Matrix metalloproteinase-2 (MMP-2, gelatinase A, EC 3.4.24.24) belongs to a family of zinc-dependent proteases secreted from a variety of cell types, including vascular endothelial and smooth muscle cells. MMP-2 is expressed in various cells and tissues, including the vascular smooth muscle and the endothelium. It is mainly known for its capacity to digest specific extracellular matrix proteins, such as collagen (types I, IV, V, VII, and X), elastin, fibronectin, and laminin-5. Thus, basal MMP-2 activity is associated with physiological remodelling, tissue repair, and angiogenesis. Tissue inhibitors of MMPs (TIMPs, particularly TIMP-2) regulate MMP-2 activity. MMP-2 is expressed in various cells and tissues, including the vascular smooth muscle and the endothelium. It is mainly known for its capacity to digest specific extracellular matrix proteins, such as collagen (types I, IV, V, VII, and X), elastin, fibronectin, and laminin-5. Thus, basal MMP-2 activity is associated with physiological remodelling, tissue repair, and angiogenesis. Tissue inhibitors of MMPs (TIMPs, particularly TIMP-2) regulate MMP-2 activity.

Imbalance in the expression of MMP-2/TIMP-2 leading to excessive matrix degradation by MMP-2 has been correlated with tissue injury, tumor invasion, inflammation, atherosclerosis, and atherosclerotic plaque rupture. Recently, a novel pathway of platelet aggregation was discovered that is mediated by the release of MMP-2 from activated platelets, which suggests that MMP-2 may have as-yet-unknown regulatory actions in the cardiovascular system. In seeking a role for MMP-2 in the regulation of vascular reactivity that is unrelated to long-term extracellular matrix breakdown, we investigated whether MMP-2 could mediate the activation of inactive precursors of vasoactive peptides/proteins, such as big endothelin-1[1–38] (big ET-1).

Similar to MMP-2, big ET-1 is produced and secreted by the endothelium and the vascular smooth muscle. Big ET-1 is essentially inactive and requires activation to produce vasoconstriction. Serine and aspartic proteases, as well as metalloproteinases, can cleave big ET-1, yielding peptides with various vasoconstrictor potencies. In the cardiovascular system, zinc-dependent endoproteases, termed endothelin-converting enzymes (ECEs), may mediate the vasoconstrictor effects of big ET-1. ECEs cleave big ET-1 at the Val21–Trp22 bond, yielding the potent vasoconstrictor peptide ET-1[1–21]. In the airway, chymase from mast cells was recently shown to cleave big ET-1 at the Tyr23–Gly24 peptide bond, yielding ET-1[1–31], which is a vasoconstrictor of tracheal and vascular smooth muscle and could be involved in allergic inflammation. All these observations reveal different pathways of activation of big ET-1. Vascular Matrix Metalloproteinase-2 Cleaves Big Endothelin-1 Yielding a Novel Vasoconstrictor

Carlos Fernandez-Patron, Marek W. Radomski, Sandra T. Davidge

Abstract—Matrix metalloproteinase-2 (MMP-2, gelatinase A) and its tissue inhibitor (TIMP-2) are mainly known for their roles in the (patho)physiological remodeling of the vasculature, angiogenesis, tissue repair, tumor invasion, inflammation, and atherosclerotic plaque rupture. A mechanism of action of MMP-2 is the proteolytic breakdown of specific extracellular matrix proteins. The amino acid sequences in interstitial collagen (Gly-Leu/Ile) and laminin-5 (Ala-Leu) that are cleaved by MMP-2 are homologous to a region (Gly13-Leu19) within human big endothelin-1[1 to 38] (big ET-1). Big ET-1 requires cleavage to an active form to produce vasoconstriction. We tested the hypothesis that vascular MMP-2 can cleave big ET-1, thus generating a vasoconstrictor peptide. In perfused rat mesenteric arteries with an intact endothelium, inhibition of vascular MMP-2 with TIMP-2 reduced (by 16.2±4.2%) the vasoconstrictor effects of big ET-1 (50 pmol). However, when the endothelium was mechanically removed, TIMP-2 abolished (>90%) the vasoconstriction of big ET-1, and this effect was mimicked by an anti–MMP-2 antibody. Incubation of big ET-1 with recombinant human MMP-2 resulted in the specific cleavage of the Gly32–Leu33 bond of big ET-1. Moreover, the resultant peptide ET-1[1 to 32] exerted greater vasoconstrictor effects than big ET-1. We conclude that vascular MMP-2 contributes to the vasoconstrictor effects of big ET-1 by cleaving big ET-1 to yield a novel and potent vasoconstrictor, ET-1[1 to 32]. These data implicate, for the first time, the endogenous MMP-2/TIMP-2 system in the regulation of vascular reactivity. (Circ Res. 1999;85:906–911.)

Key Words: metalloproteinase ■ endothelin ■ vasculature
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Gly-Leu/Ile) 5 and laminin-5 (Ala-Leu) 6 that are cleaved by 

a vasoconstrictor peptide.

Immunodetection and gelatin zymography of MMP-2 from rat mesenteric arteries were performed as described previously. 1,7

Western Blot and Gelatin Zymography

Identification of MMP-2 in rat mesenteric arteries. A, Western blot (WB) and gelatin-zymography gels demonstrating that MMP-2 is dominant gelatinase in rat mesenteric arteries (lanes 1 and 2, respectively). Gelatin-zymography typically detected quantities of gelatinolytic enzymes (35 to 70 pg per band) that were beyond detection limits of Western blotting. Gelatin-zymography also permitted visualization of gelatinases in both latent and active forms, because latent gelatinases are activated during zymographic development. 1 Std indicates standard mixture of gelatinolytic MMPs from conditioned medium of HT-1080 fibrosarcoma cells that expressed both MMP-2 and MMP-9 (lane 3). B, In situ zymographic detection revealed gelatinolytic activity (bright spots) mainly in arterial media and intima (magnification ×40). C, Fluorescence immunohistochemical analysis showing distribution of MMP-2 in a rat mesenteric artery (magnification ×40).

ET-1. It is likely that these pathways are differentially expressed, depending on the localization of the respective big ET-1-activating protease and the tissue targeted by the active form of big ET-1 that is generated. Interestingly, a region exists within human big ET-1 (Gly32-Leu33) that is homologous with the amino acid sequences in interstitial collagen (Gly-Leu/Ile)5 and laminin-5 (Ala-Leu)6 that are cleaved by MMP-2 during some (patho)physiological processes. Therefore, we hypothesized that vascular MMP-2 could specifically cleave big ET-1 at the Gly32-Leu33 bond, thus generating a vasoconstrictor peptide.

Materials and Methods

Western Blot and Gelatin Zymography

Immunodetection and gelatin zymography of MMP-2 from rat mesenteric arteries were performed as described previously. 1,7

In Situ Zymography

Regions of the arterial wall with net MMP-2 proteolytic activity were localized by in situ zymography as follows. The gelatin coating of a negative Polaroid film served as an immobilized substrate for in situ zymography. Each cryostat section of the mesenteric artery was layered with 2 μg of highly (>95%) purified, solubilized collagen type IV (Calbiochem). Then, each cryostat section was covered with a piece (~25 mm²) of Polaroid film, with the gelatin-coated side of the film facing the cryostat section. This setting was further topped with a coverslip and left in a humidified chamber. After 48 hours, proteolytic activity was recorded (Olympus BX40 microscope). As a control, some of the sections were supplemented with TIMP-1 (2 μmol/L, Chemicon), which inhibits both MMP-2 and MMP-9. 19

Chemical and Enzymatic Reactions

To study the interactions between big ET-1 and MMP-2, the general strategy was to incubate synthetic big ET-1 with highly pure recombinant human MMP-2 (Chemicon International) for times ranging from 1 to 48 hours. The incubation products were analyzed by high-performance liquid chromatography, mass spectrometry, amino acid analysis, and bioassay. Incubations were performed at 37°C in HEPES-phosphate saline solution (pH 7.4) composed of the following (in mmol/L): NaCl 142, KCl 4.7, MgSO₄ 1.17, CaCl₂ 1.56, HEPES 10, and KH₂PO₄ 1.18.

Microperfusion Bioassay

The effect of endothelin peptides on smooth muscle reactivity was studied using rat mesenteric arteries. Animal protocols were conducted in accordance with institutional guidelines issued by the Canada Council on Animal Care. Male Sprague-Dawley rats (350 to 450 g) were anesthetized with methohexital sodium (50 mg/kg) and exsanguinated. Arteries (209 ± 12 μm, inner diameter; 1 to 1.5 mm, length) were dissected from fat tissue and adventitia. All experiments were conducted on cannulated arteries perfused at a constant temperature (37°C) and flow rate (10 μL/min) with HEPES-phosphate saline solution-glucose (5.5 mmol/L). In some experiments, the arteries were denuded of endothelium. Endothelium removal was performed mechanically using a human hair threaded through the lumen of the artery and rubbed back and forth. 20 Small volumes (1 to 5 μL) of big ET-1, ET-1[1–21], MMP-2 cleavage products of big ET-1, or specified drugs were injected into the perfusion line toward the mesenteric artery by using a high-performance liquid chromatography injection valve (Rhodyne Model 9725L, Mandel Scientific Co) provided with a 20-μL loop.

Statistics

Results are the mean±SEM of at least 3 independent experiments. They were analyzed using 1-way ANOVA. When significant differences were found, the Tukey multiple comparisons’ test was used (Jandel SigmaStat statistical software). P<0.05 was considered statistically significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Identification and Involvement of MMP-2 in the Vasoconstrictor Effects of Big ET-1 on Rat Mesenteric Arteries

MMP-2 was the dominant gelatinolytic metalloproteinase detected on the rat mesenteric arteries, as confirmed by

Statistics

Results are the mean±SEM of at least 3 independent experiments. They were analyzed using 1-way ANOVA. When significant differences were found, the Tukey multiple comparisons’ test was used (Jandel SigmaStat statistical software). P<0.05 was considered statistically significant.

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gelatin-zymography and Western blotting (Figure 1A). Net MMP-2 proteolytic activity was detected on medial smooth muscle cells (Figure 1B), suggesting that the MMP-2 activity localized therein was higher than the activity of the TIMPs. Furthermore, MMP-2 immunoreactivity was predominant on smooth muscle cells of the intima and media (Figure 1C).

Big ET-1 (0.5 to 50 pmol), when infused through the arteries, resulted in dose-dependent vasoconstriction. In arteries with an intact endothelium, coinfusion of TIMP-2 (0.17 to 10 pmol) with big ET-1 (50 pmol) partially inhibited the vasoconstrictor effects of big ET-1, with a maximum effect of 16.2 ± 4.2% (P < 0.05; n = 3). In contrast, in endothelium-denuded arteries, coinfusion of TIMP-2 abolished the vasoconstrictor effects of big ET-1 (Figure 2). TIMP-2 exerted no significant effect on the vasoconstrictor response to ET-1[1–21] (data not shown). MMP-2–dependent conversion of big ET-1 was inhibited in the presence of TIMP-2 (Figure 4E). No further peptides of big ET-1 were generated when big ET-1 and MMP-2 were incubated for 48 hours, suggesting the presence of a single cleavage site on big ET-1. Microsequence and mass spectrometry analyses identified the peptides X and Y as the fragments Leu33–Ser38 and Cys1–Gly32 of big ET-1, respectively, confirming that the cleavage site on

MMP-2 Cleaves Big ET-1, Yielding Novel Peptides

We next investigated whether MMP-2 could cleave big ET-1 and generate a bioactive peptide. Big ET-1 was incubated with recombinant human pro-MMP-2 (6% of its gelatinolytic activity was due to MMP-2, as determined by gelatin-zymography). This resulted in partial conversion of big ET-1 into 2 fragments (X and Y; Figure 4, A through C), that were well resolved chromatographically from big ET-1 and ET-1[1–21]. When big ET-1 was incubated with activated MMP-2, >90% of big ET-1 was converted into X and Y fragments (Figure 4D). However, MMP-2 did not cleave ET-1[1–21] (data not shown). MMP-2–dependent conversion of big ET-1 was inhibited in the presence of TIMP-2 (Figure 4E). No further peptides of big ET-1 were generated when big ET-1 and MMP-2 were incubated for ≤48 hours, suggesting the presence of a single cleavage site on big ET-1. Microsequence and mass spectrometry analyses identified the peptides X and Y as the fragments Leu33–Ser38 and Cys1–Gly32 of big ET-1, respectively, confirming that the cleavage site on
big ET-1 was the MMP-2–susceptible Gly 32 -Leu 33 bond (Table). Cleavage at Gly 32 -Leu 33 was also observed when human big ET-1 was replaced by rat big ET-1 [1–39], rein-
forcing the specificity of MMP-2 for this peptide bond.

Peptide Y (3685 Da) was larger than ET-1 [1–21] (2492 Da) but smaller than big ET-1 (4284 Da), and it was termed ET-1 [1–32]. ET-1 [1–32], but not peptide X (Leu 33 -Ser 38 ), comprised the full sequence of ET-1 [1–21], thus containing the binding domain to endothelin receptors.

ET-1 [1–32] Is a Novel Vasoconstrictor

ET-1 [1–32] induced dose-dependent vasoconstriction of the mesenteric arteries (Figure 5) that lasted >20 to 30 minutes after its infusion (Figure 6). Furthermore, the vasoconstrictor effects of ET-1 [1–32] were significantly (P < 0.05) greater than those of its precursors, big ET-1 and ET-1 [1–21], such that ET-1 [1–32] ED 50 = 4.0 ± 1.1 pmol < ET-1 [1–21] ED 50 = 27.7 ± 8.2 pmol < big ET-1 ED 50 = 45.7 ± 5.1 pmol (Figure 4). Preinfusion of the selective ET A receptor antagonist N-acetyl-[D-Trp 16 ]-ET-1 (20 nmol) before the infusion of ET-1 [1–32] (2 pmol) inhibited vasoconstriction to this peptide by 15 ± 1% (n = 3). Preinfusion of the artery with PD 142893 (20 nmol), a nonselective antag-
onist of ET A and ET B receptors, 21 abolished the vasoconstrictor effects of ET-1 [1–32] but not those of phenylephrine (Figure 6).

Discussion

We investigated the impact of vascular MMPs on the vaso-
constrictor effects of big ET-1 on rat mesenteric arteries. Zymography and Western blot analysis revealed that MMP-2 was the major gelatinase expressed in these arteries. In situ zymography showed that the net gelatinolytic activity in the arteries colocalized with immunoreactive MMP-2. To study the significance of vascular MMP-2 for the vasoconstrictor effects of big ET-1, TIMP-2 (an endogenous inhibitor of MMPs with preferred effects on MMP-21) or an MMP-2–
specific antibody were administered together with big ET-1. The selective inhibition of MMP-2 significantly reduced the vasoconstrictor effects of big ET-1 but not ET-1 [1–21]. These data clearly indicate that vascular MMP-2 contributed to the vasoconstrictor effects of big ET-1 on arteries.

To investigate the mechanism of this action of MMP-2, we incubated big ET-1 with recombinant human MMP-2. The reaction resulted in the specific cleavage of big ET-1 at the Gly 32 -Leu 33 bond (Table). Cleavage at Gly 32 -Leu 33 was also observed when human big ET-1 was replaced by rat big ET-1 [1–39], reinforcing the specificity of MMP-2 for this peptide bond. Peptide Y (3685 Da) was larger than ET-1 [1–21] (2492 Da) but smaller than big ET-1 (4284 Da), and it was termed ET-1 [1–32]. ET-1 [1–32], but not peptide X (Leu 33 -Ser 38 ), comprised the full sequence of ET-1 [1–21], thus containing the binding domain to endothelin receptors.

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Physicochemical Characterization of X and Y in Comparison With Their Precursor, Big ET-1

<table>
<thead>
<tr>
<th>Peptide</th>
<th>X</th>
<th>Y</th>
<th>Big ET-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC ret time,* min</td>
<td>9.75</td>
<td>24.31</td>
<td>23.66</td>
</tr>
<tr>
<td>NH₂-terminal sequence</td>
<td>Leu-Gly-Ser-Pro-</td>
<td>Cys-Ser-Cys-Ser-Ser-Cys-Ser-Pro-</td>
<td>Cys-Ser-Cys-Ser-Cys-Ser-Pro-</td>
</tr>
<tr>
<td>Mass, Da§</td>
<td>616.26 ± 0.24</td>
<td>3684.97 ± 0.28</td>
<td>4282.97 ± 0.30</td>
</tr>
<tr>
<td>Deduced sequence</td>
<td>Leu²⁵-Ser³⁸</td>
<td>Cys³⁵-Gly³⁸</td>
<td>Cys³⁵-Ser³⁸</td>
</tr>
</tbody>
</table>

*Retention (ret) time on preparative high-performance liquid chromatography (HPLC) separation. 
†First 8 amino acids; Cys, Cysteins were not detected by automated microsequencing but deduced by mass-analysis and comparison with the theoretical NH₂-terminal sequence of big ET-1. 
§Experimentally determined monoisotopic peptide mass.

Pharmacological studies of TIMP-2 and MMP-2 antibody showed that the interaction between big ET-1 and vascular MMP-2 was more prominent in the mesenteric arteries denuded of endothelium than in those with an intact endothelium. These observations are of high biological significance. In contrast to ECE, which is expressed mainly by the vascular endothelium, the net proteolytic activity and immunoreactivity of MMP-2 was more pronounced in the intimal and medial vascular smooth muscle cells of the arteries studied. These results agreed with recent reports showing that MMP-2 concentrates along the basement membrane and on those smooth muscle cells in close proximity to the intima. Thus, an activation of big ET-1 to ET-1[1–21] may be favored in the endothelium, where ECE activity is high. In contrast, conversion of big ET-1 into ET-1[1–32] by vascular MMP-2 may be favored in vascular smooth muscle cells, where the activity of ECE is lower.

Interestingly, concerted overexpression of MMP-2 and big ET-1 can be observed at sites of tissue remodeling and repair under conditions of tissue injury, inflammation, and cancer. Moreover, vascular pathologies such as hypertension, atherosclerosis, atherosclerotic plaque rupture, and restenosis are also associated with the increased expression of MMPs and endothelins. Endothelial injury and increased generation of these mediators in the vessel wall–platelet microenvironment are likely to promote vasospasm, platelet activation, smooth-muscle-cell migration, proliferation, and exaggerated expansion of the extracellular matrix. In line with this, recent results from our laboratory suggest that the generation of ET-1[1–32] occurred in conditions associated with the generation of thrombin, a key enzyme in the response to tissue injury (C. Fernandez-Patron, M.W. Radomski, and S.T. Davidge, unpublished data, 1999). Indeed, we found that a rapid release of bioactive MMP-2 occurred in response to thrombin-stimulation in rat mesenteric and aorta arteries. Furthermore, the vasoactive effects of thrombin were inhibited by TIMP-2 and abolished by endothelin receptor antagonists. Thus, we propose that an activation of big ET-1 to ET-1[1–32] by vascular MMP-2 may contribute to the vasoactive effects of thrombin.

The discovery of the MMP-2–dependent pathway for the generation of ET-1[1–32] may have important pharmacological significance. A large effort has been devoted to the development of synthetic selective inhibitors of MMPs. These compounds are now being clinically tested for their ability to ameliorate the course of inflammatory and cancer diseases. Therefore, selective inhibition of MMP-2 may represent a new pharmacological strategy for regulating vascular reactivity in pathological conditions in which MMP-2 and big ET-1 are overexpressed.

We have not identified a significant expression of gelatinolytic MMPs other than MMP-2 in rat mesenteric arteries. However, MMPs such as MMP-1 and MMP-9 share many substrates with MMP-2 and may also cleave big ET-1. In addition, an increasing number of different pathways of activation of big ET-1 exist (eg, via ECE, mast cell chymase, and MMP-2) leading to different bioactive ET-1 peptides (eg, ET-1[1–21], ET-1[1–31], and ET-1[1–32]). Further studies are needed to clarify the contribution of ET-1[1–32] relative to ET-1[1–21] and ET-1[1–31] in vascular biology and disease. It is likely that these peptides play different roles in (patho)physiology, depending on the tissue expression of the respective big ET-1-activating proteases.

In summary, we found that vascular MMP-2 contributed to the vasoconstrictor effects of big ET-1 on rat mesenteric arteries via the generation of a novel vasoconstrictor peptide, ET-1[1–32]. This pathway may have important significance for the physiology, pathology, and pharmacology of the vessel wall.
Acknowledgments
This work was supported by grants from the Medical Research Council of Canada (MRC: MT-13404 [and MT-14074 to M.W. Radomski]) and the Heart and Stroke Foundation of Canada (to S.T. Davidge). C. Fernandez-Patron is a postdoctoral fellow of the Alberta Heritage Foundation for Medical Research (AHFMR), the Canadian Hypertension Society, and the Medical Research Council of Canada. S.T. Davidge is a scholar of AHFMR and the Heart and Stroke Foundation of Canada. M.W. Radomski is a scholar of AHFMR.

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Circ Res. 1999;85:906-911
doi: 10.1161/01.RES.85.10.906

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