and activity of furin in SMCs, which appears essential in activation of latent pro-MMP2. However, furin seems to only partially contribute to activation of pro-MT1MMP. In support, ANGII increased expression and activity of furin in SMCs, only while being co-cultured with ECs. Similarly, in isolated rat aorta furin activation depended on the presence of endothelium (Figure 6A and B). In addition, selective block of AT1R in co-cultured ECs, as well as ET\textsubscript{A} inhibition in SMCs, decreased ANGII-mediated increase in expression and activity of furin in SMCs.
This suggests that a stimulus, most likely endothelin-1, triggered by ANGII/AT1R in ECs increases expression and activity of furin in SMCs. To support the role of furin in activation of latent pro-MMP2 in SMCs, we demonstrate that furin antisense oligonucleotide abolished activation of latent pro-MMP2 (Figure 4B). Similarly, a less selective synthetic furin inhibitor, which also inhibits other furin-like PCs, also abolished the activation of latent pro-MMP2. In case of MT1MMP, furin antisense oligonucleotide decreased the activation of latent pro-MT1MMP significantly ($P < 0.05$), but $\approx 50\%$ of activity still remained (Figure 4A), whereas combination of the antisense with furin inhibitor abolished activation of latent pro-MT1MMP. The latter suggests that latent pro-MT1MMP can be activated with other furin-like PCs. This is consistent with previous reports pointing to partial furin-independent pathways of latent pro-MT1MMP activation in vascular SMCs.$^{14,16}$

MT1MMP is the major known proteolytic activator of latent pro-MMP2. A functional interaction of MT1MMP exists with $\alpha v \beta 3$ integrin, which is essential to ensure that the latent pro-MMP2 interacts with the active MT1MMP on the cell membrane, where the latent pro-MMP2 is proteolytically activated.$^{16,17}$ $\alpha v \beta 3$ integrin is also activated from its inactive precursor pro-$\alpha v \beta 3$. Kappert et al.$^{14}$ convincingly showed that the activation of pro-$\alpha v \beta 3$ integrin is abolished by synthetic furin and a furin-like PC inhibitor and that the subsequent activation of latent pro-MMP2 in aortic SMCs is abolished. Our findings extend those findings of Kappert et al.$^{14}$ by demonstrating a prevailing (to other PCs) role of furin in activation of pro-$\alpha v \beta 3$ in SMCs (Figure 4C), because the single antisense approach was similarly effective as the combination of the antisense with a pharmacological inhibitor. Both treatments reduced the $\alpha v \beta 3$ signal almost to baseline values. This leads us to conclude that MT1MMP activity alone is not sufficient to activate latent pro-MMP2 in conditions where lack of furin activity hinders activation of pro-$\alpha v \beta 3$. Most importantly, the endothelium remains pivotal in ANGII-triggered activation of latent pro-MT1MMP via potentially complex routes: firstly, via regulation of furin and secondly, via regulating other furin-like PCs. This strengthens the central role of the endothelium in activation of latent pro-MMP2 via regulation of furin and limiting the activation of pro-MT1MMP and pro-$\alpha v \beta 3$.

Taken together, our findings propose a novel role of endothelium in the activation of latent pro-MT1MMP and pro-MMP2 under ANGII stimulation. ANGII-stimulated endothelium, via paracrine action of endothelin-1, enhances expression and activity of SMC furin thus activating pro-$\alpha v \beta 3$ and pro-MT1MMP—key regulators in the activation of pro-MMP2 (Figure 7). In conclusion, ANGII-stimulated endothelium may contribute to adverse overactivation of MMP2 in rat aorta and presumably enhanced degradation of elastic fibres—a critical step in development of aortic stiffness. AT1 and ETA receptors may be influential therapeutic targets in this regard.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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