TIMP3 is the primary TIMP to regulate agonist-induced vascular remodelling and hypertension

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Received 3 December 2012; revised 11 March 2013; accepted 12 March 2013; online publish-ahead-of-print 21 March 2013

Time for primary review: 31 days

Aims
Hypertension is accompanied by structural remodelling of vascular extracellular matrix (ECM). Tissue inhibitor of metalloproteinases (TIMPs) inhibits matrix metalloproteinases (MMPs) that degrade the matrix structural proteins. In response to a hypertensive stimulus, the balance between MMPs and TIMPs is altered. We examined the role of TIMPs in agonist-induced hypertension.

Methods and results
We subjected TIMP-knockout mice to angiotensin II (Ang II) infusion, and found that Ang-II-induced hypertension in TIMP1−/−, TIMP2−/−, and TIMP4−/− mice was comparable to wild-type (WT) mice, but significantly suppressed in TIMP3−/− mice. Ex vivo pressure myography analyses on carotid and mesenteric arteries revealed that Ang-II-infused TIMP3−/− arteries were more distensible with impaired elastic recoil compared with the WT group. The acute response to vasoconstriction and vasodilation was intact in TIMP3−/− mesenteric and carotid arteries. Mesenteric arteries from TIMP3−/−−Ang II mice exhibited a reduced media-to-lumen ratio, suppressed collagen and elastin levels, elevated elastase and gelatinase proteolytic activities compared with WT-Ang II. TIMP3−/−−Ang II carotid arteries also showed adverse structural remodelling. Treatment of mice with doxycycline, a matrix metalloproteinase inhibitor, improved matrix integrity in mesenteric and carotid arteries in TIMP3−/−−Ang II and differentially regulated elastin and collagen levels in WT-Ang II vs. TIMP3−/−−Ang II.

Conclusion
Our study demonstrates a critical role for TIMP3, among all TIMPs, is preserving arterial ECM in response to Ang II. It is critical to acknowledge that the suppressed Ang-II-induced hypertension in TIMP3−/− mice is not a protective mechanism but owing to adverse remodelling in arterial matrix.

Keywords
Tissue inhibitor of metalloproteinase • Hypertension • Vascular remodelling • Extracellular matrix

1. Introduction
Hypertension can lead to multiple-organ complications such as chronic kidney disease, cardiac hypertrophy, and stroke. Blood pressure is determined mainly by cardiac output and peripheral vascular resistance, which is regulated by the constriction and dilation of peripheral arteries. Hypertension is associated with vascular remodelling characterized by rearrangement of vascular wall components, including extracellular matrix (ECM) proteins. Arterial ECM is primarily comprised elastin and collagen which provide significant structural support and recoil properties for the arteries.1,2 Vascular remodelling entails degradation and reorganization of the ECM; however, an imbalance in these two processes can result in pathological remodelling.

Degradation of existing structural proteins of the ECM occurs through the proteolytic function of matrix metalloproteinases (MMPs) which is kept in check by their physiological inhibitors, tissue inhibitor of metalloproteinases (TIMPs). Among the four TIMPs identified so far, TIMP3 is the only TIMP that is ECM-bound and could thereby exert tissue-specific effects.3,4 Further, a polymorphism in TIMP3 (A-915G) showed a significant association with hypertension.5 However, the direct role of TIMP3 in hypertension remains to be understood.

In this study, we investigated the role of TIMP3 in angiotensin II (Ang II)-induced hypertension and remodelling of resistance and small conduit arteries that are involved in mediating the hypertensive response. Ang II exerts direct effects on vascular remodelling and...
Our findings indicate that TIMP3 deficiency results in adverse structural remodelling of resistance and small conduit arteries which was inhibited by doxycycline. Therefore, while the absence of TIMP3 suppresses AngII-induced hypertension, this was not a protective effect but was associated with degradation of the structural ECM in the vasculature which could lead to adverse long-term outcomes.

2. Methods

2.1 Experimental animals and procedures

Wild-type (WT), TIMP1−/− and TIMP2−/− mice were purchased from Jackson Laboratories, and TIMP−/− mice from Texas Institute for Genomic Medicine. TIMP3−/− mice were generated as described.9 All mice are in pure C57BL/6 background. Alzet micro-osmotic pumps (model 1002, Durect Co.) were implanted dorsally and subcutaneously in anaesthetized (2% isoflurane) 8-week old male mice to deliver 1.5 mg/kg/day of Ang II (Sigma-Aldrich) or saline (control) for 14 days.6,9,10 Doxycycline was delivered by daily oral gavage (50 mg/kg/day).11 All experiments were conducted in accordance with the guidelines of the University of Alberta Animal Care and Use Committee and the Canadian Council of Animal Care.

2.2 Blood pressure measurement

Blood pressure was measured non-invasively in conscious mice using the tail-cuff method (iITC Life Science, Woodland Hills, CA, USA). Mice were placed in acrylic restrainers and their body temperature was maintained. Mice were acclimatized during at least three sessions (1–2 h/session) over 1 week prior to baseline blood pressure measurements. Tail-cuff systolic blood pressure was measured every second day starting 1 week prior to the pump implantation. The data were analysed using the iITC software (iITC Life Science Blood Pressure System). All measurements were performed at the same time of the day and in double-blinded manner. Inter-observer and intra-observer variability were assessed (coefficient of variance = 2.9 and 1.3%, respectively). At each time point, blood pressure measurements were averaged from three readings for each mouse.

2.3 Histological analyses

After 2 weeks of Ang II or saline infusion, mice were perfused-fixed at 80 mmHg with paraformaldehyde after a PBS wash as previously described.12 This pressure, although lower than the systolic pressure in the mice at the time of euthanasia, was used in all groups for consistency and to prevent the small arteries from collapsing during tissue collection. Subsequently, carotid and mesenteric arteries were dissected out, further fixed in 10% buffered formalin for 48 h, and paraffin-embedded. Using 5 μm paraffin-embedded sections, carotid arteries were stained with Gomori trichrome (to visualize collagen fibres) or Verhoeff–Van Gieson (VVG, to visualize elastic fibres), and mesenteric arteries with penta-chrome to visualize structural changes. Media-to-lumen ratio was determined as follows. The outer cross-sectional area of the mesenteric arteries (at the outer elastic lamina) and the lumen cross-sectional area were calculated. Medial area was obtained as the difference between these two cross-sectional areas. The ratio of medial to lumen cross-sectional area is reported as the media-to-lumen ratio.

2.4 Ex vivo vascular function

Tissue dissections were performed in ice-cold physiological saline solution (PSS), composition (in mmol/L): 10 HEPES, 5.5 glucose, 1.56 CaCl2, 4.7 KCl, 142 NaCl, 1.17 MgSO4, 1.18 KH2PO4, pH 7.5. Arteries, first-order mesenteric or carotid, were cleaned of all surrounding adipose, and connective tissues and mounted on two glass canulae in a two-bath pressure myograph (Living Systems, Burlington, VT, USA). Vessels were given a 40-min equilibration period during which they were exposed to a stepwise increase in pressure from 60 to 80 mmHg with regular changes of the PSS bathing solution. Pressure was set to 60 mmHg for measurement of active experimental responses, because this pressure did not cause myogenic responses. Following the equilibration period, a cumulative concentration–response curve (CCRC) was constructed in one vessel to increasing concentrations of the adrenergic agonist phenylephrine (PE, 0.001–100 μmol/L). To investigate vasodilator responses to methylcholine (MCh), a CCRC (0.0001–100 μmol/L) was constructed in the second vessel following constriction with PE (1 μmol/L).

2.5 Ex vivo vascular passive characteristics

Vessels were equilibrated in Ca2+-free PSS in the presence of papaverine (0.1 μmol/L) to initiate complete dilation. Passive characteristics were then assessed using pressures from 0 to 140 mmHg in mesenteric arteries and 0 to 180 mmHg in carotid arteries. Ex vivo analysis of circumentral wall stress and wall strain (change in diameter/original diameter) in carotid and mesenteric arteries were calculated as previously described.13 As the stress–strain relationship for the small arteries is non-linear, a tangential or incremental elastic modulus (Einc) was calculated as follows. An exponential curve was fitted to stress–strain data from each animal using the equation: \( Y = Y_0 \exp(k \times x) \), where \( Y \) is the circumferential stress, \( Y_0 \) is the circumferential stress at 4 mmHg, \( k \) is the rate constant, and \( x \) is the circumferential strain. Deriving this equation, we find that \( E_{inc} = kY_0 \), and therefore \( E_{inc} \) changes for any given value of circumferential stress. For any given circumferential stress, \( E_{inc} \) is directly proportional to the rate constant (\( k \)), and as such for each curve, \( k \) can be used as a measure of \( E_{inc} \).13 We have reported the rate constants for the stress–strain curves as a measure of \( E_{inc} \). An increase in the rate constant (leftward shift in the curve) would indicate an increase in arterial stiffness (reduced expandability) and, conversely, a decrease in the rate constant (rightward shift in the curve) would indicate an increase in arterial elasticity or expandability.

2.6 Protein extraction, western blot analysis, and gelatin zymography

Total protein was extracted from carotid and mesenteric arteries using an electric homogenizer (Omni THQ) in EDTA-free RIPA buffer for in vivo gelatin zymography and western blot analyses.14 For enzymatic activities, protein was extracted using CytoBuster extraction buffer (Novagen, EMD Chemicals, Inc), including protease inhibitor (Calbiochem) and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein was quantified using the BioRad DC protein assay. Mesentric and carotid arteries from three mice were pooled to obtain sufficient protein for one sample in vitro protein analyses, or enzymatic activity assays. Western blot analysis was performed to detect the protein levels of mature collagen type I (COL1α1, rabbit polyclonal antibody, sc-8784-R, Santa Cruz Biotechnology) and α-elastin (Polyclonal rabbit anti mouse α-Elastin, Abcam Inc., Cambridge, MA, USA) as previously described.

2.7 mRNA expression analysis by TaqMan real-time PCR

RNA was extracted from flash-frozen mesenteric or carotid arteries using the Trizol protocol. Subsequently, reverse transcription was performed to generate cDNA from 1 μg RNA, and mRNA expression levels of genes of interest were determined by TaqMan real-time PCR as previously described.15 All samples were run in triplicates in 384-well plates, and HPRT was used as an internal control. The primers and probes for the reported genes are as previously reported,19 primers/probe cocktail for α-elastin was purchased from Applied Biosystems (Mm00514670_m1).
2.8 Statistical analysis
All averaged values are represented as mean ± SEM. All data were normally distributed (Kolmogorov–Smirnov test for Gaussian distribution). Stress–strain relationships were analysed by comparing the averaged rate constants (mean ± SEM) between groups by Student’s t-test. The functional data in ex vivo vascular experiments are the percentage maximal vasoconstrictor or vasodilator response calculated as a percentage of constrictor tone. Statistical difference in the continuous variables between groups was determined by a two-way analysis of variance (ANOVA), with Bonferroni post-test for multiple comparisons. Comparisons between different genotypes were performed using multiple comparison ANOVA test followed by Student–Neuman–Keuls post-test. Statistical significance was recognized at P < 0.05. All other statistical analyses were performed using SPSS software (Chicago, IL, USA, Version 10.1).

3. Results

3.1 TIMP3 deficiency markedly impacts the Ang-II-induced hypertensive response
In WT mice, Ang II triggered a rise in blood pressure within 2 days of osmotic pump implantation, which reached a plateau by day 10 (Figure 1A). We investigated the role of each TIMP in the Ang-II-induced hypertensive response by similarly subjecting TIMP1−/−, TIMP2−/−, TIMP3−/−, and TIMP4−/− mice to 2 weeks of Ang II infusion. Body weights were comparable among genotypes after 2 weeks of saline (WT: 24.9 ± 1.1 g; TIMP1−/−: 24.1 ± 0.4 g; TIMP2−/−: 24.2 ± 0.2 g; TIMP3−/−: 24.6 ± 0.9 g; TIMP4−/−: 24.6 ± 0.8 g) or Ang II infusion (WT: 25.3 ± 0.4 g; TIMP1−/−: 23.7 ± 0.3 g; TIMP2−/−: 24.2 ± 0.2 g; TIMP3−/−: 23.7 ± 0.3 g, and TIMP4−/−: 24.7 ± 0.3 g). We found that while mice lacking TIMP1−/−, TIMP2−/−, or TIMP4−/− exhibited a rise in blood pressure comparable to WT mice, this response was significantly suppressed in TIMP3−/− mice (Figure 1A) as also evident by the per cent change in blood pressure (Figure 1B). TIMP3−/− mice also showed a lower baseline blood pressure (93.8 ± 1.7 mmHg) compared with WT (103.6 ± 1.6 mmHg) and other TIMP-deficient mice (TIMP1−/−: 99.2 ± 0.8; TIMP2−/−: 101.1 ± 0.3; and TIMP4−/−: 101.5 ± 0.9 mmHg).

3.2 TIMP3-deficient arteries become more distensible following Ang II infusion
To understand the underlying cause of blunted Ang-II-induced response in TIMP3−/− mice, we used the ex vivo pressure myography system to examine the mechanical properties of mesenteric-resistance arteries and carotid arteries in TIMP3−/− compared with WT mice. We measured changes in arterial diameter as a function of increasing pressure; 0–140 (mesenteric) and 180 (carotid) in 20 mmHg increments (passive curve). After 2 weeks of Ang II infusion, mesenteric arteries from both genotypes showed decreased distensibility compared with their corresponding saline group (see Supplementary material online, Figure S1A–ii). However, the pressure-dependent increase in diameter was significantly greater in TIMP3−/−-Ang II mesenteric arteries (Figure 1C) and was accompanied by a rightward shift in circular stress–strain relationship compared with WT-Ang II mesenteric arteries (rate constant = 8.58 ± 0.48 in TIMP3−/−-Ang II vs. 9.56 ± 0.89 in WT-Ang II; Figure 1Ci). Comparing the saline-treated mesenteric arteries showed a greater increase in passive pressure–diameter changes and a rightward shift in circular stress–strain relationship in TIMP3−/− compared with WT group (see Supplementary material online, Figures S1Aii and 1Bii). The circumferential stress–strain relationship is a measure of mechanical changes in the vascular wall, and a rightward shift in this curve in the TIMP3−/− mesenteric arteries indicates that TIMP3−/− mesenteric arteries are more expandable than WT mesenteric arteries.

Alterations in carotid arteries have been shown to correlate with Ang-II-induced changes in blood pressure in mice, as such we examined the mechanical properties of the carotid arteries. Two weeks of Ang II infusion resulted in a downward shift in the passive relationship in carotid arteries of both genotypes compared with their corresponding saline-infused group, although only in the TIMP3−/− group this difference reached statistical significance (see Supplementary material online, Figure S2A–ii). In addition, the protein levels of collagen and elastin, two major arterial ECM proteins, were markedly lower in TIMP3−/− mesenteric and carotid arteries rendering them more distensible compared with WT groups.

3.3 Adverse Ang-II-mediated remodelling in TIMP3-deficient mesenteric arteries
Hypertension is accompanied by vascular ECM remodelling. TIMP3 is an ECM-bound TIMP and has been demonstrated to be a key molecule in regulating ECM structure in different organs and diseases. We examined whether Ang-II-mediated ECM remodelling was altered in TIMP3−/− mesenteric arteries. Verhoeff–Van Gieson staining of perfuse-fixed mesenteric arteries showed that Ang II infusion increased the medial thickness as shown by increased media-to-lumen ratio (Figure 2A–ii). However, this ratio was significantly reduced in TIMP3−/−-Ang II mesenteric arteries compared with WT-Ang II (Figure 2A–ii) owing to reduced medial thickness and increased lumen diameter (Figure 2Aii). Consistently, the protein levels of collagen and elastin, two major arterial ECM proteins, were markedly lower in TIMP3−/−-Ang II compared with WT-Ang II mesenteric arteries (Figure 2B and C). This reduced structural support could reduce the structural integrity of the mesenteric arteries causing them to expand.

In order to determine whether the reduced ECM protein levels in TIMP3−/−-Ang II mesenteric arteries were due to enhanced proteolysis, we measured the proteolytic activities in these arteries and found that Ang II infusion increased gelatinase (Figure 3A) and elastase (Figure 3Aii) activity in both genotypes; however, this increase was significantly greater in TIMP3−/− mice. In vitro gelatin zymography further showed greater MMP2 activation in TIMP3−/−-Ang II mesenteric arteries while MMP9 levels were comparable among all groups (Figure 3B).

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Figure 1 TIMP3-deficient mice exhibit suppressed hypertensive response in vivo, and more expandable small arteries (ex vivo) following Ang II infusion. (A) Systolic blood pressure (i) and heart rate measurements (ii) in WT, TIMP1/−/−, TIMP2/−/−, TIMP3/−/−, and TIMP4/−/− mice before and after Ang II infusion. The arrow shows the start of Ang II infusion. (B) Percentage change in systolic blood pressure at 14-days post-Ang II infusion in indicated genotypes. n = 12/genotype. (C) Passive pressure myography showing pressure-dependent changes in lumen diameter of mesenteric arteries (i) and circular stress–strain relationship of mesenteric arteries (ii) after 2 weeks of Ang II infusion in TIMP3/−/− compared with WT group (i). (D) Pressure myography (i), and the circular stress–strain relationship in carotid arteries (ii) after 2 weeks of Ang II infusion in TIMP3/−/− compared with WT mice. Presented values are mean ± SEM. *P < 0.05 compared with WT-Ang II. n = 3–5/group/genotype.
Figure 2. Excess degradation of elastin and collagen in TIMP3\(^{-/-}\) AngII mesenteric arteries. (A) Representative pentachrome-stained mesenteric arteries (i), lumen diameter (ii), and media-to-lumen ratio (iii) in WT and TIMP3\(^{-/-}\) mice following saline or AngII infusion. (B) Representative western blot for collagen type 1 (i), and averaged protein content (ii). (C) Representative western blot for \(\alpha\)-elastin (i) and averaged elastin protein content (ii). For western blots, mesenteric arteries from three animals were pooled to yield sufficient protein for one sample; \(n = 6\)/saline group/genotype, 12/Ang II group/genotype. Coomassie blue staining was used as the loading control for western blots. AU, arbitrary units. Presented values are mean ± SEM. *\(P < 0.05\) compared with corresponding saline group. #\(P < 0.05\) compared with WT-Ang II.
3.4 Ang-II-mediated remodelling in TIMP3-deficient carotid arteries

In order to determine whether TIMP3-deficiency affected Ang-II-induced remodelling in other small arteries, we studied the carotid arteries. VVG staining showed disorganized and interrupted elastin fibres in TIMP3$^{-/-}$-Ang II carotid arteries (Figure 4Ai). Consistently, elastin protein levels were significantly lower (Figure 4Aii–iii); although its mRNA levels were markedly greater (Figure 4Aiv) in TIMP3$^{-/-}$-Ang II compared with WT-Ang II. Similarly, trichrome staining for ECM collagen fibres showed more disorganized arrangements (Figure 4Bi).

In assessing the proteolytic activities in the carotid arteries, we found that gelatinase activity was comparably elevated following Ang II infusion in both genotypes (Figure 5Ai), whereas the increase in elastase activity was significantly greater in TIMP3$^{-/-}$-Ang II compared with WT-Ang II group (Figure 5Aii). In vitro gelatin zymography and reduced collagen I protein levels (Figure 4Bii–iii) despite a significant elevation in mRNA levels (Figure 4Biv) in TIMP3$^{-/-}$-Ang II compared with WT-Ang II carotid arteries. These data suggest that the decrease in elastin and collagen proteins in TIMP3-deficient carotid arteries is most likely due to post-translational degradation of these proteins rather than decreased synthesis.

In assessing the proteolytic activities in the carotid arteries, we found that gelatinase activity was comparably elevated following Ang II infusion in both genotypes (Figure 5Ai), whereas the increase in elastase activity was significantly greater in TIMP3$^{-/-}$-Ang II compared with WT-Ang II group (Figure 5Aii). In vitro gelatin zymography
showed that Ang II infusion resulted in a similar rise in MMP9 levels, but a greater increase in cleaved MMP2 levels in TIMP3−/− arteries (Figure 5Bi–ii).

3.5 Doxycycline prevents the aberrant arterial extracellular matrix degradation in TIMP3−/− mice

Next, we aimed to determine whether excess proteolysis in the absence of TIMP3 is in fact the cause of the adverse ECM remodelling in TIMP3−/−-resistance arteries following Ang II infusion. Treatment with the well-known MMP inhibitor, doxycycline,20 preserved the structural integrity of mesenteric and carotid arteries in Ang-II-infused TIMP3−/− mice (Figure 6). The reduction in media-to-lumen ratio (Figure 6Ai–ii) and lumen dilation (Figure 6Aiii) observed in TIMP3−/−-Ang II mesenteric arteries was prevented, whereas structural integrity of elastin and collagen fibres was preserved with doxycycline treatment as shown by trichrome- and VVG-stained carotid arteries (Figure 6Bi–ii) and the reduced number of elastin fibre breakage (Figure 6Biii). Doxycycline treatment resulted in a marked increase in TIMP3−/−-resistance arteries following Ang II infusion. Treatment with the well-known MMP inhibitor, doxycycline,20 preserved the structural integrity of mesenteric and carotid arteries in Ang-II-infused TIMP3−/− mice (Figure 6). The reduction in media-to-lumen ratio (Figure 6Ai–ii) and lumen dilation (Figure 6Aiii) observed in TIMP3−/−-Ang II mesenteric arteries was prevented, whereas structural integrity of elastin and collagen fibres was preserved with doxycycline treatment as shown by trichrome- and VVG-stained carotid arteries (Figure 6Bi–ii) and the reduced number of elastin fibre breakage (Figure 6Biii). Doxycycline treatment resulted in a marked increase

![Figure 4](http://cardiovascres.oxfordjournals.org/)

**Figure 4** Excess degradation of vascular matrix proteins in carotid arteries of Ang-II-infused TIMP3−/− mice. (A) Representative VVG-stained carotid arteries (i), representative western blot for α-elastin (ii), quantification of elastin protein content (iii) and mRNA expression of elastin (iv) in WT and TIMP3−/− mice after saline or Ang II infusion. (B) Representative trichrome-stained carotid arteries (i), representative western blot for collagen type I (ii), quantification of collagen protein content (n = 12/group, protein from three animals/lane, averaged data from four lanes/group) (iii), and mRNA expression of pro-collagen 1-α1 (n = 5/group/genotype) (iv) in WT and TIMP3−/− mice after saline or Ang II infusion. AU, arbitrary units; RE, relative expression. Presented values are mean ± SEM. *P < 0.05 compared with corresponding saline group. #P < 0.05 compared with WT-Ang II.
in elastin and a significant reduction in collagen levels in WT-Ang II mesenteric arteries, whereas in TIMP3−/−-Ang II group, collagen levels increased while elastin levels remained unchanged (Figure 7Ai–ii). In carotid arteries, on the other hand, doxycycline treatment markedly reduced both, elastin (Figure 7Bi) and collagen protein levels (Figure 7Bii) in WT-Ang II mice but not in TIMP3−/−-Ang II mice (Figure 7Bi–ii). This differential impact of doxycycline on expression of vascular ECM proteins in WT vs. TIMP3−/− mice could underlie the resulting changes in blood pressure in these two groups. Doxycycline treatment attenuated the Ang-II-induced hypertension in WT mice while causing a small but significant increase in blood pressure in TIMP3−/− mice (Figure 7C). Overall, the protective effects of doxycycline on vascular ECM integrity of TIMP3−/−-Ang II mice indicate that the inhibitory function of TIMP3 against proteinases in the small arteries becomes more critical in the presence of a stimulus, such as elevated Ang II.

4. Discussion

Hypertension is the primary risk factor for all mortalities worldwide and contributes to approximately 7.5 million deaths annually.21,22 Hypertension is associated with vascular remodelling prior to the
onset of clinical symptoms, and as such called the ‘silent killer’. Vascular remodelling is an adaptive response to changes in the blood-pressure-induced circumferential wall stress, elastin-dependent axial stress, and blood-flow-induced wall shear stress.\(^2\) While vessel wall rigidity, which manifests as an increase in pulse wave velocity, is regarded as an independent predictor of mortality in hypertension and related diseases,\(^2\) \(\text{ex vivo}\) studies on animal models indicate that distensibility of conductance arteries (carotids) during the early stages of hypertension is associated with vascular remodelling.\(^2\) Hypertensive vascular remodelling is an active process of structural alterations, including degradation and reorganization of the vascular ECM proteins, such as collagen, elastin, proteoglycan and fibronectin.\(^2\) MMPs are a family of zinc-dependent proteases that contribute to ECM turnover by degrading existing proteins. MMPs are well regulated at levels of expression, translation, post-translational modification, and inhibition of their activated form by TIMPs.\(^2\)\(^,\)\(^2\)\(^,\) Levels of MMPs and TIMPs have been shown to be altered in hypertension in animal models\(^6\)\(^,\)\(^8\)\(^,\)\(^10\)\(^,\)\(^12\) as well as in hypertensive patients.\(^3\)\(^1\)\(^2\) \(^,\)\(^3\)\(^4\) TIMP1 and TIMP2 levels were increased in rat aorta following Ang II infusion,\(^3\)\(^5\)\(^,\)\(^3\)\(^4\) TIMP2 expression was elevated in the aorta of DOCA-salt hypertensive rats,\(^3\)\(^5\) whereas elevated plasma levels of TIMP1, MMP2, and MMP9 were detected in hypertensive patients.\(^3\)\(^6\) A population study in 1000 individuals of 50–65 years of age reported that a TIMP3 polymorphism (T-1296C), but not TIMP1 or TIMP2 polymorphisms, showed a significant association with hypertension.\(^3\) However, a direct role for TIMPs in hypertension had remained unexplored. In this study, we demonstrate that a lack of TIMP3, but not TIMP1, TIMP2, or TIMP4, results in a blunted hypertensive response.

![Figure 6](http://cardiovascres.oxfordjournals.org/)
Figure 7  Treatment with doxycycline differentially influenced elastin and collagen proteins in WT and TIMP3−/− arteries. Representative western blot and averaged protein levels for elastin (i) and collagen (ii) in mesenteric (A) and carotid arteries (B) in saline-, and Ang-II-infused WT and TIMP3−/− mice without and with doxycycline treatment, n = 3–6/group/genotype. (C) Systolic blood pressure in doxycycline-treated WT and TIMP3−/− mice before and after Ang II infusion. The data presented earlier is shown in a faded shade. n = 12/group, *p < 0.05 compared with the corresponding saline group, #p < 0.05 compared with corresponding group without doxycycline treatment.
following Ang II infusion owing to aberrant ECM degradation in resistance arteries. It is critical to note that this reduced hypertension in TIMP3−/− mice is not a protective mechanism, but the degradation of the ECM in small arteries results in pathological vasodilation which in long-term can lead to adverse outcomes such as aneurysms.37

Ang II is a well-known hypertensive agent that is up-regulated in different cardiovascular diseases.38–41 Majority of modern clinical antihypertensive drugs (ACEIs, ARBs) target the Ang II pathway; thus, it can serve as a suitable model to examine the molecular mechanisms of hypertension. In addition, we performed histological and molecular analyses in the mesenteric and carotid arteries to make a direct comparison between the hypertension phenotype and molecular alterations in arteries that are involved in blood pressure regulation.

In this study, we report that among all TIMP-deficient mice, only lack of TIMP3 resulted in a suppressed hypertensive response to Ang II infusion. TIMP3 is the only ECM-bound TIMP and has the broadest spectrum of substrates among TIMPs,42 which could explain why lack of TIMP3, but not other TIMPs, resulted in such a pronounced phenotype. Using an in vivo and ex vivo approach, we demonstrate that while acute vasoconstriction and vasodilation responses are intact in TIMP3−/− mesenteric arteries, 2 weeks of Ang II infusion resulted in structural remodelling in TIMP3−/− small arteries making them more distensible, as evident by a rightward shift in the circular stress–strain relationship, a commonly used technique to examine the mechanical properties of vessels,43,44 and an upward shift in passive pressure–diameter changes in TIMP3−/− Ang II compared with WT-Ang II mesenteric and carotid arteries. This is consistent with a loss of the ECM structural proteins, collagen and elastin,1,2 that provide strength and elastic recoil properties to the arteries, respectively,45 and increased lumen-to-media ratio and lumen diameter in the mesenteric arteries from TIMP3−/−-Ang II compared with WT-Ang II mice. The increase in mesenteric artery lumen diameter by about 20% in TIMP3−/−-Ang II compared with WT-Ang II can markedly impact blood pressure because blood pressure is inversely related to the fourth power of the lumen radius. As such, a small change in the radius of mesenteric arteries can significantly alter the blood pressure. These data collectively demonstrate that TIMP3 deficiency leads to adverse Ang-II-induced arterial ECM remodelling and impaired mechanical properties. Our findings provide evidence that vascular remodelling in the absence of TIMP3, but not other TIMPs, leads to destabilization of the vascular ECM integrity, impaired mechanical properties, and dilation of mesenteric arteries leading to impaired pressor responses. This is consistent with increased susceptibility of TIMP3−/− mice to abdominal aortic aneurysm following prolonged Ang II infusion47 despite the lower blood pressure in these mice. In addition, TIMP3 could play a critical role in vascular remodelling in diseases associated with hypotension such as sepsis, as TIMP3−/− mice exhibited markedly compromised survival in a lipopolysaccharide model of sepsis.46

TIMP3 has the broadest range of substrates among TIMPs,42 and in addition to inhibiting activated MMPs, TIMP3 can inhibit the cell surface activation of pro-MMP2 to its cleaved form,49 consistent with the observed rise in cleaved MMP2 in Ang-II-infused TIMP3−/− arteries. MMP2 has been shown to have substrate specificity for elastin and fibrillar collagen.48–50 Here we demonstrate that the elevated protease activities in TIMP3−/−-Ang II arteries contributes to the adverse vascular ECM remodelling in these mice because treatment with doxycycline, a broad spectrum MMP inhibitor shown to preserve vascular remodelling,51,52 protected the architecture and integrity of the mesenteric and carotid arteries in these mice. Doxycycline treatment markedly increased the elastin protein content and reduced collagen levels in WT-Ang II group, consistent with previous reports that doxycycline reduces collagen levels53 and preserves elastin fibres.54 The resulting increase in elastin-to-collagen ratio in doxycycline-treated WT-Ang II mesenteric arteries could reduce stiffness of these arteries and therefore reducing the hypertensive response. The protective effects of doxycycline on structural arrangement and organization of ECM proteins in TIMP3−/−-Ang II small arteries were associated with increased collagen levels in the mesenteric arteries which could contribute to enhanced stiffness and therefore the rise in blood pressure in this group. It is important to note that this increase in blood pressure with preservation of the arterial ECM integrity in TIMP3−/− mice is indeed a beneficial outcome as inhibition of excess protease activities prevented formation of abdominal aortic aneurysm in TIMP3−/− mice following chronic Ang II infusion.37

In summary, our study compares the causal role of the four TIMPs in agonist-induced hypertensive response and provides in vivo and ex vivo evidence that TIMP3 plays a primary role in vascular ECM remodelling thereby impacting the hypertensive response. This study also provides critical information that reduced hypertensive response in the absence of TIMP3 is not a protective mechanism but preludes the onset of more profound vascular complications and adverse outcomes.

4.1 Limitations

In this study, we assessed the changes in protein levels and the enzymatic activities in small arteries at the end point of our study. A time-course analysis of these parameters would provide insight into the order of events. In addition, given the strong impact of age and gender on blood pressure, role of TIMP3 in regulation of blood pressure in settings including these two factors will need to be explored.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

The authors thank the technical support from the Cardiovascular Research Center (CVRC) core facility at University of Alberta.

Conflict of interest: none declared.

Funding

This work was supported by Canadian Institute of Health Research (CIHR) grant to Z.K. (84279), G.Y.O. (86602), and S.T.D. (97838). R.B. and V.K. are supported by Alberta Innovates-Health Solution (AI-HS) studentship. Z.K. is an AI-HS Scholar.

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