Aortic Remodeling After Transverse Aortic Constriction in Mice Is Attenuated With AT₁ Receptor Blockade

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Objective—Although hypertension is the most common risk factor for thoracic aortic diseases, it is not understood how increased pressures on the ascending aorta lead to aortic aneurysms. We investigated the role of angiotensin II type 1 receptor activation in ascending aortic remodeling in response to increased biomechanical forces using a transverse aortic constriction (TAC) mouse model.

Approach and Results—Two weeks after TAC, the increased biomechanical pressures led to ascending aortic dilatation and thickening of the medial and adventitial layers of the aorta. There was significant adventitial hyperplasia and inflammatory responses in TAC ascending aortas were accompanied by increased adventitial collagen, elevated inflammatory and proliferative markers, and increased cell density attributable to accumulation of myofibroblasts and macrophages. Treatment with losartan significantly blocked TAC-induced vascular inflammation and macrophage accumulation. However, losartan only partially prevented TAC-induced adventitial hyperplasia, collagen accumulation, and ascending aortic dilatation. Increased Tgfb2 expression and phosphorylated-Smad2 staining in the medial layer of TAC ascending aortas were effectively blocked with losartan. In contrast, the increased Tgfb1 expression and adventitial phospho-Smad2 staining were only partially attenuated by losartan. In addition, losartan significantly blocked extracellular signal–regulated kinase activation and reactive oxygen species production in the TAC ascending aorta.

Conclusions—Inhibition of the angiotensin II type 1 receptor using losartan significantly attenuated the vascular remodeling associated with TAC but did not completely block the increased transforming growth factor-β1 expression, adventitial Smad2 signaling, and collagen accumulation. These results help to delineate the aortic transforming growth factor-β signaling that is dependent and independent of the angiotensin II type 1 receptor after TAC. (Arterioscler Thromb Vasc Biol. 2013;33:2172-2179.)

Key Words: aortic aneurysm, thoracic • receptor, angiotensin, type 1 • transforming growth factors

Thoracic aortic aneurysms leading to acute aortic dissections are a common cause of premature deaths in the United States.¹ Hypertension is the most common risk factor for thoracic aortic disease; >75% of patients with thoracic aortic aneurysms or acute aortic dissections have hypertension.² Despite the common association of hypertension and thoracic aortic disease, it is not understood how increased pressures on the ascending aorta lead to thoracic aortic aneurysms and aortic dissections.

An established model system to study aortic disease is acute angiotensin II (Ang II) infusion in mice, which leads to aortic disease characterized by aortic aneurysm formation and subsequent dissection/rupture in the suprarenal and ascending thoracic aorta.³ With Ang II infusion, the medial layer of the ascending thoracic aorta thickens with expansion between the elastic lamellae.⁴ Additionally, the adventitial fibroblasts secrete proinflammatory cytokines, including interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1), which recruit monocytes to the aortic wall, which further activate fibroblast proliferation and myofibroblast activation, adventitial thickening, and additional cytokine production.⁵

Another established mouse model of thoracic aortic aneurysms is the Marfan syndrome mouse, which has been engineered to harbor an Fbm1 missense mutation. Studies in this mouse model have suggested that promiscuous activation of transforming growth factor-β (TGF-β) from stores in the microfibrils in the extracellular matrix drives aortic aneurysm formation.⁶ Blocking TGF-β signaling using a TGF-β...
neutralizing antibody or an Ang II type 1 receptor (AT₁) blocking agent, losartan, prevents aneurysms in the Marfan syndrome mouse model.⁶

Transverse aortic constriction (TAC), created by contracting the aortic lumen between the innominate artery and the left carotid artery, is a common experimental method to induce pressure overload cardiac hypertrophy and heart failure. Although TAC initially leads to compensatory hypertrophy of the heart, over time the response to the chronic hemodynamic overload becomes maladaptive, resulting in cardiac dilatation and heart failure.⁷,⁸ Although studies have shown increased aortic diameter and thickening of the aortic wall, the molecular signaling pathways responsible for this remodeling have not been identified with TAC.⁸ Evidence of increased TGF-β signaling has been suggested by increased phosphorylated (p) Smad2 levels in medial smooth muscle cells (SMCs). Parallel activation of extracellular signal–regulated kinase (ERK1/2) signaling is also present in this model. Inhibition of both TGF-β and ERK1/2 signaling decreased the extent of aortic dilatation with TAC.

Because Ang II can activate ERK1/2 signaling and increase expression of TGF-β in vascular SMCs, we sought to determine whether signaling through the AT₁ receptor was responsible for both the increased TGF-β and ERK1/2 signaling in the ascending aorta of the TAC mouse model.⁸ Our results indicate that inhibition of the AT₁ receptor using losartan significantly attenuated the vascular remodeling associated with TAC but did not completely block the increased TGF-β1 expression, adventitial Smad2 signaling, and collagen accumulation. Thus, these results help to delineate aortic TGF-β signaling that is dependent and independent of the AT₁ receptor after TAC.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**TAC Induces Ascending Aortic Dilatation and Remodeling of the Aortic Wall**

Twelve-week-old C57BL/6 male mice were subjected to an established TAC protocol (Figure IA in the online-only Data Supplement) that reproducibly causes left ventricular hypertrophy.⁷ Doppler analysis performed 1 week after TAC demonstrated that the right carotid artery flow velocity ratio was 5- to 10-fold higher than the left carotid artery.⁹ Echocardiography identified an increase in the ascending aortic diameter proximal to the aortic band of 23% (±2.5%) in TAC mice compared with sham-operated mice (referred to as controls), similar to the dilatation observed in previous studies (Figure 1A)⁸

Aortas from the TAC and control mice were harvested with all 3 layers of the aorta preserved (intima, media, and adventitia). Microscopic examination of these aortas revealed no evidence of dissecting aneurysms or intramural thrombus. At 2 weeks after TAC, we measured a significant increase in the thickness of both the adventitia and media of the ascending aorta compared with control aortas (Figure 1B and 1C). Histomorphometric analysis confirmed increased medial and adventitial area (P<0.05; Figure 1D). The cell density (total cell number divided by total area) did not significantly change in the medial layer, whereas the adventitial layer had a significant increase in cell density (Figure 1E).

To further analyze the aortic remodeling with TAC, various components of the ascending aorta were further characterized. Verhoeff-van Gieson staining of elastin showed no

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Transverse aortic constriction (TAC) induces ascending aortic dilatation and remodeling in mice. A, Representative echo-image of the ascending aorta (measurement indicated by lines). Top bar graph shows the flow velocity ratio of the right carotid artery (RC) vs the left carotid artery (LC) 1 week after TAC. The mean diameter of the ascending aortic lumen at 2 weeks after TAC is also shown. Sham indicates sham operated; and TAC, transverse aortic constriction. B, Hematoxylin and eosin (H&E) staining of representative cross-sections of mouse ascending aorta from mice 2 weeks after sham operation or TAC at 200× magnification. C, Bar graphs show total aortic wall thickness. D, Bar graphs show total medial area (left) and total adventitial area (right). E, Bar graphs show total medial cell density (left) and total adventitial cell density (right). Ascending aortic sections were from mice 2 weeks after being sham operated (white bar) or after TAC (black bar). n=5 per group. *P<0.05. **P<0.01.
elastin fiber fragmentation with TAC (Figure 2A). The percentage of elastin content in the medial layer was decreased with TAC, possibly because of the increased medial area or thinning of the elastin fibers ($P < 0.05$; Figure 2B). Sirius red staining identified increased collagen content in the ascending aorta at 2 weeks after TAC, which was most pronounced in the adventitial layer ($P < 0.05$; Figure 2C). Medial and adventitial thickness, as well as collagen and elastin content of the descending aorta, did not change after TAC (data not shown).

The cell density of adventitial layer in the ascending aorta increased after TAC when compared with controls. Immunostaining for the nuclear cell mitosis marker, phospho-histone H3 (Ser10) confirmed increased number of proliferating cells in the adventitial layer after TAC ($P < 0.05$; Figure 2D). The number of cells that express α-smooth muscle actin, a marker for myofibroblasts, was also significantly increased in the adventitial layer after TAC ($P < 0.05$; Figure 2E). Immunofluorescence using an antibody to fibroblast marker antibody, a marker for fibroblast, was significantly increased in the adventitia after TAC (Figure 2A, bottom). However, the percentage of cells that stained for phospho-histone H3 and α-smooth muscle actin in the medial layer of the ascending aorta did not significantly change ($P > 0.05$; Figure 2F and data not shown), suggesting that media or accumulation of matrix proteins between the elastin layers. These results suggest that adventitial thickening after TAC is driven in part by myofibroblast proliferation and collagen accumulation. It is notable that these histological changes with TAC are similar to the changes observed in the ascending aortic with Ang II infusion.3

We have previously shown that Ang II infusion in mice leads to aortic production of IL-6 and MCP-1, which are cytokines that promote vascular inflammation and macrophage recruitment in cooperation with adventitial fibroblasts.3 Quantitative polymerase chain reaction analysis of RNA harvested from the ascending aortas of TAC and control mice showed a 10-fold increase in IL-6 expression and a 15-fold increase in MCP-1 expression in TAC aortas ($P < 0.05$; Figure 3A; Figure II in the online-only Data Supplement). Immunostaining of aortic cross-sections from the ascending aorta demonstrated that the levels of IL-6 or MCP-1 were significantly increased in the adventitial and medial layers of TAC aortas compared with controls ($P < 0.05$; Figure 3B). Macrophage recruitment to the aortas was also confirmed through immunostaining with monoclonal antibodies to CD68 and macrophage/monocyte antibody monoclonal, and positively stained cells were detected predominantly in the adventitial layer (Figure 3C). Inflammatory cells produce proteases,5 and Mmp2 and Mmp9 expressions were increased by 10- and 9-fold, respectively, in TAC mice as compared with controls ($P < 0.05$; Figure 3D). Therefore, a local inflammatory response occurs in the ascending aorta after TAC that is similar to that observed with Ang II infusion.

Figure 2. Characterization of transverse aortic constriction-induced remodeling in mice. A, Representative cross-sections of mouse ascending aorta from sham-operated and TAC mice with Verhoeff-van Gieson staining (VVG; for elastin), Sirius red staining (for collagen), anti-α-smooth muscle actin staining (anti-α-SMA; for smooth muscle cells), anti-fibroblast marker antibody (ERTR7) staining (for adventitial fibroblasts), and antiphospho-histone H3 staining (anti-PH3; for proliferative cells). B–F, Bar graphs show the quantification of percentage of elastin area, adventitial collagen area, percentage of anti-α-SMA positive cells in the adventitia, and percentage of anti-PH3 positive cells in the media. Ascending aortic sections were from sham-operated mice (white bar) and TAC mice for 2 weeks (black bar). n=5 per group. *$P < 0.05$. **$P < 0.01$. 

[Image of Figure 2]
Inhibition of AT<sub>1</sub> Activation Attenuates Remodeling of the Aorta With TAC But Does Not Completely Block TGF-β1 Expression and Adventitial Smad2 Signaling

Because pressure-induced remodeling of the ascending aorta leads to histological changes similar to those observed with Ang II infusion, we sought to test the hypothesis that this remodeling is driven by the activation of AT<sub>1</sub> receptor by treating the TAC mice with the AT<sub>1</sub> blocker losartan. Losartan was administered by oral at a dose of 0.6 g/L in drinking water for 3 days before TAC, and mice were continued on oral therapy for 2 weeks after TAC. Doppler analysis demonstrated that there was no statistical difference in arterial flow velocity ratios between TAC animals in the treatment or placebo groups. Echocardiograms also showed that systolic flow velocities in the ascending aortas of TAC mice were 4.9- to 5.3-fold higher than those of sham-operated mice with or without losartan treatment. Systolic flow velocities in the ascending aortas of mice in the losartan treated and placebo groups were not significantly different (Figure 3B in the online-only Data Supplement).

Echocardiography revealed that losartan treatment attenuated ascending aortic dilatation associated with TAC (Figure 4, upper right side). Morphological and histological analysis revealed that losartan treatment also significantly attenuated ascending aortic wall thickness, medial hypertrophy, adventitial cellular hyperplasia, and collagen accumulation but did not block these histological changes completely (Figure 4). Losartan also significantly reduced the induction of Mcp1, IL6, Mmp2, and Mmp9 expression in TAC ascending aortas (Figure 3A and 3D; P <0.05). Inflammatory responses and macrophage recruitment, as determined by anti-IL6, MCP-1, CD68, and macrophage/monocyte antibody monoclonal staining, were also dramatically decreased in the adventitial layer of the ascending aorta after losartan treatment (Figure 3B and 3C). These data indicate that the inflammatory response after TAC was effectively inhibited when AT<sub>1</sub> receptor activation was blocked with losartan. However, losartan did not completely inhibit aortic enlargement or medial and adventitial thickening.

Because losartan treatment with TAC attenuated but did not completely prevent adventitial hyperplasia and collagen accumulation, we sought to determine whether TGF-β1 signaling independent of AT<sub>1</sub> activation could be responsible for these
changes. Tgfb2 (TGF-β2) expression increased with TAC, and this increase was effectively blocked by losartan treatment. However, Tgfb1 (TGF-β1) expression was also induced by TAC, and its expression was attenuated but not completely blocked by losartan (Figure 5A). Induction of Col1a1 and Col1a3 expression was also incompletely blocked in the TAC aortas with losartan treatment (Figure 5B). Immunoblot analysis showed increased levels of phosphorated (p) Smad2 in the ascending aortas of TAC mice. Immunostaining of ascending aortic tissues detected increased number of nuclear pSmad2-positive cells in both the medial and adventitial layers after TAC (as quantitated by percentage of pSmad2-positive cells in media divided by total media area; percentage of pSmad2-positive cells in adventitia divided by total adventitia area). Although losartan treatment dramatically decreased nuclear pSmad2 staining in the media, positive pSmad2-staining cells remained significantly more abundant in the adventitia in comparison with sham-operated controls (Figure 5D; P<0.05). These observations suggest that residual Tgfb1 expression and adventitial Smad2 signaling independent of AT1 receptor activation may account for some of the aortic histopathologic changes induced by TAC.

**Activation of AT1 Receptor With TAC**

Previous studies have demonstrated that losartan attenuated both canonical (Smad dependent) and noncanonical (predominantly ERK1/2) TGF-β-signaling cascades in a TGF-β- and AT1 receptor–dependent manner. To further characterize signaling through the AT1 receptor with pressure-induced aortic remodeling in mice, ascending aortic tissue was harvested 2 weeks after TAC, and Agtr1a (encoding AT1 receptor) expression was measured by quantitative polymerase chain reaction. Agtr1a message levels were elevated 3-fold 2 weeks after TAC in comparison with controls (Figure 5E; P<0.05). Inhibition of AT1 signaling by losartan treatment significantly decreased Agtr1a message levels in the TAC ascending aortas to levels similar to controls (Figure 5E; P<0.05). To determine the effect of mechanical stress induced by TAC on the activation of ERK1/2, a kinase that can be activated by AT1, immunoblotting was performed with antiphospho (p) ERK1/2 antibody. TAC significantly increased pERK1/2 levels in ascending aortic tissue, and this activation was significantly attenuated by losartan (Figure 5F; P<0.05).

Increased production of reactive oxygen species (ROS) via NAD(P)H oxidase has been shown to occur with ascending
aortic aneurysm formation after Ang II infusion. To determine whether ROS are increased in aortic SMCs in response to TAC, we performed in situ dihydroethidium staining using frozen ascending aortic sections from TAC and control animals. Dihydroethidium is an ROS-sensitive nuclear dye. Widespread and enhanced DHE staining was observed in the TAC ascending aortic regions compared with controls (Figure IIIA in the online-only Data Supplement). The intensity of DHE staining was significantly attenuated in TAC mice after losartan treatment and reduced to levels similar to control. Cyba (encoding p22-PHOX) encodes the α subunit of cytochrome b(-245), which is a component of the NAD(P)H oxidase that is markedly upregulated after Ang II infusion. Cyba expression was increased in TAC aortas compared with controls (Figure IIIB in the online-only Data Supplement; P<0.05), and losartan effectively reduced Cyba expression to that of controls (Figure IIIB in the online-only Data Supplement; P<0.05). Thus, pERK1/2 levels, ROS production, and Cyba expression are
increased in the ascending aorta after TAC and are reduced by losartan, supporting the hypothesis that AT1 receptor activation is central to the pathological changes that occur after TAC.

Discussion

Aortic remodeling after TAC is characterized by medial thickening, adventitial hyperplasia, and collagen deposition. In this study, we demonstrate that TAC leads to inflammatory changes in the ascending aorta that are similar to those observed with Ang II infusion, including increased expression of Il6 and Mcp1 and macrophage accumulation. Furthermore, our data also indicate that an AT1 receptor antagonist, losartan, effectively blocked these TAC-induced inflammatory changes. Because the observed inflammatory changes are similar to those associated with Ang II infusion and were effectively blocked by losartan treatment, these data support the conclusion that losartan effectively blocked AT1 signaling associated with TAC. Despite this evidence of effective AT1 blockade on TAC-induced inflammatory responses, other features of aortic remodeling associated with TAC were attenuated but not completely blocked with losartan treatment, including increased lumen diameter and thickening of the medial and adventitial layer. It is important to note that the dose of losartan used in this study was comparable to the dose used in other studies investigating the role of AT1 receptor in mice. Therefore, this model provides insight into AT1-dependent and independent aortic remodeling with TAC.

Our results indicate that Tgfb1 expression was attenuated but not completely blocked by losartan treatment. Similarly, molecular changes downstream of TGF-β signaling, such as myofibroblast proliferation and activation, Col1a1 and Col3a1 expression, adventitial collagen accumulation, and nuclear pSmad2 accumulation, were attenuated but not completely blocked by losartan. Induction of Tgfb2 expression and pSmad2 accumulation in the media were both effectively blocked by losartan, suggesting that these components of TGF-β signaling are dependent on AT1 activation. Activation of α-smooth muscle actin expression is an important marker of myofibroblast differentiation, which contributes to vascular remodeling through cell proliferation, cell migration, and synthesis of extracellular matrix proteins. Our data suggest that TAC-induced TGF-β1 expression and activation in adventitial fibroblasts may be responsible for myofibroblast differentiation, activation, and fibrosis in the adventitia that is independent of AT1 activation. It is important to note that losartan did attenuate adventitial myofibroblast activation and collagen deposition. Our data show that losartan may attenuate Ang II–driven monocyte recruitment and previous studies have shown that monocytes help drive fibroblast proliferation, myofibroblast activation, adventitial thickening, and cytokine production. Because losartan effectively blocked monocyte recruitment with TAC, this monocyte-driven component of myofibroblast activation was most likely blocked.

The observed AT1–independent, pressure-induced fibroblast to myofibroblast transition after TAC is similar to a well-characterized transition in response to mechanical stress. TGF-β1 is synthesized as a propeptide that binds to the latent TGF-β1–binding protein-1, which is part of the extracellular matrix. Transmission of forces on cell via integrin receptors leads to release and activation of latent TGF-β1 in the extracellular matrix, and the released TGF-β1 promotes the differentiation of fibroblasts to myofibroblasts. Therefore, AT1-independent myofibroblast transition and collagen deposition in the adventitial layer with TAC could result from locally increased TGF-β1 attributable to mechanical stress on the aortic wall. Increased expression of Tgfb1 was also identified in the aorta after TAC. The autocrine production of TGF-β by myofibroblasts has been shown to be important for proper wound healing, and the biomechanical forces could also increase autocrine production of TGF-β. The remodeling of vein grafts driven by increased hemodynamic stress is also characterized by increased Tgfb1 expression and myofibroblast activation in the adventitial layer.

Previous studies have shown that TGF-β–mediated ERK1/2 activation is the predominant driver of aneurysm progression in Marfan syndrome. ERK1/2 activation occurs after TAC, but we did not determine whether this activation is driven by AT1 versus TGF-β signaling. We did show that losartan effectively blocks TAC-induced ERK1/2 activation and nuclear pSmad2 accumulation in medial SMCs. Further studies are needed to determine whether ERK1/2 signaling is attributable to AT1 activation or downstream of AT1–driven TGF-β signaling. It is notable that exposure of SMCs to Ang II leads to rapid EKR1/2 activation. At the same time, Ang II increases TGF-β expression in SMCs, and Ang II can also lead to Smad signaling that is not dependent on TGF-β.

Our data indicate that activation of the AT1 receptor is responsible for many aspects of aortic remodeling induced by TAC. However, the molecular mechanism by which the AT1 receptor is activated by increased-intraluminal pressure is unknown. AT1 is a G-protein–coupled receptor. The β-arrestins (β-arrestin-1 and -2) function to regulate agonist-mediated G-protein–coupled receptor signaling by mediating both receptor desensitization and resensitization processes. Recent work suggests that load-induced membrane stretch on cardiomyocytes activates AT1 receptor signaling in a ligand-independent manner. This ligand-independent activation of the AT1 receptor occurs via transient receptor potential channels that depend on G protein coupling and the recruitment of β-arrestin to act as a biased agonist for the AT1 receptor. Mechanical stretch of cardiomyocytes also increases inward potassium currents, by ligand-independent mechanical activation of AT1 receptors. Therefore, the ligand-independent activation of the AT1 receptor after TAC could result from a similar mechanical activation attributable to the increased mechanical forces induced by the increased pulsatile stretch on aortic SMCs. Alternatively, the increased mechanical stress on SMCs could induce ROS formation and increase the sensitivity of SMCs to Ang II activation. AT1 activation could potentially further increase ROS production and AT1 activation in a feedback loop. Finally, local production of Ang II attributable to mechanical stretch may be augmented by increased SMC expression of renin–angiotensin pathways genes.

In summary, this study indicates that AT1 receptor activation plays a critical role in the ascending thoracic aortic remodeling that occurs because of increased biomechanical
stress associated with TAC (Figure IV in the online-only Data Supplement). This study also illustrates that pressure-induced aortic remodeling involves increased expression of Tgfb1 and Tgfb2, as well as activation of pSmad2. Activation of these TGF-β signaling components is dependent on AT1 receptor activation in the media but occurs partially independently of AT1 receptor activation in the adventitia. Furthermore, we have identified histopathologic and molecular changes in the medial versus the adventitial layer of the aorta that are differentially dependent on AT1 activation. These findings have important implications for understanding the role of AT1 and TGF-β signaling in the aorta with increased pressures.

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Disclosures

None.

References


Significance

Although hypertension is the most common risk factor for thoracic aortic diseases, it is not understood how increased pressures on the ascending aorta may lead to thoracic aortic aneurysms and aortic dissections. This is the first study to investigate the role of angiotensin II type 1 receptor activation in ascending aortic remodeling in response to increased biomechanical forces, using the transverse aortic constriction mouse model. Inhibition of the angiotensin II type 1 receptor significantly attenuated the vascular remodeling associated with thoracic aortic constriction but did not completely block increased transforming growth factor expression and signaling and collagen accumulation in the adventitial layer of the aorta.
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Supplemental Figure I. Confirmation of Transverse Aorta banding using echocardiogram Measurement.  

A. Schematic graph of mouse transverse aortic constriction (TAC).  
B. Representative echocardiograms showing the maximum systolic velocities in the ascending aorta of sham operated and 2 weeks of TAC mice with or without losartan treatment. These recordings demonstrate a successful banding.
Supplemental Figure II. Q-PCR analysis of IL-6 and Mcp-1 expression in ascending aortic tissues from sham-operated (white bars), TAC (black bars) and TAC mice treated with losartan for 2 weeks (grey bar). Gene expression levels were normalized to Dimt 1(18S rRNA). n = 5 per group, *, P<0.05.
Supplemental Figure III. TAC without and with losartan treatment alters angiotensin II type I receptor (AT₁) signaling and reactive oxygen species formation. A. In situ DHE staining. Transverse cryosections (7 µm) of ascending aorta were prepared from sham, 2 weeks of TAC and 2 weeks of losartan treated TAC mice and incubated in DHE (oxidation is shown in red). Nuclei were stained with DAPI (blue). Original magnification: 400X. B. Cyba expression levels by Q-PCR analysis in ascending aortic tissues from sham-operated (white bar), 2 weeks of TAC (black bar) and TAC with losartan treatment for 2 weeks (gray bar) mice. Gene expression levels were normalized to Gapdh (n = 5 per group). *, P<0.05.
Supplemental Figure IV. Summary of the role of Losartan in the transverse aortic constriction (TAC) induced ascending aortic remodeling.
Materials and Methods

Mice
Twelve week old male C57BL/6 mice were underwent TAC using a standard surgical protocol. Studies were performed 2 weeks after TAC, prior to the maladaptive cardiac failure associated with TAC. Animals were cared for according to the NIH Guide for the Care and Use of Laboratory Animals. All animal experiments were performed under protocols approved by the University of Texas Health Science Center at Houston in accordance with NIH guidelines.

Transverse Aortic Constriction (TAC)
All mouse experimental procedures were approved by the Institutional Animal Care and Use Committee of Medical Animal Resource Center, University of Texas Health Science Center at Houston. C57BL/6 male mice (10–12 weeks of age, 18–23 g, Jackson Laboratories, Bar Harbor, ME, USA) are used in the surgical preparation. Prior to anesthesia each animal receives a dosage of buprenorphine (0.1-2.5 mg/kg subcutaneous injection). The animal is then anesthetized using 2% isoflurane in 0.5 -1.0 L/min 100% oxygen. The neck and chest areas are prepared by shaving and removing hair, cleansing the skin with surgical soap and wiping with 70% ethanol. This procedure is repeated three times. Prior to surgery, all instruments are sterilized in a dry bead sterilizer. The anesthetized animal is placed in a supine position and a 5mm section of the trachea is carefully exposed by mid-neck incision and retraction of muscle tissue. This allows visualization for insertion of the endotracheal tube which is a polyethylene size 90 tubing beveled on the edge for ease of entrance through the larynx. The tongue is carefully manipulated as the endotracheal tube is inserted into the trachea with visibility through a dissecting microscope, viewing the trachea and entrance of the endotracheal tube. Once the proper position is confirmed, the endotracheal cannula is connected to a volume-cycled rodent ventilator (CWE, Inc.) which runs on supplemental 100% oxygen with a tidal volume approximately 0.15-0.25ml and a respiratory rate of 100-125 breaths per minute. Once steady breathing is established, an incision is made through the ventral chest skin to mid-thorax after which the thorax is opened to mid-sternum. This partial thoracotomy is followed by retracting the sternal edges with a retractor (fine science tools). The thymus is then retracted to expose the transverse aorta. Between the right innominate and left carotid artery, an aortic constriction is placed by tying a 6-0 suture black braided nonabsorbable silk suture against a 3mm length of 27 gauge needle (Supplemental Figure 1A). After two knots, the 27 gauge needle is promptly removed which yields a constriction of approximately 0.3mm as the outer diameter of the 27 gauge needle. This produces a 60-80% aortic constriction which can be double checked using the doppler flow option on the Vevo ultrasound machine. The outflow is then briefly (1-2s) pinched off on the respirator to allow re-inflation of the lungs. The retractor is removed and the ribs are drawn together and sutured using 5-0 Vicryl suture. Once the chest is closed, the outflow is briefly pinched off again to ensure proper breathing. The skin is then closed using 5-0 non-absorbable sutures, which will be removed within 10 days post-surgery. Once all sutures are in place, anesthesia is stopped and the animal is allowed to recover and will be removed from the ventilator. The sham group mice were performed the same operation but did not ligate the aorta. The animal will be monitored closely for any abnormal signs of pain or labored breathing before being returned to the animal room. In the case of any signs of pain, the animal will receive another dosage of buprenorphine (0.1-2.5 mg/kg subcutaneous injection) every 6-12 hours when needed.

Mouse Doppler echocardiography procedure
The successful TAC procedure was validated by Doppler Imaging. Specifically, the TAC procedure, which produces a 60-80% aortic constriction, was monitored for such constriction by a specially designed non-invasive Doppler flow probe. The flow characteristics indicate the magnitude of constriction; can be easily accessed by measuring the relative flow velocities in each of the carotid arteries, post-aortic constriction. This non-invasive Doppler procedure was performed 1 and 2 weeks post-TAC procedure. Only mice with a right carotid (RC)/left carotid (LC) flow ratio within a certain range were included for further analysis. For example, a moderate degree of pressure overload leads to a ratio of 5-8, whereas a tighter constriction resulting in severe pressure overload leads to a ratio of 8-10. A sham animal (operated but not ligated), however, is expected to have a ratio of ~1. Therefore, RC to LC flow velocity ratio reached a gradient greater than 5-10-fold higher by Doppler indicated a successful transverse constriction.

High resolution ultrasound was performed with a Vevo 770 imaging system using a 40 MHz 704 probe (VisualSonics, Toronto, Canada). Mice were anesthetized by inhalation of 1.5-4% isoflurane-oxygen mixture while echocardiography and respirations were continuously monitored on a warmed platform. The anterior chest was denuded using depilatory cream prior to application of ultrasound gel and imaging. The dimensions of the ascending aorta (in mm) were measured in a single plane using right parasternal views at four levels (annulus, sinuses of Valsalva, sinotubular junction and ascending aorta). The flow velocities in the aorta were recorded. After stabilization of the signal (1 min), peak systolic velocity (V, cm/sec) was recorded as the mean of three to five cardiac cycles. Images were then exported to Sante DICOM Editor software (SanteSoft LTD, Athens Greece) for further analysis. In all studies, at least five animals per group were used. All scanning and analysis was performed by an experienced ultrasound technician who was blinded to the mouse genotype.

**Histomorphometric study**

After Doppler study confirmed increase pressures and echocardiography assessed the ascending aortic diameter, animals were anesthetized and perfusion fixed with 5 ml of 1XPBS, followed by 5 ml of 4% paraformaldehyde for 3 min under physiological pressure. Ascending aorta, descending aorta, left and right carotid arteries were excised and further fixed overnight. The tissues were then embedded transversely in paraffin and cut at 5 µm thickness. Cross-sections were stained by haematoxylin and eosin (H&E) following standard protocol. For morphometric analyses, images of H&E stained cross-sections were be taken and recorded with a camera connected to a light microscope at 100x and 400X magnifications. The luminal, medial and adventitial areas of each cross-section were analyzed using Image J software. The medial area was defined as the area between the external and internal elastic lamina. Because the intimal thickness was not increased, the luminal area was the same as the internal elastic lamina area. The adventitia area was defined as the area between the external elastic lamina and the tunica externa, the outermost layer of the vessel. Sections were also stained with Sirius red to determine the collagen content and with Verhoeff-van Gieson elastin staining to detect the expression of elastin. For all these area measurements, two sections from a sample from one mouse were chosen at random. Five mice for each time point were analyzed and averaged.

**Immunohistochemistry and immunofluorescence**

Immunohistochemical staining was performed on paraffin-embedded sections with primary antibodies against Phospho-Histone H3 (PH3, Upstate) and Smooth muscle-α actin (α-SMA, Sigma-Aldrich), followed by biotinylated secondary antibody according to the manufacturer’s protocol. Staining was treated with peroxidase-conjugated biotin–avidin complex using VECTASTAIN ABC-AP kit (Vector Laboratories) and visualized by DAB (Vector Laboratories). Slides were counterstained with hematoxylin. For immunofluorescence staining, slides containing frozen mouse aortic cross sections (6-μm) were immediately fixed with 4%
paraformaldehyde for 30 minutes, blocked using 0.1% Triton-X, 5% normal serum of the species producing the highly cross-absorbed Alexa Fluor 568–conjugated secondary antibody (Invitrogen) for 30 minutes at 37°C and then incubated with primary antibodies at the following concentrations: 1:100 anti-CD68 (eBioscience), 1:100 anti-fibroblast ER-TR7 (Bachem), 1:50 anti–MCP-1 (ECE.2; Abcam), 1:200 anti–IL-6 (eBioscience), and 1:200 anti-macrophage MOMA-2 (Abcam). Incubations were performed for overnight at 4°C. After washing, secondary antibodies were added at a dilution of 1:200 for 1 hour at 37°C. Slides were then DAPI stained (Vector Laboratories).

**In situ DHE oxidation**
Aortas from sham- or TAC mice were dissected, OCT embedded, and frozen in a methybutane/ethanol/dry ice bath. Frozen sections (8-μm) were mounted on glass slides, rinsed in PBS, and incubated in 10 μm DHE (37°C, 30 minutes; Invitrogen). Then slides were DAPI stained, mounted, and photographed using a Texas red filter (488-nm excitation, 610-nm emission).

**RNA Extraction and Quantitative Real-time PCR**
RNA was extracted from each mouse ascending aortic tissue using Trizol (Invitrogen) according to the manufacturer’s protocol. 0.5 μg RNA was reverse transcribed using the High Capacity cDNA Archive Kit (Life Technologies) according to the manufacturer’s protocol. For quantitative real-time PCR analysis of mRNA expression, TaqMan probes were purchased from Applied Biosystems and analyzed using an Applied Biosystems Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. Experiments were performed in triplicate. All of the Q-PCR data in this study were replicated in independent assays with two different endogenous controls: Gapdh and D1mt 1(18S rRNA). Only Q-PCR data generated using the GAPDH control are shown.

**Immunoblot analyses**
Protein lysates were prepared from each mouse aortic tissue following standard protocol. Briefly, aortic tissues were homogenized and lysed in RIPA buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Protein (25μg) for each sample was separated on Tris–HCl gel (Bio-Rad, Hercules, CA) by SDS-PAGE, followed by transfer to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were immunoblotted with primary antibody and the appropriate horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA). Immunoblots blots were visualized by the enhanced chemiluminescence technique (GE Healthcare, Piscataway, NJ). The following dilution of antibody was used: total ERK (cell Signaling), 1:1000; anti-phosphorylated ERK (cell Signaling), 1:1000; anti-phosphorylated Smad2 (cell Signaling), 1:1000; anti-phosphorylated Smad3 (cell Signaling), 1:1000; anti-GAPDH (Fitzgerald Industries, Acton, MA), 1:2000.

**Losartan treatment**
Losartan Potassium (sc-204796A, Santa Cruz) was administered by oral at a dose of 0.6g/l in drinking water (n=6) for 3 days prior to TAC. Mice were continued on oral therapy for 2 weeks after TAC and then sacrificed.

**Statistical analysis**
Data are expressed as the means ± standard deviation. Statistical differences between the data at different time point were analyzed by a Student’s t-test. Morphometric analysis of aorta and carotid artery was done by one-way ANOVA. Differences were considered statistically significant at values of P <0.05.
References