





ORIGINAL RESEARCH

Myogenic Vasoconstriction Requires Canonical $G_{q/11}$ Signaling of the Angiotensin II Type 1 Receptor

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BACKGROUND: Blood pressure and tissue perfusion are controlled in part by the level of intrinsic (myogenic) arterial tone. However, many of the molecular determinants of this response are unknown. We previously found that mice with targeted disruption of the gene encoding the angiotensin II type 1a receptor (AT1AR) (*Agtr1a*), the major murine angiotensin II type 1 receptor (AT1R) isoform, showed reduced myogenic tone; however, uncontrolled genetic events (in this case, gene ablation) can lead to phenotypes that are difficult or impossible to interpret.

METHODS AND RESULTS: We tested the mechanosensitive function of AT1R using tamoxifen-inducible smooth muscle-specific AT1aR knockout (smooth muscle-*Agtr1a*^{-/-}) mice and studied downstream signaling cascades mediated by $G_{q/11}$ and/or β -arrestins. FR900359, Sar11e411e8-angiotensin II (SII), TRV120027 and TRV120055 were used as selective $G_{q/11}$ inhibitor and biased agonists to activate noncanonical β -arrestin and canonical $G_{q/11}$ signaling of the AT1R, respectively. Myogenic and Ang II-induced constrictions were diminished in the perfused renal vasculature, mesenteric and cerebral arteries of smooth muscle-*Agtr1a*^{-/-} mice. Similar effects were observed in arteries of global mutant *Agtr1a*^{-/-} but not *Agtr1b*^{-/-} mice. FR900359 decreased myogenic tone and angiotensin II-induced constrictions whereas selective biased targeting of AT1R- β -arrestin signaling pathways had no effects.

CONCLUSIONS: This study demonstrates that myogenic arterial constriction requires $G_{q/11}$ -dependent signaling pathways of mechanoactivated AT1R but not G protein-independent, noncanonical pathways in smooth muscle cells.

Key Words: angiotensin II type 1a receptor ■ arterial smooth muscle ■ biased ligands ■ myogenic vasoconstriction

See Editorial by Chen and Sonkusare

Elevation of intravascular pressure causes constriction (myogenic tone) of small arteries and arterioles, and this response is a key element in regulating blood flow. This response was first described by Bayliss¹ and has been observed in various microvascular beds.² Many cardiovascular disorders are associated with dysfunctional arterial myogenic response and they include hypertension, chronic heart failure, ischemic stroke, and

diabetes.³⁻⁷ Despite the functional importance of the myogenic response, the nature of sensing intraluminal pressure that causes this response has remained elusive.

Myogenic vasoconstriction is mediated by pressure-dependent depolarization of vascular smooth muscle (SM) cells, an event that augments Ca^{2+} influx through voltage-dependent $Ca_v1.2$ channels.⁸⁻¹³ $G_{q/11}$ -coupled receptors (GPCRs) are thought to function as the upstream

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CLINICAL PERSPECTIVE

What Is New?

- Our study using novel tamoxifen-inducible smooth muscle-specific angiotensin II receptor type 1a knockout mice (smooth muscle-*Agtr1a*^{-/-}) demonstrates that myogenic arterial constriction requires canonical G_{q/11}-dependent signaling pathways of mechanoactivated AT1R but not G protein-independent, noncanonical pathways in smooth muscle cells.

What Are the Clinical Implications?

- Our study will foster understanding and development of new diagnostic and therapeutic options for hypertension.
- Our study also will provide valuable new insights for researchers working on blood pressure control-related signaling mechanisms and for clinicians treating patients with hypertension.

Nonstandard Abbreviations and Acronyms

AT1R/AT1aR/AT1bR	angiotensin II type 1/1a/1b receptor
GRK	G protein-coupled receptor kinase
MA	mesenteric arteries
SM	smooth muscle
SMMHC	smooth muscle myosin heavy chain

sensor of membrane stretch.¹⁴ We previously found that mice with targeted disruption of the gene encoding the angiotensin II (Ang II) type 1a receptor (*Agtr1a*), the major murine AT1 receptor isoform, showed reduced myogenic tone.¹⁵ However, uncontrolled genetic events (in this case, global gene ablation) can lead to phenotypes that are difficult or impossible to interpret in terms of cardiovascular function. This is particularly relevant for mouse models of the renin-angiotensin system. For example, mice with body-wide elimination of angiotensin-converting enzyme have systolic blood pressures that average 35 mm Hg below that of wild-type mice. Surprisingly, renal arterioles and small arteries were thickened, a paradoxical finding given the very low systolic blood pressure that could have been caused by developmental processes and/or mouse strains. The renal changes in angiotensin-converting enzyme knockout mice were completely unanticipated but have also been noted in angiotensinogen and Ang II receptor knockout mice.¹⁶ AT1Rs are known to couple primarily to classical G_{q/11} proteins to activate multiple downstream

signals, including protein kinase C, extracellular signal-regulated kinases, Raf kinases, tyrosine kinases, receptor tyrosine kinases (epidermal growth factor receptor, platelet-derived growth factor, insulin receptor) and reactive oxygen species.¹⁷ The AT1R activation also stimulates G protein-independent signaling pathways such as β -arrestin-mediated mitogen-activated protein kinase activation and Src-Janus kinase-signal transducer and activator of transcription.¹⁷ Recently, it has been shown that the activation of intracellular signaling by mechanical stretch of the AT1R does not require the natural ligand Ang II^{15,18,19} but requires the activation of the transducer β -arrestin.¹⁸ Interestingly, mechanical stretch appears to allosterically stabilize specific β -arrestin-biased active conformations of AT1R to promote noncanonical downstream signaling mediated exclusively by the multifunctional scaffold protein, β -arrestin.²⁰ Whether this noncanonical β -arrestin effector pathway plays a role in myogenic and ligand-dependent vasoconstriction has yet to be ascertained.

This study explored the specific function of AT1R in the regulation of myogenic tone and whether downstream signaling pathways are dependent on canonical G_{q/11} and/or noncanonical alternative signaling pathways. In this regard, we generated mice with cell specific deletion of SM AT1a receptors (SM-*Agtr1a* mice) and studied the effects of biased GPCR agonists and G_{q/11} protein inhibition on tone development in 3 distinct vascular beds (renal, cerebral, and mesenteric circulation). We found that the SM AT1aR coupled toward the canonical G_{q/11} signaling pathway is required for the myogenic response in all 3 vascular beds. Our data argue against involvement of noncanonical G protein-independent alternative signaling downstream of the AT1R to cause myogenic vasoconstriction.

METHODS

Data and analytic methods will be made available to other researchers upon reasonable request to the corresponding author.

Mouse Model

We used the SM myosin heavy chain (SMMHC)-Cre-ER^{T2} transgenic mouse line expressing Cre recombinase in SM cells under control of the SMMHC promoter²¹ and a mouse line bearing a floxed allele of the *Agtr1a* gene (*Agtr1a*^{fllox}), encoding the major murine AT1 receptor isoform (AT1aR)²² to generate SMMHC-Cre+*Agtr1a*^{fllox/fllox} (SM-*Agtr1a*^{fl/fl}) mice (Figure S1A). Genotyping was performed by polymerase chain reaction analysis of tail DNA as described previously.²¹ Amplification of the SMMHC-Cre gene was performed in a multiplex polymerase chain reaction with the primers TGA CCC CAT CTC TTC ACT CC (SMWT1), AAC TCC ACG ACC ACC TCA TC (SMWT2), and AGT CCC TCA CAT CCT CAG GTT (phCREAS1).²³ The following primers (5'-3') were

used to identify *Agtr1a*^{fllox} alleles: forward GCT TTC TCT GTT ATG CAG TCT and reverse ATC AGC ACA TCC AGG AAT G. Knockout (SM-*Agtr1a*^{-/-}) was induced in adult (12–16 weeks) SM-*Agtr1a*^{fl/fl} male mice by intraperitoneal injection with tamoxifen (30 µg/mg body weight) on 5 consecutive days. Isolated arteries were usually obtained after 2 to 3 days after tamoxifen treatment. Tamoxifen treatment of mice did not influence myogenic tone (SM-*Agtr1a*^{+/+} cerebral arteries without tamoxifen: 12.35±2.16%, n=6 vessels, each from individual mice; SM-*Agtr1a*^{+/+} cerebral arteries with tamoxifen: 13.23±1.07%, n=4 vessels, each from individual mice). Figure S1B shows reduction of AT1aR expression in vascular SM cells of SM-*Agtr1a*^{-/-} arteries. The same antibody did not detect AT1R expression across the vessel wall from global *Agtr1a*^{-/-} mice¹⁵ (Figure S1C). We also studied adult (12–16 weeks) male mice with global AT1a receptor deficiency (*Agtr1a*^{-/-}),^{15,24,25} and with global AT1b receptor deficiency (*Agtr1b*^{-/-}).²⁶ Age-matched male mice were used as controls in the experiments. Animal care followed American Physiological Society guidelines, and all protocols were approved by local authority (LAGeSo, Berlin, Germany) and the animal welfare officers of the Max Delbrück Center for Molecular Medicine (approval reference number X 9011/16). All procedures conformed to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Mice were maintained in the Max Delbrück Center animal facility in individually ventilated cages (Tecniplast Deutschland, Hohenpeißenberg, Germany) under standardized conditions with an artificial 12-hour dark-light cycle, with free access to standard chow (0.25% sodium; SSIFF Spezialitäten, Soest, Germany) and drinking water. Animals were euthanized by cervical dislocation and randomly assigned to the experimental procedures.

Materials

Antibody to α -SM actin (α -SMA, #ab8211) was from Abcam (Cambridge, MA). Anti-AT1R (#PA5-20812) and donkey antirabbit IgG (H+L) secondary antibody (A10040) were purchased from Thermo Fisher Scientific (Waltham, MA). 4',6-diamidino-2-phenylindole (DAPI, #D9542) was purchased from Sigma-Aldrich Co. (St. Louis, MO). Ang II (#A9525), SII (#sc-391239A), and tamoxifen (#H7904) were from Sigma-Aldrich Co (82024 Taufkirchen, Germany). TRV120055 (#JT-71995) and TRV120056 (#JT-71996) were from Synpeptide Co., Ltd (Shanghai, China). TRV120027 (#HY-P2141A) was from MedChemExpress (Monmouth Junction, NJ).

Mesenteric and Cerebral Arteries

After mice were killed, the mesenteric bed and brain were removed and placed into cold (4 °C), gassed

(95% O₂-5% CO₂) physiological saline solution (PSS) of the following composition (mmol/L): 119 NaCl, 4.7 KCl, 25 NaHCO₃, 1.2 KH₂PO₄, 1.6 CaCl₂, 1.2 MgSO₄, 0.03 EDTA, and 11.1 glucose. Third- or fourth-order mesenteric arteries (MA) and middle cerebral arteries or posterior cerebral arteries were dissected and cleaned of adventitial connective tissue.^{15,27–29}

Pressure Myography

Vessel myography was performed as previously described.^{14,15,21,28} MA or cerebral arteries were mounted on glass cannula and superfused continuously with PSS (95% O₂-5% CO₂; pH, 7.4; 37 °C). The vessels were stepwise pressurized to 20, 40, 60, 80, or 100 mm Hg using a pressure servo control system (Living System Instrumentation, Burlington, VT). We measured the inner diameter of the vessels with a video microscope (Nikon Diaphot, Düsseldorf, Germany) connected to a personal computer for data acquisition and analysis (HaSoTec, Rostock, Germany).^{15,27–30} Arteries were equilibrated for 45 to 60 minutes before starting experiments. A 60-mmol/L KCl challenge was performed before any other intervention.

Isolated Perfused Kidneys

Isolated kidneys were perfused in an organ chamber using a peristaltic pump at constant flow (0.3–1.9 mL/min) of oxygenated (95% O₂ and 5% CO₂) PSS.¹⁵ Drugs (Ang II or biased agonists) were added to the perfusate. Perfusion pressure was measured by a pressure transducer after an equilibration period of 60 to 90 minutes. Data were recorded and analyzed by a Powerlab acquisition system (AD Instruments, Colorado Springs, CO). Ang II-induced pressor effects were normalized to the maximal pressor effect induced by KCl (60 mmol/L).^{14,15,27}

Immunofluorescence

SM-*Agtr1a*^{+/+} and SM-*Agtr1a*^{-/-} mice MA were dissected and further fixed in 4% formaldehyde and embedded in Tissue-Tek O.C.T. compound to be frozen in liquid nitrogen. Tissues were then sectioned and permeabilized in 1% Triton X-100 in PBS. Sections were stained with the primary antibody overnight at 4 °C. After washing with PBS for 3×5 minutes, the secondary antibody and DAPI were applied for 2 hours at room temperature. Fluorescence images were captured by use of Olympus FV1000 confocal microscopy and images were analyzed by ImageJ analysis software.

Statistical Analysis

Data are presented as means±SEM. Statistically significant differences in mean values were determined by

Student unpaired *t* test or 1-way analysis of variance. $P < 0.05$ were considered statistically significant.

RESULTS

AT1aR Is Essential for Pressure-Induced Response in the Renal Circulation

We evaluated myogenic tone in mouse renal circulation, a highly myogenic bed regulating blood flow to

the kidneys and consequently sodium excretion and systemic blood pressure. Renal vascular resistance of isolated perfused kidneys was determined by measuring perfusion pressure at fixed levels of flow. The perfusion pressure increased with flow rate in kidneys of wild-type *Agtr1a*^{+/+} mice, reaching a value of about 160 mm Hg at a flow rate of 1.9 mL/min, which is in the physiological range (between 1.5 and 3.5 mL/min pulsatile flow)³¹ (Figure 1A). Kidneys from *Agtr1a*^{-/-} mice developed significantly less pressure at the same

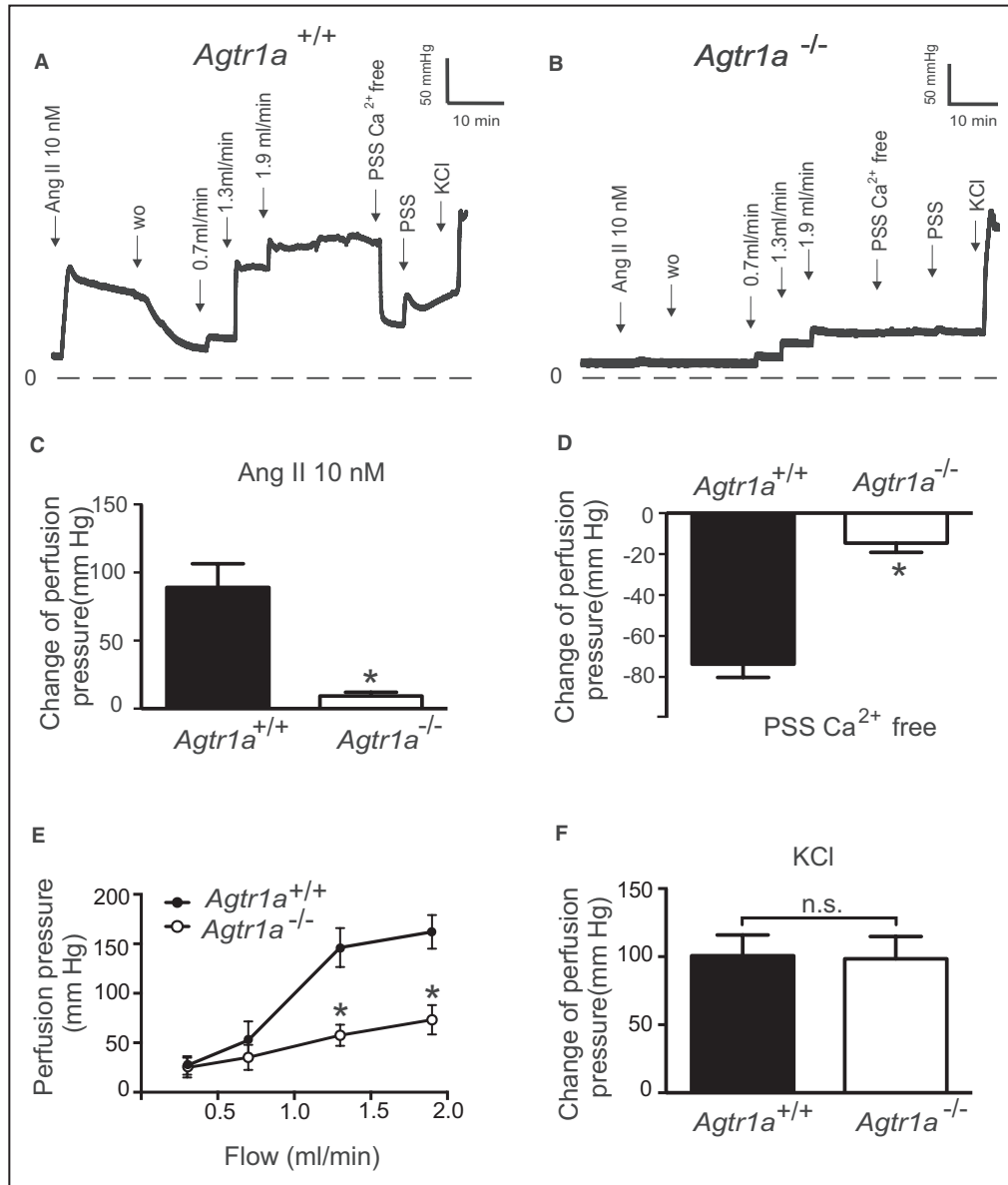


Figure 1. Vasoregulation in isolated perfused kidneys of *Agtr1a*^{-/-} mice.

A and **B**, Original recordings of perfusion pressure in kidneys of *Agtr1a*^{+/+} (**A**) and *Agtr1a*^{-/-} mice (**B**). **C**, Increase in the perfusion pressure induced by 10 nmol/L Ang II. **D**, Myogenic tone assessed by exposure to Ca²⁺ free PSS. **E**, Perfusion pressure at flow rates of 0.3, 0.7, 1.3, and 1.9 mL/min. **F**, Increase in the perfusion pressure induced by 60 mmol/L KCl. $n=6$ *Agtr1a*^{+/+} kidneys from 6 mice and $n=7$ *Agtr1a*^{-/-} kidneys from 7 mice for all panels. * $P < 0.05$. Ang II indicates angiotensin II; n.s., not significant; PSS, physiological saline solution; and wo, wash-out.

flow rate (Figure 1B and 1E). 60 mmol/L KCl-induced increases in perfusion pressure were not altered in *Agtr1a*^{-/-} kidneys (Figure 1F). At a flow rate of 1.9 mL/min, pressure in *Agtr1a*^{-/-} kidneys was ≈100 mm Hg lower than in *Agtr1a*^{+/+} kidneys. Ang II (10 nmol/L) increased perfusion pressure by ≈80 mm Hg in kidneys of *Agtr1a*^{+/+} mice but had no effect in kidneys of *Agtr1a*^{-/-} mice (Figure 1C); this is indicative of AT1aRs mediating Ang II-dependent vasoconstriction. Removal of

external Ca²⁺ nearly abolished flow-induced myogenic constriction in perfused kidneys of *Agtr1a*^{+/+} mice but had no effect in kidneys of *Agtr1a*^{-/-} mice (Figure 1D), indicating AT1aRs mediate also myogenic constriction of mouse renal arterioles. Of note, there was no difference in myogenic tone and Ang II vasoconstrictions between *Agtr1b*^{-/-} versus *Agtr1b*^{+/+} kidneys (Figure 2).

Next, we focused on kidneys from SM-*Agtr1a*^{-/-} mice (Figure 3). At a flow rate of 1.9 mL/min, pressure

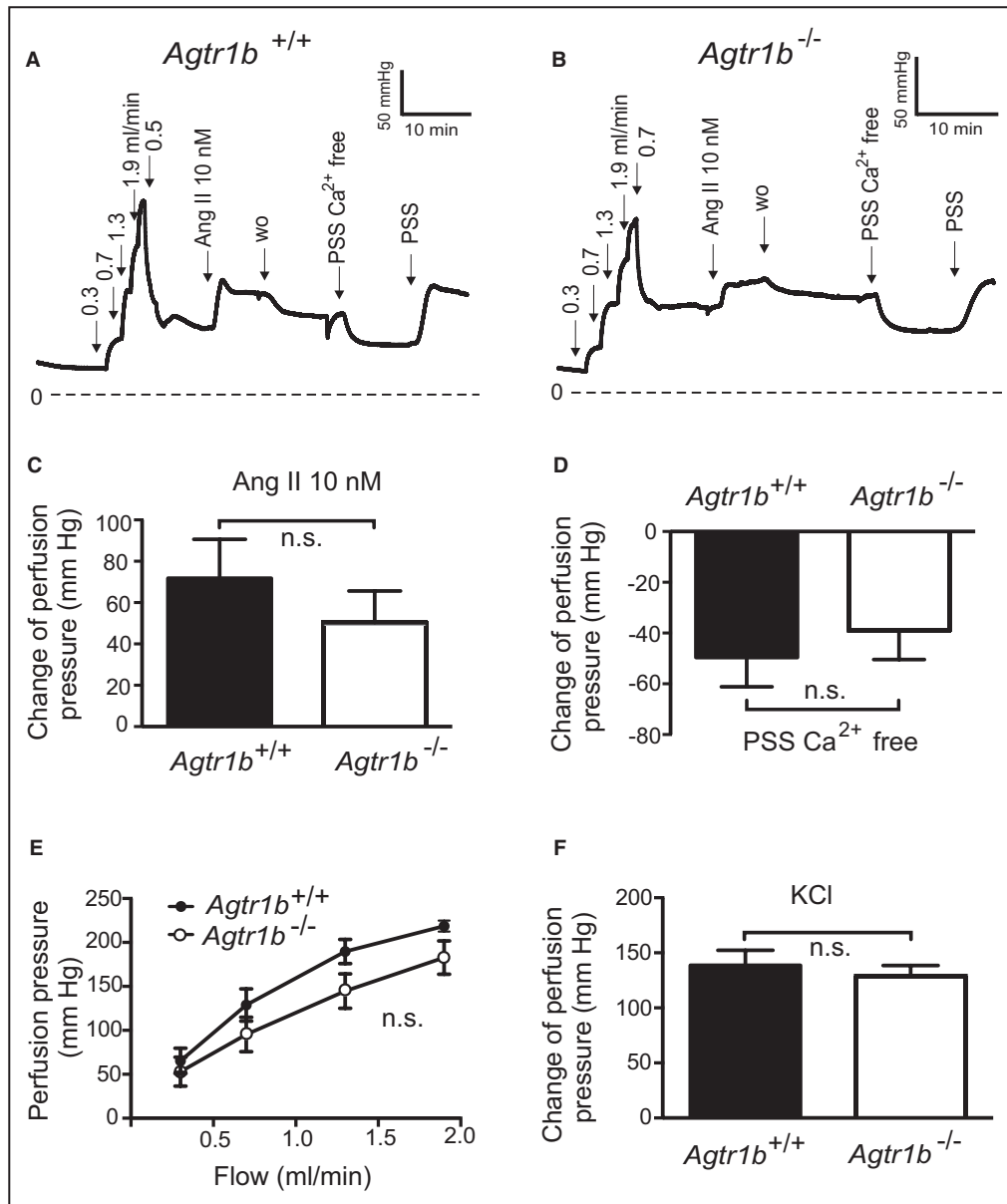


Figure 2. Vasoregulation in isolated perfused kidneys of *Agtr1b*^{-/-} mice.

A and **B**, Original recordings of the perfusion pressure in kidneys of *Agtr1b*^{+/+} (**A**) and *Agtr1b*^{-/-} mice (**B**). **C**, Increase in perfusion pressure induced by 10 nmol/L Ang II. **D**, Change of pressure assessed by exposure to Ca²⁺ free PSS. **E**, Perfusion pressure at flow rates of 0.3, 0.7, 1.3, and 1.9 mL/min. **F**, Increase in perfusion pressure induced by 60 mmol/L KCl. n=6 *Agtr1b*^{+/+} kidneys from 5 mice and n=6 *Agtr1b*^{-/-} kidneys from 3 mice for all panels. Ang II indicates angiotensin II; n.s., not significant; PSS, physiological saline solution; and w.o., washout.

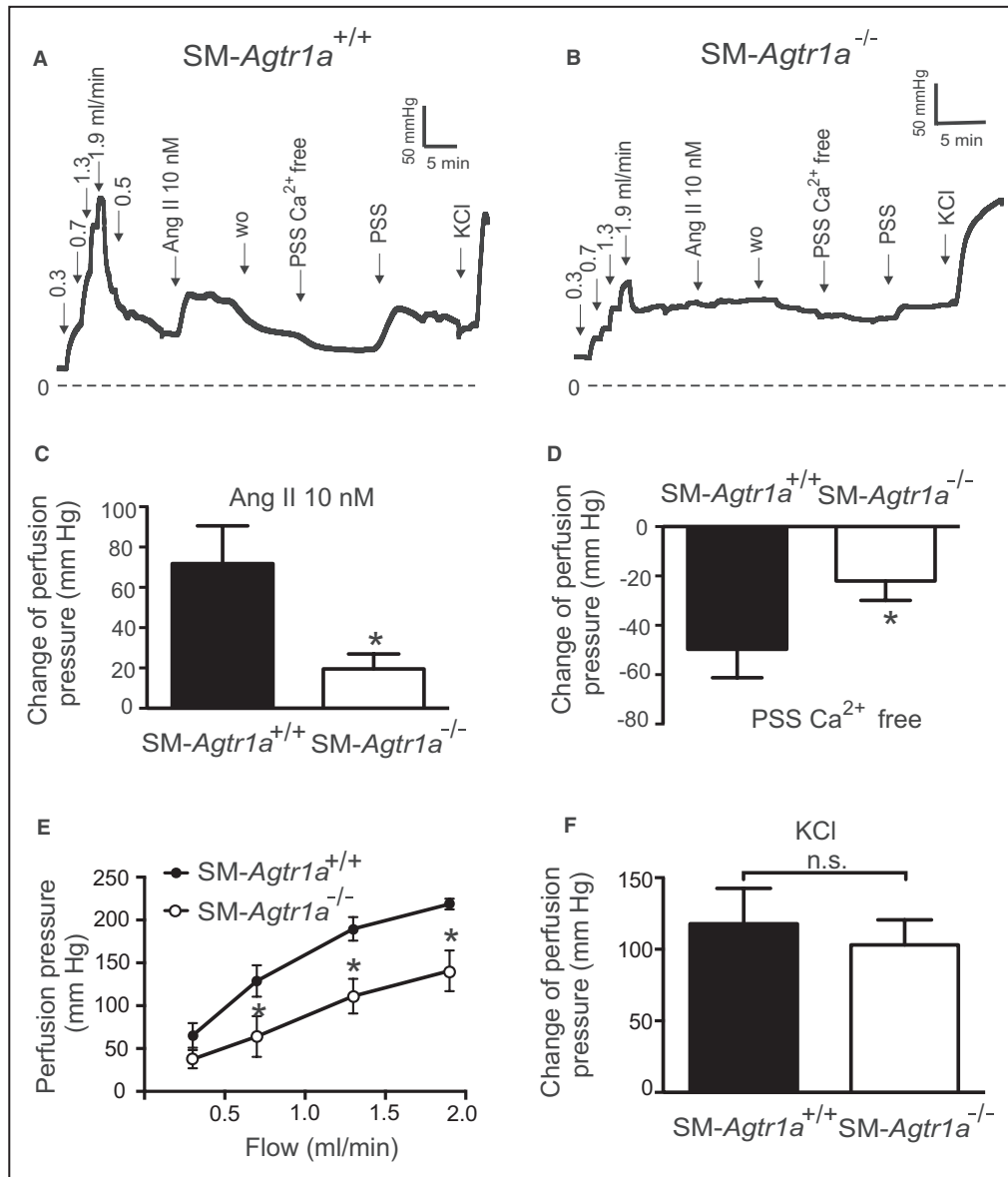


Figure 3. Vasoregulation in isolated perfused kidneys of SM-Agtr1a^{-/-} mice.

A and **B**, Original recordings of the perfusion pressure in kidneys of SM-Agtr1a^{+/+} (**A**) and SM-Agtr1a^{-/-} mice (**B**). **C**, Increase in perfusion pressure induced by 10 nmol/L Ang II. **D**, Change of pressure assessed by exposure to Ca²⁺ free PSS. **E**, Perfusion pressure at flow rates of 0.3, 0.7, 1.3, and 1.9 mL/min. **F**, Increase in perfusion pressure induced by 60 mmol/L KCl. n=6 SM-Agtr1a^{+/+} kidneys from 5 mice and n=6 SM-Agtr1a^{-/-} kidneys from 6 mice for all panels. *P<0.05. Ang II indicates angiotensin II; n.s., not significant; PSS, physiological saline solution; SM, smooth muscle; and w.o., washout.

in SM-Agtr1a^{-/-} kidneys was ≈90 mm Hg lower than in SM-Agtr1a^{+/+} kidneys (Figure 3A, 3B, and 3E). SM-Agtr1a^{-/-} kidneys showed largely reduced myogenic vasoconstriction as assessed by exposure of the kidneys to Ca²⁺ free PSS (Figure 3B and 3D), whereas wild-type kidneys showed strong myogenic vasoconstrictions (Figure 3A and 3D). Sixty mmol/L KCl-induced increases in perfusion pressure were normal in SM-Agtr1a^{-/-} kidneys (Figure 3F). Ang II (10 nmol/L) induced weaker increases in perfusion pressure in kidneys of SM-Agtr1a^{-/-} mice compared with controls

(Figure 3C). Together, these results reveal a key role of AT1aR but not AT1bR in the flow-induced myogenic response and Ang II-evoked constriction of the mouse renal vasculature.

AT1aR Contributes to Myogenic Constriction in Mesenteric and Cerebral Arteries

We first monitored myogenic constriction in resistance-sized MA using videomicroscopy. MA were exposed to

stepwise (20 mm Hg) increases in intraluminal pressure (20–100 mm Hg) in the presence and absence of external Ca^{2+} (1.6 mmol/L) to determine active and passive vessel diameters, respectively. Figure 4 shows representative recordings of MA from *SM-Agtr1a*^{+/+} mice (Figure 4A) and *SM-Agtr1a*^{-/-} mice (Figure 4B) and myogenic vasoconstriction was defined as the diameter difference in the presence and absence of external Ca^{2+} (1.6 mmol/L) at each pressure step.¹⁵ Increases in intraluminal pressure generated active tension that counteracted further dilation of the vessels at 60 to 80 mm Hg in MA from *SM-Agtr1a*^{+/+} mice, reaching peak constrictions of ~50 μm at 80 to 100 mm Hg

(Figure 4A). In contrast, MA from *SM-Agtr1a*^{-/-} mice produced only ~35% of the constriction observed in wild-type arteries (Figure 4B and 4C). Ang II strongly constricted arteries from *SM-Agtr1a*^{+/+} mice but had no effect on arteries from *SM-Agtr1a*^{-/-} mice (Figure 4D); the latter did constrict in response to 60 mmol/L KCl (Figure 4E). This study observed a marked reduction in AT1aR expression in the media of *SM-Agtr1a*^{-/-} MA compared with wild-type (Figure S1B), in keeping with this receptor mediating myogenic constriction in MA. Next, we studied the function of AT1aRs in cerebral arteries. Vessels were equilibrated at 15 mm Hg (30 minutes) and following an assessment of KCl-induced

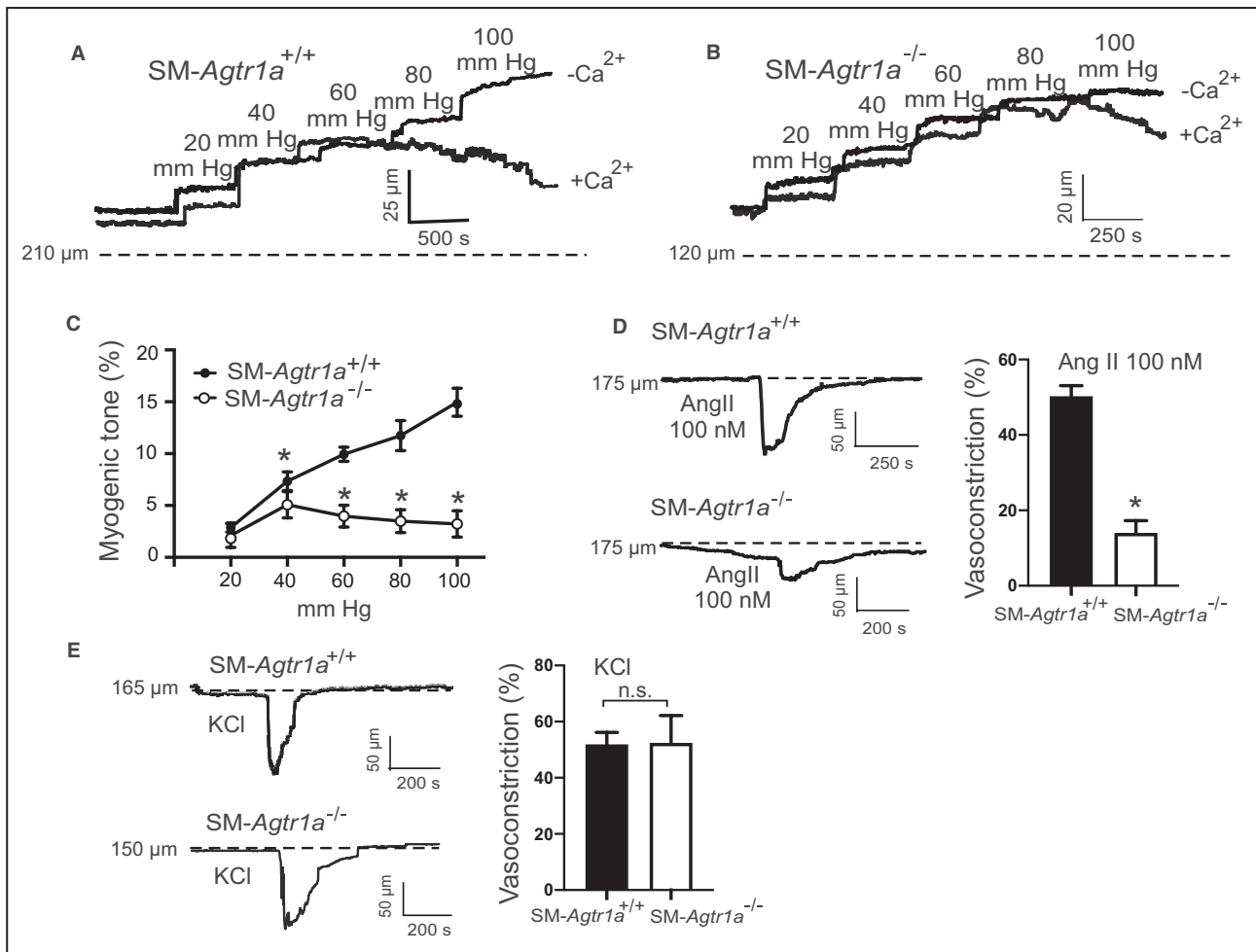


Figure 4. Myogenic tone in mesenteric arteries.

A and B, Representative recordings of MA diameter during a series of pressure steps from 20 to 100 mm Hg in 20 mm Hg increments in control conditions (+ Ca^{2+}) and in Ca^{2+} free solution (- Ca^{2+}). Arteries were isolated from *SM-Agtr1a*^{+/+} (**A**) and *SM-Agtr1a*^{-/-} mice (**B**). Note the increase in active constriction over the entire pressure range from 60 to 100 mm Hg in vessels from *SM-Agtr1a*^{+/+}, but not from *SM-Agtr1a*^{-/-} mice. Vasodilation in Ca^{2+} -free solution was observed in *SM-Agtr1a*^{+/+} but not in *SM-Agtr1a*^{-/-} arteries ($P < 0.05$). **C,** Average myogenic tone of mesenteric arteries in PSS expressed as dilation of vessels induced by external Ca^{2+} free solution (0 Ca/EGTA; *SM-Agtr1a*^{+/+}, $n = 9$, and *SM-Agtr1a*^{-/-}, $n = 6$ vessels, each from individual mice for both groups). **D,** Response to Ang II and **(E)** response to 60 mmol/L KCl in MA of *SM-Agtr1a*^{+/+} and *SM-Agtr1a*^{-/-} mice. MAs were pressurized to 80 mm Hg. Responses are expressed as relative changes in vessel inner diameter. *SM-Agtr1a*^{+/+}, $n = 5$ vessels, and *SM-Agtr1a*^{-/-}, $n = 4$ vessels, from 5 and 4 mice, respectively. * $P < 0.05$. Ang II indicates angiotensin II; MA, mesenteric arteries; PSS, physiological saline solution; and SM, smooth muscle.

constriction, arteries were pressurized to 80 mm Hg (Figure S2A). Ang II constrictions and myogenic constriction was significantly decreased in SM-*Agtr1a*^{-/-} arteries compared with wild-type (Figure S2A through S2D). Both wild-type and SM-*Agtr1a*^{-/-} arteries produced similar constrictions when exposed to 60 mmol/L KCl (Figure S2E). The results demonstrate a key role of AT1aR in the myogenic response of mouse cerebral arteries.

G_{q/11} Protein Dependent Signaling Pathway Is Responsible for Myogenic Tone

To explore the role of G_{q/11} and β-arrestin signaling pathways downstream of AT1R, we used the biased agonists TRV120055, SII, and TRV120027 to activate G_{q/11} and β-arrestin signaling pathways, respectively.^{32–36} We found that TRV120055 increased vascular tone in MA (Figure 5A and 5B), whereas SII and TRV120027 had no effect (Figure 5C through 5F). Similarly, TRV120055 and TRV120056 (another biased G_{q/11} coupled AT1R agonist) enhanced dose-dependent perfusion pressure in isolated kidneys (Figure 6A and 6C), whereas SII had no effect (Figure 6B and 6C). The removal of external Ca²⁺ abolished agonist-induced vasoconstrictions in perfused kidneys (Figure 6D), indicating AT1aRs mediate vasoconstriction via canonical G_{q/11} but not noncanonical β-arrestin pathways. The biased G_{q/11} coupled AT1R agonist TRV120055 and TRV120056 equally modified perfusion pressure (Figure 6E). Viability of the isolated perfused kidneys was assessed by 60 mmol/L KCl (Figure 6F). To confirm the results, we next examined the effects of FR900359, a selective G_{q/11}-protein inhibitor.^{37–39} FR900359 abolished both myogenic and Ang II-dependent constrictions in renal arterioles (Figure 7), MA (Figure 5G and 5H) and cerebral arteries (Figure S2F). These results indicate that myogenic vasoconstriction is mediated through the mechanosensitive AT1aR and the canonical G_{q/11} signaling pathway.

DISCUSSION

AT1Rs Are Primary Mechanosensors in Intact Arteries

Multiple GPCRs have been proposed to act as mechanosensors to regulate myogenic tone in resistance

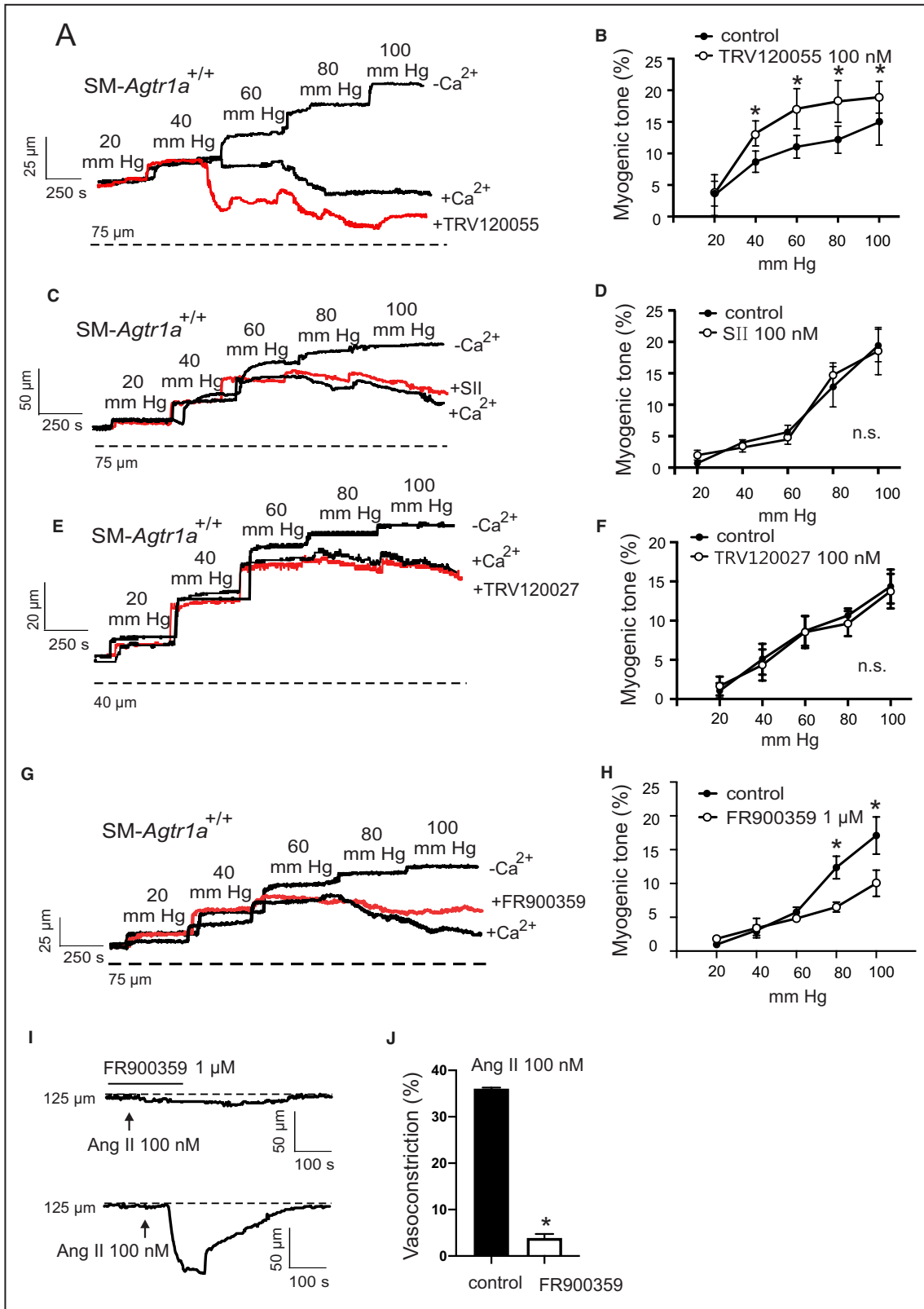
arteries. Although stretch induces activation of purinergic P2Y6 UDP receptors, thromboxane A2 receptors and sphingosine-1-phosphate receptors in certain vascular beds,^{40–42} the AT1R remains one of the best characterized mechanosensor in the vasculature.^{19,43} Humans express a single type of AT1R, whereas 2 isoforms (AT1aR and AT1bR) are present in rodents.^{44,45} Using *Agtr1a*^{-/-} mice and inverse AT1R agonist, our previous data suggested that ligand-independent AT1aR activation is required for myogenic response in resistance MA and renal arterioles.¹⁵ Two recent studies reported also a possible role of AT1bRs in myogenic constriction in certain vessels.^{46,47} We found that Ang II and myogenic constrictions were normal in *Agtr1b*^{-/-} perfused kidneys, which is consistent with AT1A receptor being the major murine AT1 receptor isoform in the renal circulation.⁴⁸ All data were, however, obtained in global mutant mice, which often display compensatory mechanisms for the lack of AT1Rs and can lead to phenotypes that are difficult or impossible to interpret in terms of cardiovascular function. For example, AT1aR and AT1bR are expressed at similar levels in cerebral parenchymal arterioles and genetic knockout of AT1aR (but not AT1bR) blunted the ability of these vessels to generate myogenic tone.⁴⁹ The latter effect is opposite to cerebral arteries where genetic knockout of AT1bR blunted the ability to develop myogenic tone.⁴⁶ To overcome these potential limitations, we generated tamoxifen-inducible SM-*Agtr1a* (SMMHC-Cre+*Agtr1a*^{fllox/fllox}) mice for careful phenotypic investigation. We found that myogenic constriction was impaired in cerebral, mesenteric, and renal arteries isolated from SM AT1aR-deficient mice. Our data provide firm evidence that AT1Rs play a key role as mechanosensors mediating myogenic constriction in the murine vasculature.

AT1Rs Downstream Signaling to Cause Vasoconstriction

We next explored downstream signaling pathways mediated by G_{q/11} and/or β-arrestin of AT1R in the vascular response. In cell culture, osmotic cell stretch has been found to increase the binding affinity and potency of the β-arrestin-biased agonist TRV120023 with no effect on the balanced agonist Ang II through AT1R to induce a conformation change of β-arrestin 2, similar to that induced by β-arrestin-biased agonists.²⁰

Figure 5. Enhancement of the vascular tone by TRV120055.

A, C, E, and G, Representative recordings of mesenteric artery diameter during a series of pressure steps from 20 to 100 mm Hg in 20 mm Hg increments in control conditions (+Ca²⁺), TRV120055 100 nmol/L (**A**), SII 100 nmol/L (**C**), TRV120027 100 nmol/L (**E**), FR900359 1 μmol/L (**G**) and in Ca²⁺-free solution. **B, D, F, and H,** Average myogenic constriction of mesenteric arteries in drug-free physiological saline solution and in PSS containing 100 nmol/L TRV120055 (**B**), 100 nmol/L SII (**D**), TRV120027 100 nmol/L (**F**) and 1 μmol/L FR900359 (**H**) (n=6, 4, 5 and 4, respectively, each from individual mice). **I and J,** Response to Ang II in MA in drug-free PSS and PSS in presence of FR900359 at 80 mm Hg (n=6 each from individual mice). *P<0.05. Ang II indicates angiotensin II; n.s. indicates not significant; and SII, Sar11le41le8-angiotensin.



Similarly, hypo-osmotic stretch induced β -arrestin-biased signaling of AT1Rs in the absence of G protein activation.¹⁸ We failed to observe β -arrestin-mediated

enhancement of myogenic constriction with the β -arrestin biased agonists SII and TRV120027 in intact arteries (mesenteric and renal arteries: Figure 8). The

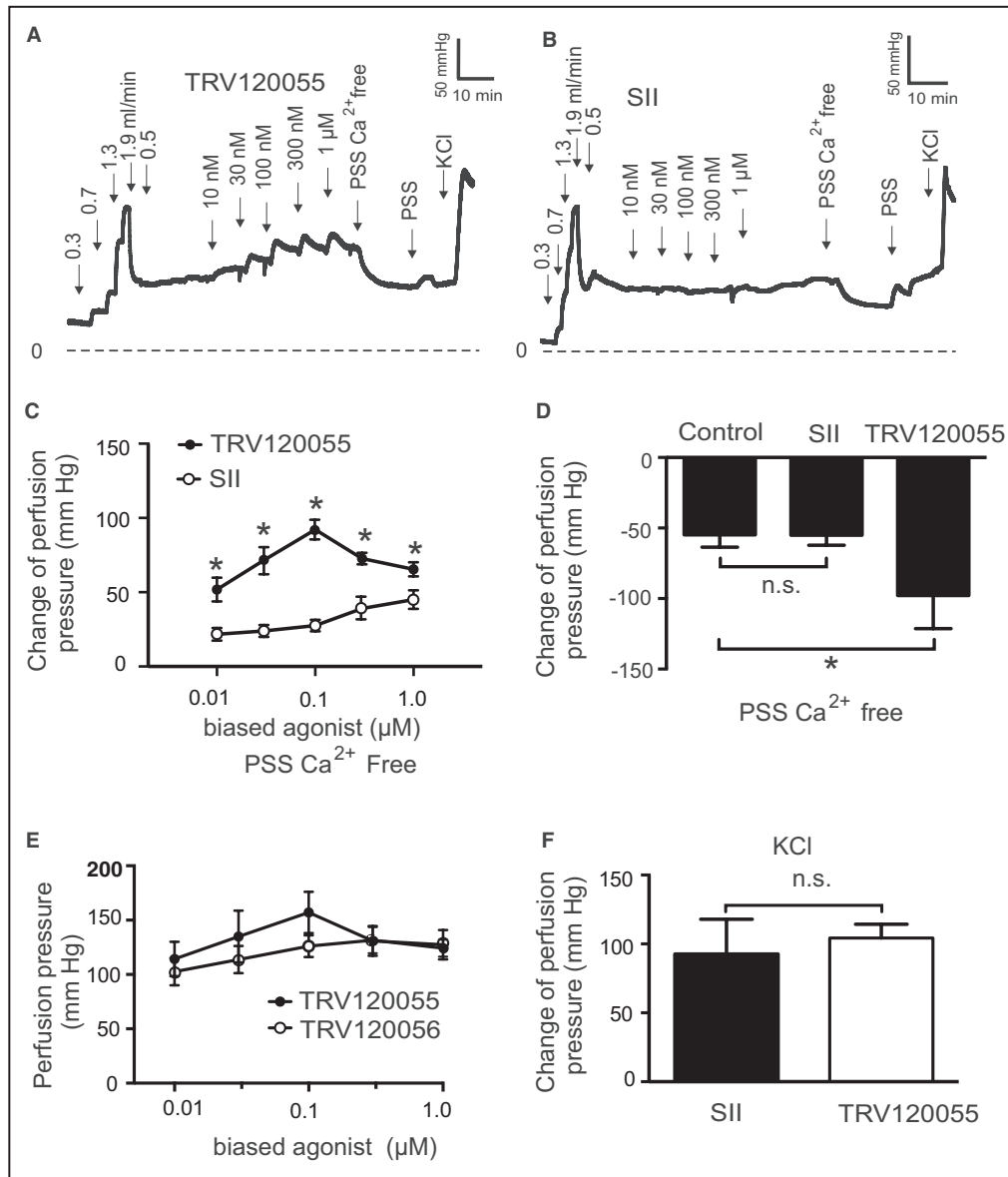


Figure 6. Function of biased AT1R agonists to vasoregulation in isolated perfused kidneys from SM-Agtr1a^{+/+} mice.

A and **B**, Original recordings of perfusion pressure in response to various flow rates (in mL/min), TRV120055 (**A**) or Sar1Ile4Ile8-SII (**B**), Ca²⁺ free perfusion solution (PSS Ca²⁺ free) and reexposure of the kidneys to PSS. **C**, Increase in perfusion pressure induced by TRV120055 and SII in various concentrations (10 nmol/L to 1 μmol/L). **D**, Change of perfusion pressure assessed by exposure of the kidneys to Ca²⁺ free PSS at the presence of TRV120055 or Sar-Ile II at the concentration of 100 nmol/L. **E**, Dose-response relationships for TRV120055 and TRV120056. **F**, Increase in perfusion pressure induced by 60 mmol/L KCl. TRV120055, TRV120056, SII. n=6 kidneys from 5 mice in each group; n=6 kidneys from 5 mice in the control group. *P<0.05. AT1R, angiotensin II type 1 receptor; Control, SM-Agtr1a^{+/+} without biased ligand; n.s., not significant; PSS, physiological saline solution; and SM, smooth muscle.

discrepancy might be caused by differences between hypo-osmotic cell swelling and tensile stretch on the SM cell layer in intact arteries to cause mechanoactivation of AT1Rs in situ. AT1R is one of the best characterized GPCR enabling biased receptor signaling. It can be activated in either a canonical G protein-dependent signaling mode^{14,50} or noncanonical β-arrestin-mediated

signaling mode.^{18,20} In line, we found that the natural biased agonist Ang II was able to increase G protein signaling of mechanoactivated AT1R receptors to enhance the vasoconstrictor response. A recent study reported that GPCRs, including AT1R, can promote a direct interaction between Gai protein subtype family members and β-arrestins, regardless of their canonical

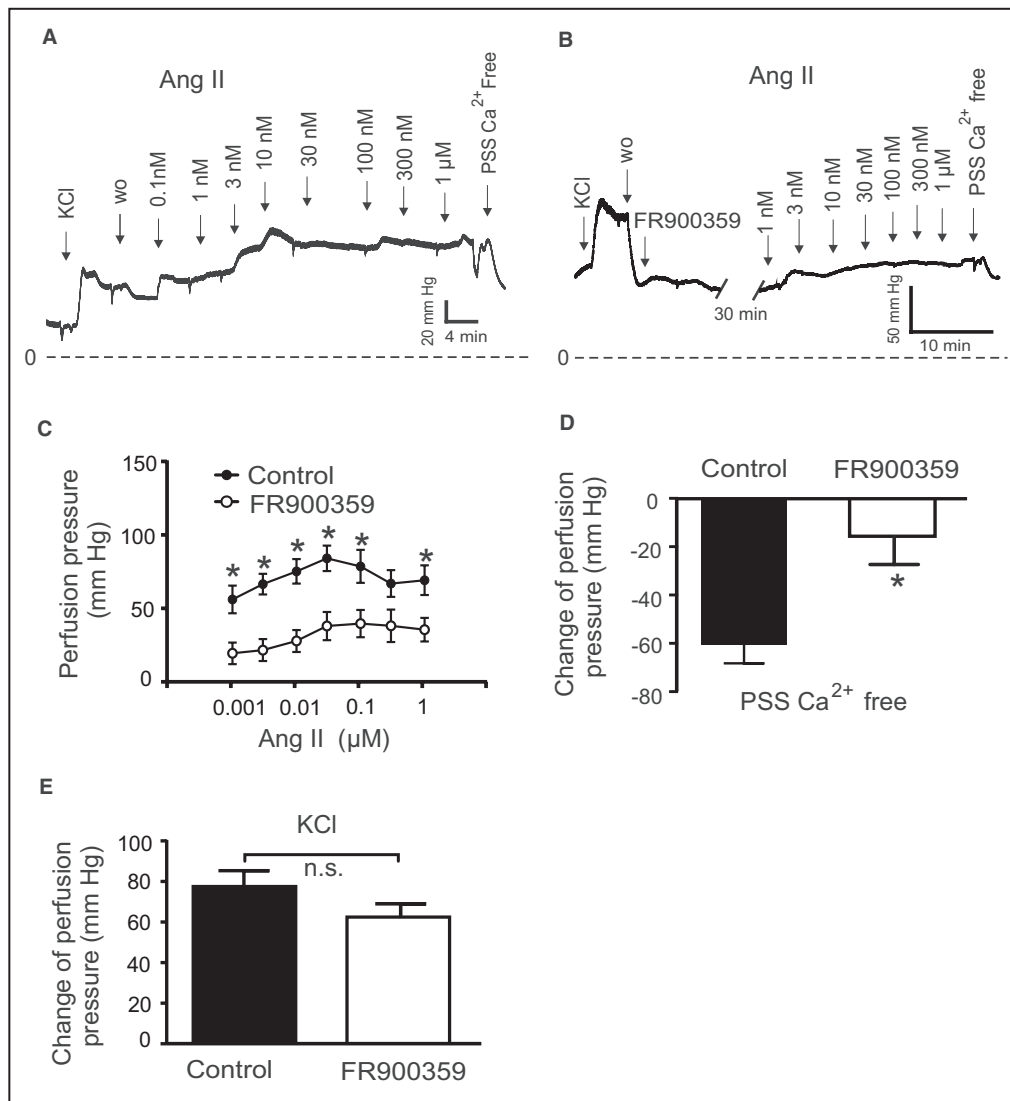


Figure 7. Vasoregulation in isolated perfused kidneys of SM-*Agtr1a*^{+/+} mice pretreated with 300 nmol/L G_{q/11} blocker FR900359.

A, Original recordings of perfusion pressure in kidneys of *Agtr1a*^{+/+} mice in response to various concentrations of Ang II (**B**) same as (**A**) but pretreated with 300 nmol/L FR900359 for 30 minutes. **C**, Increases in perfusion pressure induced by Ang II (1 nmol/L to 1 μmol/L). **D**, Myogenic tone assessed by exposure of the kidneys to Ca²⁺-free PSS. **E**, Increase in perfusion pressure induced by 60 mmol/L KCl. n=15 SM-*Agtr1a*^{+/+} kidneys from 12 mice and n=6 SM-*Agtr1a*^{+/+} kidneys from 4 mice pretreated with FR900359 for all panels. **P*<0.05. Ang II indicates angiotensin II; n.s., not significant; PSS, physiological saline solution; SM, smooth muscle; and w.o., wash-out.

G protein subtype coupling, that might be prerequisite for certain β-arrestin signaling pathways.⁵¹

We hypothesized that G_{q/11} signaling contributes to myogenic tone in MA and renal arteries and consistent with this idea, we found that the vasoconstrictor responses were strongly increased by the G_{q/11} AT1R biased agonists TRV120055 and TRV20056 (Figure 8). Moreover, we found that the G_{q/11} blocker FR900359 inhibited both myogenic tone and Ang II-induced constrictions in MA and renal arterioles (Figure 8). The data imply that myogenic vasoconstriction requires

canonical G_{q/11} signaling of the AT1R. Consistently, myogenic tone is increased in the absence of regulator of G-protein signaling 2, which is an endogenous terminator of Galpha_{q/11} (Gα_{q/11}) signaling.^{14,30} The data align with findings indicating that mechanically activated AT1Rs generate diacylglycerol, which in turn activates protein kinase C to induce the actin cytoskeleton reorganization necessary for pressure-induced vasoconstriction.⁵² Finally, our conclusions are supported by findings indicating that another G_{q/11}-protein inhibitor YM 254890 profoundly reduced myogenic tone in MA⁴³ (but see

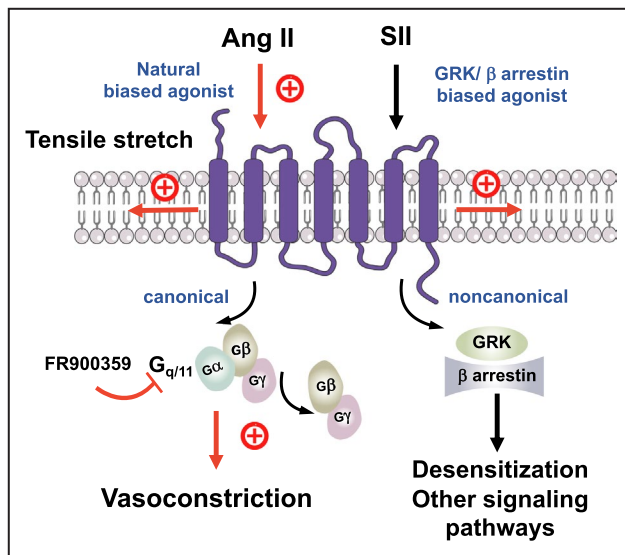


Figure 8. Schematic illustration of angiotensin II type 1a receptor (AT1aR) biased signaling cascade regulating myogenic arterial tone.

Canonical $G_{q/11}$ signaling pathway of the AT1R (purple blue) causes myogenic vasoconstriction whereas noncanonical β -arrestin-biased signaling is not involved in this process. $G_{q/11}$ proteins are heterotrimeric G proteins, which are made up of alpha (α), beta (β), and gamma (γ) subunits. The alpha subunit is attached to either a guanosine triphosphate (GTP) or guanosine diphosphate (GDP), which serves as an on-off switch for the activation of the G-protein. Upon activation of the AT1aR by either ligand-independent mechanical stretch or the natural-biased ligand Ang II, the $G\beta\gamma$ complex is released from the $G\alpha$ subunit after its GDP-GTP exchange for canonical G protein signaling to cause myogenic and/or humoral (Ang II-mediated) vasoconstriction. This pathway is inhibited by the $G_{q/11}$ inhibitor FR900359. Although, GRKs and arrestins play a role in multiple noncanonical signaling pathways in cells, this pathway is unlikely engaged by mechanoactivated AT1Rs in response to tensile stretch or their natural ligand angiotensin II to cause vasoconstriction. Ang II indicates angiotensin II; GRK, G protein-coupled receptor kinase; and SII, Sar11le41le8-angiotensin.

Ref. 50). Our study provides firm evidence that AT1Rs coupled to $G_{q/11}$ signaling is an essential component of dynamic mechanochemical signaling in arterial vascular SM cells causing myogenic tone (Figure 8).

Signaling of most GPCRs via G proteins is terminated (desensitization) by the phosphorylation of active receptor by specific kinases (GPCR kinases) and subsequent binding of β -arrestins that selectively recognize active phosphorylated receptors. Although, GPCR kinases and β -arrestins also play a role in multiple non-canonical signaling pathways in the cell, both GPCR initiated and receptor independent,^{53,54} our study demonstrates that this pathway is not involved in the myogenic response (Figure 8). Thus, blood pressure-lowering effects of β -arrestin biased AT1R agonists, for example, Trevena 120027,⁵⁵ are unlikely caused by direct effects of this GPCR in arterial SM cells.

CONCLUSIONS

In summary, we provide new and firm evidence for a mechanosensitive function of AT1R in myogenic constriction in mesenteric, renal, and cerebral arteries, that is, in 3 different highly myogenic vascular beds. Our study clearly shows that mechanical stress activates AT1R in arterial SM cells, which subsequently triggers canonical $G_{q/11}$ signaling, irrespective of GPCR kinases/ β -arrestin signaling, to cause myogenic vasoconstriction. Our results argue against the idea of multiple mechanosensors coupled to noncanonical β -arrestin pathways generating myogenic arterial tone. These findings lay ground for future studies to characterize the molecular mechanisms of mechanoactivated AT1R coupled to $G_{q/11}$ signaling in intact arteries, which may reveal new molecular targets for drug development to alleviate increased or dysregulated arterial tone in hypertension and other cardiovascular diseases.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Figures S1–S2

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SUPPLEMENTAL MATERIAL

Supplemental Figures

Figure S1. Conditional deletion of AT1a receptors in vascular smooth muscle cells of arteries. **A:** Schematic representation of the mouse allele containing loxP sequences, and the floxed allele after the action of Cre recombinase. **B:** Immunofluorescence staining results show that AT1R (red) is highly expressed in the mesenteric artery of SM-*Agtr1a*^{+/+} mice. In SM-*Agtr1a*^{-/-} mouse mesenteric artery, the expression of AT1R is specifically reduced in smooth muscle cells. **C:** Expression of AT1R is completely reduced in the mesenteric artery of global *Agtr1a*^{-/-} mice. DAPI (4',6-diamino-2-phenylindole) nuclear staining, alpha-SMA (alpha smooth muscle actin), Scale bar: 40 μ m.

Figure S2. Myogenic tone in cerebral arteries. **A, B:** Representative recordings of middle/posterior cerebral arteries diameter at the pressure of 80 mmHg in control conditions (WT), Ang II 100 nmol/L, and in Ca²⁺ free solution. **C:** Myogenic tone (at 80 mmHg) expressed as dilation of vessels induced by external Ca²⁺ free solution. **D, E:** Response to Ang II (**D**) and 60 mM KCl (**E**) in middle/posterior cerebral arteries of SM-*Agtr1a*^{+/+} and SM-*Agtr1a*^{-/-} mice. SM-*Agtr1a*^{+/+}, n=6 vessels from 6 mice, and SM-*Agtr1a*^{-/-}, n=6 vessels from 6 mice. **F:** Average myogenic constriction of cerebral arteries in physiological salt solution (PSS) in the absence and presence of 1 μ mol/L FR900359 (n=6 each, from 6 mice each). **G:** Response to Ang II in the absence and presence of 1 μ mol/L FR900359 at 80 mmHg (n=6 each, from 6 mice each). *p<0.05; n.s., not significant.

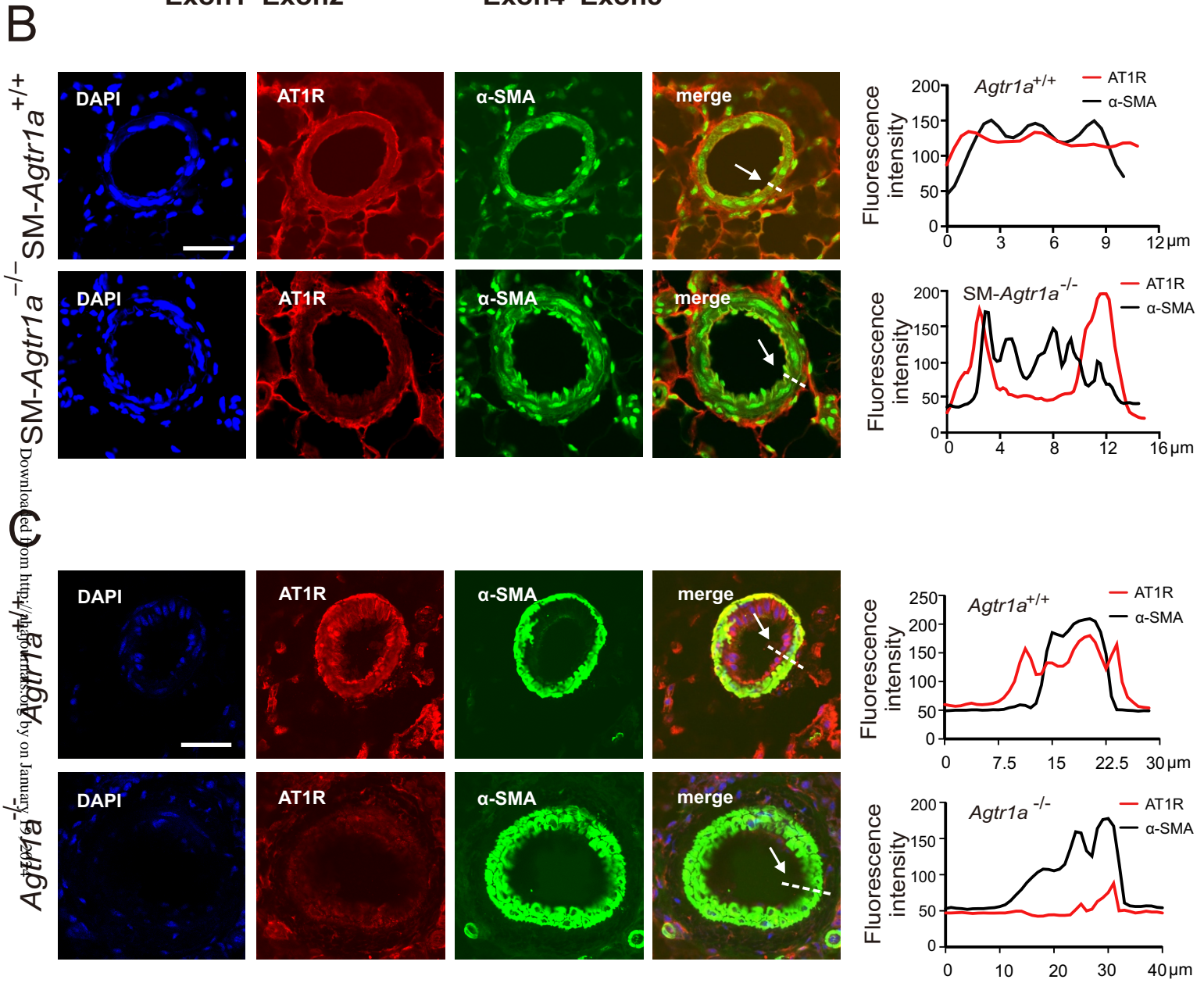
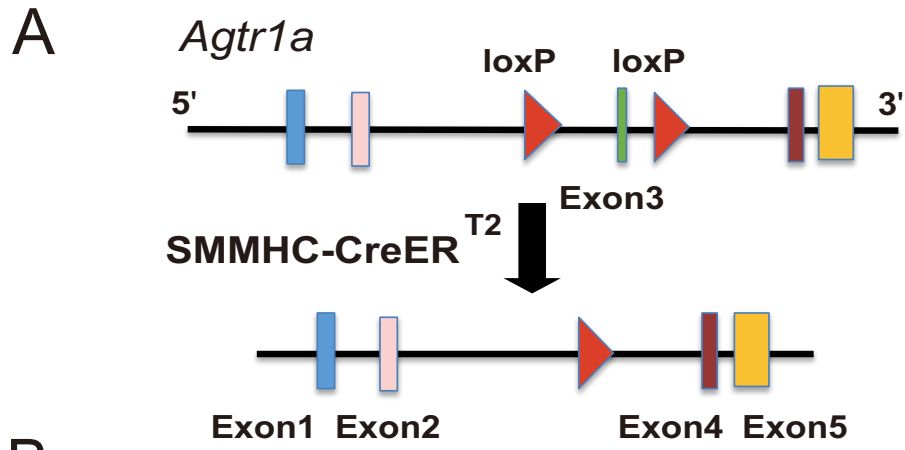


Figure S1

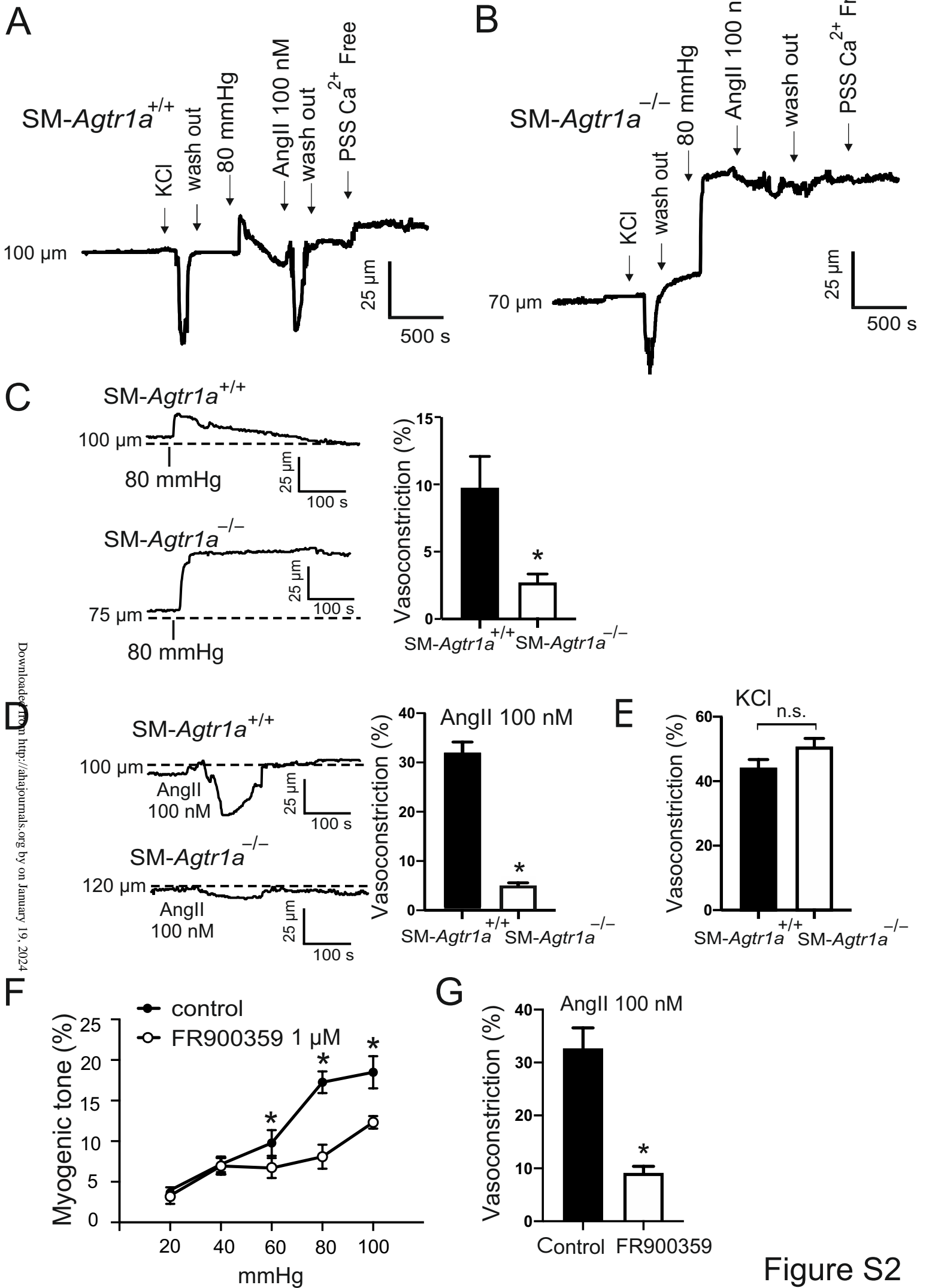


Figure S2