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# Reversal of Aortic Enlargement Due to Increased Biomechanical Forces Requires AT1R Inhibition in Conjunction with AT2R Activation

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## Abstract

**Objective—**Pharmacological inhibition of the angiotensin II type 1 receptor (AT1R) with losartan can attenuate ascending aortic remodeling induced by transverse aortic constriction (TAC). In this study, we investigated the role of the angiotensin II type 2 receptor (AT2R) and Mas receptor (MasR) in TAC-induced ascending aortic dilation and remodeling.

**Approach and Results—**Wild-type C57BL/6J mice were subjected to sham or TAC surgeries in the presence and absence of various drugs. Aortic diameters were assessed by echocardiography and central blood pressure was measured in the ascending aorta two weeks post-operation, and histology and gene expression completed. An angiotensin converting enzyme inhibitor (ACEi), captopril, decreased systolic blood pressure to the same level as losartan, but did not attenuate aortic dilation, adventitial inflammation, medial collagen deposition, elastin breakage or *Mmp9* expression when compared with TAC mice. In contrast, co-administration of captopril with an AT2R agonist, compound 21, attenuated aortic dilation, medial collagen content, elastin breaks and *Mmp9* expression, whereas co-administration of captopril with a MasR agonist (AVE0991) did not reverse aortic dilation and lead to aberrant aortic remodeling. An AT2R antagonist, PD123319, reversed the protective effects of losartan in TAC mice. Treatment with compound 21 alone showed no effect on TAC-induced aortic enlargement, blood pressure, elastin breakage or *Mmp9* expression.

**Conclusions—**Our data indicate that when AT1R signaling is blocked, AT2R activation is a key modulator to prevent aortic dilation that occurs with TAC. These data suggest that ACEi may not be as effective as losartan for slowing aneurysm growth since losartan requires intact AT2R signaling to prevent aortic enlargement.

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**Keywords**

angiotensin II receptors; ascending aortic remodeling; biomechanical stress; transverse aortic constriction

## Introduction

Renin angiotensin system (RAS) plays pivotal role in regulating blood pressure and maintaining body water-electrolyte balance. Infusion of the predominant peptide hormone in RAS, angiotensin II (Ang II), can trigger aortic aneurysms in mice.[1] Ang II initiates its biological effects mainly through binding to Ang II type 1 receptor (AT1R) and type 2 receptor (AT2R). Another important peptide, Ang (1-7), is generated from angiotensin converting enzyme 2 (ACE2) proteolysis of Ang II and ACE proteolysis of Ang (1-9), and binds to Mas receptor (MasR). These receptors are widely expressed in the aortic endothelium, smooth muscle cells and macrophages.[2–6]

Ang II signaling has an established role in aortic aneurysm formation. Ang II infusion in hyperlipidemic mice induces suprarenal aortic aneurysms and ruptures. Ang II infusion at high doses in wild-type mice induces ascending aortic aneurysms.[7–9] In fact, increased Ang II level have been found both in plasma and ascending aortic tissues from patients with thoracic aortic aneurysm.[10] Additionally, smooth muscle cell-specific fibulin-4 gene knock-out (*Fbln4*$^{SMKO}$) mouse model has increased levels of Ang II in aortic tissue and thoracic aortic disease can be rescued in these mice by administration of the ACE inhibitor (ACEi), captopril.[11] The importance of Ang II signaling through the AT1R is supported by studies demonstrating that AT1R blockers (ARBs) decrease aortic dilation in mouse models[11–14] and patients with Marfan syndrome (MFS).[15–17] In contrast, AT2R inhibition exaggerates aortic disease in Ang II infused hyperlipidemic mice and AT2R gene (*Agtr2*) deletion augments aortic root enlargement in a Marfan mouse model (*Fbn1*$^{C1039G/+}$ mice).[18–20] Growing evidence shows a vasoprotective role of AT2R activation through inhibiting proliferation and inflammation of endothelial cells and vascular smooth muscle cells and promoting apoptosis in neointimal formation and restenosis models.[3, 4, 21, 22] A recent study tested the effect of a selective non-peptide AT2R agonist, compound 21 (C21),[23] on the aortic root enlargement in the *Fbn1*$^{C1039G/+}$ mouse model and showed no beneficial effect of C21 on the aortic aneurysm growth or aortic wall remodeling.[24]

Whether AT2R activation is beneficial in the attenuating aortic aneurysm growth due to hypertension has not been explored. Hypertension is the major risk factor for thoracic aortic disease in humans. We previously showed that signaling through the AT1R contributes to aortic remodeling in a mouse model of acute hypertension, resulting from transverse aortic constriction (TAC) in the arch between the innominate artery and left common carotid artery.[12] Two weeks after TAC, the ascending aorta enlarges and significant remodeling occurs characterized by aortic wall thickening due to medial and adventitial widening, along with increased expression of the extracellular matrix remodeling associated matrix metalloproteinase-9 (*Mmp9*). A prominent component of TAC-induced aortic remodeling is vascular inflammation characterized by increased recruitment of macrophages to the

adventitia layer of the aorta.[12] When losartan was administrated shortly before and during TAC, ascending aortic dilation, adventitial inflammation and collagen accumulation were all attenuated.

We sought to determine if increased AT1R signaling driving aortic enlargement and remodeling with TAC was due to increased Ang II levels and characterize the involvement of other Ang II receptor signaling in this aortic remodeling. We initially blocked Ang II production with an ACEi, captopril, and assessed aortic remodeling with TAC. In contrast to losartan, captopril did not attenuate TAC-induced aortic enlargement and remodeling. These results lead to additional studies to interrogate the role of AT2R and MasR activation in TAC-induced aortic enlargement and remodeling. Our current study reveals that AT2R is the key modulator in TAC-induced aortic enlargement when the AT1R signaling is blocked with losartan, and its effect is associated with decreased elastin breaks, medial collagen deposition and *Mmp9* expression but independent of the degree of TAC-induced hypertension or adventitial inflammation.

## Materials and Methods

The authors declare that all supporting data are available within the article and its online supplementary files.

All experimental procedures were designed in accordance with the National Institutes of Health guidelines and approved by the Animal Welfare Committee and the Center for Laboratory Animal Medicine and Care in the University of Texas Health Science Center at Houston. Ten to eleven-week-old male C57BL/6J wild-type mice were purchased from Jackson Laboratory, Bar Harbor, ME, USA. At the age of 12 weeks, mice weighing 23-31grams (27.5±1.9g) were randomized to treatment with or without various drugs in the drinking water for three days, then underwent TAC or sham operation. Treatment was continued for two weeks post-operation. Losartan (sc-204796A, Santa Cruz Biotechnology, 0.6g/L)[12], captopril (sc-200566A, Santa Cruz Biotechnology, 75mg/L)[11], AT2R agonist C21 (a gift from Vicore Pharma, 300μg/kg/day),[25] AT2R antagonist PD123319 (P186-10MG, Sigma-Aldrich, 3mg/kg/day)[20] and MasR agonist AVE0991 (a gift from Sanofi-Aventis, Frankfurt/Main, 576μg/kg/day)[26] were administrated 1 hour prior operation to two weeks post-operation via intraperitoneal injection. An illustration of the signaling pathways and the drug targets are presented in Supplemental figure 1A.

### TAC and Sham Surgery

Mice were anesthetized by 0.3-0.5L/min pure oxygen with 2% isoflurane and placed supine on 38°C heating pad. After intubation with a 22 gauge venous catheter connected to a rodent ventilator, the machine was set at a respiratory rate of 125-150 breaths/min with a tidal volume of 6-8μL/g, according to the body weight and respiratory depth of the animal. Ketoprofen (dose of 5 mg/kg) and bupivacaine (dose < 2.5 mg/kg) were administrated before an upper partial sternotomy incision (about 1cm) was made in the second intercostal space. A 6-0 silk suture was coiled under the aortic arch between the innominate artery and the left common carotid artery and ligated with a 27 gauge needle inserted into the aortic arch inside the ligation. The needle was then promptly removed in order to yield a constriction of

0.41mm in the outer diameter. Sham-operated mice underwent the same procedure but without ligation the suture around aortic arch. Lung was re-inflated before the skin was closed. By two weeks, the mice demonstrated evidence of mild heart failure as indicated by decreased stroke volume, ejection fraction and fraction shortening (Supplemental figure 1B to F).

## Echocardiography

Echocardiography measurement (Vevo 3100 imaging system (MX550D, 40MHz transducer, VisualSonics, Toronto, Canada) were performed 2 weeks post-operation. Briefly, mice were weighed and anesthetized by 0.5-1.0L/min room air with 2% isoflurane via nose cone. The heart rate was closely monitored and the body temperature was maintained around 38.5°C using the heating system. Mouse aortic root and ascending aorta were imaged in B-mode. Left ventricular function derived from short axis parasternal planes was imaged using M-mode. Three measurements were taken of maximal internal diameter at the aortic root and ascending aorta. Left ventricular function measurements were gained from three different cardiac cycles and averaged. Data were analyzed by operator blinded to the treatment groups.

## Invasive Blood Pressure Measurement

Following echocardiography analyses, intraluminal blood pressure measurements were performed using a Millar pressure catheter (SPR-1000, 1.0F, Oakville, Ontario, Canada) inserted into the right common carotid artery. Mice were intubated and placed on a ventilator using the same conditions as in TAC and sham surgery except replacing pure oxygen with room air. The 1.0F catheter was inserted into the ascending aorta to monitor the blood pressure. For 14-week-old wild-type C57BL/6J male mice, the estimated location of the insertion was 10-12mm from the distal bifurcation of right common carotid artery. Stable pressure tracings were recorded for 5 minutes at a PCU-2000 pressure signal conditioner and PowerLab 4/35 station (ADInstruments Inc., Colorado Springs, CO, USA), and systolic and diastolic blood pressure were averaged from the midterm 4 minutes record. After blood pressure measurements, the ascending aortas were harvested and randomly used for histology (formalin fixation) or RNA (flash frozen with liquid nitrogen). Note that 5% of the TAC mice died acutely after blocking the right common carotid artery with the blood pressure catheter and these blood pressure measurements were excluded from the analyses but the aortas were harvested for analyses.

## Histology

After intraperitoneal injection with Avertin (2.5%, 350mg/kg), animals were perfusion fixed with 20mL 1×phosphate buffered saline buffer (PH=7.4) followed by 20mL 10% formalin for 5 minutes through left ventricle under physiological pressure. Ascending aortas were harvested and further fixed in 10% formalin overnight, then embedded in paraffin and cut into 5μm thickness. Cross-sections were stained by hematoxylin and eosin (H&E) following standard protocol. For morphometric analyses, images of sections were taken and recorded with a camera connected to a light microscope at 40×, 100× and 400× magnifications. The whole aortic wall, medial and adventitial areas were analyzed using Image J software. The medial area was defined as the area between the external and internal elastic lamina. The

adventitial area was defined as the area between the external elastic lamina and the organized tissue in the the outermost area of the vessel. Medial elastin breaks and adventitial macrophage cell numbers were counted in the aortic segments of at least two nonconsecutive sections stained by Movat pentachrome stain (MPS-1, ScyTek Laboratories) and immunostaining using anti-Mac-2 antibodies (1ug/ml, CL8942B-3, Cedarlane), respectively. Sections were also stained with Sirius red (KTPSRPT, American MasterTech) to determine the collagen content and anti-CD45 immunostaining (1.25ug/ml, ab10558, Abcam). Alpha-smooth muscle actin staining (1:70,000, A2547, Sigma-Aldrich) was used to identify the smooth muscle cells in the media and myofibroblasts in the adventitia. Quantitative analyses were performed by three individuals blinded to the group information. Five to seven mice were analyzed for each group and averaged.

### RT-qPCR

Mice were perfused with 20mL ice incubated phosphate buffered saline (PH=7.4) for 5 minutes through left ventricle, and ascending aortic tissues were harvested, rapidly cleaned and flash frozen with liquid nitrogen. Trizol (15596026, Invitrogen) was utilized for the extraction of RNA. RNA was reverse transcribed using the cDNA Archive Kits (Life Technologies) following the manufacturer's protocol. TaqMan probes were purchased from Thermo Fisher Scientific and real-time PCR analyses were performed on a Roche LightCycler 96 System. *Gapdh* was used as endogenous control. Experiments were performed in triplicate and repeated three times with similar results.

### Statistical analysis

All data are expressed as mean ± standard deviation. Nonparametric statistical tests were conducted. Statistical differences between two groups were analyzed using unpaired Mann-Whitney analysis. For three or more groups, Kruskal–Wallis analysis was performed with Dunnett post-tests to compare between specific groups. Analyses were carried out using the GraphPad Prism 7.0.

## Results

### TAC-induced Aortic Enlargement in the absence of Ang II and activation of AT2R and MasR

We previously demonstrated that losartan could attenuate the enlargement and remodeling that occurs in the ascending aorta with TAC.[12] To determine if increased Ang II production was responsible for the AT1R activation with TAC, we treated twelve weeks old mice with the ACEi, captopril, 72 hours prior to TAC surgery and two weeks after surgery. Captopril failed to attenuate aortic root or ascending aortic enlargement when compared with TAC group , even though it significantly decreased the TAC-induced increase of systolic blood pressure (SBP, $p<0.001$, Figure 1A and B). To further evaluate the differences in TAC-induced aortic remodeling with exposure to captopril, macrophage accumulation in the adventitial aorta, collagen accumulation, elastin breaks and *Mmp9* expression were assessed.[12, 27] Captopril did not decrease any of these parameters (Figure 1C to F, Supplemental figure 2, 3 and 4). Based on these results, blocking Ang II production with an ACEi failed to block TAC-induced aortic enlargement and remodeling as effectively as selective inhibition of the AT1R receptor.

Captopril blocks not only signaling through AT1R, but also signaling through AT2R (Supplemental figure 1A). Furthermore, since captopril also prevents Ang (1-7) production, the MasR signaling is also blocked. As both the AT2R and MasR can block inflammation, proliferation, reactive oxygen production and apoptosis,[28, 29] one or both of these receptors could act in conjunction with blockade of the AT1R to prevent TAC-induced aortic enlargement. To characterize the role of the AT2R and MasR in the captopril-treated TAC mice, mice were treated with agonists for these receptors, C21 and AVE0991, respectively. Co-administration of captopril and C21 was able to reverse the aortic dilation (root, $p<0.001$; ascending aorta, $p<0.01$, Figure 1A), despite an increase in SBP in this group when compared with the captopril treated TAC mice. In contrast, captopril plus AVE0991 had no effect on the ascending aorta or aortic root enlargement compared with untreated or captopril-treated TAC mice (Figure 1A); SBP was increased to a similar level as in the captopril plus C21 TAC mice (Figure 1B). As previously reported, captopril preserved heart function after TAC,[30] and this protection was maintained when either AVE0991 or C21 treatment was added to the captopril treatment (Supplemental figure 5). Even though a lower dose of AVE0991 (576μg/kg/day) was used in the current study than other mouse studies, [31, 32] captopril plus AVE0991 completely prevented macrophage infiltration into the aortic adventitia compared with TAC alone or captopril-treated TAC mice (Figure 1C and D), whereas captopril plus C21 augmented adventitial inflammatory response when compared with TAC only mice ($p<0.05$, Figure 1C and D). Captopril plus C21 significantly reduced elastin breaks in the media layer (Figure 1C and E) and decreased *Mmp9* expression ($p<0.01$, Figure 1F) in the TAC mice, while captopril plus AVE0991 did not alter these parameters compared with TAC only mice.

Interestingly, co-treatment of captopril and AVE0991 completely blocked TAC-induced adventitial macrophage infiltration but there was an accumulation of cells still present in the adventitia (Figure 1C and D). CD45 staining and smooth muscle α-actin staining showed that the recruited adventitial cells were not leukocytes or myofibroblasts (Supplemental figure 2). Additionally, there was a significant increase of collagen accumulation in the adventitial layer and decrease of smooth muscle α-actin in the medial layer when compared with sham operated mice (Supplemental figure 2 and 3).

## Further analyses of the role of the AT2R in TAC-induced aortic remodeling

We sought to further characterize the role of AT2R in TAC-induced aortic remodeling. Since the non-peptide specific AT2R antagonist, PD123319, prevents AT2R signaling effectively in aortic disease mouse model,[20] TAC mice were treated with both losartan and PD123319. PD123319 reversed the beneficial effects of losartan on the aortic dilation, adventitial inflammation, medial collagen deposition, elastin breaks and also *Mmp9* expression (Figure 2A, C, D, E and F, Supplemental figure 4). Surprisingly, PD123319 abolished the anti-hypertensive effect of losartan and increased SBP from 140.3mmHg (losartan group) to 186.1mmHg ($p<0.001$), which was a higher level than the untreated TAC group ($p<0.05$, Figure 2B). These treatments did not alter heart function when compared to the TAC treated mice (Supplemental figure 6).These results reveal that AT2R activation acts in conjunction with AT1R blockade in preventing TAC-associated aortic enlargement and remodeling. To test the hypothesis that stimulation of AT2R alone might be beneficial in preventing aortic

remodeling in TAC mice, we treated TAC mice with C21 alone. Unexpectedly, C21 showed no anti-hypertensive effect in TAC mice and pathological aortic remodeling and dilation still occurred with C21 treatment (Figure 3A, B, C, E and F). Heart function was minimal decreased in C21-treated TAC mice when compared to TAC alone (Supplemental figure 7). Interestingly, C21 alone significant decreased adventitial inflammation and medial collagen accumulation associated with TAC ($p<0.01$, Figure 3C and D, Supplemental figure 4).

## Discussion

TAC-induced ascending aortic dilation and remodeling could be attenuated with AT1R blockade, losartan.[12] In this study, we demonstrate that AT1R blocking agents do not prevent TAC-induced aortic enlargement and remodeling unless AT2R signaling is activated based on the following: (1) captopril is not as effective as losartan in TAC-induced aortic remodeling but captopril plus C21 treatment significantly attenuates aortic enlargement and decreases *Mmp9* expression; (2) TAC-induced remodeling of the aorta is attenuated with losartan treatment but this rescue is reversed when AT2R signaling is blocked with PD123319. Our results are consistent with other studies that also illustrated the importance of AT2R signaling preservation when the AT1R is blocked in other aortic disease mouse models. When the $Agtr2^{-/-}$ mouse was crossed into the $Fbn1^{C1039G/+}$ mouse model, the loss of *Agtr2* significantly augmented aortic root enlargement, and losartan treatment had minimal effect on these double mutant mice.[19] Other studies have also shown that AT2R inhibition could reverse protective effects of ARBs in cardiovascular system. Long-term treatment with losartan resulted in relaxation of isolated aortic rings from spontaneously hypertensive rats (SHR) due to AT2R-mediated nitric oxide (NO) production, and this vasorelaxation was abrogated by PD123319.[33] In old SHR, cardiac remodeling, perivascular fibrosis and aortic hypertrophy were attenuated by the ARB candesartan and these effects were reversed by PD123319.[34] Interestingly, a recent study showed C21 treatment had no additional benefit in terms of blocking aortic enlargement when given with losartan treatment in the $Fbn1^{C1039G/+}$ mice.[24] In contrast to our data, the administration of an ACEi, enalapril, plus C21 was also not effective in blocking aortic enlargement in this mouse model. These differences could potentially be due to a higher dose of C21 (0.5mg/kg/day) used in that study than the dose for these studies (0.3mg/kg/day in our study). Taken together, these results show the importance of maintaining AT2R signaling with AT1R blockade to prevent aortic enlargement.

Our data on hypertensive remodeling, along with previous studies of aortic enlargement occurring with Ang II infusion or in the $Acta2^{-/-}$ mice, indicate that activation of AT1R plays a role in aortic enlargement. Similar to our results, Ang II-induced suprarenal aortic enlargement is increased with AT2R deficiency or PD123319 treatment in wild-type mice.[18] In the same study, PD123319 augmented Ang II-induced abdominal aortic aneurysms in both wild-type and AT2R deficient mice, but AT2R deficiency and PD123319 treatment had no enhanced effect on Ang II-induced thoracic aortic aneurysms or atherosclerosis. These results suggest that PD123319 treatment in the absence of AT2R had different effects in the thoracic versus abdominal aorta. At the same time, we cannot rule out that PD123319 reverses the beneficial effects of losartan with TAC due to off target properties beyond blocking AT2R.

Similar to the results in the *Fbn1*$^{C1039G/+}$ mouse model, augmentation of AT2R signaling alone did not prevent TAC-induced aortic enlargement despite the fact that C21 decreased adventitial inflammation. C21 has a beneficial effect in cardiovascular diseases by reducing inflammation. C21 significantly decreases plasma MCP-1 and several pro-inflammatory markers in myocardial infarction model in rats,[28] and prevents endothelial inflammation and leukocyte adhesion in a high fat diet-induced vascular inflammation in apolipoprotein E knockout mice.[4] C21 inhibits nuclear factor-kappa B (NF-κB) activity and decreases *Mcp1* and *Il6* expression when dermal fibroblasts and endothelial cells are exposed to TNF-α, and pre-treatment of PD123319 abolishes this effect.[21] C21 also decreases inflammatory response and NF-κB activation in a femoral artery injury mouse model via AT2R-mediated increase in PPARγ activity.[3] Although C21 is ineffective in blocking aortic enlargement associated with TAC or in the *Fbn1*$^{C1039G/+}$ mouse model, C21 may block aortic enlargement in the *Acta2* deficient mice based on its effect in blocking NF-κB signaling.[14] In this mouse, increased basal NF-κB signaling augments the expression of the AT1R, making the aortic SMCs sensitive to a 100-fold lower dose of exogenous Ang II. Losartan attenuates aortic enlargement in the *Acta2*$^{-/-}$ mice, suggesting that AT1R signaling is in part responsible for aortic dilatation. Since NF-κB signaling can trigger increased AT1R expression, and C21 blocks NF-κB signaling, this mouse model could potentially be responsive to C21 treatment.[14] Excitingly, a recent study indicates that decreasing NF-κB signaling is one of the targets of C21 that prevents elastase-induced abdominal aortic aneurysm.[35] Moreover, our data reveals that systemic AT2R activation with C21 seems to play no role in lowering systolic blood pressure, which is consistent with data showing that C21 has no anti-hypertensive effect in SHR.[36] Further studies show that C21 decreases blood pressure only under nonphysiological conditions, such as female deoxycorticosterone acetate-salt hypertensive rat[37] or anaesthetic SHR.[23] However, neither short-term nor long-term systemic administration of AT2R agonist shows any anti-hypertensive effect in multiple studies reviewed recently.[38]

Losartan has been shown to reduce aortic dilation in MFS patients, making it a good alternative choice to β-blocker, which have been considered the standard treatment for MFS patients for over 20 years.[16, 39] ARBs also prevent aortic enlargement in a number of mouse models, including *Acta2*$^{-/-}$ mice,[14] *Fbn1*$^{C1039G/+}$ mice,[13, 19, 24] *Fbln4*$^{SMKO}$ mice[11] and TAC mice.[12] However, the continued suppression of endothelium-dependent NO pathway in losartan-treated the *Fbn1*$^{C1039G/+}$ mice implies a potential failure in long-term trials.[40] And a clinical trial shows losartan has no additional benefit in limiting aortic root enlargement compared with placebo to β-blocker therapy in MFS patients.[41] Moreover, losartan treatment in a large clinical trial showed higher risk of aortic-annulus enlargement in MFS patients (p=0.002), and non-significant but more aortic events in 3 years follow up when compared with atenolol therapy (p=0.10).[16] ARBs have been used to block fibrosis,[42] and losartan was effective in blocking TAC-induced collagen accumulation in the media but not the adventitia in this study. Losartan treatment in *Fbn1*$^{C1039G/+}$ mice and MFS clinical trials were designed to assess only aortic growth, not progression to dissection. Therefore, whether the decreased aortic collagen accumulation associated with losartan treatment identified in this study impacts the risk for aortic dissection needs to be addressed.

A limitation of current study is the use of TAC to model hypertensive aortic remodeling in mice. Since the acute increases of intraluminal biomechanical stress induced by TAC could only reproduce part of the progression of thoracic aortic disease, specifically TAC leads to aneurysm formation but not dissection, these results do not address of the role of these receptors in aortic dissection. TAC also alters cardiac function over time (Supplemental figure 1). In our study, the most significant change associated with the various drug treatments was fact the captopril, with or without C21, improved cardiac function but decrease blood pressure (Supplemental figure 5). At the same time, captopril plus C21 blocked aortic enlargement whereas captopril alone did not, thus despite these hemodynamic changes, our results indicate a role for AT2R activation in attenuating aortic growth when AT1R is blocked. Finally, losartan prevents aortic root enlargement in *Fbn1*$^{C1039G/+}$ mice, but crossing the *Agtr1a*$^{-/-}$ mice into the *Fbn1*$^{C1039G/+}$ mice does not prevent aortic root dilation.[43] Thus, our results using drugs to manipulate the RAS receptors maybe not be replicated with genetic manipulation of these same receptors.

Interestingly, our study finds significant and unique pathological changes in aortas from captopril plus AVE0991-treated TAC mice. Macrophage accumulation in the adventitia is defined as one of the main features associated with vascular remodeling,[44] along with aortic dilation,[45] and both can be attenuated by losartan in the TAC mice.[12] It has been shown Ang II infusion-induced thoracic and suprarenal aortic dissection is due to IL-6/MCP-1 mediated adventitial inflammation, and further *in vitro* experiment via co-culture of monocytes and aortic adventitial fibroblasts-produced conditional medium show exaggerate differentiation of monocytes into macrophages.[46] These results reveal leukocyte/macrophage-fibroblast interaction enhances vascular inflammation and may contribute to aortic destabilization. However, in present study, co-treatment of captopril and MasR agonist blocked adventitial inflammation without affecting adventitial collagen deposition (Supplemental figure 2 and 3) when compared with TAC mice. Furthermore, there was an accumulation of cells in the adventitial layer that were not leukocytes or myofibroblasts, and possibly fibroblasts. These findings are notable because the aortic remodeling is distinctly different than that observed with alterations of AT1R or AT2R signaling.

In summary, our results provide evidence that AT2R signaling is required for AT1R blockade to attenuate aortic enlargement and remodeling that occurs with increased biomechanical forces in TAC mice. We further show that the impact of AT2R on aortic remodeling is independent of its anti-hypertensive or anti-inflammatory effects but correlated with elastin breaks and *Mmp9* expression. These findings have important implications as to which drugs should be used to slow or prevent aortic enlargement. Based on our data, ACEi may not be as effective as β-adrenergic blocking agents or losartan for slowing aortic growth and losartan needs signaling through the AT2R to work optimally in preventing aortic enlargement.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

Z.Z. and A.M.P. designed the study, performed TAC surgery, echocardiography measurement, Millar measurement, data analysis and wrote the manuscript; S.W. and E.A. contributed to the Millar measurement, histology analysis and data analysis; A.J. performed qPCR analyses; J.C., P.Z. and C.S.K. contributed to data analysis and discussion; D.M.M. conceptualized the project, secured funding, supervised the work and assembled the manuscript. All authors read and approved the manuscript in its final form.

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## Nonstandard Abbreviations and Acronyms

| | |
|---|---|
| **TAC** | transverse aortic constriction |
| **RAS** | renin angiotensin system |
| **Ang II** | angiotensin II |
| **AT1R** | angiotensin II type 1 receptor |
| **AT2R** | angiotensin II type 2 receptor |
| **MasR** | Mas receptor |
| **ACEi** | angiotensin converting enzyme inhibitor |
| **ARB** | angiotensin II type 1 receptor blocker |
| **C21** | compound 21 |
| **SBP** | systolic blood pressure |
| **SHR** | spontaneously hypertensive rat |
| **IL6** | interleukin-6 |
| **MCP1** | monocyte chemoattractant protein-1 |
| **MMP2** | matrix metalloproteinase-2 |
| **MMP9** | matrix metalloproteinase-9 |

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## Highlights

- Captopril is not as effective as losartan in attenuating the aortic dilation and remodeling that occurs with acute increases in blood pressure in the ascending aorta due to transverse aortic constriction (TAC), but captopril plus C21 treatment significantly prohibits the pathological changes in the ascending aorta.
- TAC-induced remodeling of the aorta is rescued by losartan treatment but completely reversed to baseline while AT2R signaling is blocked with PD123319.
- Augmentation of AT2R signaling alone did not prevent TAC-induced aortic enlargement despite the fact that C21 decreased adventitial inflammation.
- Co-treatment of captopril and MasR agonist completely blocked TAC-induced adventitial macrophage infiltration without affecting aortic enlargement and remodeling when compared with untreated TAC mice.

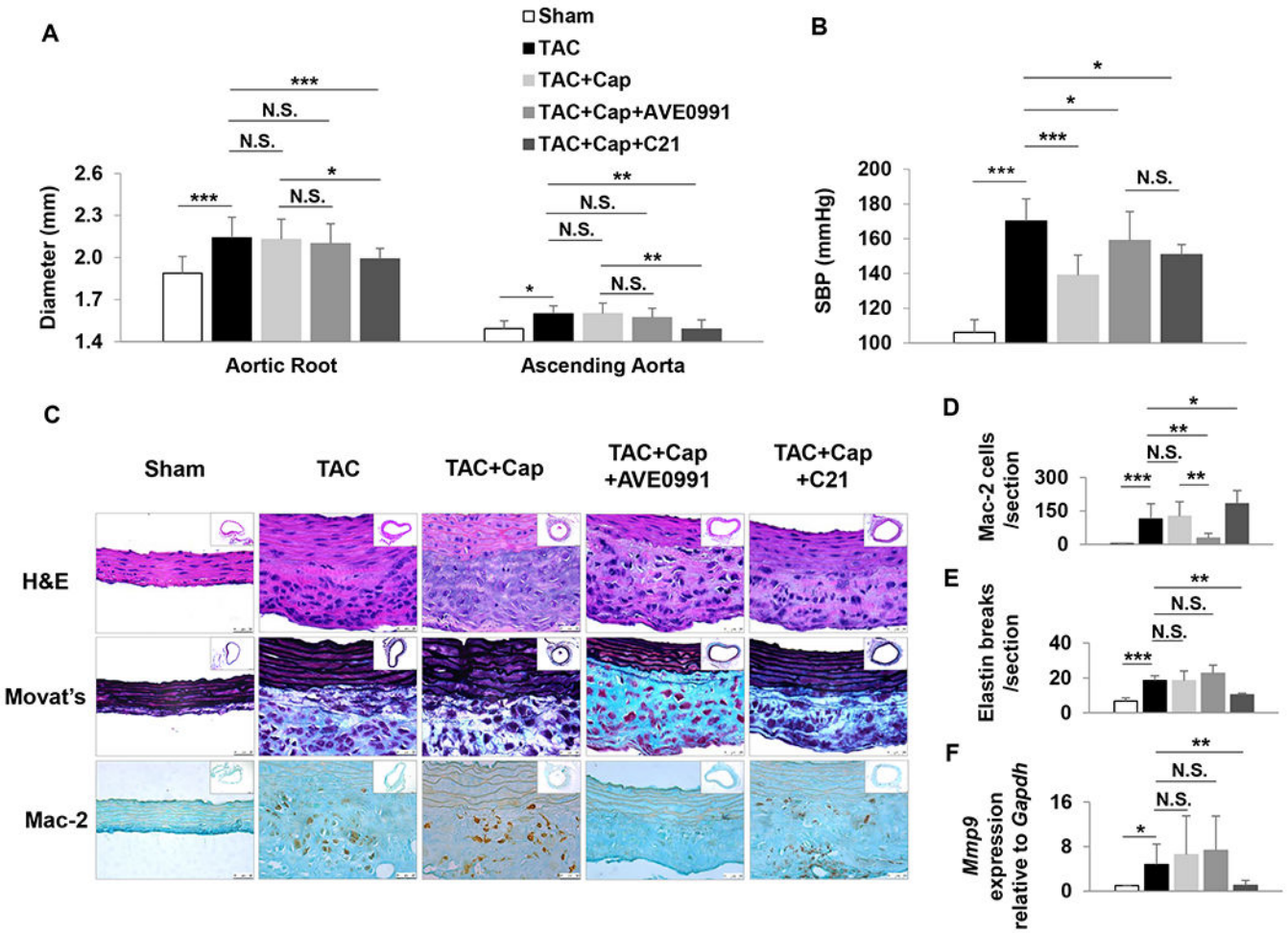


**Figure 1.**

Effects of captopril, captopril plus AVE0991 or C21 on TAC-induced aortic dilation, blood pressure, adventitial macrophage infiltration, elastin breaks and *Mmp9* mRNA expression. (**A**) TAC-induced aortic root and ascending aortic dilation could be rescued by captopril plus C21, but not captopril or captopril plus AVE0991. (**B**) C21 and AVE0991 increased systolic blood pressure in the context of captopril treatment to similar levels compared with captopril in TAC mice, via Millar catheterization measurement. (**C**) Representative images of H&E, Movat's and Mac-2 staining of sections from the ascending aortas. (**D**) Quantitative analysis of Mac-2 positive cells in sections from the ascending aortic adventitia. (**E**) Quantitative analysis of elastin breaks in sections from the ascending aortic medial layer via Movat's staining. (**F**) *Mmp9* mRNA expression in the ascending aortas. N≥5 of each group in **A** to **E**. N≥3 of each group in **F**. TAC, transverse aortic constriction; Cap, captopril; C21, compound 21; SBP, systolic blood pressure; N.S., non-significant. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

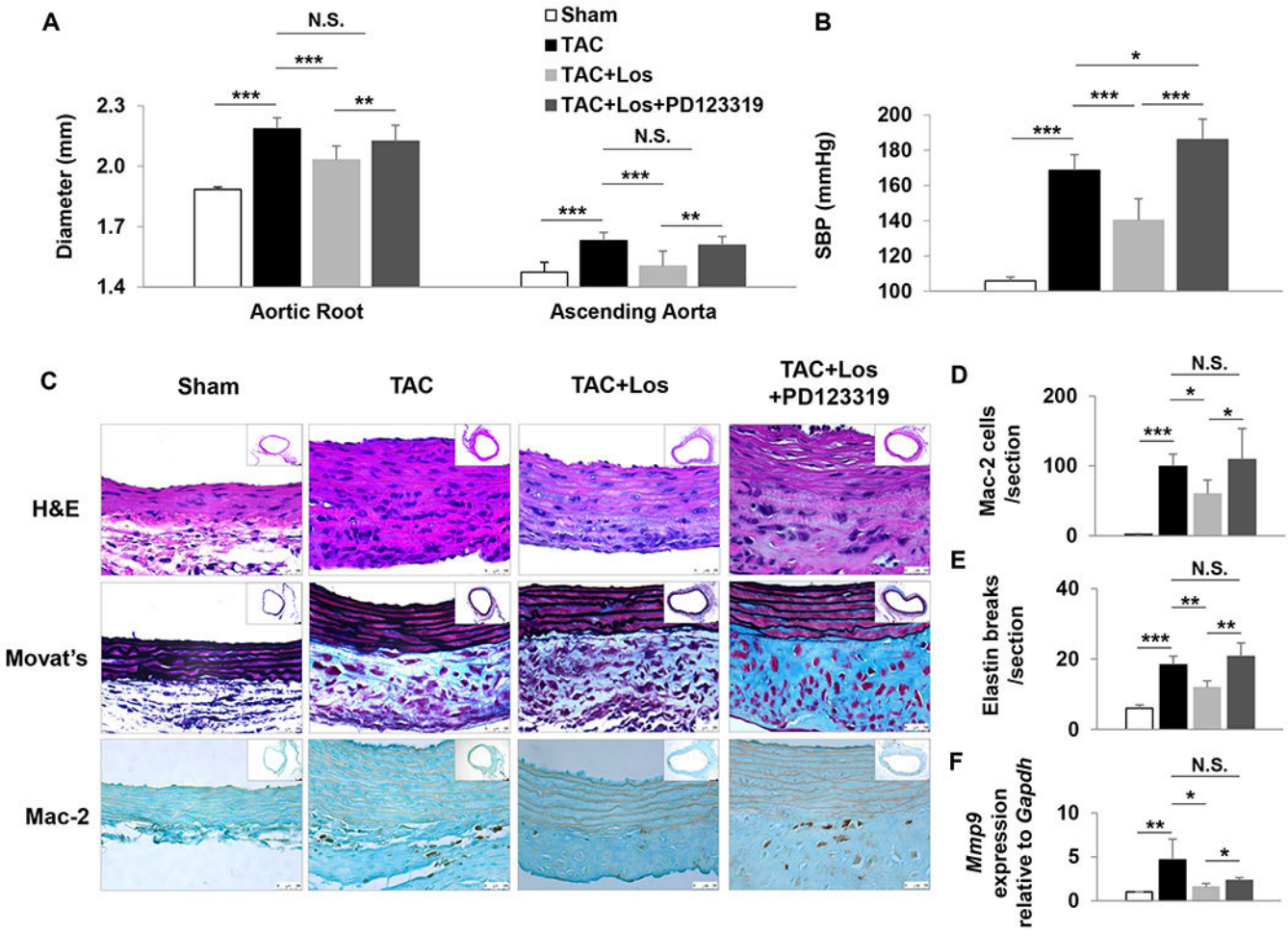


**Figure 2.**

Effects of losartan, losartan plus PD123319 on TAC-induced aortic dilation, blood pressure, adventitial macrophage infiltration, elastin breaks and *Mmp9* mRNA expression. PD123319 abolished anti-dilatory (**A**) and anti-hypertensive (**B**) effects of losartan in TAC mice. (**C**) Representative images of H&E, Movat's and Mac-2 staining of sections from the ascending aortas. (**D**) Quantitative analysis of Mac-2 positive cells in sections from the ascending aortic adventitia. (**E**) Quantitative analysis of elastin breaks in sections from the ascending aortic medial layer via Movat's staining. (**F**) *Mmp9* mRNA expression in the ascending aortas. N≥5 of each group in **A** to **E**. N≥3 of each group in **F**. TAC, transverse aortic constriction; Los, losartan; SBP, systolic blood pressure. N.S., non-significant. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

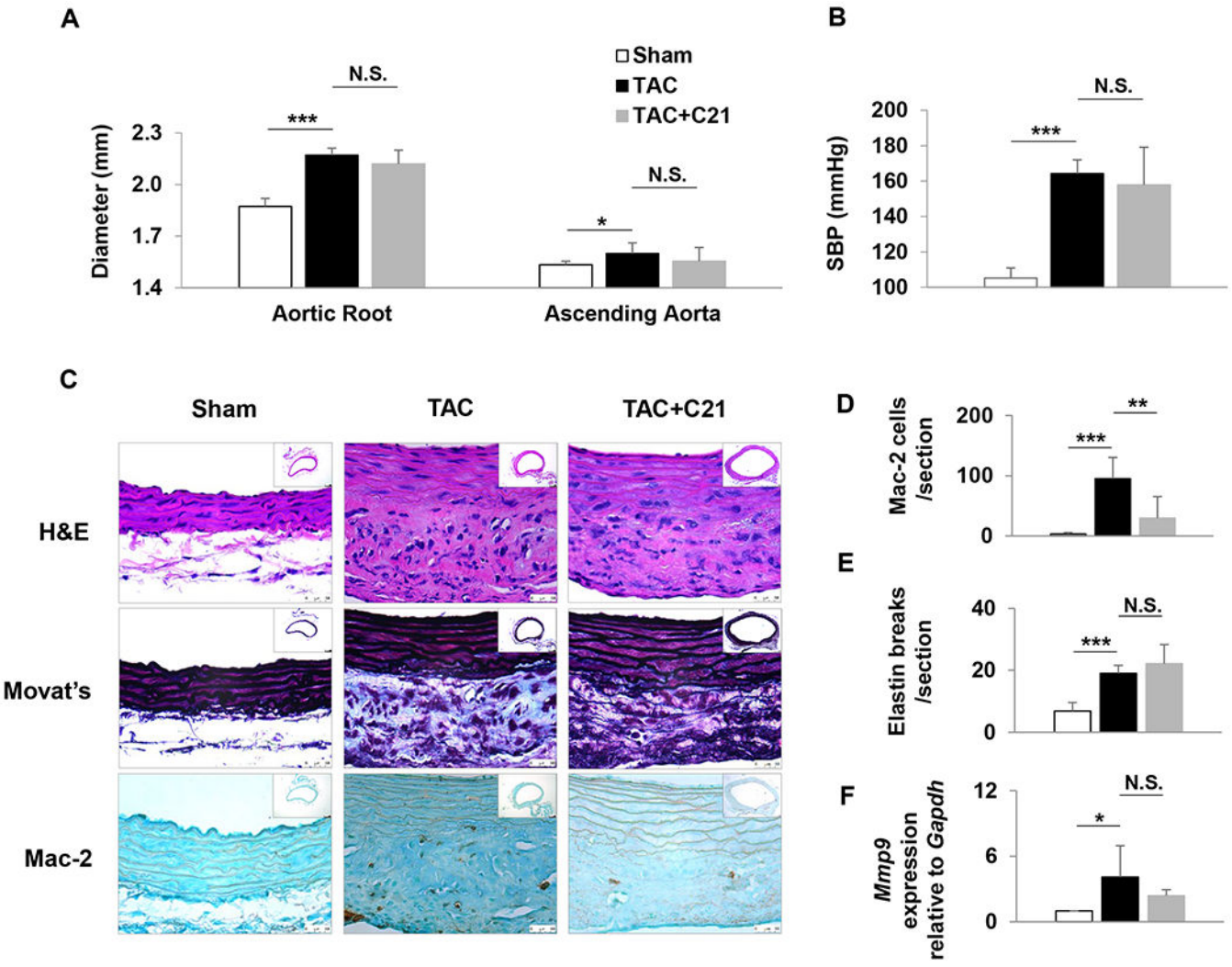


**Figure 3.**

Effects of C21 on TAC-induced aortic dilation, blood pressure, adventitial macrophage infiltration, elastin breaks and *Mmp9* mRNA expression. C21 treatment showed no effect on TAC-induced aortic enlargement (**A**) despite significantly inhibiting adventitial macrophage infiltration (**D**) when compared with untreated TAC mice. (**B**) C21 did not significantly decrease the blood pressure in TAC mice. (**C**) Representative images of H&E, Movat's and Mac-2 staining of sections from the ascending aortas. (**D**) Quantitative analysis of Mac-2 positive cells in sections from the ascending aortic adventitia. (**E**) Quantitative analysis of elastin breaks in sections from the ascending aortic medial layer via Movat's staining. (**F**) *Mmp9* mRNA expression in the ascending aortas. N≥5 of each group in **A** to **E**. N≥3 of each group in **F**. TAC, transverse aortic constriction; C21, compound 21; SBP, systolic blood pressure; N.S., non-significant. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.