Angiotensin II Type 1A Receptors in Vascular Smooth Muscle Cells Do Not Influence Aortic Remodeling in Hypertension

Matthew A. Sparks, Kelly K. Parsons, Johannes Stegbauer, Susan B. Gurley, Anuradha Vivekanandan-Giri, Christopher N. Fortner, Jay Snouwaert, Eric W. Raasch, Robert C. Griffiths, Timothy A.J. Haystead, Thu H. Le, Subramaniam Pennathur, Beverly Koller, Thomas M. Coffman

Abstract—Vascular injury and remodeling are common pathological sequelae of hypertension. Previous studies have suggested that the renin-angiotensin system acting through the type I angiotensin II (AT1) receptor promotes vascular pathology in hypertension. To study the role of AT1 receptors in this process, we generated mice with cell-specific deletion of AT1 receptors in vascular smooth muscle cells using Cre/Loxp technology. We crossed the SM22α-Cre transgenic mouse line expressing Cre recombinase in smooth muscle cells with a mouse line bearing a conditional allele of the Agtr1a gene (Agtr1a^floxl^), encoding the major murine AT1 receptor isoform (AT1A). In SM22α-Cre^+Agtr1a^floxl^ (SMKO) mice, AT1A receptors were efficiently deleted from vascular smooth muscle cells in larger vessels but not from resistance vessels such as preglomerular arterioles. Thus, vasoconstrictor responses to angiotensin II were preserved in SMKO mice. To induce hypertensive vascular remodeling, mice were continuously infused with angiotensin II for 4 weeks. During infusion of angiotensin II, blood pressures increased significantly and to a similar extent in SMKO and control mice. In control mice, there was evidence of vascular oxidative stress indicated by enhanced nitrated tyrosine residues in segments of aorta; this was significantly attenuated in SMKO mice. Despite these differences in oxidative stress, the extent of aortic medial expansion induced by angiotensin II infusion was virtually identical in both groups. Thus, vascular AT1A receptors promote oxidative stress in the aortic wall but are not required for remodeling in angiotensin II–dependent hypertension. (Hypertension. 2011;57:00-00.)  ● Online Data Supplement

Key Words: angiotensin II • hypertrophy • hyperplasia • aorta • smooth muscle • hypertension

The renin-angiotensin system (RAS) is a principal regulator of blood pressure homeostasis; dysregulation of this system commonly contributes to human hypertension. Accordingly, pharmacological inhibitors of the RAS, including angiotensin converting enzyme inhibitors and angiotensin receptor blockers, can effectively lower blood pressure in a significant proportion of patients with essential hypertension. Moreover, these agents also attenuate end-organ damage, decreasing cardiovascular morbidity, and slowing the progression of chronic kidney injury. It has been suggested that RAS inhibitors provide protection against complications of hypertension beyond their effects to lower blood pressure, indicating nonhemodynamic, cellular actions of angiotensin II to promote tissue damage. However, in some clinical trials, end-organ protection by RAS inhibition has been accompanied by more effective reduction of blood pressure. Moreover, studies in animal models have suggested that the antihypertensive actions of RAS inhibitors are critical for preventing cardiac hypertrophy and progressive kidney injury.

The vascular system is a major target of damage in hypertension. Expansion of arteries and arterioles in the kidney, also called nephrosclerosis, is the most common renal pathological lesion accompanying hypertension and is an important cause of chronic kidney disease in blacks. Vessel remodeling with changes in compliance is also seen in the aorta and other vascular beds in hypertension, in which the RAS has potent actions to influence vascular structure and function. For example, angiotensin II causes systemic vasoconstriction by activa-
tion of angiotensin II type 1 (AT₁) receptors in vascular smooth muscle cells (VSMCs). Along with their effects on vascular tone, AT₁ receptors may also stimulate growth and hypertrophy of VSMCs, thereby directly contributing to vascular remodeling in hypertension. It has been suggested that nonhemodynamic actions of AT₁ receptors, including enhanced generation of reactive oxygen species (ROS), may promote changes in vascular structure that perpetuate the development of hypertension. Further, angiotensin II receptor blockers reverse vascular remodeling in patients with hypertension, suggesting a direct role for vascular AT₁ receptors in this process.

Nonetheless, the precise role of AT₁ receptors in individual tissues is difficult to discern through experiments using pharmacological inhibitors or conventional gene targeting, in which actions of AT₁ receptors are abrogated in all tissues, and changes in blood pressure may further confound interpretations. Accordingly, we generated mice with cell-specific deletion of AT₁A receptors from smooth muscle cells in conduit vessels that are subject to hypertensive remodeling. During angiotensin II–dependent hypertension, we find reduced oxidative stress in vascular segments lacking AT₁A receptors, but vascular remodeling is unaffected.

**Methods**

**Generation of Experimental Animals**

A mouse line with a conditional Agtr1a allele was generated using homologous recombination in embryonic stem cells as described (Gurlay et al, unpublished data; also see online data supplement, available at http://hyper.ahajournals.org). SM22α-Cre mice were purchased from The Jackson Laboratory (stock No. 004746). Mice were bred and maintained in the Association for Assessment and Accreditation of Laboratory Animal Care–accredited animal facilities at the Durham VA Medical Center according to National Institutes of Health guidelines. All of the animal studies were approved by the Durham Veterans Affairs Medical Center Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals had free access to standard rodent chow and water unless specified. Eight- to 12-week-old male mice and littermate controls were used for experiments.

**Isolation of Preglomerular Vessels for Receptor Expression Analysis**

Afferent arterioles and interlobular arteries were isolated from kidneys using a modified iron oxide–sieving technique according to Chatziantoniou et al. The enriched preparation of preglomerular arterioles and arterioles was transferred to a tube containing RNA later and stored at 4°C for 24 hours, then at −80°C.

**Measurement of AT₁A mRNA Levels and RT-PCR**

Relative levels of mRNA for the AT₁A receptor in various tissues were determined by real-time RT-PCR with the ABI Prism 7700 sequence determination system as described. Tissues were harvested, and total RNA was determined using TRI Reagent (Sigma-Aldrich) per manufacturer instructions. The number of copies of the PCR template in the starting sample is calculated using the Sequence Detector Software incorporated in the ABI Prism 7700 Sequence Detector System. For each experimental sample, the amounts of the target and of the endogenous control were determined from the appropriate standard curves or relative values were determined using the ΔΔCT method (Applied Biosystems).

**Isometric Force Measurements in Aortic and Mesenteric Rings**

Aortic and mesenteric artery rings were mounted in a wire myograph as described previously. Dose-response curves were generated for phenylephrine and angiotensin II. Forces are expressed as a percentage of the maximal response to phenylephrine, and this was equivalent between groups.

**Assessment of Acute Vasoconstrictor Responses**

Our previous studies showed that vasoconstrictor responses to acute administration of angiotensin II are almost completely extinguished in mice with complete AT₁A receptor deficiency. Therefore, to determine the veracity of the deletion of vascular AT₁A receptors, we examined acute pressor responses to angiotensin II as described previously. At 5-minute intervals, increasing doses (0.1, 1, and 10 µg/kg) of angiotensin II (Sigma-Aldrich) or 10 µg/kg of epinephrine (Sigma-Aldrich) were injected intravenously while intra-arterial pressures were continuously monitored.

**Blood Pressure Measurements in Conscious Mice**

Blood pressures were measured in 8- to 12-week-old male conscious SM22α-Cre Agtr1a flox/flox (SMKO) and control mice using radiotelemetry, as described previously. During the measurement period, mice were housed in a monitoring room where quiet is maintained and no other experiments are performed. Arterial blood pressures were collected, stored, and analyzed using Dataquest A. R. T. software (version 4.0; Transom Medical). Measurements were recorded over a 10-second interval every 5 minutes at baseline and during 21 days, where angiotensin II was infused chronically (1000 ng/kg/min) by osmotic mini-pump (Alzet).

**Analysis of Oxidized Amino Acids in Aortae**

After 28 days of angiotensin II infusion, thoracic aortae were dissected from control and SMKO mice and immediately placed in antioxidant buffer (100 µmol/L diethylen tetramino pentaacetic acid (metal chelator), 50 µmol/L butylated hydroxytoluene (lipid-soluble antioxidant), 10 µL/mL protease inhibitor (Halt Protease Inhibitor Cocktail; Pierce) in 50 mmol/L sodium phosphate buffer, pH 7.4) at −80°C. Amino acids were isolated from the acid hydrolysate using a solid-phase column (Supelclean ENVI ChromP column; Supelco Inc.) as described. Oxidized amino acids were quantified by liquid chromatography-electrospray ionization tandem mass spectrometry using multiple reaction monitoring mode. Under these chromatography conditions, authentic compounds and isotopically labeled standards were baseline separated and exhibited retention times identical to those of analytes derived from tissue samples. 3-nitrotyrosine and dityrosine were detected by characteristic liquid chromatography–electrospray ionization tandem mass spectrometry using multiple reaction monitoring mode. The ratio of the peak areas of the analyte with corresponding 13C internal standard were used to quantify levels of oxidized amino acids in tissue. Results are normalized to protein content of tyrosine, the precursor of 3-nitrotyrosine and dityrosine.

**Measurement of Hydrogen Peroxide Production in Aortae**

Hydrogen peroxide (H₂O₂) of freshly prepared thoracic aorta from both SMKO and control mice (n = 3 for both) were measured with the Amplex Red H₂O₂ Assay Kit (Molecular Probes). Thoracic aortae were harvested, and adventitial tissue was dissected free in ice-cold Krebs-Henseleit buffer. After opening the aorta with scissors and washing out the blood, the aorta was incubated in the reaction mixture for 60 minutes in the dark at 37°C. The supernatant was then read in a fluorescent spectrophotometry according to the protocol provided by the manufacturer. The fluorescent values were normalized to the protein content measured in each sample (BioRad).

**Measurement of Medial Thickness, Medial Area, and Luminal Area of Aortae**

The extent of vascular pathology was assessed by measuring medial thickness of descending thoracic aorta. Two centimeters of descending aorta was dissected in animals that were fixed and perfused with
4% paraformaldehyde and placed in 10% formalin overnight. Tenmicrometer sections were obtained after paraffin embedding. Sections were stained with hematoxalin and eosiin, and photographs were taken at 40× and 10× (Zeiss Axio Imager; QImaging MicroPublisher 5.0 MP color camera). Medial thickness, medial area, and luminal area of the aorta were measured from 4 random sections throughout the specimen and quantified using MetaMorph in a blinded fashion.

**Statistical Analysis**

The values for each parameter within a group are expressed as mean±SEM. For comparisons between control and SMKO groups, statistical significance was assessed using an unpaired 2-tailed Student t test. A paired 2-tailed Student t test was used for comparisons within groups. P values <0.05 were considered significant. For blood pressure tracings measured over multiple days, comparisons within groups.

**Results**

**Generation of Mice With Deletion of AT1 Receptors From Smooth Muscle**

We performed successive intercrosses between the SM22α-Cre line and mice homozygous for the conditional “floxed” Agtr1a allele (Agtr1a<sup>flox/flox</sup>) to generate SMKO mice and SM22α-Cre<sup>−/−</sup>Agtr1a<sup>flox/flox</sup> (control) mice for experiments. To confirm elimination of AT1α receptors from various vascular beds, levels of expression for AT1α receptor mRNA were measured by real-time RT-PCR. Segments of aorta were isolated from SMKO and control mice, and the adventitia and endothelium were removed by dissection. As shown in Figure 1A, mRNA for the AT1α receptor was easily detected in aortae from control mice but not from SMKO mice (<i>P</i>&lt;0.0005). Similarly, AT1α mRNA expression in mesenteric arteries, with intact endothelium and adventitia, was decreased by 60% in SMKO compared with control mice (<i>P</i>&lt;0.05; Figure 1A). Thus, in SMKO mice, AT1α receptors are efficiently eliminated from VSMCs in conduit vessels. Further, no difference was seen in the relatively low levels of AT1β receptor expression in aorta between the groups (Figure 1B).

**Vasoconstrictor Responses Measured Ex Vivo**

To functionally verify the efficiency of deletion of the AT1α receptor from VSMCs, the contractile response of isolated vessels was assessed ex vivo. Isometric force was first measured after exposure to phenylephrine and then was independently measured to angiotensin II. Forces are expressed as a percentage of the maximal response to phenylephrine, which was equivalent between groups. As shown in Figure 2, the contractile response to angiotensin II was significantly reduced by ~75% in aortae from the SMKO compared with control mice (<i>P</i>&lt;0.0005) consistent with the absence of AT1α receptor mRNA (Figure 1A). Similarly, there was a corresponding reduction of ~65% in the mesenteric artery segments from SMKO compared with control mice (<i>P</i>&lt;0.05; Figure 2).

**AT1α mRNA Expression in Resistance Arteries**

To examine AT1α receptor expression in a preparation of resistance arteries, preglomerular arterioles were isolated from kidneys using the iron oxide–sieving technique. Based on microscopic examination (Figure 3A) and augmented mRNA expression of VSMC markers such as α-smooth muscle actin (Figure 3B), there was marked enrichment for preglomerular vessels using this approach. However, unlike results with the aorta or mesenteric arteries, expression of AT1α mRNA in preglomerular arterioles isolated from SMKO mice was preserved at levels that were not significantly different from controls, indicating lack of efficient excision of the floxed Agtr1a gene in these segments (<i>P</i>=NS; Figure 3C).

**Acute Responses to Vasoconstrictors**

We next compared acute vasoconstrictor responses in SMKO and control mice in vivo. As shown in Figure 4, we observed robust acute vasoconstriction in the controls in response to escalating doses of angiotensin II from 0.1 to 10 μg/kg. The magnitude of vasoconstriction was dose proportional and virtually identical in SMKO and control mice. Preservation of a normal vasoconstrictor response to angiotensin II in the SMKO mice is consistent with their unmodified expression of AT1α receptors in resistance vessels (Figure 3C).
Blood Pressure Homeostasis Is Not Affected in SMKO Mice

Radiotelemetry units were implanted into 8- to 12-week-old male SMKO and control mice to measure blood pressure in the conscious, unrestrained state. Mean arterial pressures measured over 5 days were virtually identical between SMKO and control mice fed a normal-salt (0.4% NaCl) diet (116 ± 1100 mm Hg; n = 11 in each group). Moreover, diurnal variation of blood pressure was not affected in SMKO mice (data not shown).

Blood Pressure Responses in Angiotensin II–Dependent Hypertension

The preceding studies suggest that AT1A receptors are effectively deleted from VSMCs in the aorta in SMKO mice, but their blood pressure responses to angiotensin II are preserved. Thus, we reasoned that the SMKO mice would be a useful model for separating the relative contributions of hypertension from direct actions of AT1A receptors in VSMCs to aortic remodeling. To induce vascular remodeling, osmotic mini-pumps were implanted subcutaneously to infuse angiotensin II at 100 ng/kg/min while blood pressures were continuously monitored. As shown in Figure 5, the blood pressure responses to chronic angiotensin II infusion were very similar in the SMKO and control mice. Mean arterial pressure increased significantly by 30 mm Hg in both groups and remained elevated to an equivalent extent throughout the period of the infusion. Further, there was no difference in MAP between the groups averaged for the duration of angiotensin II administration (157 ± 153 mm Hg).

Vascular Oxidative Stress

To examine levels of oxidative stress specifically in the vasculature, we assessed oxidation of proteins in the vascular wall. To this end, we isolated vascular wall proteins from thoracic aortic segments of control and SMKO mice. After hydrolyzing the proteins with acid, the samples were subjected to western blot analysis.

Figure 2. VSMC-specific AT1A receptor deletion leads to diminished acute vascular angiotensin II responses in conduit vessels ex vivo. Isometric force was measured in abdominal aorta and mesenteric artery ex vivo in response to increasing doses of phenylephrine, followed by 100 nmol/L angiotensin II. Forces are shown as a percentage of the maximal response to phenylephrine, which was equal between groups. The contractile response to angiotensin II was reduced significantly in both the abdominal aorta (* P < 0.05 SMKO mice; n = 5 vs control mice; n = 5) and mesenteric artery (# P < 0.01 SMKO mice; n = 6 vs control mice; n = 5).

Figure 4. Intact response to acute vascular angiotensin II (Ang II) response in vivo. Acute vasoconstrictor responses to angiotensin II and epinephrine were measured in anesthetized mice. Blood pressure was measured continuously while increasing doses of angiotensin II (0.1 to 10 µg/kg) were administered at 5- to 10-minute intervals. No difference was seen in peak pressor response at any dose between SMKO and control mice (P = NS at 0.1 µg/kg, 1 µg/kg, and 10 µg/kg doses; n = 6 for both groups). MAP indicates mean arterial pressure.
We then determined the content of the 2 oxidized amino acids, 3-nitrotyrosine and dityrosine, “molecular signatures” characteristic of peroxynitrite-mediated oxidation, by isotope dilution liquid chromatography tandem mass spectrometry. As shown in Figure 6A and 6B, increased levels of both 3-nitrotyrosine and dityrosine were detected in aortic segments from control mice after angiotensin II infusion. This increase was significantly attenuated in SMKO mice by ∼50% for dityrosine (137±27 versus 74±10 μmol/mol tyrosine; \( P<0.05 \)) by ∼80% for 3-nitrotyrosine (7819±1676 versus 1768±450 μmol/mol tyrosine; \( P<0.005 \)). To examine ROS generation in the vasculature, we measured local production of hydrogen peroxide using amplex red in freshly prepared thoracic aorta from control and SMKO mice at baseline. H₂O₂ generation was significantly lower in aortic segments from SMKO mice (0.59±0.4 μmol/L/mg protein) than in control mice (4.72±1.1 μmol/L/mg protein; \( P<0.05 \)). Together, these data indicate that oxidative stress in the aortic wall is significantly attenuated in the SMKO mice.

Vascular Responses in Angiotensin II–Dependent Hypertension

To determine the extent of vascular remodeling associated with the angiotensin II infusion, medial thickness and medial-to-luminal area ratio of thoracic aortic sections were measured by morphometry. As shown in Figure 7, there were no differences between control and SMKO mice at baseline, suggesting that the absence of AT₁A receptors in VSMCs does not significantly impact normal development and structure of the aorta. After 4 weeks of angiotensin II infusion, there was significant remodeling of the aorta in control mice, reflected by an increase in medial thickness from 27.7±1.9 μm at baseline to 50.5±2.4 μm (Figure 7E; \( P<0.0005 \)) and an increase in medial-to-lumen ratio from 0.38±0.08 at baseline to 0.67±0.03 (Figure 7F; \( P<0.05 \)). Similar increases in medial expansion (26.6±2.9 μm versus 47±4 μm; \( P<0.005 \)) and medial-to-lumen ratio (0.36±0.07 versus 0.62±0.05; \( P<0.05 \)) were seen in the SMKO mice with angiotensin II infusion, such that after angiotensin II infusion, these parameters were virtually identical in SMKO and control mice (Figure 7C–7F).

Discussion

Vascular remodeling and injury are typical features of end-organ damage from hypertension, contributing to clinical morbidity and mortality.¹⁴ In the kidney, vascular lesions, interstitial fibrosis, and arteriosclerosis are the defining characteristics of hypertensive nephrosclerosis, a common cause of chronic kidney disease.¹² In larger vessels such as the aorta, vascular remodeling in hypertension produces changes in compliance resulting in increased pulse pressure, which has been associated with enhanced cardiovascular risk.³²⁻³³ Medial thickening of the carotid artery has been similarly
associated with increased cardiovascular risk and is commonly used as a surrogate marker for vascular outcomes in clinical trials.34

The RAS is a major determinant of vascular function and pathology.35 For example, angiotensin II acting through the AT1 angiotensin receptor causes potent vasoconstriction.16 This vasoconstrictor response is mediated by activation of AT1 receptors in VSMCs, triggering increased intracellular calcium leading to myosin phosphorylation.36 Along with these physiological effects, activation of the RAS also promotes vascular remodeling in patients with hypertension.18 In smaller vessels, these structural changes can have hemodynamic consequences.18,37 Further, activation of the RAS may also impact the development of atherosclerosis38 and aortic aneurysms.39 Many of the vascular consequences of AT1 receptor activation seem to emanate from direct effects in VSMCs, but these have largely been characterized in cultured cell systems.40,41 The precise contribution of AT1 receptors in VSMCs to vascular physiology and pathology in vivo has been difficult to define because pharmacological antagonists or conventional gene knockouts lower blood pressure and produce broad inhibition of AT1 receptors across all tissues, including VSMCs. Accordingly, to examine their actions in isolation in the intact animal, we developed a mouse model to eliminate expression of AT1A receptors specifically in smooth muscle using Cre-Loxp technology.

To excise the floxed Agtr1a allele from smooth muscle, we used the Sm22α-Cre transgenic mouse line expressing Cre recombinase under control of the promoter of the Sm22α gene.42 We found that AT1A receptors were efficiently deleted from aorta and early branches of the mesenteric arteries in SMKO mice (Figure 1). However, there was little or no excision from resistance vessels, reflected by preserved acute pressor responses to angiotensin II (Figure 4) and normal levels of AT1A receptor mRNA expression in preglomerular vessels isolated from SMKO mice (Figure 3C). Further, the extent of excision from small resistance arteries could not be appreciably enhanced when the Sm22α-H9251-Cre transgene was crossed onto an Agtr1aflx/null background (data not shown).

Although previous studies have suggested that the Sm22α-Cre transgene was crossed onto an Agtr1aflx/null background (data not shown). Previous studies have suggested that the Sm22α-Cre transgene was crossed onto an Agtr1aflx-null background (data not shown). Although previous studies have suggested that the Sm22α-Cre transgene was crossed onto an Agtr1aflx/null background (data not shown).

One of the key pathways linked to AT1 receptors in VSMCs is the generation of ROS.46 In this regard, AT1...
receptors activate NADPH oxidases (Nox1 and Nox4) in VSMCs, generating ROS such as superoxide anion and H$_2$O$_2$, which may contribute to the pathogenesis of hypertension. Further, stimulation of ROS production by AT$_1$ receptors in cultured VSMCs has been associated with cellular hypertrophy. To examine the capacity of AT$_{1A}$ receptors to promote oxidative stress in vivo, we compared indices of oxidative stress between SMKO and control mice before and during chronic angiotensin II infusion. At baseline, we found reduced levels of hydrogen peroxide production by isolated aortic segments from SMKO compared with control mice. In addition, we found evidence for marked reductions in the levels of oxidized tyrosine residues (Figure 6). Excess O$_2^-$ produced by NOX enzymes or uncoupled endothelial nitric oxide synthase can form H$_2$O$_2$ or react with NO to form the highly reactive oxidant peroxynitrite. Along with contributing to oxidative stress, this reaction extinguishes the beneficial actions of NO in the vasculature. Moreover, peroxynitrite can oxidize tyrosine residues in the vascular wall, and this covalent alteration provides a footprint to assess the extent of oxidative stress over time. Our findings of decreased 3-nitrotyrosine and dityrosine in SMKO mice after angiotensin II administration are consistent with decreased peroxynitrite formation by either of these mechanisms.

Together, our data indicate a key role for AT$_1$ receptors in VSMCs to promote oxidative stress, as evidenced by the profound reduction in 3-nitrotyrosine and dityrosine in hypertension, independent of any concomitant effects of elevated blood pressure per se. As discussed above, there exists ample evidence suggesting that generation of ROS by AT$_1$ receptor activation in VSMCs may have direct consequences on vascular structure and function. In this regard, several studies have shown that administration of potent antioxidant agents can attenuate angiotensin II–dependent hypertension. Our studies suggest that reduced oxidative stress in large conduit arteries alone is not sufficient to lower blood pressure. Instead, this may require more robust ROS inhibition in resistance arteries, the central nervous system, or the kidney. Moreover, the absence of AT$_{1A}$ receptors and the dramatic diminution of oxidative stress in the aortic wall of the SMKO mice did not result in detectable attenuation of medial hypertrophy compared with control mice (Figure 7). This indicates that direct actions of AT$_{1A}$ receptors in VSMCs, including generation of oxidative stress, are not required to induce aortic remodeling in this setting, and it is consistent with previous reports describing dissociations between levels of ROS generation and cardiac or aortic remodeling.

Because the severity of hypertension was similar in SMKO and control mice, we suggest that elevated blood pressure is the major mechanism driving medial expansion in this setting. This is in line with previous studies by our group and others documenting the dominant actions of blood pressure to drive end-organ damage in the heart and vasculature.

**Perspectives**

Other factors, such as elevated blood pressure, may play a dominant role in hypertensive vascular remodeling because the extent of hypertension was very similar between the groups in our study. Alternatively, AT$_1$ receptor actions, including ROS generation, in other cell lineages such as endothelium or circulating inflammatory cells may have significant roles in hypertensive vascular remodeling. Further, our studies do not rule out the possibility that pathways linked to AT$_1$ receptors in VSMCs may contribute to the pathogenesis of more complex vascular lesions such as atherosclerosis or aneurysms, in which oxidative stress has also been implicated.

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**Disclosures**

None.

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CHBPR: AT₁A RECEPTORS IN VASCULAR SMOOTH MUSCLE CELLS DO NOT INFLUENCE AORTIC REMODELING IN HYPERTENSION

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SUPPLEMENT MATERIALS AND METHODS

Generation of experimental animals- A mouse line with a conditional Agtr1a allele was generated using homologous recombination in embryonic stem cells.\(^1\) Two loxp sites were placed in positions flanking exon 3 of the Agtr1a gene (Supplemental Figure 1A), which contains the entire protein encoding sequence, 3' untranslated region, and poly-adenylation signal, such that Cre-mediated recombination renders the gene completely non-functional. The targeting vector was introduced into ES cells by electroporation as described previously\(^2\) and the transfected ES cells were cultured in media containing G418 for positive selection. Correctly modified ES cells were identified by Southern analysis. Prior to injection of the correctly modified ES cell lines into blastocysts, the neomycin cassette was removed by transient transfection with plasmid vectors expressing Flp recombinase (pCAGGS-Flpe-IRES) and CMV-GFP. GFP-expressing cells were identified, sorted by FACS, colonies plated, expanded and split into duplicate 12-well plates. Clones that had undergone the appropriate Flp-mediated recombination with removal of the neomycin cassette were identified by Southern analysis (supplemental Figure 1D). Appropriately modified ES cell lines were then expanded and injected into blastocysts to generate chimeras. Germ-line transmitting chimeras were bred with wild-type C57Bl/6 mice to generate inbred C57Bl/6 Agtr1aflox/+ mice. In order to delete AT1a-receptors from VSMCs, we used a Sm22α-Cre transgenic mouse wherein Cre expression is driven by the Sm22α promoter.\(^3\)

X-galactosidase staining- In order to verify appropriate, tissue-specific expression of Cre recombinase, SM22α-Cre transgenic mice were inter-crossed with Rosa26-lacZ reporter mice. Tissues, except mesentery, were harvested and fixed in formalin and sectioned at 10µm. Mesentery was cleaned in xylenes and mounted with Feramount. Tissues were stained for β-galactosidase activity using a LacZ Staining Kit (InvivoGen) according to manufacturer’s instructions. As shown in Supplemental Figure 2A-C, X-gal stained tissues from SM22α-Cre+/Rosa26-lacZ+ mice demonstrated robust LacZ-staining in smooth muscle cells in the aorta, as well as mesenteric and medium-sized branches of the renal artery, compared to the absence of any specific staining in Cre- controls or in non-smooth muscle cell lineages (supplemental Figure 2D-F).

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**Supplemental Figure S1. Generation of mice with a conditional Agtr1a allele.** (A) Targeting vector containing PGK-Neo cassette for positive selection with loxp sites engineered in positions flanking exon 3 of the Agtr1a gene (black triangles). FRT sites are depicted by open triangles and novel PstI and EcoRV recognition sites are shown. Probes A and B were used for Southern analysis. (B) Correctly targeted Agtr1a<sup>flox</sup> allele after exposure to Flp recombinase to remove PGK-Neo cassette. (C) Null allele is generated after exposure of Agtr1a<sup>flox</sup> to Cre recombinase. Positions of diagnostic PCR fragments are shown. (D) Confirmation of genomic structure of Agtr1a<sup>flox</sup> allele by Southern analysis. Genomic DNA from ES cell clones was digested with BamH1 and probed with Probe B. The 8.5 kb band is the endogenous Agtr1a locus. The 4.3 kb band is the targeted floxed locus containing neo. The 6.25 kb band is the targeted floxed locus with neo removed by Flp. *Indicates clones with correct rearrangement. Chimeric mice were generated from correctly targeted ES lines using standard techniques.
Supplemental Figure S2. Verification of smooth muscle specific Cre recombinase expression. Specific expression of the SM22α-Cre transgene was verified by inter-crossing with Rosa26-lacZ reporter mice. Specific x-gal staining was seen in the smooth muscle layer of aorta (A), large vessels of the kidney (B) and throughout the first branches of the mesentery (C) from Cre⁺ Rosa26-lacZ⁺ mice. However no x-gal staining was seen in aorta (D), kidney (E) or mesentery (F) of Cre⁻ Rosa26-lacZ⁺ mice.