**Matrix Metalloproteinase**

**Chronic Matrix Metalloproteinase Inhibition Retards Age-Associated Arterial Proinflammation and Increase in Blood Pressure**

Mingyi Wang, Jing Zhang, Richard Telljohann, Liqun Jiang, James Wu, Robert E. Monticone, Kapil Kapoor, Mark Talan, Edward G. Lakatta

**Abstract**—Age-associated arterial remodeling involves arterial wall collagen deposition and elastin fragmentation, as well as an increase in arterial pressure. This arterial remodeling is linked to proinflammatory signaling, including transforming growth factor-β1, monocyte chemoattractant protein 1, and proendothelin 1, activated by extracellular matrix metalloproteinases (MMPs).1–5 MMPs are a family of zinc-dependent endopeptidases that includes gelatinases MMP-2/-9, collagenases MMP-7/-13, and matrilysin MMP-7.6 In general, these proteinases are capable of degrading numerous extracellular matrix proteins but also can process a number of bioactive molecules, which tightly control the turnover of the extracellular matrix.6–12 MMP gene polymorphism is closely associated with hypertension and atherosclerosis.13,14 MMPs trigger a local proinflammatory signaling loop that disrupts arterial extracellular structure and causes vasoconstriction.4,12,14–16 MMP type II (MMP-2) relays signaling of monocyte chemoattractant protein 1 (MCP-1) and triggers transforming growth factor-β1 (TGF-β1) activation, which, in a feed-forward manner, activates both MMP-2 and MCP-1.4 Canonically, activation of this signaling loop with aging not only results in increased cellularity and thickening of the arterial intima but also causes elastin network fracture, the release of soluble fibrillin 1, and collagen deposition in the arterial wall.4,6,7,11 Noncannonically, however, MMP catalytic action resembles that of endothelin-convert enzyme, enhancing the conversion from the “big” inactive proendothelin 1 (pro–ET-1, 1-31) to the “small” active vasoconstrictor endothelin 1 (ET-1, 8–32).

Advancing age is a major risk factor for hypertensive and atherosclerotic complications attributed, in part, to an increase in local arterial wall inflammatory signaling that is linked to an enhanced activity of extracellular matrix metalloproteinases (MMPs).1–5 MMPs are a family of zinc-dependent endopeptidases that includes gelatinases MMP-2/-9, collagenases MMP-7/-13, and matrilysin MMP-7.6 In general, these proteinases are capable of degrading numerous extracellular matrix proteins but also can process a number of bioactive molecules, which tightly control the turnover of the matrix.5–12 MMP gene polymorphism is closely associated with increases in arterial calcification and stiffness in humans with hypertension and atherosclerosis.13,14

MMP mRNA, protein, and activity are markedly increased in the aged arterial wall of rodents, nonhuman primates, and humans.6,11,15,16 MMPs trigger a local proinflammatory signaling loop that disrupts arterial extracellular structure and causes vasoconstriction.4,12,14–16 MMP type II (MMP-2) relays signaling of monocyte chemoattractant protein 1 (MCP-1) and triggers transforming growth factor-β1 (TGF-β1) activation, which, in a feed-forward manner, activates both MMP-2 and MCP-1.4 Canonically, activation of this signaling loop with aging not only results in increased cellularity and thickening of the arterial intima but also causes elastin network fracture, the release of soluble fibrillin 1, and collagen deposition in the arterial wall.4,6,7,11 Noncannonically, however, MMP catalytic action resembles that of endothelin-convert enzyme, enhancing the conversion from the “big” inactive proendothelin 1 (pro–ET-1, 1-31) to the “small” active vasoconstrictor endothelin 1 (ET-1, 8–32).

Key Words: aging ■ arterial remodeling ■ matrix metalloproteinase inhibitor ■ endothelin 1 ■ transforming growth factor-β1 ■ets-1 ■ monocyte chemoattractant protein 1 ■ blood pressure

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MMP inhibition effectively reduces age-associated increases in aortic gelatinase and interstitial collagenase activity in situ

In vitro polyacrylamide gel electrophoresis gelatin zymographs to detect gelatinase activity of rat aorta (Figure S1A, available in the online-only Data Supplement) show that the renatured gelatinase, MMP-2, is increased with aging, whereas the renatured MMP-9 is rarely detected. Importantly, gelatin zymographs of the arterial wall in situ indicate that aortic gelatinase activity (green color) increases with aging (Figure 1A), consistent with previous reports. In situ aortic gelatinase activity is markedly decreased by chronic treatment with the PD166793 group (24Mi) compared with the placebo group (24M; Figure 1A).

Polyacrylamide gel electrophoresis casein zymography (Figure S1B), which was used to detect activity of collagenases MMP-1, MMP-13, and matrilysin MMP-7 based on their molecular weights, shows that the renatured MMP-13 activity increases with aging (the lowest weak bands), which is consistent with the level of its protein expression (Figure S1C). Importantly, an MMP-13 antibody-capture assay indicates that PD166793 treatment markedly reduces aortic MMP-13 activity in 24Mi compared with 24M group rats (Figure S1D).

Casein zymograms in situ confirm that the capacity to digest casein (red color) increases with aging (Figure 1B) and also shows that age-associated enhanced digestive capability is markedly inhibited in the 24Mi compared with 24M group (Figure 1B). In addition, collagen zymograms in situ further show that the capacity to digest collagen increases with aging, in particular in the thickened intima (green color, Figure 1C), and also shows that age-associated enhanced digestive capability is markedly inhibited in 24Mi compared with the 24M group (Figure 1C).

MMP inhibition reduces age-associated aortic extracellular matrix remodeling

Morphological analysis (Table S1, available in the online-only Data Supplement) indicates that intimal thickness, medial thickness, and intimal medial thickness significantly increase with age from 16 to 24 months (24M vs 16 month-old group). Although the age-associated increase in intimal thickness, medial thickness, and intimal medial thickness is not substantially reduced, histochemical staining and morphometric analysis of the elastin fraction/density reveal that age-associated degradation (decreased elastin density) in the arterial wall are prevented in 24Mi versus 24M (Figure 2A). Furthermore, Western blot analysis demonstrates that the formation and release of the elastin microfibril-apparatus breakdown product, soluble fibrillin 1, are completely abolished in 24Mi (Figure 2B). Collagen I immunolabeling shows that aortic collagen type I deposition, which increases with age, is reduced in 24Mi (Figure 2C). This finding is further confirmed by Western blot analysis (Figure 2C).

MMP inhibition reduces age-associated aortic inflammation

It is known that the proinflammatory MCP-1/TGF-β1 signaling loop is involved in arterial collagen disorders with...
aging.\textsuperscript{4,7,8,11} Western blot analysis (Figure 3A) shows that the active dimer form of MCP-1\textsuperscript{23,24} is increased in the arterial wall with advancing age and is markedly decreased in 24Mi. Immunostaining demonstrates that activated TGF-\(\beta\)1 is increased in the arterial wall with advancing age, particularly in the thickened intima, and this is markedly decreased in 24Mi (Figure 3B). Western blot analysis further confirms these findings (Figure 3C). Immunostaining and Western blot analyses show that the amount of activated p-SMAD2/3 and the number of stained VSMC nuclei for p-SMAD2/3, effective downstream signaling molecules of the TGF-\(\beta\)1 cascade, are also markedly increased.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig1}
\caption{Matrix metalloproteinase (MMP) activation in situ. \textbf{A}, Fluorescence micrographs of in situ gelatin zymograms (green, \(\times400\)) and average intensity of the cleaved gelatin signal (\textit{lower panel}; \(n=3\) per group). \textbf{B}, Fluorescence micrographs of in situ casein zymograms (red, \(\times400\)) and average intensity of the cleaved casein signal (\textit{lower panel}; \(n=3\) per group). \textbf{C}, Fluorescence micrographs of in situ collagenase zymograms (green, \(\times200\)) and average intensity of the cleaved collagen signal (\textit{lower panel}; \(n=3\) per group). \(*P<0.05\) vs 16 month-old group; \#\(P<0.05\) vs 24 month-old placebo group. L indicates lumen; M, media.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig2}
\caption{Aortic extracellular matrix remodeling. \textbf{A}, Photomicrographs of elastic fibers (dark blue) in elastic Van Gieson stained with elastin protein in aortic sections with (\textit{left panels}, \(\times400\)). Average elastin fraction (EF; \textit{right panel}, \(n=5\) per group). \textbf{B}, Western blots of fibrillin 1 (\textit{left panels}) and average data (\(n=3\) per group, \textit{right panel}). \textbf{C}, Photomicrographs of collagen I immunostaining (brown, \(\times200\), \textit{left panels}) of aortic sections and average data (\textit{right panel}, \(n=4\) per group). \(*P<0.05\) vs 16 month-old group; \#\(P<0.05\) vs 24 month-old placebo group. L indicates lumen; M, media.}
\end{figure}
with aging, and these increases are significantly reduced by MMP inhibition (Figure 3D).

**MMP Inhibition Modifies Posttranslational Processing of Arterial Vasoconstrictor ET-1**

Prior in vitro studies document that MMP-2 has a potent capacity to cleave latent "big" pro–ET-1 to the "small" form of activated ET-1, a much more effective vasoconstrictor than the precursor. Figure 4A shows that arterial small ET-1 protein expression is upregulated with aging, particularly within the intima and innermost media, confirming a recent report. Chronic MMP inhibition significantly decreases small ET-1 (Figure 4B). Note that, that the small active form of ET-1 is undetectable in 16-month rat aorta.

**MMP Inhibition Reduces Expression of the Proinflammatory Transcription Factor Ets-1**

A growing body of evidence indicates that ET-1 signaling and its effects to increase collagen production via TGF-β1 and MCP-1 upregulation are orchestrated by the transcription factor ets-1. Immunostaining shows that the number of ets-1–stained VSMC nuclei (activated form) within the aortic wall in vivo is markedly increased in the 24M compared with the 16 month-old group (Figure 4C). This age effect is abolished by MMP inhibition (Figure 4C, right). Western blot analysis further confirms this finding (Figure 4D).

**MMP Inhibition Blocks the ET-1/Ets-1 Signaling in Aortic VSMCs In Vitro**

Previous studies demonstrate that MMP-2 enhances expression of inflammatory signaling molecules TGF-β1 and MCP-1, which is orchestrated, in part, by the nuclear transcriptional factor ets-1 in VSMCs. We next explored whether MMP inhibition blocks the ET-1/ets-1 proinflammatory signaling in aortic VSMCs in vitro. In early passage aortic VSMC expression of ets-1 protein is increased in a dose-dependent manner by treatment with active ET-1 (Figure 5A). Importantly, the ability of pro–ET-1 to increase both ets-1 transcription and its translation is also markedly reduced by MMP inhibition (Figure 5B and C). To demonstrate a role of ets-1 in arterial proinflammatory signaling, we overexpressed ets-1 in VSMCs via an adenovirus harboring a full-length ets-1 cDNA (Figure 5D, top). Overexpression of ets-1 substan-
ially increases both active MCP-1 and TGF-β1 (Figure 5D, middle).

**Age-Associated Increase in Arterial Pressure Is Lowered by MMP Inhibition**

An increase in arterial pressure is an important functional readout of age-associated arterial wall proinflammation.\(^1\)\(^-\)\(^2\)\(^5\) Figure 6 demonstrates that the anti-inflammatory effects of MMP inhibition (Figures 1–5) are accompanied by a marked blunting of the increase in blood pressure that occurs between 16 and 24 months in this rat strain.

**Other Effects of MMP Inhibition**

MMP inhibition did not affect either diet or body weight of rats compared with the placebo group (Table S2). Notably, MMP inhibition similarly diminishes age-associated coronary extracellular matrix remodeling and proinflammation (Table S1 and Figure S2).

**Discussion**

MMP activation is an element within an age-associated proinflammatory signaling circuit. Active MMP functions as an effective molecular scissor with broad structural/func-

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**Figure 4.** Endothelin (ET) 1 expression and cleavage and transcriptional factor, ets-1, expression. **A**, Immunolabeling of aortic wall ET-1 (brown color, \(\times 200\)). **B**, Western blots of arterial wall ET-1 (left panel) and average data (right panel, \(n=3\) per group). \(^*P<0.05\), 24 month-old inhibitor treatment group vs 24 month-old placebo group (24M). **C**, Photomicrographs of aortic wall ets-1 staining (brown, \(\times 400\)). **D**, Western blots of ets-1 (left panels) and average data (\(n=3\) per group). \(^*P<0.05\) vs 16 month-old group; \(^#P<0.05\) vs 24M group. L indicates lumen; M, media.

**Figure 5.** Proinflammation cascade within vascular smooth muscle cells (VSMC). **A**, Western blots of ets-1 (left panels) and average data (right panel, \(n=3\) independent experiments). \(^*P<0.05\) vs control. **B**, RT-PCR analysis (\(n=4\) independent experiments). \(^#P<0.05\) vs PD166793 treatment. **C**, Representative Western blots of ets-1. **D**, Overexpression of ets-1 in VSMCs increases activated monocyte chemoattractant protein (MCP) 1 and transforming growth factor (TGF)-β1 protein.
The interaction between treatment groups and age on arterial pressure in linear mixed-effects models is highly statistically significant for both SBP (*P=0.0008) and DBP (#P=0.0082).

Our results demonstrate, for the first time, that PD166793, a broad-spectrum MMP inhibitor, retards a local inflammatory signaling loop and alleviates adverse extracellular matrix remodeling in both the aortic and coronary arterial walls and that these effects are accompanied by a blunting of the age-associated increase in blood pressure. PD166793 in the 10- to 100-μmol/L range has global MMP inhibitory activity, high affinity for MMP-2 and -13 and lower affinity for MMP-1, -7, and -9.21,26,27 Note that PD166793 does not exhibit inhibitory activity of other proteases, for example, angiotensin-converting enzyme, endothelin-converting enzyme, or tissue necrosis factor-α convertase.21,28,29 A 5-mg/kg dose of PD166793 was selected for our study because this dose produces plasma drug levels of 100 μmol/L after 4 months and markedly reduces the aforementioned MMP activities in rats.21 Indeed, the present findings show that injection of this dose after 4 months markedly retards an age-associated increase in blood pressure in rats and inhibits activity of aortic gelatinases and collagenases in situ.

Our results show that the activities of both MMP-2 and MMP-13 increase within the aortic wall with aging. It is known that activated MMP-2 binds to elastin fibers and increases the cleavage of elastin, resulting in the dissolution of the structural microfibril-associated apparatus and the release of fibrillin.1,8,10,30 Activated MMP-2 also binds to the basement membrane of VSMCs, enabling their migration, and to the basement membrane of endothelial cells, enabling their desquamation.4,9,31 Activated MMP-13 attaches to and cleaves intact type I or III collagen, resulting in a release of collagen ½- and ¾-length fragments.31–33 These fragments are further cleaved into growth factor-like “matrikines,” which activate MMPs in a feed-forward manner.34,35 MMP inhibition maintains the intact scaffold of the aorta via reduction of the activity of gelatinase and interstitial collagenase (Figure S3).

MMPs not only cleave elastin and collagen fibers but also cleave latent TGF-β1, releasing soluble latent TGF-β binding protein 1, to initiate the processes of TGF-β1 activation, which results in SMAD2/3 phosphorylation and production of collagen in VSMCs.4,7,36 The present study confirms and extends previous findings that MMP increases pro–ET-1 activity, enhances ets-1 activation, and that, in turn, increases both MCP-1 and TGF-β1 activation, reinforcing their downstream molecule SMAD-2/3 phosphorylation and collagen production in VSMCs.4,15,16 MMP inhibition also diminishes the age-associated increase in arterial fibrosis via intervening on the proinflammatory signaling loop. Our findings suggest that MMP inhibition restores a balance of collagen production and cleavage within the arterial wall, resulting in a retardation of age-associated arterial disorders. Importantly, Figure S3 implicates each of these cleaved products generated by MMPs as an element of the proinflammation circuitry of either the angiotensin II (Ang II) or ET-1 cascade within the arterial wall with aging.

An age-associated increase in arterial pressure is a clinical hallmark of aging and results from joint effects of multiple factors, including intimal-medial thickening, arterial proinflammatory responses, and vasoconstriction from Ang II and ET-1 effects.1–3,37,38 The components of the renin-angiotensin-aldosterone system, including angiotensinogen, angiotensin-converting enzyme, angiotensin II, its receptor Ang II type 1, and aldosterone protein and signaling, are increased in the aged arterial wall.5,8,10,39–42 So too, the present study, as well as others, show that ET-1 protein and its activity are increased in the arterial wall with aging.15,41,42 MMP inhibition reduces the age-associated increase in arterial blood pressure likely, in part, via blockade of the ET-1 and Ang II–associated proinflammatory signaling loop and matrix remodeling. These effects contribute to decreased vasoconstriction and blood pressure, even without having significant effects on the intimal-medial thickening. In addition, PD166793 reduces production and activity of cardiovascular reactive oxygen species, which also modulate blood pressure.43,44

Ang II enhances ET-1 expression in the arterial wall.45 Blockades of the renin-angiotensin-aldosterone system signaling, including angiotensin-converting enzyme inhibitor, Ang II type 1 receptor antagonists, and aldosterone blockers, all are capable of retarding age-associated arterial disorders, including increases in intimal-medial thickness, arterial stiffness, and blood pressure.46–48 Inhibition of ET-1 also alleviates endothelial dysfunction and arterial stiffness with aging and hypertension, in part via inhibition of MMP-2 activation.49,50 In addition, inhibition of reactive oxygen species production, and TGF-β1/MMP-2 activation, by deletion of the gene p66Shc, nitrite supplementation, or exercise, substantially improves age-associated endothelial dysfunctions and arterial stiffening.51–54

Perspectives
MMP activation in age-associated arterial remodeling is a convergence point of multiple inflammatory stress pathways,
including Ang II, ET-1, and mechanical forces. Our unique study provides proof of concept that MMP inhibition can attenuate the extent and rate of adverse arterial remodeling that accompanies aging. Although the currently available MMP inhibitors have undesirable adverse effects, including the delay of wound healing and impairment of angiogenesis, as well as skeletal muscle damage,\textsuperscript{35,36} advances in structural adjustment of existing inhibitors to increase selectivity, remove toxicity, and improve bioavailability in newer versions show promise. Thus, MMP inhibition may offer a future preferable therapeutic approach to maintain arterial health during aging. Molecular components and signaling networks of age-associated arterial remodeling are recapitulated in young subjects with hypertension and atherosclerosis, and arterial aging is a chronic process intimately linked to subclinical arterial diseases, including hypertension and atherosclerosis.\textsuperscript{2}

Thus, MMP inhibition may also offer a potential therapeutic approach to retard the development of this age-associated arterial disease.

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**Disclosures**

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Chronic Matrix Metalloproteinase Inhibition Retards Age-associated Arterial Proinflammation and Increase in Blood Pressure


Short title: MMP Inhibition Retards Arterial Aging

Equal contribution #

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Expanded Methods

**Blood pressure measurement**
Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured monthly via a tail-cuff method according to the manufacturer’s guidelines (RTBP 1007, Kent Scientific Co).

**Tissue harvesting**
Rats were sacrificed with an overdose of sodium pentobarbital, and loose aortic adventitia were removed and remaining aortic tissues were harvested according to the protocol described previously [1].

**Histology and morphometry**
Elastica van Gieson (EVG) and Sirius Red staining for paraffin aortic sections were performed as reported in prior studies [1]. High-resolution digital images of a cross-section of thoracic aorta and epi-coronary arteries stained with EVG or Sirius Red methods were acquired through a Leica microscope. Morphometric evaluation was performed with use of a computerized imaging analysis system (Metamorph, University Imaging) by investigators who were blinded to the treatment protocol. Thoracic elastin area was determined by the respective area of EVG per arterial wall cross-sections; and elastic fraction (EF) of stained area to target area. The thickness of the thoracic intima (IT), corresponding to the layer between the lumen of surface and internal elastic lamina (IEL), was measured from images taken at 400X magnification, thoracic medial thickness (MT) was calculated from the IEL to external elastic lamina (EEL) from images taken at 200X magnification, and intimal-medial thickness of thoracic aorta is a sum of IT and MT. Coronary collagen content was expressed as the Sirius Red stained area per arterial wall cross-section; and proportion of stained area to target area (CF). Coronary luminal area (CLA) and coronary wall area (CWA) were measured from images taken at 400X magnification.

**Immunohistochemistry and immunofluorescence**
Immunostaining was performed as reported in prior studies [1]. The source and characteristics of primary antibodies used are listed in online supplemental Table S3.

**Polyacrylamide gel electrophoresis (PAGE) and in situ zymography**
PAGE gelatin and casein zymography to detect MMP activity were performed according to instructions of the manufacturer (Invitrogen, Carlsbad, CA). In situ zymography to detect MMP activity within the arterial wall, was performed according to the modified protocol previously described [1]. In detail, to localize net in situ activities of MMPs by zymography, FITC-labeled DQ gelatin (Molecular Probes, Eugene, OR) and Collagen type I (AnaSpec, Inc. Fremont, CA), and BODIPY labeled DQ casein intra-molecularly quenched (Molecular Probes, Eugene, OR) was used as a substrate. Fresh aortas were collected and rinsed in cold PBS to remove outside connective tissue. Aortas were then immersed in ornithine carbamyl transferase (OCT) (Tissue-Tek, Torrance, CA) and quick-frozen into a block of dry ice. The thoracic aortic segments in the OCT blocks were cut into 10 :mol/L sections using a cryostat (Leica, Wetzler, Germany) and collected sequentially. In situ zymography was performed by modification of a combination of methods previously described [1, 2, 3]. quenched FITC-labeled DQ gelatin (500ng/mL), BODIPY-labeled casein (500ng/mL), or FITC-conjugated collagen type I (1000ng/ml) was mixed (1:1) with reaction buffer (0.05 mol/L Tris-HCl, 0.15 M NaCl, 5 mM CaCl$_2$, and 0.2 mmol/L NaN$_3$, pH 7.6). A drop of the liquid mixture was added over each tissue section, and a silated coverslip was applied. Slides were incubated in light-protected humidified chambers at 37°C for 24 hours for measurement of cleaved collagen signal or 48 hours for cleaved gelatin
and casein signals, respectively. At the end of the incubation period and without fixation or washes, lysis of the substrate was assessed by fluorescence microscopy. As a negative control for in situ zymography, the frozen sections were processed as above but without the FITC-labeled DQ gelatin, collagen type I, or BODIPY-labeled DQ casein.

**Aortic MMP-13 activity assay**

Specific detection of aortic MMP-13 activity was performed according to a modified protocol provided by the manufacturer (Cat# 72019, AnaSpec, Inc., CA). Total homogeneous aortic protein (20µg) were pulled down by MMP-13 antibody coated miroplates for 2 hours, activated by 4-aminophenylmercuric acetate (APMA) at 37°C for 40 min, and then an MMP-13 substrate solution was added and incubated for 16 hours and measured for relative fluorescence intensity.

**VSMC isolation, culture, and treatment**

Vascular smooth muscle cells (VSMC) were enzymatically isolated and cultured as previously described [4]. FXBN thoracic aortas were rinsed in Hanks balanced salt solution (HBSS) containing 50µg/mL penicillin, 50µg/mL streptomycin and 0.25µg/mL amphotericin B (Gibco). After digestion for 30 min in 2mg/mL collagenase I solution (Worthington Biomedical, Freehold, New Jersey) at 37°C, the adventitia and intima were removed from the vessel media layer, which was placed overnight in complete medium (DMEM plus 10% FCS). On day 2 the vascular media was further digested with 2mg/mL collagenase II/0.5mg/mL elastase (Sigma) for 1 hour at 37°C, and the isolated cells were washed and plated in complete medium.

Early passage VSMCs utilized for treatment were plated in a 60 mm culture dish at a density of 4x10⁵ in 10% DMEM for 24 hours. Cells were starved in 0.1% DMEM for 24 hours prior to treatment. Cells were pretreated with 100nmol of PD166793 for 1 hour and pro-endothelin-1 (big) and endothelin-1 (small) (Sigma) were then added at a concentration of 100 nmol/L for 24 hours. Cells were then washed with cool PBS and harvested for isolation of protein and RNA. In all cases, >95% of cells stained positive for α-smooth muscle actin.

**Generation of recombinant adenoviruses and VSMC infection**

The mouse ets-1 (1463 bp) full-length cDNA fragment was amplified by polymerase chain reaction (PCR) with primers containing Bgl II and Sal I restriction sites at the 5’ and 3’ ends, respectively. The upstream primer is 5’-aga,tct, cat,ccc,gcc,ggc,c-3’ and the downstream primer is 5’-gtc,gac,tag,tca,gca,tcc,gg-3’. The amplified PCR fragments were ligated to pGEM-T vector and sequenced. The ets-1 was cut by Bgl II and Sal I and then ligated to pAdTrack-CMV, which has a reporter gene expressing green fluorescent protein. The adenoviral backbone plasmid pAdEasy-1 and shuttle plasmid pAdTrack-CMV were a gift from Dr Bert Vogelstein (John Hopkins Oncology Center Baltimore Maryland, USA). A replication-defective, recombinant adenovirus encoded ets-1 together with green fluorescent protein was constructed by homologous recombination as described in previous reports [5, 6, 7]. An adenoviral vector expressing green fluorescent protein (GFP) served as a control virus. Standard viral amplification and cesium chloride purification methods were used to amplify and purify these adenoviruses. The titer for each adenovirus in HEK293A cells was determined via a dilution assay.

Adenoviral infection of VSMC was performed with a multiplicity of infections (MOI) of 100. After 48 hours of adenoviral infection, total cellular proteins were isolated for Western blot.

**qRT-PCR**

Quantitative Real-time PCR (q-PCR) of ets-1 was performed using the SYBRGreen PCR-based protocol in a 384-well plate format (Applied Biosystems). All the primers used for Real Time PCR analysis have been designated using Primer Express software 1.5 (Applied Biosystems, Foster City, CA), and synthesized by Invitrogen Life Technologies (Carlsbad, CA). The primer sequence sizes are: Ets-1, forward GGTATTCGAGCATGCTCAGTTG; and, backward
TCTGGAGAGGGTCCCGGAGAAT (provided by Drs. Yumei Zhan & Peter Oettgen, Division of Molecular and Vascular Medicine Beth Israel Deaconess Medical Center, Harvard Medical School); B2M, forward TGCTACGTGTCTCAGTTCCA, backward GCTCCTTCAGAGTGACGTGT.

RNA was extracted from cultured VSMC using Trizol reagent. RNA (2µg) was reverse transcribed for 30 minutes at 48°C using random hexonucleotides according to the manufacturer’s instructions (Applied Biosystems Foster City CA). Real-time PCR was performed according to the SYBRGreen PCR protocol (Applied Biosystems Foster City CA). Each sample was tested in quadruplicate. The reaction conditions were: 10 min at 95°C (one cycle); 30 sec at 95°C; 30 sec at 60°C; and 30 sec at 72°C (40 cycles). Gene-specific PCR products were continuously measured by an ABI PRISM 7900 HT Sequence Detection System (PE Applied Biosystem Norwalk, CT). The PCR product sizes were verified by agarose gel electrophoresis. Samples were normalized to the expression of the “housekeeping” gene, B2M. Data are expressed using the formula: quantity=10^{-(Ct-Y \text{ intercept})/\text{slope value}} , where Ct represents the threshold cycle value.

**Western blot analysis**

For Western Blotting, fifteen µg of whole cell or arterial lysates were resolved by SDS-PAGE and transferred onto PVDF membrane (Immobilon). The transferred membranes were incubated in PBS containing primary antibodies (online supplemental Table 1) at 4°C for 24 hours. HRP-conjugated IgG (Amersham Pharmacia Biotech, Buckinghamshire, GB) were used as secondary antibodies and detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). The average densitometric analysis of the bands was obtained on the protein extracts from the aortae of three or four rats of each age group, or from VSMC of at least three rats. β-actin immunoblotting has been used as a protein loading control since its expression does not significantly change with age.

**Statistical analysis**

All results are expressed as the mean ± SEM. Statistical analyses was performed via a Student T-test when two groups were analyzed, or via an ANOVA, followed by a Bonferroni post hoc test, for multiple comparisons. For systolic and diastolic blood pressure (SBP and DBP), the repeated-measures data are analyzed using a linear mixed-effects model [8]. A p value of <0.05 was taken as statistically significant.
Results

MMP inhibition similarly diminishes age-associated coronary extracellular matrix remodeling and proinflammation

To explore the effects of MMP inhibition on smaller vessels, we performed immunostaining and morphometric analysis of epi-cardial coronary arteries of left ventricles. The coronary artery lumen becomes dilated with aging, its wall becomes thickened, and perivascular collagen deposition increases. Chronic MMP inhibition (24Mi) markedly retards the age-associated increase in lumen dilatation, wall area, and collagen deposition (Figure S2 & Table S2). Importantly, MMP inhibition also retards age-associated increases in profibrotic signaling molecules SMAD2/3 phosphorylation in the epi-cardial coronary wall (Figure S3 & Table S3).
References

### Table S1 Aortic Remodeling

<table>
<thead>
<tr>
<th>Parameter</th>
<th>16M (n=12)</th>
<th>24M(n=12)</th>
<th>24Mi (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IT (µM)</td>
<td>3.87 ± 1.20</td>
<td>5.84 ± 1.52*</td>
<td>4.76 ± 1.43</td>
</tr>
<tr>
<td>MT (µM)</td>
<td>102 ± 8</td>
<td>115 ± 18*</td>
<td>110 ± 9*</td>
</tr>
<tr>
<td>IMT (µM)</td>
<td>105 ± 7</td>
<td>121 ± 18*</td>
<td>115 ± 9*</td>
</tr>
</tbody>
</table>

One-way ANOVA. *p<0.05 vs. 16M. IT=intimal thickness; MT=medial thickness; Intimal-medial thickness=IMT.

### Table S2 Coronary Remodeling

<table>
<thead>
<tr>
<th>Parameter</th>
<th>16M (n=12)</th>
<th>24M(n=12)</th>
<th>24Mi(n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW(g)</td>
<td>518±56</td>
<td>588±35*</td>
<td>588±28*</td>
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<tr>
<td>HW(g)</td>
<td>1.12±0.11</td>
<td>1.25±0.05</td>
<td>1.28±0.48</td>
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<td>HW/BW(mg/g)</td>
<td>2.17±0.19</td>
<td>2.14±0.09</td>
<td>2.18±0.18</td>
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<tr>
<td>CWA (µM²)</td>
<td>120±58</td>
<td>252±73</td>
<td>88±45</td>
</tr>
<tr>
<td>Peri-coll(µM²)</td>
<td>153±51</td>
<td>316±129*</td>
<td>305±191*</td>
</tr>
<tr>
<td>CF(%)</td>
<td>0.27±0.07</td>
<td>0.45±0.05*</td>
<td>0.34±0.08*#</td>
</tr>
<tr>
<td>CLA(µM²)</td>
<td>195±71</td>
<td>416±275*</td>
<td>170±45</td>
</tr>
</tbody>
</table>

BW=body weight; HW=heart wet weight; CWA=epi-coronary wall area; peri-coll=peri-ep-coronary collagen area; CF=collagen fraction; and CLA=epi-coronary lumen area. One-way ANOVA. *p<0.05 vs. 16M; #P<0.05 vs. 24M.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specie</th>
<th>Titer Blotting</th>
<th>Titer Staining</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>M</td>
<td>1:1000</td>
<td>1:50</td>
<td>Chemicon Intern. Inc., CA</td>
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<tr>
<td>TGF-β1</td>
<td>R</td>
<td>1:200</td>
<td>1:50</td>
<td>Santa Cruz, CA</td>
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<td>M</td>
<td>1:600</td>
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<td>Miuipore</td>
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<td>MMP-7</td>
<td>M</td>
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<td>MMP-13</td>
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<td>p-SMAD2/3</td>
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<td>Collagen I</td>
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<td>1:50</td>
<td>Rockland Immunochemicals, Inc., PA</td>
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<td>ETS-1</td>
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<td>1:50</td>
<td>Santa Cruz, CA</td>
</tr>
<tr>
<td>ET-1</td>
<td>G</td>
<td>1:200</td>
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<td>Santa Cruz, CA</td>
</tr>
<tr>
<td>β-actin</td>
<td>M</td>
<td>1:10000</td>
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</table>

R=rabbit; M=mouse; and G=goat.
**Figure S1.** MMP expression and activity. A) Example of PAGE gelatin zymograms (upper panel) and average data of activated MMP-2 (lower panel, n=3/group). *p<0.05, vs. 16M. B) PAGE casein zymograms. *p<0.05, vs. 16M. C) Western blots of MMP-1, -3, -7, and -13 (n=3/group). *p<0.05, vs. 16M. D) Aortic MMP-13 activity (n=3/group). *p<0.05, 24Mi vs. 24M.
Age-Associated Arterial Proinflammatory Signaling

Figure S3. Simplified diagram of the age-associated arterial proinflammatory signaling circuit modified from a prior report [9]. Activated MMPs promote an arterial proinflammatory signaling loop (red lines) that facilitate destruction of the arterial wall scaffold (green lines) and vasoconstriction (yellow intermittent lines), responsible for the age-associated arterial phenotypes (increases in arterial stiffening and blood pressure). Notably, the novel molecules-linked to MMP inhibition in vivo were highlighted (italic front) ET-1=endothelin-1; Ang II=angiotensin II; LTBP=latent TGF binding protein; LAP=latent associated protein; TGF-β1=transforming growth factor-beta 1; SMAD=homologs of both the drosophila protein, mothers against decapentaplegic (MAD) and the Caenorhabditis elegans protein SMA; and MCP-1=monocyte chemoattractant protein-1.