Hypertension

RENIN-ANGIOTENSIN SYSTEM

# Direct Actions of $AT_1$ (Type 1 Angiotensin) Receptors in Cardiomyocytes Do Not Contribute to Cardiac Hypertrophy

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**ABSTRACT:** Activation of $AT_1$ (type 1 Ang) receptors stimulates cardiomyocyte hypertrophy in vitro. Accordingly, it has been suggested that regression of cardiac hypertrophy associated with renin-Ang system blockade is due to inhibition of cellular actions of Ang II in the heart, above and beyond their effects to reduce pressure overload. We generated 2 distinct mouse lines with cell-specific deletion of $AT_{1A}$ receptors, from cardiomyocytes. In the first line (C-SMKO), elimination of $AT_{1A}$ receptors was achieved using a heterologous *Cre recombinase* transgene under control of the *Sm22* promoter, which expresses in cells of smooth muscle lineage including cardiomyocytes and vascular smooth muscle cells of conduit but not resistance vessels. The second line (R-SMKO) utilized a *Cre* transgene knocked-in to the *Sm22* locus, which drives expression in cardiac myocytes and vascular smooth muscle cells in both conduit and resistance arteries. Thus, although both groups lack $AT_1$ receptors in the cardiomyocytes, they are distinguished by presence (C-SMKO) or absence (R-SMKO) of peripheral vascular responses to Ang II. Similar to wild-types, chronic Ang II infusion caused hypertension and cardiac hypertrophy in C-SMKO mice, whereas both hypertension and cardiac hypertrophy were reduced in R-SMKOs. Thus, despite the absence of $AT_{1A}$ receptors in cardiomyocytes, C-SMKOs develop robust cardiac hypertrophy. By contrast, R-SMKOs developed identical levels of hypertrophy in response to pressure overload–induced by transverse aortic banding. Our findings suggest that direct activation of $AT_1$ receptors in cardiac myocytes has minimal influence on cardiac hypertrophy induced by renin-Ang system activation or pressure overload. **(*Hypertension*. 2021;77:393–404. DOI: 10.1161/HYPERTENSIONAHA.119.14079.)**

• **Data Supplement**

**Key Words:** hypertension ■ hypertrophy ■ myocyte, cardiac ■ renin-angiotensin system ■ transgenes

Hypertension is a major public health problem causing significant risk of end-organ damage including stroke and cardiovascular disease.[1] Left ventricular hypertrophy (LVH) is a frequent complication of chronic hypertension, increasing risk for heart failure and cardiovascular mortality.[2] The renin-Ang (angiotensin) system (RAS) plays a prominent role in blood pressure (BP) regulation and hypertension pathogenesis.[3] Components of the RAS are expressed in organ systems and cell lineages contributing to BP regulation. In addition to its effects to cause hypertension, Ang receptors may directly promote end-organ damage in target tissues. In this regard, it has been suggested that actions of Ang II mediated by its receptors in the kidney, heart, and central nervous system may contribute to morbidity and mortality above and beyond increases BP.

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## Novelty and Significance

### What Is New?

- The absence of AT1 (type 1 angiotensin) receptors in cardiomyocytes does not impair the development of cardiac hypertrophy in 2 distinct experimental models.
- Increases in blood pressure driven by changes in peripheral vascular resistance are a critical determinant of cardiac hypertrophy in hypertension.

### What Is Relevant?

- Blood pressure control is paramount to reducing the development of cardiac hypertrophy in hypertension.

### Summary

We generated 2 novel mouse models in which the $AT_{1A}$ receptor was knocked out specifically in cardiomyocytes and vascular smooth muscle. Each mouse had differential hypertensive responses to Ang II–induced hypertension. The mice that developed cardiac hypertrophy, had blood pressure responses indistinguishable to wild-type controls. Whereas, mice with an attenuated blood pressure response to Ang II–induced hypertension had similarly reduced cardiac hypertrophy development. In the mice with protection of cardiac hypertrophy with Ang II infusion, cardiac hypertrophy was induced using a pressure overload model. Thus, demonstrating the primacy of pressure increases in the development of heart hypertrophy.

### Nonstandard Abbreviation and Acronyms

| | |
|---|---|
| **ACE** | angiotensin-converting enzyme |
| **Ang** | angiotensin |
| **$AT_1$** | type 1 angiotensin |
| **BP** | blood pressure |
| **LVH** | left ventricular hypertrophy |
| **RAS** | renin-angiotensin system |
| **TAC** | transverse aortic constriction |

The classical effects of the RAS are mediated by Ang II acting via $AT_1$ (type 1 angiotensin) receptors. This activation triggers a cascade of classic physiological responses, including vasoconstriction, activation of sympathetic nervous system, and stimulation of sodium reabsorption by the kidney, which all contribute to elevated BP in hypertension.[4] $AT_1$ receptor blockers and ACE (angiotensin-converting enzyme) inhibitors are effective and widely used antihypertensive agents.[5] RAS blockade using Ang receptor blockers and ACE inhibitors are not only effective at lowering BP but also attenuating end-organ damage, such as reducing LVH, and decreasing cardiovascular mortality.[6,7]

The clinical evidence indicating a key contribution of RAS activation to development of LVH in hypertension is clear-cut.[8–10] However, the mechanism of this effect is potentially complex as $AT_1$ receptor activation triggers an array of cellular, vascular, and neuroendocrine responses that each might distinctly promote the development of hypertrophy. For example, activation of $AT_1$ receptors triggers signaling pathways that have been linked to LVH,[11] and indeed, Ang II induces hypertrophy of cultured cardiac myocytes.[12,13] Furthermore, models of forced overexpression of wild-type (WT) and constitutively activated $AT_1$ receptors in cardiomyocytes have consistently been shown to induce hypertrophy in vivo.[14,15] In contrast, several studies using mice completely deficient in $AT_{1A}$ receptors, the major murine $AT_1$ receptor, suggest that BP elevation and after-load have the predominant role to drive Ang II–dependent LVH.[16,17] However, it is difficult to specifically isolate cardiac and vascular actions in systems where $AT_1$ receptors are absent in all tissues.

In this study, we reexamine this issue, taking advantage of 2 mouse models lacking $AT_1$ receptors in cardiac myocytes, but with divergent expression of $AT_1$ receptors in resistance vessels. Thus, although $AT_1$ receptor signaling is abrogated in cardiac myocytes in both groups, pressor responses to Ang II are preserved in one group (conduit vessel smooth muscle AT1A receptor knockout [C-SMKO]) but are absent in the other (resistance vessel smooth muscle AT1A receptor knockout [R-SMKO]). We find no evidence for a significant impact of direct effects of $AT_1$ receptor signaling in cardiomyocytes to promote hypertrophy. Instead, the extent of hypertrophy seems to be predominantly driven by pressure.

## METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### Details of Mice

A mouse line with a conditional *Agtr1a*$^{flox}$ allele was generated using homologous recombination in embryonic stem cells, as described previously.[18,19] To delete the $AT_{1A}$ receptor from vascular smooth muscle cells, we crossed inbred C57BL/6 transgenic mouse lines expressing *Cre* recombinase *specifically in smooth muscle cells under the control of the sm22α* promoter (KI*sm22α-Cre,* Strain Name B6.129S6-*Tagln*$^{tm2(cre)Yec/J}$,

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Stock Number 006878, The Jackson Laboratory, Bar Harbor, ME) or the (Tg*sm22α-Cre,* Strain Name B6Tg[Tagln-cre]1Her, Stock Number 004746, The Jackson Laboratory, Bar Harbor, ME). Therefore, we generated 2 separate mouse models, KI*sm22α -Cre*$^{+}$*Agtr1a*$^{flox/flox}$ (R-SMKO) mice and Tg*sm22α -Cre*$^{+}$*Agtr1a*$^{flox/flox}$ (C-SMKO), both on inbred C57BL/6 background. All experiments were performed on 8- to 24-week old male mice. In all experimental models, mice were randomized and researchers were blinded to genotype. Membrane-targeted *tdTomato (mT)/*membrane–targeted *EGFP (mG*) mice with loxP sites flanking the membrane-targeted *tdTomato* cassette followed by an N-terminal membrane-tagged version of *EGFP* were purchased from The Jackson Laboratory (Strain Name B6.129[Cg]-*Gt[ROSA]26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo/J}$, Stock Number 007676) and crossed with either the constitutively expressed *Tagln-Cre* or KI*sm22α-Cre* recombinase transgenic lines as above. Mice possessing the *mTmG* transgene normally express red fluorescence protein (*mT*) in all tissues. However, when *Cre* recombinase is present, the *mT* cassette is deleted, triggering expression of the membrane-targeted *EGFP (mG*).[20] Assessment of red and green fluorescence were conducted in male mice at 8 weeks of age.

All experimental mice were bred in an Association for Assessment and Accreditation of Laboratory Animal Care international accredited animal facility at the Duke University and Durham Veterans Affairs medical centers under National Institutes of Health guidelines for care and use of laboratory animals and housed with free access to standard rodent chow and water unless otherwise specified.

## BP Measurements in Conscious Mice

BPs were measured in conscious, unrestrained 12- to 20-week-old male C-SMKO, R-SMKO, and WT mice using radiotelemetry (PA-C10), as described previously.[16] Arterial BP was collected, stored, and analyzed using Dataquest A.R.T software (version 4.0; Data Sciences International, Saint Paul, MN). Mice were allowed to recover for 7 days after telemetry implantation to reestablished normal circadian rhythms.[21] After recovery, telemetry data were collected continuously with sampling every 5 minutes for 10-s intervals. Baseline measurements were recorded for 7 consecutive days while mice ingested a normal sodium diet. On day 8, an osmotic minipump (Alzet, Cupertino, CA) infusing Ang II (Sigma Aldrich, St. Louis, MO) at a rate of 1000 ng/(kg·min) were implanted subcutaneously and BP measurements continued for at least 18 days in all 3 groups of mice (WT, C-SMKO, R-SMKO).

## Real-Time Reverse Transcription Polymerase Chain Reaction of B-Type Natriuretic Peptide and $AT_{1A}$ mRNA Levels in Left Ventricle

RNA was extracted from the left ventricle of mice at baseline (uninfused) and after 4 weeks of Ang II infusion (1000 ng/kg/min) using an RNeasy Fibrous mini kit (Qiagen), cDNA was made using the High-Capacity cDNA Reverse Transcription Kit (4368814, ThermoFisher Scientific). Real-time quantitative polymerase chain reaction (PCR) was performed using PerfeCTa quantitative PCR FastMix II (Quanta) using the ΔCT method. Primers were from Taqman (ThermoFisher Scientific)-endogenous control 18s- 4352930E, Mm01255770_g1 BNP (B-type natriuretic peptide; Nppb). As previously described, relative levels of mRNA for the $AT_{1A}$ receptor were measured from the heart in WT, C-SMKO, and R-SMKO in male mice averaging 15±2 weeks of age, and there was no difference in age between groups.[22] RNA was isolated and reverse transcription performed using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). SYBR Green-based quantitative PCR was carried out using the SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). For $AT_{1A}$ receptor expression, the amount of target gene relative to endogenous control was determined by the change in threshold cycle method. The following primer sequences were used: type 1 angiotensin Forward-5′-ACTCACAGCAACCCTCCAAG-3′, type 1 angiotensin Reverse-5′-ATCACCACCAAGCTGTTTCC-3′ (Amplicon size: 236bp), GAPDH Forward-5′-TCACCACCATGGAGAAGGC-3′, GAPDH Reverse-5′-GCTAAGCAGTTGGTGGTGCA-3′ (Amplicon size: 168bp). Control tubes lacking cDNA and containing RNA were included in each run.

## Analysis of Oxidized Amino Acids in Heart

After 28 days of Ang II infusion, the ventricles were dissected from WT and C-SMKO mice and immediately placed in antioxidant buffer (100 μmol/L diethylene 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.[23] 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 retention time and specific ion transitions in the multiple reaction monitoring mode. The ratio of the peak areas of the analyte with corresponding 13C internal standard was 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.

## Pressure Overload

Animal studies were carried out according to approved protocols and animal welfare regulations of Duke University Medical Center's Institutional Review Boards. Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg). Chronic pressure overload was induced by transverse aortic constriction (TAC) in R-SMKO and WT mice for 8 weeks, whereas in sham-operated mice the suture was passed around the aorta but not ligated as previously described.[24] TAC gradients (Figure S1 in the Data Supplement) and mortality (40%) were not different between WT controls and R-SMKO mice (Figure S3). Mice with pressure gradients of ≤15 mm Hg were excluded from analysis as previously described.[25]

## Histopathologic Analysis

After 28 days of Ang II infusion, C-SMKO hearts were harvested, weighed, fixed in formalin, sectioned, and stained with Masson trichrome. The severity of cardiac pathology was determined

by a pathologist (P. Ruiz) without knowledge of genotypes based on the presence and severity of component abnormalities, including cellular infiltrate, myocardial cell injury, vessel wall thickening, and fibrosis. Grading for each component was performed by using a semiquantitative scale where 0 was normal and 1–3+ represented mild through severe abnormalities. The total cardiac injury score for each heart was a summation of the component injury scores across 5 domains scored 0 to 3 (interstitial Infiltrate, myocyte injury, vascular medial/fibrointimal changes, interstitial fibrosis, chronic vascular injury grade, total score: 0–15). For quantitation of fibrosis, 6 to 9 images were randomly captured for each sample. Percentage of fibrosis area (blue color on Masson trichrome stained sections) were measured by using a computer image analysis system (National Institutes of Health image J, 1.8.0) and averaged for each animal and then across each group by F. Rianto without knowledge of the genotype or treatment. Cardiomyocyte diameters were obtained by measuring the shortest perpendicular axis of a transverse section of a single cardiomyocyte using a computer image analysis system (National Institutes of Health image J, 1.8.0). Analysis of hematoxylin and eosin–stained sections was performed by F. Rianto without knowledge of genotype or treatment condition. Diameters of between 100-250 cardiomyocytes were measured for each sample from different areas at ×40 magnification.

### Statistical Analysis

The values for each parameter within a group are expressed as the mean±SEM. For comparisons between groups with normally distributed data, statistical significance was assessed using 2-way ANOVA followed by Sidak adjusted for multiple comparisons. For comparisons within groups, normally distributed variables were analyzed by a paired *t* test, whereas non-normally distributed variables were analyzed by the Wilcoxon signed-rank test. Normality was determined using the Shapiro-Wilk W test. A $P$ value of $<0.05$ was considered statistically significant.

## RESULTS

### Mice With Cell-Specific Deletion of AT1 Receptors in Heart and Vascular Smooth Muscle Cells

Generation of mice with cell-specific deletion of the $AT_{1A}$ receptor from smooth muscle and myocyte cell lineages using the KI*Sm22α-Cre* (R-SMKO) and (transgene) *Sm22α-Cre* (C-SMKO) has been described previously.[19,26] We examined cardiac expression of Cre recombinase in both lines using the *mTmG* dual-reporter mouse line[20] and found equivalent, robust GFP (green fluorescent protein) fluorescence in hearts of both KI*sm22α-Cre-mTmG* and Tg*Sm22α-Cre-mTmG* mice, which was absent in WT *Cre*-negative-*mTmG* mice (Figure 1A, 1B, and 1C), indicating robust *Cre* recombinase *activity in cardiomyocytes. Successful excision of Agtra$^{flox}$* allele from heart was confirmed in reverse transcription PCR analysis of $AT_{1A}$ receptor mRNA expression shown in Figure 1D and 1E. Although $AT_{1A}$ receptor mRNA was easily detected in the hearts of WT mice, levels of $AT_{1A}$ mRNA were substantially reduced by >95% in hearts of both C-SMKO ($P$=0.02) and R-SMKO mice ($P$<0.0001). Thus, cardiac expression of $AT_{1A}$ receptors is similarly and dramatically reduced in both C- and R-SMKOs.

Using *mTmG* mice, we have previously demonstrated robust Cre expression and coincident deletion of $AT_{1A}$R mRNA in large arteries of both C-SMKO[19] and R-SMKO[26] mice, whereas Cre expression in small resistance vessels such as preglomerular arterioles was only observed in R-SMKO mice.[26] In this regard, expression of $AT_{1A}$ receptors in preglomerular vessels of C-SMKOs was not different from WT controls.[19] Furthermore, acute vasoconstrictor responses to Ang II are dramatically attenuated in R-SMKO[26] mice but are relatively preserved in C-SMKOs,[19] indicating functional absence of $AT_{1A}$ receptors from resistance vessels in R-SMKOs. These differing patterns of vascular *Cre* expression have been seen by others in KI*sm22α*- and Tg*sm22α*-Cre mouse lines.[27,28] Taken together, our findings indicate virtually complete deletion of $AT_{1A}$ receptors from myocardium and larger, conduit arteries in both groups, whereas efficient deletion from resistance vessels is only seen in R-SMKOs.

### Cardiac Hypertrophy With Chronic Ang II Infusion Follows Elevated BP

To induce cardiac hypertrophy, Ang II 1000 ng/(kg·min) was infused subcutaneously in 12- to 20-week old male WT, C-SMKO, and R-SMKO mice for 28 days using osmotic minipumps. Radiotelemetry units were also implanted to measure intraarterial pressure continuously in the conscious, unrestrained state. Heart-to-body weight ratios were determined in groups of WT, C-SMKO, and R-SMKO mice without Ang II to establish baseline values for each group. No differences in heart weight to body weight ratio were detected between groups that did not receive Ang II infusion (Figure 2A). As shown in Figure 2A, after 4 weeks of Ang II infusion, heart-to-body weight ratios were significantly increased to a similar extent in WT (44%; versus uninfused) and C-SMKO (51% versus Uninfused) mice. By contrast, Ang II caused only a modest increase of 23% in Heart-to-body weight ratio in the R-SMKO mice versus uninfused, and this was significantly less than the extent of cardiac hypertrophy in the other 2 groups WT, 44% and C-SMKO, 51%). We did not detect any differences in cardiac injury or cardiomyocyte diameter between WT and C-SMKO mice after 4 weeks of Ang II infusion (Figure 6A and 6B). Thus, although both C- and R-SMKOs lack $AT_{1A}$ receptors in the heart, C-SMKOs developed robust cardiac hypertrophy with Ang II infusion that was not different from WTs, whereas cardiac hypertrophy was significantly attenuated in the R-SMKO group.

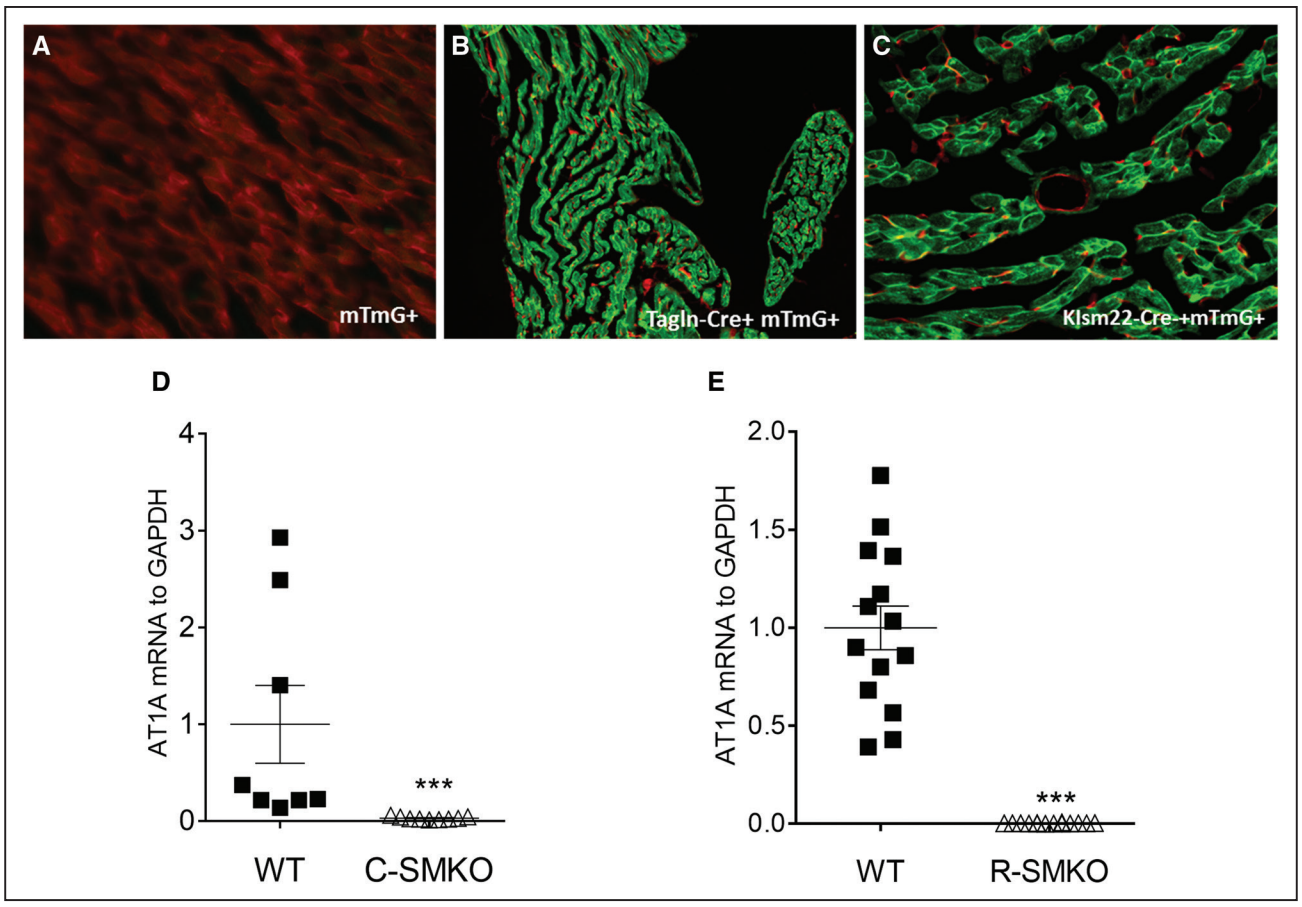


**Figure 1. Verification of cardiomyocyte-specific Cre recombinase expression and $AT_{1A}$ deletion.**
Cre expression patterns of both the *TagIn*-Cre *and KIsm22*-Cre mouse lines were verified by intercrossing with the *mTmG* reporter mouse line, which contains a Cre-activated GFP (green fluorescent protein) transgene. **A**, GFP expression indicated by green fluorescence was not seen in cardiac myocytes from $Cre^{negative}$ $mTmG^+$ mice. **B**, Robust GFP expression reflecting Cre activity was observed in cardiomyocytes from *TagIn*-$Cre^+$ $mTmG^+$ and (**C**) in KI*sm22-Cre*$^+$ *mTmG*$^+$ mice. **D** and **E**, $AT_{1A}$ receptor is efficiently deleted from the hearts of both C-SMKO and R-SMKO mice. Quantitative reverse transcription polymerase chain reaction performed on whole left ventricle in C-SMKO, R-SMKO, and wild-type (WT) mice. **D**, mRNA for the $AT_{1A}$ receptor was easily detected from the hearts of WT mice (n=8) but not hearts of C-SMKO mice (n=10; WT1±0.4 vs C-SMKO 0.03±0.0005 $AT_{1A}$ to GAPDH mRNA, arbitrary units, ***$P$=0.02). **E**, Similarly, $AT_{1A}$ receptor mRNA expression was virtually absent in the hearts of R-SMKO mice (control; n=14, R-SMKO n=14; control 1±0.11 vs R-SMKO 0.003±0.0005 $AT_{1A}$ to GAPDH mRNA, arbitrary units, ***$P$<0.0001, unpaired *t* test*). Data* are expressed as mean±SEM.

We also measured systolic BP at baseline and with Ang II infusion (Figure 2B). R-SMKO mice had lower systolic BP at baseline (123±5 mm Hg) compared with both WT (137±3 mm Hg) and C-SMKO (143±3 mm Hg) mice (Figure 2B). Compared with baseline, Ang II caused significant increases in mean systolic BP in WT of 30±4 mm Hg and C-SMKO of 37±7 mm Hg. The magnitude of systolic BP elevation was similar between these 2 groups. In contrast, mean systolic BP only increased by 14±2 mm Hg in R-SMKO mice with Ang II infusion, which was significantly less than WTs or C-SMKOs, reflecting the key role of $AT_{1A}$ receptors in resistance vessels in Ang II–dependent hypertension. Moreover, there was a correlation between achieved average systolic BP and heart/body weight ratio (Figure 2C). Thus, in Ang II hypertension, the relative extent of cardiac hypertrophy in the groups mirrored the mean increases in BP and not presence or absence of $AT_{1A}$ receptors in the heart.

## Cardiac BNP mRNA Expression

As transcription of the BNP gene is characteristically enhanced with cardiac hypertrophy,[29,30] we measured mRNA levels of BNP in the various experimental groups using real-time reverse transcription quantitative PCR. Levels of BNP mRNA were measured in ventricular tissues from mice in each of the experimental groups after 4 weeks of Ang II infusion, compared with respective WT controls that did not receive Ang II infusion. As shown in Figure 3, there were no differences in mRNA levels of BNP at baseline between the 3 experimental groups, while chronic infusion of Ang II caused significant increases in BNP expression in WT and C-SMKO but not R-SMKO hearts compared with their respective uninfused control hearts. However, after Ang II infusion, BNP mRNA levels in R-SMKO mice were significantly lower than WT and C-SMKO mice.

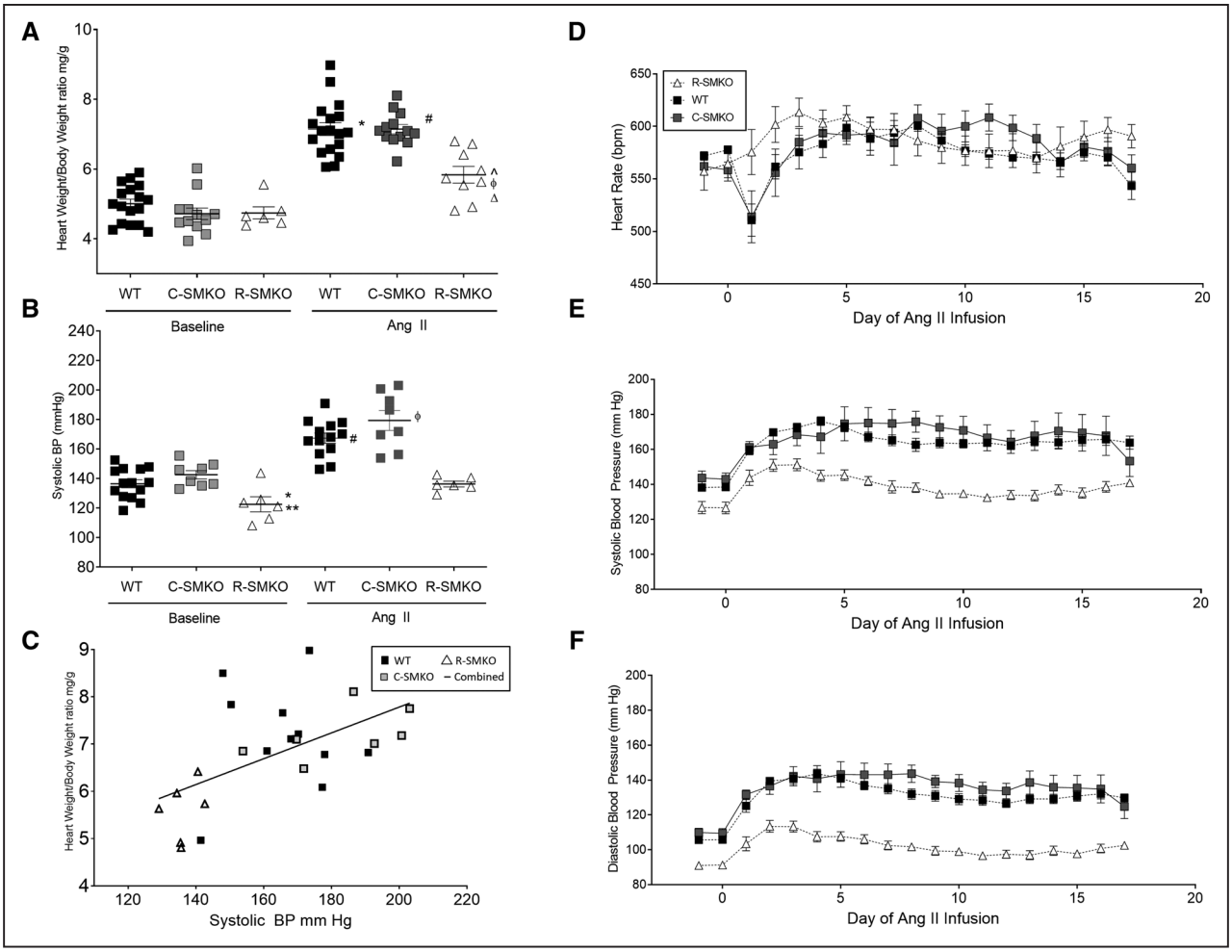


**Figure 2. Reduction in cardiac hypertrophy and systolic blood pressure (SBP) observed in mice lacking $AT_{1A}$ receptors in resistance vessels.**

**A**, Heart-to-body weight ratios were calculated at baseline in wild-type (WT; n=17), C-SMKO (n=12), and R-SMKO (n=6) mice and separate groups after 28 d of Ang (angiotensin) II infusion; WT (n=18), C-SMKO (n=13), and R-SMKO (n=9). No differences in heart-to-body weight ratio were detected between the 3 groups at baseline, and Ang II infusion caused significant increases in heart-to-body weight ratio in all groups: WT: 5.0±0.1–7.1±0.2 mg/g *$P$<0.0001; C-SMKO: 4.7±0.2–7.1±0.1 mg/g #$P$<0.0001; and R-SMKO: 4.7±0.2–5.8±0.2 mg/g ^$P$=0.01, analyzed by 2-way ANOVA with Sidak test for multiple comparisons. After Ang II, heart-to-body weight ratios were significantly lower in R-SMKOs compared with both WT and C-SMKO (R-SMKO: 5.8±0.2 vs WT: 7.1±0.2, ϕ$P$<0.0001 vs ΔC-SMKO: 7.1±0.1 mg/g, $P$<0.0001). **B**, Systolic BP was measured via radiotelemetry in WT (n=14), C-SMKO (n=8), and R-SMKO (n=6) mice. At baseline, R-SMKO have had lower systolic BP than WT and C-SMKO mice (123±5 vs137±3 and 143±3 mm Hg, *$P$=0.01, **$P$=0.003). Compared with baseline, Ang II infusion caused significant increases in systolic BP in WT (137±3–167±3 mm Hg; #$P$<0.0001) and C-SMKO (143±3 to 179±7 mm Hg; ϕ$P$<0.0001) but not R-SMKO (123±5 to 136±2 mm Hg; $P$=nonsignificant) mice (daily averaged). **C**, Correlation between average systolic BP during Ang II–induced hypertension and cardiac hypertrophy. The degree of cardiac hypertrophy as measured by heart-to-body weight ratio (mg/g). Across the experimental groups, cardiac hypertrophy was significantly correlated with average SBP during the Ang II infusion period. Combined, solid line: $r$=0.55, $R^2$=0.31, $P$=0.005. Pearson correlation. **D**, Twenty-four–hour heart rates, (**E**) SBP, and (**F**) diastolic BP (DBP) of WT, C-SMKO, and R-SMKO at baseline and after Ang II–induced hypertension. Daily heart rates are higher during days 1 and 2 of Ang II–induced hypertension in R-SMKO compared with C-SMKO and WT mice (589±51 vs 535±24 vs 536±54 bpm, $P$=0.005 R-SMKO to C-SMKO, $P$=0.002 R-SMKO to WT). SBP and DBP were significantly decreased at baseline and throughout the entire Ang II period, similar to averages in **B**.

## Diminished Oxidative Stress in Hearts Lacking $AT_{1A}$ Receptors in Cardiomyocytes

Activation of $AT_1$ receptors in cardiomyocytes stimulates free radical generation, which may contribute to end-organ damage in hypertension.[31,32] To examine levels of oxidative stress in the heart, we assessed oxidation of proteins in WT and C-SMKO mice to assess if downstream $AT_{1A}$ receptor effects are altered in the hearts even with a similar degree of hypertension after Ang II infusion. We determined the content of the 2 oxidized amino acids, 3-nitrotyrosine, and dityrosine, as molecular signatures of peroxynitrite-mediated oxidation using isotope dilution liquid chromatography–tandem mass spectrometry.[19,23] As shown in Figure 4, levels of both dityrosine (162±29 versus 68±22 μmol/mol tyrosine) and 3-nitrotyrosine

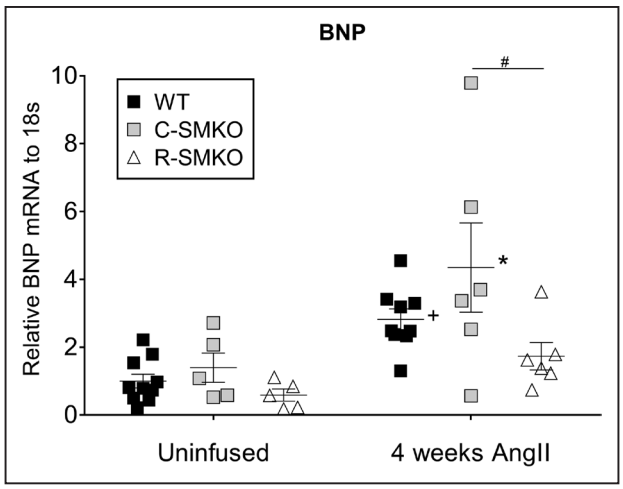


**Figure 3. Levels of heart BNP (B-type natriuretic peptide) before and after chronic Ang (angiotensin) II.**
Left ventricular (LV) BNP mRNA expression was measured using real-time reverse transcription–quantitative polymerase chain reaction at baseline and after 4 wk of Ang II infusion in wild-type (WT), C-SMKO, and R-SMKO mice and normalized to 18s mRNA levels within each sample. For comparisons between groups, all values were normalized to the uninfused WT group. In uninfused mice, there were no differences in BNP levels between WT (n=10), C-SMKO (n=5), or R-SMKO (n=5) mice. After 4 wk of chronic Ang II infusion, BNP mRNA levels in LV increased significantly in all groups (WT [n=9], C-SMKO [n=6], and R-SMKO [n=6]; +$P$=0.0001 for Ang II effect, F[1, 35]=18.2). However, after Ang II infusion, BNP values in R-SMKO were significantly lower than C-SMKO mice (#$P$=0.04). Error bars represent SEM. Statistics were performed using a 2-way ANOVA with Sidak post-test for multiple comparisons.

(4899±2022 versus 569±125 µmol/mol tyrosine) were significantly reduced in the hearts from C-SMKO compared with WT mice (Figure 4B). This indicates a significant role for $AT_1$ receptors in cardiomyocytes to promote oxidative stress in the heart, yet, these actions do not seem to affect the development of cardiac hypertrophy, since heart weights and BNP levels in Ang II–infused C-SMKOs were similar to WTs (Figures 2 and 3).

## Absence of Cardiomyocyte $AT_{1A}$ Receptors Does Not Affect Responses to TAC

To examine cardiac hypertrophy responses in R-SMKO mice in a model where pressure load could be directly controlled, we utilized TAC. As shown in Figure 5A, significant increases in LV weight/tibia length were observed in both WT (6.0±0.6 versus 8.6±0.4) and R-SMKO (5.6±0.1 versus 9.2±0.8) mice after TAC compared with matched sham WTs. There was no difference in LV weight/tibia length between WT and R-SMKO after 8 weeks of TAC (8.6±0.4 versus 9.2±0.8). Thus, in contrast to the Ang II–infusion model, the absence of $AT_{1A}$ receptors in cardiomyocytes in R-SMKO mice does not diminish ventricular weight after TAC. We measured fractional shortening, as a reflection of ventricular function, at baseline and 8 weeks after TAC. As depicted in Figure 5B, TAC was associated with significant reductions of fractional shortening in R-SMKO mice (55±1.2% versus 37±6.3%). However, WT mice had reduced fractional shortening but this was not significant (53±1.3% versus 47±3.1%). Although fractional shortening after TAC was numerically lower in R-SMKO than WTs (37±6.3% versus 47±3.1%), this difference between genotypes at 8 weeks did not achieve statistical significance. In these studies, we also used echocardiography to assess posterior and septal wall thicknesses at baseline and after TAC. As shown in Figure 5C, WT mice (n=7) had significant increases in ventricular wall thickness by echocardiography after TAC (2.3±0.08 versus 2.7±0.09 mm). Likewise, ventricular wall thickness also tended to be increased in R-SMKO mice (n=7) 8 weeks after TAC (2.5±0.09 versus 2.7±0.15 mm) but this did not reach statistical significance. There was no significant difference in ventricular wall thickness between the WT and R-SMKO groups at 8 weeks after TAC. We measured the end-diastolic dimension (EDD) at baseline and after 8 weeks of TAC in both WT and R-SMKO mice (Figure 5D). At baseline,

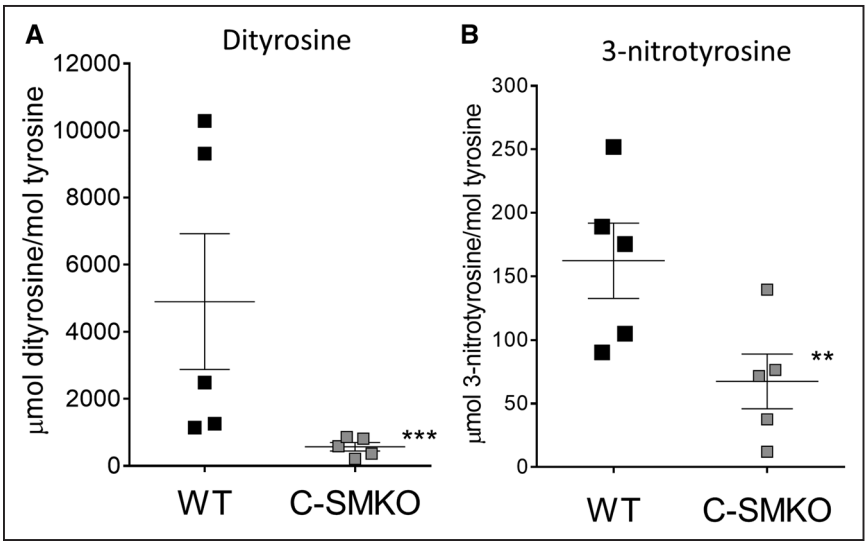


**Figure 4. C-SMKO mice have reduced oxidative stress in cardiac myocytes after 4 wk of Ang (angiotensin) II infusion.**
Oxidized amino acids in hearts were quantified by liquid chromatography–electrospray ionization tandem mass spectrometry using multiple reaction monitoring mode in wild-type (WT; n=5) and C-SMKO (n=5) mice after 4 wk of Ang II. Compared with WT mice, markedly diminished levels of (**A**) dityrosine (162±29 vs 68±22 µmol/mol tyrosine; ***$P$=0.03) and (**B**) 3-nitrotyrosine (4899±2022 vs 569±125 µmol/mol tyrosine; **$P$=0.008) were detected in the hearts of C-SMKO mice after Ang II infusion. Error bars represent SEM.

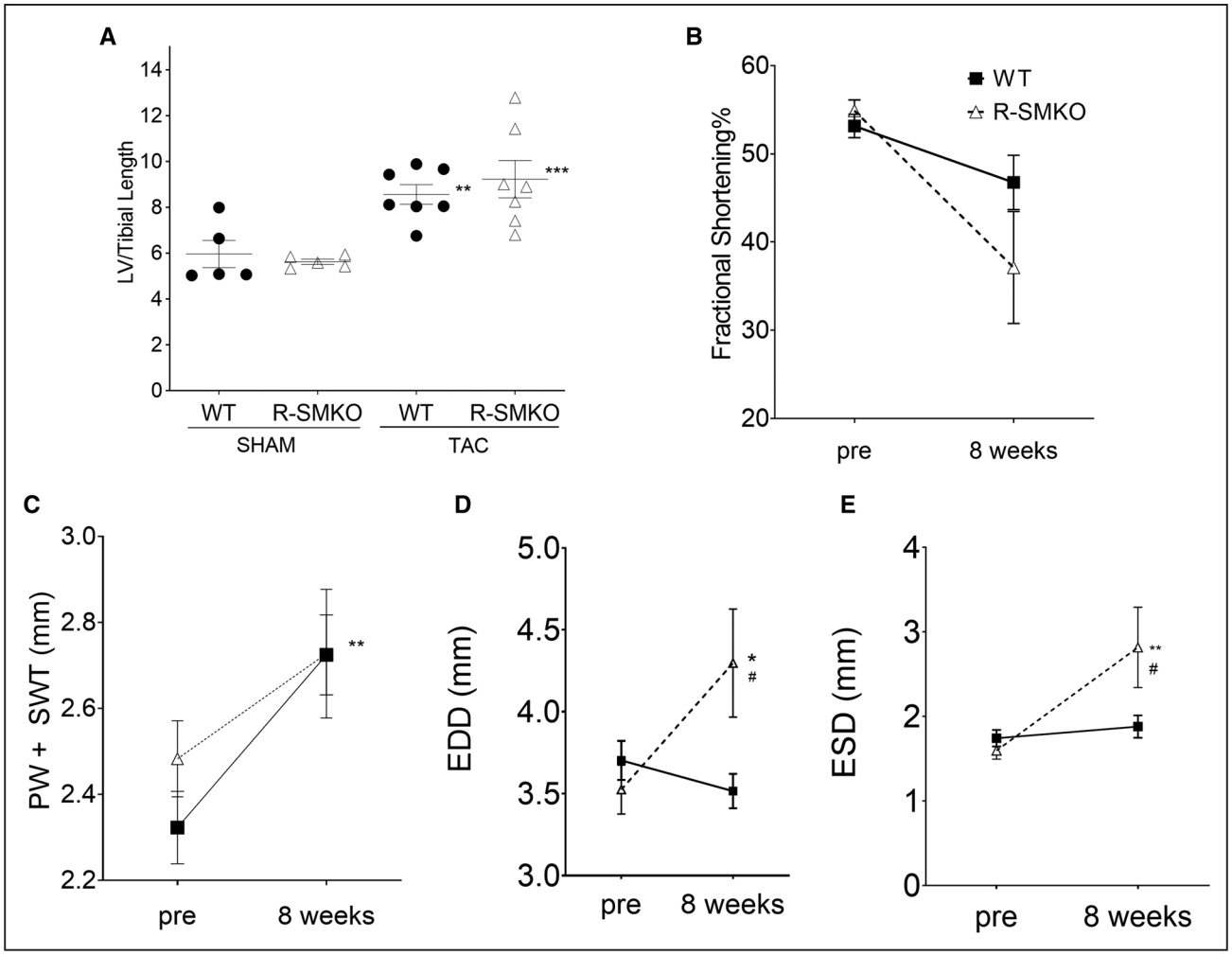


**Figure 5. Transverse aortic banding (TAC) causes hypertrophy and reduced cardiac function in R-SMKO mice.**
TAC was performed in wild-type (WT; n=7) and R-SMKO (n=7) mice. As controls, sham surgeries were carried out in separate groups of WT (n=5) and R-SMKO (n=5). **A**, TAC induced similar levels of cardiac hypertrophy assessed as left ventricular (LV) weight-to-tibia length in WT and R-SMKO mice. ***P*=0.02; ****P*=0.004. **B**, Fractional shortening (FS) fell in both WT and R-SMKO mice 8 wk after TAC but this reduction only achieved statistical significance in R-SMKOs (***P*=0.02). There was no significant difference in FS between WT and R-SMKO mice after 8 wk exposure to TAC. **C**, Cardiac hypertrophy was also assessed by echocardiography as the sum of posterior wall (PW) and septal wall (SW) thicknesses. There were numerical increases in this parameter in both groups, but this only achieved statistical significance in WT mice (***P*<0.01). There was no significant difference in ventricular wall thickness between the WT and R-SMKO groups at 8 wk after TAC. **D**, End-diastolic dimension (EDD) was measured at baseline and after 8 wk of TAC in both WT and R-SMKO mice. A baseline, there was no difference in EDD between WT and R-SMKO (*P*=nonsignificant [NS]) and 8 wk of TAC had no effect on EDD in the WT group. However, EDD increased significantly in R-SMKO after TAC (**P*=0.04) and EDD post-TAC was significantly greater in R-SMKOs than WT mice (#*P*=0.02). **E**, At baseline, we did not detect differences in end-systolic diameter (ESD) between WT and R-SMKO (*P*=NS). TAC did not affect ESD WT mice (*P*=NS) but caused a significant increase in ESD in R-SMKO mice (***P*=0.01) such that ESD was significantly greater after 8 wk of TAC in R-SMKOs compared with WT (#*P*=0.03). Error bars represent SEM. Statistics performed with 2-way ANOVA (repeated measures) with Sidak multiple comparisons.

we did not detect a difference in EDD between WT and R-SMKO (3.7±0.1 versus 3.5±0.2 mm). After 8 weeks of TAC, the EDD did not change in WT mice (3.7±0.1–3.5±0.1 mm). However, the EDD increased in R-SMKO mice pre to 8 weeks (3.5±0.2–4.3±0.3 mm). Thus, EDD was greater after 8 weeks of TAC in R-SMKOs compared with WT (4.3±0.3 versus 3.5±0.1 mm). We also examined end-systolic diameter (ESD) in WT and R-SMKO mice (Figure 5E). At baseline, we did not detect a difference in ESD between WT and R-SMKO (1.6±0.07 versus 1.5±0.09 mm). After 8 weeks of TAC, the ESD did not change in WT mice (1.7±0.1–1.9±0.1 mm). However, the ESD increased in R-SMKO mice pre to 8 weeks of TAC (1.6±0.1–2.8±0.5 mm). Thus, ESD was greater after 8 weeks of TAC in R-SMKOs compared with WT (2.8±0.4 versus 1.9±0.1 mm). With regard to histopathology, no difference in severity of fibrosis was observed between WT and R-SMKO mice. However, R-SMKO mice had larger cardiomyocyte diameter compared with controls (Figure 6C/6D). These findings further indicate that while the absence of $AT_{1A}$ receptors in cardiomyocytes does not prevent development of hypertrophy and

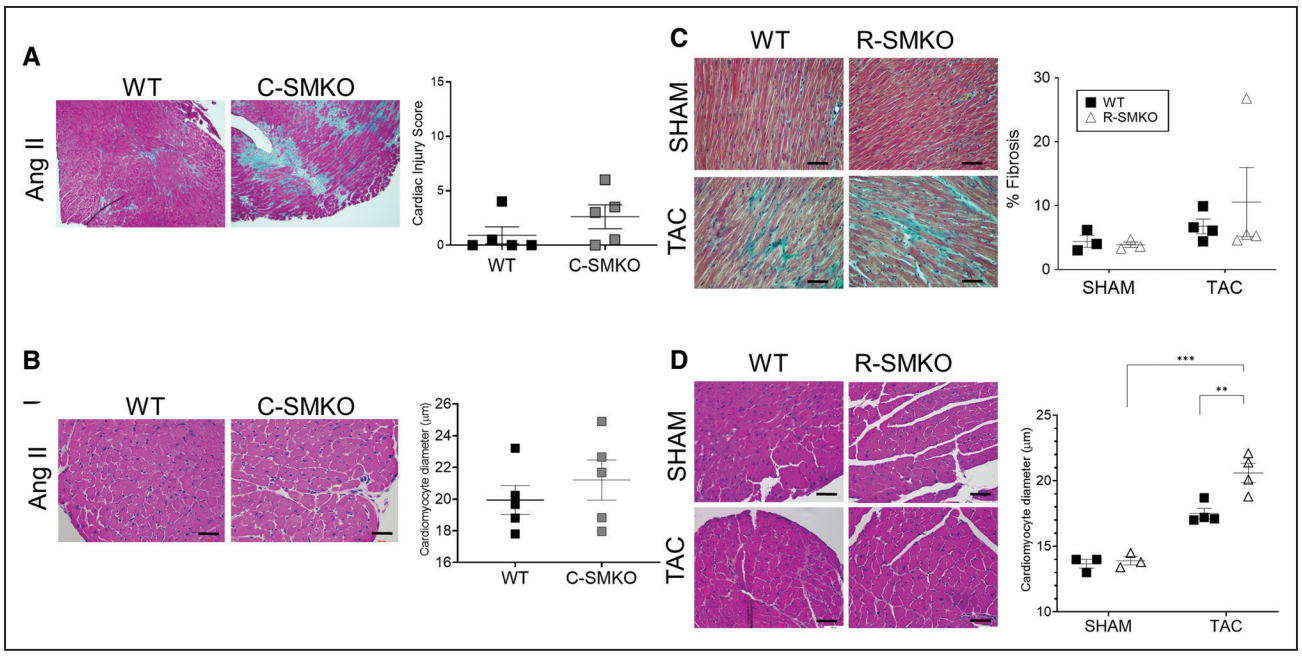


**Figure 6. Assessment of cardiac histology of C-SMKO mice after Ang (angiotensin) II–induced hypertension and in R-SMKO mice after transverse aortic constriction (TAC).**

**A**, Histolopathological analysis of hearts from wild-type (WT; n=5) and C-SMKO (n=5) mice was performed after 28 of Ang II–induced hypertension. Masson trichrome staining showed only minimal to mild cardiac injury in WT and C-SMKO with no difference between groups (WT: 0.9±0.78 vs C-SMKO 2.6±1.2 cumulative cardiac injury score). Representative ×10 images of WT and C-SMKO hearts. **B**, Cardiomyocyte diameter was assessed in hematoxylin and eosin–stained hearts from Ang II–infused WT (n=5) and C-SMKO (n=5) mice. There was no difference in cardiomyocyte diameter between Ang II infused WT and C-SMKO mice (WT 20±0.9 vs C-SMKO 21±1.3 μm; *P*=nonsignificant [NS]). **C**, The extent of cardiac fibrosis in hearts WT and R-SMKO mice from sham surgery and TAC groups stained with Masson trichrome were determined using a computer image analysis system. Scale bar=50 μm. Scale bar=50 μm. More fibrosis was seen after TAC, but these differences did not achieve statistical significance in any of the groups (WT:sham: 4.4±0.9 vs TAC 6.7±1.2% fibrosis; R-SMKO:sham: 3.9±0.4 vs TAC 10.6±5.4% fibrosis). **D**, Cardiomyocyte diameter was assessed in hematoxylin and eosin stained hearts from WT and R-SMKO mice 8 wk after sham surgery or TAC. Scale bar=50μm. There was no difference in cardiomyocyte diameter in sham mice (WT 14±0.3 vs R-SMKO 14±0.3 μm; *P*=NS). Cardiomyocyte diameter increased in both WT and R-SMKO mice after 8 wk of TAC (WT 17±0.4 μm; *P*=0.06 vs WT sham; R-SMKO 21±0.7 μm; ***P*=0.02 vs R-SMKO sham). After TAC, cardiomyocyte diameter was significantly greater in R-SMKO compared with WT mice (***P*=0.002). Error bars represent SEM. Statistics performed with 2-way ANOVA with Sidak multiple comparisons.

in this TAC model, severity of cardiomyopathy may actually be enhanced. WT mice develop concentric hypertrophy after acute pressure overload, whereas R-SMKO mice develop ventricular dilation and eccentric hypertrophy. Thus, $AT_{1A}$ receptors in cardiomyocytes may provide protection against adverse remodeling in this acute pressure overload model.

## DISCUSSION

Activation of the RAS potently induces hypertension and its associated end-organ damage.[3,33] The major pathological consequences of RAS activation are mediated by $AT_1$ receptors. Along with their actions to increase BP, $AT_1$ receptors may contribute to complications of hypertension through direct actions in target tissues to promote injury.[34] For example, $AT_1$ receptors are expressed in cardiac myocytes where they can have potentially maladaptive actions, such as induction of cellular hypertrophy[35] and activation of pathways associated with fibrosis and inflammation.[36,37]

LVH is one of the most common complications of hypertension and is associated with significant increase in cardiovascular risk.[38,39] In humans, a role for $AT_1$ receptors in development of LVH has been demonstrated by clinical trials showing better efficacy of Ang receptor blockers in preventing or attenuating LVH relative to other classes of antihypertensive agents.[40,41] Moreover, findings of enhanced regression of LVH with $AT_1$ receptor blockade at comparable levels of BP control seem to support the importance of direct $AT_1$ receptor actions to promote hypertrophy independent of BP.[10,41] However, studies from our laboratory and others utilizing $AT_{1A}$ receptor-null mice[16,42] have suggested that $AT_1$ receptors drive the development of cardiac hypertrophy primarily by increasing BP rather than through direct cellular actions.[43]

In this study, we reexamined the relative impact of hemodynamic and cellular effects of the $AT_1$ receptor in cardiac hypertrophy using newly developed mouse models.[19,26] We generated 2 mouse lines, both lacking $AT_{1A}$ receptors in the heart but with divergent vascular

responses to Ang II due to the presence (C-SMKO) or absence (R-SMKO) of $AT_1$ receptors in vessels mediating Ang II–dependent control of peripheral vascular resistance.[19,26] These models provide a unique opportunity to precisely assess the contributions of $AT_1$ receptors actions in cardiac myocytes compared with their effects to increase vascular resistance and pressure load.

We found that robust cardiac hypertrophy developed in C-SMKOs, lacking $AT_{1A}R$ in cardiac myocytes but with preserved expression in resistance vessels. By contrast, hypertrophy was attenuated in R-SMKOs, which fail to develop full-fledged hypertension because of impaired vascular responses to Ang II. Thus, eliminating $AT_{1A}$ receptors from myocardium is not sufficient to protect from cardiac hypertrophy when BP is elevated. Moreover, by increasing pressure load directly with TAC, hypertrophy could easily be induced in the R-SMKOs that had been protected in the Ang II infusion model. R-SMKO mice also had evidence of eccentric rather than concentric hypertrophy, along with increased cardiomyocyte diameters, when subjected to pressure overload. One explanation for this might be loss of $AT_1$ receptor–dependent, noncanonical β-arrestin signaling in cardiomyocytes perhaps leading to cytotoxicity and worsening heart function. β-arrestin signaling through the $AT_1$ receptor is cytoprotective and promotes cardiac contractility.[44,45]

The dominant role of pressure to drive hypertrophy was further corroborated by the strong positive correlation between BP and heart weight across all groups (Figure 2C). Our findings also highlight the key contribution of $AT_1$ receptor-induced peripheral vasoconstriction to cardiac hypertrophy.

The development of LVH is associated with a well-characterized molecular responses including enhanced transcription of the BNP gene.[46] Moreover, elevated BNP level is a useful clinical biomarker in LVH and heart failure.[47,48] We compared BNP expression in WT, C- and R-SMKO mice at baseline and after chronic infusion of Ang II. BNP levels increased with Ang II infusion and were highest in WT and C-SMKO mice, but diminished in R-SMKOs (Figure 3). Furthermore, we found significant correlations between BNP expression, BPs, and extent of cardiac hypertrophy across the various mouse lines. Therefore, BNP mRNA levels in Ang II–dependent hypertension are driven by pressure load, with no evidence for an appreciable influence of $AT_1$ receptors actions in cardiac myocytes on BNP gene expression.

Activation of $AT_1$ receptors stimulates generation of reactive oxygen species.[49] Although it has been suggested that these free radicals may participate in physiological $AT_1$ receptor signaling,[4] Ang II–dependent oxidative stress also contributes to tissue damage and end-organ injury in hypertension.[32] Thus, we were interested in understanding the relationship between $AT_1$ receptor–mediated oxidative stress in the heart and development of cardiac hypertrophy in our models. To this end, we measured 3-nitrotyrosine and dityrosine in heart tissue from WT and C-SMKO mice after Ang II infusion as both of these lines develop marked and equivalent cardiac hypertrophy with Ang II (Figure 2), but $AT_{1A}$ receptor expression is absent in the C-SMKOs. These oxidized amino acids provide a molecular signature of reactive oxygen species generated in tissues.[50] Although there were prominent levels of 3-nitrotyrosine and dityrosine levels in WT hearts, these were dramatically reduced in C-SMKOs indicating a critical role for $AT_1$ receptors in cardiomyocytes to promote cardiac oxidative stress in Ang II hypertension (Figure 4). Nonetheless, despite the marked attenuation of free radical generation in C-SMKOs, cardiac hypertrophy occurred unabated consistent with dominant effects of pressure load in this process.

There is ample clinical evidence indicating superiority of RAS blockade in prevention and regression of LVH compared with other therapies for hypertension. Our studies suggest that this enhanced efficacy cannot be explained by blockade of $AT_1$ receptor actions in the heart, including attenuation of oxidative stress. Instead, they highlight the importance of pressure load contributed in part by $AT_1$ receptor–dependent increases in peripheral vascular resistance. It follows that the beneficial actions of RAS antagonists in LVH may relate to the specific characteristics of their BP lowering, such as enhanced reduction of central arterial pressures or sustained duration of antihypertensive effects. We acknowledge that with extended RAS activation over many years in humans with hypertension, pathological consequences of direct cardiac actions of $AT_1$ receptors might become more important. Nonetheless, the lack of any detectable impact of cardiac $AT_1$ receptors on development of hypertrophy in animal models with sustained elevation of Ang II levels is striking and suggests a limited capacity to promote LVH.

## PERSPECTIVES

Cardiac hypertrophy is one of the most common complications of hypertension and is associated with increased cardiovascular risk. Clinical studies show that blockade of the renin-Ang system promotes regression of cardiac hypertrophy, and it has been suggested that these effects are above and beyond the impact of BP lowering. In addition, Ang II consistently causes hypertrophy of cultured cardiomyocytes. Using novel genetically modified mice, we show here that cardiac hypertrophy is primarily driven by increased pressure and not direct activation of $AT_1$ receptors in cardiomyocytes.

### ARTICLE INFORMATION

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## Disclosures

None.


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