Differential Effects of Mechanical and Biological Stimuli on Matrix Metalloproteinase Promoter Activation in the Thoracic Aorta

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Background—The effect of multiple integrated stimuli on vascular wall expression of matrix metalloproteinases (MMPs) remains unknown. Accordingly, this study examined the influence of the vasoactive peptide angiotensin II (Ang II) on wall tension–induced promoter activation of MMP-2, MMP-9, and membrane type-1 MMP (MT1-MMP).

Methods and Results—Thoracic aortic rings harvested from transgenic reporter mice containing the MMP-2, MMP-9, or MT1-MMP promoter sequence fused to a reporter gene were subjected to 3 hours of wall tension at 70, 85, or 100 mm Hg, with or without 100 nM Ang II. Total RNA was harvested from the aortic rings, and reporter gene transcripts were quantified by quantitative real-time polymerase chain reaction to measure MMP promoter activity. MT1-MMP promoter activity was increased at both 85 and 100 mm Hg, compared with baseline tension of 70 mm Hg, whereas treatment with Ang II stimulated MT1-MMP promoter activity to the same degree at all tension levels (P < 0.05). Elevated tension and Ang II displayed a potential synergistic enhancement of MMP-2 promoter activation at 85 and 100 mm Hg, whereas the same stimuli caused a decrease in MMP-9 promoter activity (P < 0.05) at 100 mm Hg.

Conclusions—This study demonstrated that exposure to a relevant biological stimulus (Ang II) in the presence of elevated tension modulated MMP promoter activation. Furthermore, these data suggest that a mechanical-molecular set point exists for the induction of MMP promoter activation and that this set point can be adjusted up or down by a secondary biological stimulus. Together, these results may have significant clinical implications toward the regulation of hypertensive vascular remodeling. (Circulation. 2009;120[Suppl 1]:S262–S268.)

Key Words: metalloproteinases • aorta • wall stress • angiotensin

Although the development and progression of vascular remodeling, such as that which can occur with hypertension and with aneurysmal disease, is a multifactorial process, it is now becoming evident that the activation of key proteolytic pathways contributes to the structural changes that occur within the extracellular matrix. One mechanism by which extracellular remodeling within the vascular wall is regulated is through the induction and activation of the matrix metalloproteinases (MMPs). The MMPs constitute a large family of enzymes that are proteolytically diverse, and cause-effect relationships exist between the induction of certain MMP types and pathological vascular remodeling with regard to atherosclerosis, hypertension, and aortic aneurysm formation. For example, a class of MMPs generically termed the gelatinases and consisting of primarily MMP-2 and MMP-9 have been implicated in vascular remodeling in several disease states. Because MMPs cause dynamic and significant changes within the extracellular space, it follows that these enzymes are tightly controlled at the transcriptional, translational, and post-translational levels. However, it remains unclear to what degree mechanical and/or biological stimuli modulate the induction of MMP promoter activity.

In a recent report from this laboratory, aortic rings harvested from transgenic mice expressing MMP promoter-reporter gene constructs were used in an ex vivo ring apparatus to examine the role of isolated tension on MMP-2 and MMP-9 promoter activation. The study demonstrated that a physiologically relevant mechanical stimulus was sufficient to induce MMP-2 promoter activity, whereas the MMP-9 promoter was found to be unresponsive. Furthermore, these initial data suggested that a mechanical-molecular set point may exist for induction of MMP-2, which may have a significant impact on the prognostic and therapeutic management of hypertensive patients.

To further examine the role of a mechanical stimulus on the regulation of MMP promoter activation, the present study...
explored 2 objectives. First, it tested the hypothesis that promoter activity of a known activator of MMP-2, the membrane type 1 MMP (MT1-MMP) gene,7 should be coregulated with MMP-2 in response to mechanical stimuli. The MT-MMPs, of which MT1-MMP is considered prototypical, proteolytically process a wide range of extracellular proteins, cytokines, and growth factors—all relevant to the vascular remodeling process.8 In addition, increased levels of MT1-MMP have been identified in vascular remodeling in both animal and clinical studies.9,10 Thus, the induction of MT1-MMP would not only cause increased proteolytic potential but could also amplify extracellular remodeling through post-translational modification of other MMPs such as MMP-2.

In addition to hemodynamic forces, MMP expression may be induced by neurohormonal stimuli, and 1 of the most extensively studied vasoactive peptides is angiotensin II (Ang II). This hormone has been shown to contribute to numerous cardiovascular disease states, including atherosclerosis, hypertension, and left ventricular dysfunction,11—13 by eliciting endothelial dysfunction14 and/or a potent mitogenic and hypertrophic response in vascular smooth muscle cells.15,16 Evidence also suggests that Ang II can directly contribute to matrix remodeling by inducing MMP expression.13,17 Understanding how these discrete forces may be integrated to initiate and propagate vascular remodeling may identify novel therapeutic strategies; however, very little is known about the collective influence of mechanical and hormonal stimuli. Accordingly, the second objective of this study was to evaluate the regulation of MMP promoter activation under the influence of both increased wall tension and Ang II.

Methods

MMP Reporter Strains

Male and female reporter mice carrying a transgene construct consisting of an MMP promoter fragment linked to a reporter gene were used. A 5-kb fragment of the rat MMP-2 gene extending from −1686 to the second exon was linked to the β-galactosidase gene lacZ to create the transgenic CD-1[MMP-2:β-gal] reporter mouse line.18 The rabbit MMP-9 gene segment from base pairs −522 to +19 was fused to lacZ to generate the CD-1[MMP-9:β-gal] reporter mice.19 Alternatively, the MT1-MMP reporter strain linked the firefly luciferase gene Luc to the human MT1-MMP promoter genomic fragment spanning from −3364 base pairs to the first intron. This construct was subsequently inserted on an FVB background. In the MMP-2 and MMP-9 reporter strains, transgene expression was confirmed by reaction of tail tissue with the β-galactosidase substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Additionally, polymerase chain reaction (PCR) amplification of tail-clip DNA with primers specific for lacZ or Luc was performed in all 3 reporter strains to verify transgene expression. A concordant response to elevated tension between the native MMP promoters and the MMP promoter-reporter gene construct in these mouse strains has been previously established.6

Experimental Design

Wall tension was applied to segments of murine descending thoracic aorta using a vascular ring ex vivo method as previously described.12,20 Wall tension values consistent with mildly hypertensive mean arterial pressures were applied to excised aortic rings that were concomitantly treated with Ang II. Aortic tissue was homogenized and lacZ or Luc gene expression was measured by quantitative real-time PCR (QPCR) to directly evaluate MMP promoter activity.

All mice were maintained according to the National Institutes of Health Guide to the Care and Use of Laboratory Animals, and this animal protocol was approved by the Medical University of South Carolina Institutional Animal Care and Use Committee.

Preparation of Aortic Rings

After induction of general anesthesia with 2% isoflurane, the mice underwent a left posterolateral thoracotomy. The descending thoracic aorta was harvested and immediately placed in cold Krebs-Hanseleit buffer (118 mmol/L NaCl, 4.6 mmol/L KCl, 1.2 mmol/L KH2PO4, 25 mmol/L NaHCO3, 2.5 mmol/L CaCl2, 0.5 mmol/L Na2-EDTA, 11 mmol/L glucose, 1.2 mmol/L MgSO4, pH 7.4). Extraneous connective tissue was removed, and aortas were denuded of endothelium to minimize the potential for altered shear stress to influence gene expression. The vessel was then divided into 3-mm-long segments and mounted on parallel wires in a water jacketed tissue bath system (Radnoti, Monrovia, Calif; 25 mL) maintained at 37°C and connected to an isometric force transducer (Radnoti). The vessels were equilibrated on the ring apparatus for 30 minutes at zero tension, and the aerated buffer (95% O2/5% CO2) was exchanged every 30 minutes. Micrometer measurements at zero tension and calculations set forth by He et al21 and Lalli et al22 were used to derive the mean internal circumference and cross-sectional area of the vessels. For each aortic ring, tension was digitally measured and graphically recorded (BioBench; National Instruments, Austin, Tex).

Defining Optimal Tension and Experimental Pressure Gradients

Before applying tension to the aortic rings to examine promoter activity, the native tension parameters of the tissues were defined. The passive, active, and optimal tension values for the MMP-2 and MMP-9 reporter strains have been previously defined.6 The same experimental procedure was performed to define the optimal tension for the MT1-MMP reporter strain. Briefly, to quantify the passive tension, vessel segments (n=8) were sequentially stretched in 0.1-g increments and allowed to equilibrate, during which time the extracellular matrix microfibrils would adapt and relax, demonstrating a decay in the transduced vessel tension. The percent decline in passive tension at each level of applied tension was compared with a standard of 10% using a 1-sample t test. The passive tension of the vessel was identified when the transduced vessel tension decayed less than 10%. Similarly, active tension was then defined in fresh aortic rings stabilized at a given tension increment by stimulating contraction with 100 mmol/L KCl (n=8). Peak tension generation was recorded, and comparisons among active tension values were conducted by 1-way ANOVA with a post hoc Tukey multiple comparisons test.

The point at which all passive tension was overcome and the smooth muscle cells were able to maximally contract was identified as the optimal tension. This value varies among mouse strains,23,24 Therefore, optimal tension was converted to a pressure measurement to ensure equivalent application of tension across aortic rings from each mouse strain.21,22 The optimal tension for the MMP-2 and MMP-9 reporter mouse strains has been previously defined at 0.7 g, and normalization for cross-sectional area led to a pressure value of 70 mm Hg. The MT1-MMP mouse strain was defined as a moderate pressure of 0.28±0.01 mm Hg and ring surface area of 7.33±0.05 mm2. Using these measurements, optimal tension for the MT1-MMP reporter mouse line was also found to be equivalent to 70 mm Hg.

Inducing MMP Promoter Activity

Aortic rings were stabilized at zero tension and equilibrated at optimal tension for 30 minutes each. Tension was then applied for 3 hours at 70, 85, or 100 mm Hg, with buffer exchange every 30 minutes (n=6 for each). Rings were treated with 100 nM Ang II at initiation of the zero tension stabilization phase and 100 nM Ang II treatment was repeated with each buffer exchange over the 3-hour duration of the experiment. This concentration of Ang II was chosen based on previous studies that reported that 100 nM would cause a minimal contractile response of the murine thoracic aorta but would...
activity at optimal tension (70 mm Hg) considered to be baseline.

Each aortic ring was subsequently stored for 24 to 48 hours in RNAlater (Ambion, Austin, Tex) then snap-frozen and stored at -80°C. Approximately 5% of aortic rings were discarded from the study because evidence of tissue destruction was observed while mounting vessel segments or during the 3-hour treatment period.

QPCR Amplification of lacZ and Luc

Aortic rings underwent rotor-stator homogenization in Buffer RLT (QIAGEN, Valencia, Calif), total RNA was extracted using the RNaseasy Fibrous Tissue MiniKit (QIAGEN), and reverse transcription to cDNA was conducted using the iScript cDNA Synthesis Kit (BioRad, Hercules, Calif). QPCR, using TaqMan chemistry with specific primers/probes, was performed with either lacZ (TaqMan Gene Expression Assays on Demand catalog No. 185757894; Applied Biosystems, Foster City, Calif) or Luc primers (Custom designed: forward-5’-AAGATTCAAAAGTGGCTGCTTGTTG-3’; reverse-5’-TGCGCTGATACCTGGAGATGGAA-3’; probe-5’/6-FAM/TTACGAAATTTGCTCTGCTGTGG/ BHQ_1/-3’; Integrated DNA Technologies, Coralville, Iowa). Results were compared with 18s rRNA used as an internal standard (TaqMan Gene Expression Assays on Demand catalog No. 4333760; Applied Biosystems). The BioRad MyiQ Single-Color Real-Time PCR Detection System was used (BioRad), with an initial denaturing cycle consisting of 10 minutes at 95°C, followed by 45 cycles of 30 seconds at 95°C and 1 minute at 60°C. The fold gene expression of lacZ or Luc was calculated by the ΔΔCt method, with promoter activity at optimal tension (70 mm Hg) considered to be baseline.

Statistical Analysis

Fold expression of lacZ or Luc was compared with baseline promoter activity by a 2-sided, 1-sample t test versus the fixed value of 1. Comparisons among treatment groups were conducted by 1-way and 2-way ANOVA. All values are reported as a mean±SEM. Stata 8 statistical software (Intercooled, College Station, Tex) was used for all calculations, and values were significant at P<0.05.

Results

Optimal tension for the MT1-MMP reporter mouse strain was 0.6 g, which was equivalent to 70 mm Hg. When aortic rings extracted from these mice were subjected to elevated pressure, promoter activity was significantly increased at both 85 and 100 mm Hg (Figure 1). On adding 100 nM Ang II, MT1-MMP promoter activity at the baseline optimal tension was increased approximately 2-fold and remained at this level despite the addition of elevated tension (Figure 1). A coregulatory interaction was identified between tension and Ang II for the MT1-MMP promoter (P=0.01).

It was previously demonstrated that an elevated pressure of 100 mm Hg caused a 1.5-fold increase in MMP-2 promoter activity. With the addition of 100 nM Ang II to aortic rings, promoter activity was significantly augmented at both 85 and 100 mm Hg as compared with baseline optimal tension. MMP-2 promoter activation at 85 mm Hg in the presence of Ang II displayed a 1.5-fold increase, whereas treatment with Ang II at 100 mm Hg affected approximately a 7-fold increase (Figure 2).

Similarly, it was reported in a previous study that aortic rings from the MMP-9 promoter-reporter mouse strain were unaffected by elevated pressure alone. However, at 100 mm Hg, the addition of 100 nM Ang II resulted in a reduction of MMP-9 promoter activity compared with both baseline optimal tension and 100 mm Hg alone (Figure 3).

Discussion

Vascular remodeling results from the complicated interplay of numerous mechanical and neurohormonal stimuli, many of which have been shown to stimulate MMP production, thereby driving extracellular matrix degradation and reorganization. Careful experimental derivation of the effect of individual stimuli has created a vast network of data concern-
The continuous influence of shear stress and wall tension not only modulates normal vascular function but is suspected to contribute to vascular dysfunction by regulating differential gene expression. Pertinent mechanosensors include the integrins, receptor tyrosine kinases, and G protein–coupled receptors (Figure 4). By creating a physical connection between the extracellular matrix and the intracellular space, integrins transduce mechanical stress to initiate signaling cascades resulting in activation of the mitogen-activated protein kinases (MAPKs). Although the receptor tyrosine kinases and G protein–coupled receptors possess ligand specificity, members of both families have demonstrated an ability to be activated by biomechanical stress alone with subsequent MAPK activation. Additionally, evidence suggests that mechanical stress may directly stimulate the generation of reactive oxygen species (ROS), also capable of activating MAPKs. Because the MAPKs have been directly implicated in the activation of various transcription factors, it therefore follows that MAPK activation in response to divergent signaling pathways may differentially activate unique subsets of transcription factors, thereby differentially regulating MMP promoter activation.

Signal Transduction for Ang II

With regard to the long-term biological effects of Ang II in the vascular system, such as hypertrophy and extracellular matrix deposition, the AT1R, a G protein–coupled receptor, is the major mediator. Through the immediate activation of protein kinase C, several nonreceptor tyrosine kinases become phosphorylated and trigger cascades resulting in MAPK activation (Figure 4). Interestingly, the AT1R can also stimulate production of ROS with subsequent activation of MAPKs. The nuclear translocation and regulation of inducible transcription factors resulting from MAPK activation plays a major role in Ang II stimulated gene expression and cellular growth. Remarkably, in addition to fostering...
protein synthesis through the MAPK signaling cascades, Ang II also induces key negative feedback mechanisms including activation of the primary negative regulator of MAPKs, MAPK phosphatase-1 (MKP-1). AT1R can also directly stimulate phosphorylation of numerous tyrosine kinases, such as phosphoinositide-3 kinase (PI3K), which activate signaling cascades with implications in gene expression. Therefore, similar to the signaling cascades evoked by mechanical stress, Ang II may trigger signaling cascades that result in the activation of a unique set of key transcription elements capable of regulating MMP gene expression.

**Potential Sites of Signaling Pathway Interactions**

Evidence outlining the intracellular signal transduction pathways for mechanical stimuli and Ang II has implicated activation of particular profiles of transcription factors, many of which have binding sequences within the MMP promoters evaluated in this study, potentially inducing or repressing MMP promoter activity (Figure 4).

In the case of MT1-MMP, tension-induced activation of MAPK can lead to activation of Ets and NF-kB, transcription factors that have binding elements within the MT1-MMP promoter region. Accordingly, the activation of MAPK may therefore contribute to the increased promoter activity seen at 85 and 100 mm Hg. On the other hand, Ang II can stimulate production of early growth response genes including Egr-1, an element whose binding site in the MT1-MMP promoter has previously been shown to regulate transcription, and may explain the increased promoter activity seen at baseline optimal tension in aortic rings treated with Ang II. Alternatively, Ang II has been shown to activate MKP-1, allowing for the activation of Ang II signaling to inhibit tension-induced transcription altogether, thus resulting in the consistent upregulation of MT1-MMP promoter activity through an Ang II-dependent transcriptional response. This is consistent with our results showing a stable amount of MT1-MMP promoter activity across all tension levels in the presence of Ang II. With regard to early hypertensive remodeling, increased MT1-MMP transcriptional activity suggests that this enzyme contributes to initiation of proteolytic remodeling of the vessel wall.

The MMP-2 promoter responded to tension alone at 100 mm Hg. The significant increase in MMP-2 promoter activity quantified at both 85 and 100 mm Hg when aortic rings were treated with Ang II suggests a synergistic effect. This response may be mediated through the activation of MAPK and transcription factors compatible with the MMP-2 promoter region such as AP-1, Ets, and c-myc. The amplified effect demonstrated by addition of Ang II may be attributed to increased input into a single signaling pathway, potentially bypassing the negative feedback initiated by Ang II. Alternatively, the AT1R can phosphorylate PI3K, initiating a pathway that activates Akt, a kinase subsequently shown to activate AP-2, a pertinent element in MMP-2 transcriptional activity. Therefore, in addition to amplifying the MAPK pathway, Ang II may activate the PI3K/Akt pathway to enhance AP-2 activity and produce a large increase in MMP-2 promoter activity over that of tension alone. A third potential pathway for heightened MMP-2 promoter activation may be derived from evidence that increasing amplitudes of cardiac myocyte stretch elicited increasing amounts of ROS generation. Likewise, greater tension application to the thoracic aorta may amplify ROS production, ultimately stimulating MMP-2 transcriptional activity. The marked elevation in MMP-2 promoter activity documented with elevated tension in the presence of Ang II, a potent hypertensive stimulus, strongly suggests that this protease may play a major role in initiating hypertensive remodeling.

Although not effected by tension alone, the combination of elevated tension and Ang II reduced MMP-9 promoter activity. Previous studies have demonstrated an increase in MMP-9 transcription in response to mechanical stress or Ang II. Each stimulus has the potential to activate several transcription factors capable of binding the MMP-9 promoter region, including NF-kB, AP-1, and Ets. The lack of response by the MMP-9 promoter in this model implies that either additional regulatory elements are required for promoter activation, or that transcriptional repressors are also induced by these stimuli to inhibit MMP-9 transcription. In early hypertensive remodeling, therefore, the role for MMP-9 appears to be limited.

**Limitations**

The goal of this project was to evaluate a single aspect of MMP regulation, gene promoter activation, as it is influenced by integrated mechanical and neurohormonal stimuli. The net MMP proteolytic activity ultimately driving vascular remodeling is influenced at both the transcriptional and translational levels, in addition to propeptide activation and endogenous inhibition. The present study demonstrates that mechanical and neurohormonal stimuli induce transcriptional activity using a promoter-reporter system. Previous work from this laboratory and others has shown that the MMP promoter-reporter gene constructs used in this study do respond to these stimuli in similar fashion to the native genes. Additionally, it has been well documented that MMP mRNA, protein levels, and zymographic activity increase concomitantly in various cardiovascular tissue systems. Although this may imply that MMPs are primarily regulated at the transcriptional level, it must be noted that this study has only assessed changes in promoter activation. Unfortunately, the small size of the murine thoracic aortic rings, coupled with the short duration of treatment, prohibited protein quantification. Therefore, the contribution of post-transcriptional regulation to overall MMP activity in this ex vivo system could not be assessed. Nevertheless, using stimuli known to induce vascular remodeling revealed 2 MMPs that undergo early transcriptional activation and identified these proteases as potential targets to attenuate extracellular matrix restructuring. Whether the protein levels of these targets follow the observed changes in transcriptional activation will require further investigation.

**Summary and Clinical Implications**

This project demonstrated that 2 discrete stimuli, elevated wall tension and Ang II, can interact to regulate MMP promoter activity and ultimately induce transcription of a subset of enzymes, including MMP-2 and MT1-MMP, which
are suspected to play a major role in initiating hypertensive vascular remodeling. Understanding the integrated influence of numerous vascular remodeling stimuli has significant therapeutic implications. Interference in the renin-angiotensin system with angiotensin-converting enzyme inhibitors and Ang II receptor blockers has already improved morbidity among patients with diabetes mellitus, numerous forms of heart disease, and peripheral vascular disease. Clariﬁcation of the interaction between Ang II and a mild hypertensive state to initiate vascular remodeling may identify new indications for Ang II inhibitory therapy or new targets for pharmacological intervention.

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Disclosures
None.

References
18. Mukherjee R, Mingoia JT, Bruce JA, Austin JS, Stroud RB, Escobar GP, McClister DM Jr, Allen CM, Alfonso-Jaume MA, Fini ME, Lovett DH, Spinale FG. Interference in the renin-angiotensin system with angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers has already improved morbidity among patients with diabetes mellitus, numerous forms of heart disease, and peripheral vascular disease. Clariﬁcation of the interaction between Ang II and a mild hypertensive state to initiate vascular remodeling may identify new indications for Ang II inhibitory therapy or new targets for pharmacological intervention.

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References


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