# Central angiotensin-II increases blood pressure and sympathetic outflow via Rho kinase activation in conscious rabbits

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# Central Angiotensin-II Increases Blood Pressure and Sympathetic Outflow via Rho Kinase Activation in Conscious Rabbits

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## Abstract

Elevated sympathetic tone and activation of the renin-angiotensin system are pathophysiologic hallmarks of hypertension, and the interactions between these systems are particularly deleterious. The importance of Rho kinase as a mediator of the effects of Angiotensin-II in the periphery is clear, but the role of Rho kinase in sympatho-excitation caused by central angiotensin-II is not well-established. We hypothesized that angiotensin-II mediates its effects in the brain by activation of the RhoA/Rho kinase pathway. Chronically instrumented, conscious rabbits received the following intracerebroventricular infusion treatments for two weeks via osmotic minipump: angiotensin-II, the Rho kinase inhibitor Fasudil, angiotensin-II plus Fasudil, or a vehicle control. Angiotensin-II increased mean arterial pressure over the course of the infusion, and this effect was prevented by co-administration of Fasudil. Angiotensin-II increased cardiac and vascular sympathetic outflow as quantified by the heart rate response to metoprolol and the depressor effect of hexamethonium; co-administration of Fasudil abolished both of these effects. Angiotensin-II increased baseline renal sympathetic nerve activity in conscious animals and impaired baroreflex control of sympathetic nerve activity; again Fasudil co-infusion prevented these effects. Each of these endpoints showed a statistically significant interaction between angiotensin-II and Fasudil. Quantitative immunofluorescence of brain slices confirmed that Rho kinase activity was increased by angiotensin-II and decreased by Fasudil. Taken together, these data indicate that the hypertension, elevated sympathetic outflow, and baroreflex dysfunction caused by central angiotensin-II are mediated by Rho kinase activation and suggest that Rho kinase inhibition may be an important therapeutic target in sympatho-excitatory cardiovascular diseases.

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## Keywords

rho-Associated Kinases; Angiotensin II; Sympathetic Nervous System; hypertension; Baroreflex; rhoA GTP-Binding Protein; Autonomic Nervous System Diseases

## INTRODUCTION

Activation of the renin-angiotensin-aldosterone system (RAAS) and sympatho-excitation are important maladaptive mechanisms in cardiovascular, renal, and metabolic disease[1–4]. In these chronic diseases, the RAAS and the sympathetic nervous system interact in a particularly deleterious, feedforward manner[5,6]. The primary effector peptide of the RAAS, angiotensin-II (AngII), acts via the AngII type 1 receptor (AT1R) in the brain to raise blood pressure, increase sympathetic outflow, and impair baroreflex function[7,8]. Recently, the intracellular mediators of AT1R signaling in both the periphery and the brain have garnered increasing interest[9–11]. Specifically, signaling in autonomic centers in the forebrain like the subfornical organ (SFO) and the paraventricular regions of the hypothalamus (PVH) is particularly important experimental models of AngII-induced hypertension and cardiovascular disease[12–16].

Many studies have shown that the RhoA/Rho kinase pathway is a crucial downstream effector of AT1R activation by AngII in the heart, endothelium, and vasculature[17–19]. RhoA/Rho kinase is particularly important in actin cytoskeleton assembly, calcium sensitization, and nitric oxide bioavailability[20,21]. Patients with diseases characterized by RAAS activation and autonomic dysfunction like hypertension and heart failure have elevated peripheral Rho kinase activity[22–25].

While logistical issues have prevented assaying central Rho kinase activity in patients, central Rho kinase activity is elevated in experimental models of hypertension and heart failure[24,26–29]. Moreover, central Rho kinase inhibition has beneficial autonomic effects in experimental models of cardiovascular disease[24,26,28–31]. Despite the importance of Rho kinase in peripheral AT1R signaling and central autonomic regulation, the interaction between the autonomic effects of central AngII and the Rho kinase pathway has never been directly tested. Therefore, in the present study, we hypothesized that AngII mediates its central effects by activation of the Rho kinase pathway, leading to baroreflex dysfunction, increased sympathetic tone, and hypertension.

## MATERIALS AND METHODS

### Animals

A more detailed description of the materials and methods can be found in the online supplemental file. Experiments were carried out on male New Zealand White rabbits ranging in weight from 3.1 to 4.5 kg (Charles River Laboratories, International, Wilmington, MA). All experiments were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

### Within-rabbits Cohort

To test the hypothesis that Rho kinase activation mediates the hemodynamic and autonomic effects of central AngII, six rabbits were instrumented with arterial pressure (AP) radiotelemetry transducers and intracerebroventricular (ICV) cannulae. Each rabbit received ICV infusions of AngII (40 ng/min; Sigma-Aldrich Corp., St. Louis, MO), AngII plus the Rho kinase inhibitor Fasudil (Fas, 175 ng/min; GeneDEPOT, Barker, TX), Fas alone, and artificial cerebrospinal fluid vehicle (Veh) via osmotic minipump in a randomized order. Mean arterial pressure (MAP) and HR were measured daily, and volume status[32], cardiac parasympathetic tone[33], cardiac sympathetic tone, global sympathetic vasomotor tone, and baroreflex function were assessed on days 10 through 14 of treatment by IV administration of FITC-sinistrin, autonomic blockers, or vasoactive drugs. This time period (days 10 through 14 of treatment) is referred to as the late phase of treatment. Between treatment infusions, rabbits received a 7–14 day washout with a Veh minipump.

### Between-rabbits Cohort

To test the hypothesis that Rho kinase activation mediates the renal sympatho-excitation caused by central AngII, rabbits were instrumented with radiotelemetry transducers, ICV cannulae, and renal sympathetic nerve electrodes. Seven days after renal nerve electrode surgery, resting RSNA and baroreflex control of RSNA were assessed. Not all chronic sympathetic nerve electrode implantations were successful (Online Figure S4B), and rabbits without RSNA bursts one week after implantation were excluded from the study. Rabbits were subsequently euthanized and perfused with paraformaldehyde, and their brains were collected for molecular analysis.

### Assessment of Rho Kinase Activity

To validate that central AngII activates Rho kinase and that this activation is inhibited by Fasudil, Rho kinase activity was assessed using a LiCor Odyssey scanner to perform quantitative immunofluorescence[34] on sagittal brain slices with antibodies raised against the 695-threonine phosphorylated form of the Rho kinase target myosin phosphatase targeting protein (p-MYPT).

### Statistical Analysis

Individual data are shown via dot plots with each dot representing one rabbit, and group data are displayed as group mean ± SEM. Statistical testing on endpoints from the within-rabbits study was performed using two-way or three-way repeated measure (RM) analysis of variance (ANOVA) with $\alpha = 0.05$. Statistical testing on endpoints from the between-rabbits study was performed using two-way ANOVA with $\alpha = 0.05$. The P values for the AngII, Fas, and AngII × Fas terms from the ANOVA are reported throughout the manuscript. Crucial to the central hypothesis of this manuscript, $P_{AngII \times Fas} < \alpha$ indicates that the response to AngII is significantly modified by Fas, i.e. that there is a non-additive interaction between AngII and Fas. This allows for statistical testing of the hypothesis that the effects of AngII are mediated by the Rho kinase pathway. In the absence of a significant interaction (i.e. $P_{AngII \times Fas} \gg \alpha$), $P_{AngII} < \alpha$ indicates that AngII significantly affects the endpoint independent of Fas. This would be the expected result for a Rho kinase-independent effect of

AngII. If the ANOVA reached statistical significance, it was followed by Bonferroni-corrected, paired t-tests of Veh versus AngII and AngII versus AngII+Fas ($\alpha = 0.025$). These t-tests were chosen as they most closely relate to the central hypothesis of the study while other t-tests (e.g., Veh vs. Fas) were foregone in order to decrease the effect of corrections for multiple comparisons on power.

## RESULTS

### Baseline Hemodynamics

We set out to evaluate the role of the Rho kinase pathway in the effect of AngII on basal hemodynamics by measuring MAP and HR daily over the course of ICV treatments (Figure 1A). Power analysis and treatment order for this cohort can be found in Online Figure S1. Compared to Veh treatment, ICV infusion of AngII significantly increased MAP in all rabbits (Figure 1B and 1C), and this increase in MAP was abolished by co-administration of Fas. The statistically significant interaction between AngII and Fas indicates that ICV AngII-mediated increases MAP are dependent on Rho kinase activation. Conversely, none of the treatments had a significant effect on HR during the entire course of the infusion (Figure 1D) or in the late phase of infusion (Figure 1E).

### Pharmacological Assessment of Autonomic Balance

Cardiac parasympathetic tone was assessed by the HR response to atropine. This was not significantly affected by any ICV treatment (Figure 2A, Online Table S1). Cardiac sympathetic tone assessed by the HR response to metoprolol was significantly increased by ICV infusion of AngII, and this cardiac sympatho-excitation was blocked by Fas co-administration (Figure 2B, Online Table S2). The statistically significant interaction between AngII and Fas indicates that the AngII-mediated cardiac sympatho-excitation depends on Rho kinase activation.

Global sympathetic vasomotor tone assessed by the MAP response to hexamethonium was significantly elevated by ICV infusion of AngII, and again co-administration of Fas blocked this elevation despite Fas alone having little effect (Figure 2C, Online Table S3). This interaction between AngII and Fas indicates that AngII elevates sympathetic vasomotor tone by Rho kinase activation.

Assessment of baroreflex function with vasoactive drugs showed that ICV AngII caused a right-shift of the cardiac baroreflex curve in these rabbits without significantly affecting any other indexes of cardiac baroreflex function like gain or range (Online Figure S2 and Table S4). Fas decreased the lower HR plateau independent of AngII, consistent with Fas-mediated augmentation of maximal vagal outflow. Assessments of volume homeostasis showed that, despite increases in MAP, extracellular fluid volume was not decreased in rabbits receiving ICV AngII, nor did AngII affect other two-compartment model parameters (Online Figure S3 and Table S5).

### Baseline RSNA

We set out to directly measure RSNA in conscious rabbits using the experimental paradigm shown in Figure 3A. The power analysis and flow diagram for this cohort is shown in Online Figure S4. Baseline hemodynamics are shown in Table S6; notably, AngII-treated rabbits in this cohort were hypertensive while HR did not significantly differ between treatment groups.

Rabbits receiving ICV AngII had significantly increased baseline RSNA when quantified as a percent of the nasopharyngeal reflex (Figure 3B and 3C) and tended to exhibit increased RSNA burst frequency and burst incidence (Figure 3D and 3E). Again, Fas alone had little effect, and the statistically significant interaction between AngII and Fas with regard to normalized RSNA and RSNA burst incidence indicates that AngII-mediated renal sympatho-excitation depends on Rho kinase activation.

### Baroreflex Control of Renal Sympathetic Nerve Activity

ICV AngII impaired baroreflex control of RSNA compared to Veh treatment, and Fas co-administration prevented this baroreflex dysfunction (Figure 4A and 4B). When RSNA was expressed relative to basal RSNA, rabbits receiving AngII showed decreased RSNA baroreflex range (Online Table S7), with the upper plateau significantly lower ($P < 0.025$ vs. Veh) and the lower plateau significantly higher ($P < 0.025$ vs. Veh). These effects depended on Rho kinase activation ($P_{AngII \times Fas} < 0.05$). Similarly, AngII treatment lowered baroreflex gain when expressed as a percentage of basal RSNA ($P < 0.025$ vs. Veh) only in the absence of Fas coadministration.

We also analyzed baroreflex curves with RSNA normalized to the nasopharyngeal reflex to account for the aforementioned differences in baseline RSNA (Figure 4C and 4D). When RSNA was normalized in this manner, the gain and the range of the RSNA baroreflex curves tended to be decreased by AngII infusion, but these trends did not reach statistical significance, indicating that these effects are at least partially related to the differences in basal RSNA. Even after normalization to the nasopharyngeal reflex, the lower plateau was significantly higher in AngII-infused rabbits compared to the Veh group ($P < 0.025$), and this increase depended on Rho kinase activation ($P_{AngII \times Fas} < 0.01$). Thus, central AngII acts via Rho kinase activation to cause deficits in the capacity for baroreflex-induced sympatho-inhibition regardless of how RSNA was normalized.

### Assessment of Rho Kinase Activity

Rho kinase phosphorylates MYPT in the brain[35], and this phosphorylation was used as a marker for Rho kinase activity. AngII treatment increased Rho kinase activity in the SFO, PVH, as well as the entire slice (Figure 5 A–D). Interestingly, in the SFO, AngII treatment increased Rho kinase in a Fas-independent manner, and Fas decreased Rho kinase activity in an AngII-independent manner, whereas in the PVH and diffusely, AngII and Fas showed a statistically significant interaction.

## DISCUSSION

In the present study we show that central infusion of AngII increases blood pressure, elevates sympathetic outflow, and blunts baroreflex function by downstream activation of Rho kinase (Figure 6). This study builds upon a previous study from our laboratory performed in the rabbit pacing model of chronic heart failure which showed that central infusion of Fas improved baroreflex function and cardiac autonomic balance in the setting of heart failure[26]. These data are also consistent with other studies in hypertensive rat models which have described central activation of Rho kinase and shown benefits from central Rho kinase inhibition[28–31]. Ito et al. showed that transfection of the nucleus tractus solitarius (NTS) of both spontaneously hypertensive and Wistar-Kyoto rats with a dominant-negative Rho kinase results in decreases in MAP, HR, and urinary norepinephrine excretion[28]. These effects were greater in the spontaneously hypertensive rats, which also had greater Rho kinase activity in the NTS compared to Wistar-Kyoto control rats. Our study also corroborates one of these studies, which found that subchronic central administration of the Rho kinase inhibitor Y27632 prevented the pressor effect of central AngII in Wistar-Kyoto rats[31]. This rat study did not include rats receiving the Rho kinase inhibitor alone and thus was unable to address the central hypothesis of the current study, nor were direct measurements of sympathetic nerve activity or baroreflex function performed.

Baroreflex function, sympathetic outflow, and AP interact in complex ways which merit further discussion. Studies of baroreceptor unloading and stimulation implicate the baroreflex as an important controller of chronic sympathetic outflow and arterial pressure[36–42]. This gives weight to the possibility that Rho kinase activation mediates sympathetic baroreflex dysfunction which underlies the sympatho-excitation and hypertension caused by central AngII. In particular, elevated RSNA is crucial to the development of hypertension because of its ability to impair the powerful homeostatic mechanism of pressure-natriuresis[43–45]. In the within-rabbits cohort, neither extracellular fluid volume nor the high-perfusion compartment volume, which is a surrogate for intravascular blood volume, were decreased by AngII treatment despite a 19 mmHg increase in AP, implicating a deficit in pressure-natriuresis with central AngII. This departure from expected pressure-mediated volume regulation corroborates the idea that elevated RSNA facilitates chronic elevations in AP during central AngII.

Because of the inherent difficulties in the normalization of RSNA, it is unclear whether the reported sympathetic baroreflex dysfunction in AngII-treated rabbits leads to elevated RSNA or if it is the increased RSNA which causes the sympathetic baroreflex to appear perturbed. Clearly, normalizing the RSNA baroreflex by the nasopharyngeal reflex instead of the baseline RSNA greatly attenuates the AngII-mediated decrease in sympathetic baroreflex sensitivity and range. But any further interpretation is prone to a classic chicken-and-egg dilemma as it is just as reasonable that the AngII-mediated baroreflex dysfunction underlies the observed increases in RSNA as it is that the AngII-mediated increase in baseline RSNA results in apparent baroreflex dysfunction. Regardless of how RSNA is quantified, AngII causes a deficit in the maximal pressor-mediated sympatho-inhibition, perhaps indicating that baroreflex dysfunction is the primary disturbance. Of course, elevations in AP *per se*

affect baroreflex function[46,47], further complicating the process of parsing cause from effect in this truly integrative system.

The activity of Rho kinase in the brain of these rabbits showed interesting differential patterns. In the SFO, which is a primary sensor and essential mediator of the effects of central and peripheral AngII[10,48], AngII and Fas significantly affected Rho kinase activity independently, with no evidence for interaction ($P_{AngII \times Fas} = 0.46$). Conversely, in the PVH, which is an important preautonomic integration center, and in the whole saggital slice, AngII and Fas interacted in their effect on Rho kinase activity. The additive nature of the effects of AngII and Fas on Rho kinase activity in the SFO may indicate that both are acting at this site via their canonical, independent mechanisms, with AngII activating RhoA via AT1R signaling while Fas directly inhibits Rho kinase. In the absence of Fas, AngII increases Rho kinase activity in the SFO and drives downstream activation of the PVH, global sympatho-excitation, and hypertension[13]; when both AngII and Fas are present, Rho kinase activity in the SFO is relatively normal and these untoward downstream effects are blocked. Thus, the interaction observed in the PVH and whole saggital slice, instead of reflecting local action of AngII and Fas, may be a reflection of system changes caused by diffuse factors like blood pressure, presympathetic network activity, sympathetic outflow, and humoral activation.

While solid evidence indicates that central AngII stimulates Rho kinase via AT1R activation[31], the intermediaries between AT1R and RhoA/Rho kinase in the brain remain to be elucidated. In the periphery, AT1R signaling stimulates the RhoGEFs Arhgef1[19] and p63RhoGEF[22], which activate RhoA/Rho kinase, and inhibits the RhoGAP p190A[49] which, in turn, inhibits RhoA/Rho kinase. It is likely that these same mediators are important in the transduction between AT1R and RhoA in the brain, but other factors may play an important role and this area merits further study.

The downstream molecular mechanisms by which Rho kinase mediates its effects are likely multiple. Rho kinase is a crucial part of feedforward AngII-mediated oxidative signaling in the endothelium and may play a similar role in sympatho-excitatory superoxide signaling in the preautonomic centers of the brain[12,18]. The RhoA/Rho kinase pathway is directly activated by reactive oxygen species[50,51] and stimulates the formation of superoxide by NADPH oxidase[17,52,53]. Closely intertwined is the effect of Rho kinase to decrease the production of sympatho-inhibitory nitric oxide, which rapidly reacts with superoxide[54]. These free radicals affect neuronal excitability by altering $K^+$ and $Ca^{2+}$ currents[10,55]. Additionally, Rho kinase is known to play a fundamental role in neurotransmitter release and dendritic spine formation[28], and thus Rho kinase inhibition might prevent the release of sympatho-excitatory neurotransmitters and impair neuroplastic conversion to a sympatho-excitatory phenotype.

The two Rho kinase isoforms (ROCK1 and ROCK2) share approximately 90% homology in their kinase domains but despite this structural similarity, they are differentially distributed and perform distinct functions[56]. Of note, ROCK1 in the hypothalamus has been shown to play an important role in metabolic regulation, which is closely related to sympathetic outflow[57]. ROCK2 is more highly expressed in the brain, and induction of heart failure in rabbits by rapid ventricular pacing increases ROCK2 levels in the rostral ventrolateral

medulla, an important brainstem autonomic center[26]. Fasudil inhibits both isoforms with approximately equal affinity[58], and thus this study is unable to address the contributions of each isoform to AngII-mediated sympatho-excitation.

At present, Fasudil is used clinically in Japan after acute ischemic stroke and for the prevention of vasospasm after surgery for subarachnoid hemorrhage[59,60]. Another Rho kinase inhibitor, ripasudil, is also approved in Japan for the treatment of glaucoma and ocular hypertension[61]. Other clinical trials have investigated or are currently investigating Rho kinase inhibitor therapy in psoriasis, diabetic retinopathy, pulmonary arterial hypertension, erectile dysfunction, amyotrophic lateral sclerosis, spinal cord injury, atherosclerosis, and chronic kidney disease[56].

While the clinical use of specific Rho kinase inhibitors is limited, over 30 million Americans take statins for their cholesterol- and cardiovascular risk-reducing effects. Statins inhibit HMG-CoA reductase, which is crucial in the synthesis of not only cholesterol but also isoprenoid intermediates. Post-translational isoprenylation is necessary for trafficking and activation of small GTPases like Rac, Ras, and RhoA[62], and thus, statins not only lower cholesterol but also reduce RhoA/Rho kinase activity. Indeed, therapeutic doses of statins decrease Rho kinase activity in patients with atherosclerosis in an LDL-independent manner[63]. Our lab has similarly found that statin treatment improves autonomic function in experimental and clinical heart failure independent of LDL-lowering effects[64–68], and clinical studies in patients with hypertension and chronic kidney disease support the sympatholytic effect of statins in humans regardless of the presence of hyperlipidemia[69–72]. Given the strong associative[73–75] and experimental[76,77] data linking autonomic function and mortality in cardiovascular disease, the sympatholytic effects of statins through Rho kinase inhibition may be an important mechanism by which these drugs reduce cardiovascular risk.

We acknowledge that the present study has several limitations. First, it is unclear if the dose of AngII falls within the limits of pathophysiology or if this is a strictly pharmacological dose. Similarly, we do not know if brain tissue levels of Fas are achievable by systemic administration of Fas in humans. Moreover, treatments were administered ICV and thus the exact location of their action in the brain cannot be truly known, although it is likely that concentrations of all agents would be higher near structures close to the ventricular system (e.g. SFO). Finally, the precise mechanisms by which Rho kinase is activated by AngII and the downstream mechanisms by which Rho kinase induces sympatho-excitation remain to be elucidated.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

## REFERENCES

1. Mann DL, Bristow MR. Mechanisms and models in heart failure: the biomechanical model and beyond. Circulation. 2005; 111:2837–2849. [PubMed: 15927992]

2. Schiffrin EL, Lipman ML, Mann JFE. Chronic kidney disease: effects on the cardiovascular system. Circulation. 2007; 116:85–97. [PubMed: 17606856]

3. Converse RL, Jacobsen TN, Toto RD, Jost CM, Cosentino F, Fouad-Tarazi F, Victor RG. Sympathetic overactivity in patients with chronic renal failure. N Engl J Med. 1992; 327:1912–1918. [PubMed: 1454086]

4. Mancia G, Dell'Oro R, Quarti-Trevano F, Scopelliti F, Grassi G. Angiotensin-sympathetic system interactions in cardiovascular and metabolic disease. J Hypertens Suppl. 2006; 24:S51–S56. [PubMed: 16601574]

5. Zucker IH. Novel Mechanisms of Sympathetic Regulation in Chronic Heart Failure. Hypertension. 2006; 48:1005–1011. [PubMed: 17015773]

6. Mancia G, Grassi G, Giannattasio C, Seravalle G. Sympathetic Activation in the Pathogenesis of Hypertension and Progression of Organ Damage. Hypertension. 1999; 34:724–728. [PubMed: 10523349]

7. Sasaki S, Dampney RA. Tonic cardiovascular effects of angiotensin II in the ventrolateral medulla. Hypertension. 1990; 15:274–283. [PubMed: 2303285]

8. Matsumura K, Averill DB, Ferrario CM. Angiotensin II acts at AT1 receptors in the nucleus of the solitary tract to attenuate the baroreceptor reflex. Am J Physiol Regul Integr Comp Physiol. 1998; 275:R1611–R1619.

9. Nguyen Dinh Cat A, Touyz RM. Cell signaling of angiotensin II on vascular tone: novel mechanisms. Curr Hypertens Rep. 2011; 13:122–128. [PubMed: 21274755]

10. Davisson RL, Zimmerman MC. Angiotensin II, oxidant signaling, and hypertension: down to a T? Hypertension. 2010; 55:228–230. [PubMed: 20008674]

11. Savalia K, Manickam DS, Rosenbaugh EG, Tian J, Ahmad IM, Kabanov AV, Zimmerman MC. Neuronal uptake of nanoformulated superoxide dismutase and attenuation of angiotensin II-dependent hypertension after central administration. Free Radic Biol Med. 2014; 73:299–307. [PubMed: 24924945]

12. Zimmerman MC, Lazartigues E, Sharma RV, Davisson RL. Hypertension caused by angiotensin II infusion involves increased superoxide production in the central nervous system. Circ Res. 2004; 95:210–216. [PubMed: 15192025]

13. Llewellyn T, Zheng H, Liu X, Xu B, Patel KP. Median preoptic nucleus and subfornical organ drive renal sympathetic nerve activity via a glutamatergic mechanism within the paraventricular nucleus. Am J Physiol Regul Integr Comp Physiol. 2012; 302:R424–R432. [PubMed: 22160544]

14. Li Y-F, Mayhan WG, Patel KP. NMDA-mediated increase in renal sympathetic nerve discharge within the PVN: role of nitric oxide. Am J Physiol Hear Circ Physiol. 2001; 281:H2328–H2336.

15. Sharma NM, Llewellyn TL, Zheng H, Patel KP. Angiotensin II-mediated posttranslational modification of nNOS in the PVN of rats with CHF: role for PIN. Am J Physiol Heart Circ Physiol. 2013; 305:H843–H855. [PubMed: 23832698]

16. Li Y-F, Wang W, Mayhan WG, Patel KP. Angiotensin-mediated increase in renal sympathetic nerve discharge within the PVN: role of nitric oxide. Am J Physiol Regul Integr Comp Physiol. 2006; 290:R1035–R1043. [PubMed: 16322353]

17. Higashi M, Shimokawa H, Hattori T, Hiroki J, Mukai Y, Morikawa K, Ichiki T, Takahashi S, Takeshita A. Long-term inhibition of Rho-kinase suppresses angiotensin II-induced cardiovascular hypertrophy in rats in vivo: effect on endothelial NAD(P)H oxidase system. Circ Res. 2003; 93:767–775. [PubMed: 14500337]

18. Burger D, Montezano AC, Nishigaki N, He Y, Carter A, Touyz RM. Endothelial microparticle formation by angiotensin II is mediated via Ang II receptor type I/NADPH oxidase/ Rho kinase pathways targeted to lipid rafts. Arterioscler Thromb Vasc Biol. 2011; 31:1898–1907. [PubMed: 21597004]

19. Guilluy C, Brégeon J, Toumaniantz G, Rolli-Derkinderen M, Retailleau K, Loufrani L, Henrion D, Scalbert E, Bril A, Torres RM, Offermanns S, Pacaud P, Loirand G. The Rho exchange factor Arhgef1 mediates the effects of angiotensin II on vascular tone and blood pressure. Nat Med. 2010; 16:183–190. [PubMed: 20098430]

20. Rikitake Y, Liao JK. Rho GTPases, statins, and nitric oxide. Circ Res. 2005; 97:1232–1235. [PubMed: 16339495]

21. Dong M, Yan BP, Liao JK, Lam Y-Y, Yip GWK, Yu C-M. Rho-kinase inhibition: a novel therapeutic target for the treatment of cardiovascular diseases. Drug Discov Today. 2010; 15:622–629. [PubMed: 20601092]

22. Calò LA, Davis PA, Pagnin E, Dal Maso L, Maiolino G, Seccia TM, Pessina AC, Rossi GP. Increased level of p63RhoGEF and RhoA/Rho kinase activity in hypertensive patients. J Hypertens. 2014; 32:331–338. [PubMed: 24356540]

23. Hata T, Soga J, Hidaka T, Idei N, Fujii Y, Fujimura N, Mikami S, Maruhashi T, Kihara Y, Chayama K, Kato H, Noma K, Liao JK, Higashi Y. Calcium channel blocker and Rho-associated kinase activity in patients with hypertension. J Hypertens. 2011; 29:373–379. [PubMed: 21063203]

24. Masumoto A, Hirooka Y, Shimokawa H, Hironaga K, Setoguchi S, Takeshita A. Possible Involvement of Rho-Kinase in the Pathogenesis of Hypertension in Humans. Hypertension. 2001; 38:1307–1310. [PubMed: 11751708]

25. Kishi T, Hirooka Y, Masumoto A, Ito K, Kimura Y, Inokuchi K, Tagawa T, Shimokawa H, Takeshita A, Sunagawa K. Rho-kinase inhibitor improves increased vascular resistance and impaired vasodilation of the forearm in patients with heart failure. Circulation. 2005; 111:2741–2747. [PubMed: 15927989]

26. Haack KKV, Gao L, Schiller AM, Curry PL, Pellegrino PR, Zucker IH. Central Rho kinase inhibition restores baroreflex sensitivity and angiotensin II type 1 receptor protein imbalance in conscious rabbits with chronic heart failure. Hypertension. 2013; 61:723–729. [PubMed: 23283363]

27. Ito K, Hirooka Y, Kishi T, Kimura Y, Kaibuchi K, Shimokawa H, Takeshita A. Rho/Rho-kinase pathway in the brainstem contributes to hypertension caused by chronic nitric oxide synthase inhibition. Hypertension. 2004; 43:156–162. [PubMed: 14732730]

28. Ito K, Hirooka Y, Sakai K, Kishi T, Kaibuchi K, Shimokawa H, Takeshita A. Rho/Rho-kinase pathway in brain stem contributes to blood pressure regulation via sympathetic nervous system: Possible involvement in neural mechanisms of hypertension. Circ Res. 2003; 92:1337–1343. [PubMed: 12791705]

29. Ito K, Kimura Y, Hirooka Y, Sagara Y, Sunagawa K. Activation of Rho-kinase in the brainstem enhances sympathetic drive in mice with heart failure. Auton Neurosci Basic Clin. 2008; 142:77–81.

30. Ito K, Hirooka Y, Hori N, Kimura Y, Sagara Y, Shimokawa H, Takeshita A, Sunagawa K. Inhibition of Rho-kinase in the nucleus tractus solitarius enhances glutamate sensitivity in rats. Hypertension. 2005; 46:360–365. [PubMed: 16051902]

31. Sagara Y, Hirooka Y, Nozoe M, Ito K, Kimura Y, Sunagawa K. Pressor response induced by central angiotensin II is mediated by activation of Rho/Rho-kinase pathway via AT1 receptors. J Hypertens. 2007; 25:399–406. [PubMed: 17211247]

32. Schiller AM, Pellegrino PR, Zucker IH. Renal nerves dynamically regulate renal blood flow in conscious, healthy rabbits. Am J Physiol Regul Integr Comp Physiol. 2016; 310:R156–R166. [PubMed: 26538235]

33. Pellegrino PR, Schiller AM, Zucker IH. Validation of pulse rate variability as a surrogate for heart rate variability in chronically instrumented rabbits. Am J Physiol Heart Circ Physiol. 2014; 307:H97–H109. [PubMed: 24791786]

34. Eaton SL, Cumyn E, King D, Kline RA, Carpanini SM, Del-Pozo J, Barron R, Wishart TM. Quantitative imaging of tissue sections using infrared scanning technology. J Anat. 2016; 228:203–213. [PubMed: 26510706]

35. Lontay B, Serfozo Z, Gergely P, Ito M, Hartshorne DJ, Erdodi F. Localization of myosin phosphatase target subunit 1 in rat brain and in primary cultures of neuronal cells. J Comp Neurol. 2004; 478:72–87. [PubMed: 15334650]

36. Lohmeier TE, Irwin ED, Rossing MA, Serdar DJ, Kieval RS. Prolonged Activation of the Baroreflex Produces Sustained Hypotension. Hypertension. 2004; 43:306–311. [PubMed: 14707159]

37. Lohmeier TE, Hildebrandt DA, Dwyer TM, Barrett AM, Irwin ED, Rossing MA, Kieval RS. Renal denervation does not abolish sustained baroreflex-mediated reductions in arterial pressure. Hypertension. 2007; 49:373–379. [PubMed: 17159083]

38. Lohmeier TE, Iliescu R. The baroreflex as a long-term controller of arterial pressure. Physiology (Bethesda). 2015; 30:148–158. [PubMed: 25729060]

39. Lohmeier TE, Iliescu R, Dwyer TM, Irwin ED, Cates AW, Rossing MA. Sustained suppression of sympathetic activity and arterial pressure during chronic activation of the carotid baroreflex. Am J Physiol Heart Circ Physiol. 2010; 299:H402–H409. [PubMed: 20511410]

40. Thrasher TN. Unloading arterial baroreceptors causes neurogenic hypertension. Am J Physiol Regul Integr Comp Physiol. 2002; 282:R1044–R1053. [PubMed: 11893608]

41. Thrasher TN. Effects of chronic baroreceptor unloading on blood pressure in the dog. Am J Physiol Regul Integr Comp Physiol. 2005; 288:R863–R871. [PubMed: 15563576]

42. Bisognano JD, Bakris G, Nadim MK, Sanchez L, Kroon AA, Schafer J, de Leeuw PW, Sica DA. Baroreflex activation therapy lowers blood pressure in patients with resistant hypertension: results from the double-blind, randomized, placebo-controlled rheos pivotal trial. J Am Coll Cardiol. 2011; 58:765–773. [PubMed: 21816315]

43. Ehmke H, Persson PB, Seyfarth M, Kirchheim HR. Neurogenic control of pressure natriuresis in conscious dogs. Am J Physiol. 1990; 259:F466–F473. [PubMed: 2396671]

44. Brand PH, Coyne KB, Kostrzewski KA, Shier D, Metting PJ, Britton SL. Pressure diuresis and autonomic function in conscious dogs. Am J Physiol. 1991; 261:R802–R810. [PubMed: 1681746]

45. Guyton A. Blood pressure control--special role of the kidneys and body fluids. Science (80-). 1991; 252:1813–1816.

46. Tan W, Panzenbeck MJ, Hajdu MA, Zucker IH. A central mechanism of acute baroreflex resetting in the conscious dog. Circ Res. 1989; 65:63–70. [PubMed: 2736740]

47. Chapleau MW, Hajduczok G, Abboud FM. PERIPHERAL CENTRAL MECHANISMS OF BAROREFLEX RESETTING. Clin Exp Pharmacol Physiol. 1989; 16:31–43.

48. McKinley MJ, Badoer E, Vivas L, Oldfield BJ. Comparison of c-fos expression in the lamina terminalis of conscious rats after intravenous or intracerebroventricular angiotensin. Brain Res Bull. 1995; 37:131–137. [PubMed: 7606488]

49. Bregeon J, Loirand G, Pacaud P, Rolli-Derkinderen M. Angiotensin II induces RhoA activation through SHP2-dependent dephosphorylation of the RhoGAP p190A in vascular smooth muscle cells. Am J Physiol Cell Physiol. 2009; 297:C1062–C1070. [PubMed: 19692654]

50. Aghajanian A, Wittchen ES, Campbell SL, Burridge K. Direct Activation of RhoA by Reactive Oxygen Species Requires a Redox-Sensitive Motif. PLoS One. 2009; 4

51. Jin L, Ying Z, Webb RC. Activation of Rho/Rho kinase signaling pathway by reactive oxygen species in rat aorta. Am J Physiol Heart Circ Physiol. 2004; 287:H1495–H1500. [PubMed: 15371261]

52. Gibson CL, Srivastava K, Sprigg N, Bath PMW, Bayraktutan U. Inhibition of Rho-kinase protects cerebral barrier from ischaemia-evoked injury through modulations of endothelial cell oxidative stress and tight junctions. J Neurochem. 2014; 129:816–826. [PubMed: 24528233]

53. Lemarie A, Bourdonnay E, Morzadec C, Fardel O, Vernhet L. Inorganic arsenic activates reduced NADPH oxidase in human primary macrophages through a Rho kinase/p38 kinase pathway. J Immunol. 2008; 180:6010–6017. [PubMed: 18424721]

54. Wirth A. Rho kinase and hypertension. Biochim Biophys Acta - Mol Basis Dis. 2010; 1802:1276–1284.

55. Zimmerman MC, Sharma RV, Davisson RL. Superoxide mediates angiotensin II-induced influx of extracellular calcium in neural cells. Hypertension. 2005; 45:717–723. [PubMed: 15699459]

56. Loirand G. Rho Kinases in Health and Disease: From Basic Science to Translational Research. Pharmacol Rev. 2015; 67:1074–1095. [PubMed: 26419448]

57. Huang H, Kong D, Byun KH, Ye C, Koda S, Lee DH, Oh B-C, Lee SW, Lee B, Zabolotny JM, Kim MS, Bjørbæk C, Lowell BB, Kim Y-B. Rho-kinase regulates energy balance by targeting hypothalamic leptin receptor signaling. Nat Neurosci. 2012; 15:1391–1398. [PubMed: 22941110]

58. Liao JK, Seto M, Noma K. Rho kinase (ROCK) inhibitors. J Cardiovasc Pharmacol. 2007; 50:17–24. [PubMed: 17666911]

59. Shibuya M, Hirai S, Seto M, Satoh S, Ohtomo E. Effects of fasudil in acute ischemic stroke: results of a prospective placebo-controlled double-blind trial. J Neurol Sci. 2005; 238:31–39. [PubMed: 16005902]

60. Suzuki Y, Shibuya M, Satoh S-I, Sugimoto Y, Takakura K. A postmarketing surveillance study of fasudil treatment after aneurysmal subarachnoid hemorrhage. Surg Neurol. 2007; 68:126–131. discussion 131–132. [PubMed: 17586012]

61. Garnock-Jones KP. Ripasudil: first global approval. Drugs. 2014; 74:2211–2215. [PubMed: 25414122]

62. Hall A. Rho GTPases and the Actin Cytoskeleton. Science (80-). 1998; 279:509–514.

63. Nohria A, Prsic A, Liu P-Y, Okamoto R, Creager MA, Selwyn A, Liao JK, Ganz P. Statins inhibit Rho kinase activity in patients with atherosclerosis. Atherosclerosis. 2009; 205:517–521. [PubMed: 19167712]

64. Haack KKV, Marcus NJ, Del Rio R, Zucker IH, Schultz HD. Simvastatin treatment attenuates increased respiratory variability and apnea/hypopnea index in rats with chronic heart failure. Hypertension. 2014; 63:1041–1049. [PubMed: 24516105]

65. Pliquett RU, Cornish KG, Peuler JD, Zucker IH. Simvastatin normalizes autonomic neural control in experimental heart failure. Circulation. 2003; 107:2493–2498. [PubMed: 12695293]

66. Gao L, Wang W, Zucker IH. Simvastatin inhibits central sympathetic outflow in heart failure by a nitric-oxide synthase mechanism. J Pharmacol Exp Ther. 2008; 326:278–285. [PubMed: 18441251]

67. Gao L. Simvastatin Therapy Normalizes Sympathetic Neural Control in Experimental Heart Failure: Roles of Angiotensin II Type 1 Receptors and NAD(P)H Oxidase. Circulation. 2005; 112:1763–1770. [PubMed: 16157767]

68. Deo SH, Fisher JP, Vianna LC, Kim A, Chockalingam A, Zimmerman MC, Zucker IH, Fadel PJ. Statin therapy lowers muscle sympathetic nerve activity and oxidative stress in patients with heart failure. Am J Physiol Heart Circ Physiol. 2012; 303:H377–H385. [PubMed: 22661508]

69. Lewandowski J, Symonides B, Gaciong Z, Si ski M. The effect of statins on sympathetic activity: a meta-analysis. Clin Auton Res. 2015; 25:125–131. [PubMed: 25739473]

70. Lewandowski J, Si ski M, Bidiuk J, Abramczyk P, Dobosiewicz A, Ciarka A, Gaciong Z. Simvastatin reduces sympathetic activity in men with hypertension and hypercholesterolemia. Hypertens Res. 2010; 33:1038–1043. [PubMed: 20668455]

71. McGowan CL, Murai H, Millar PJ, Notarius CF, Morris BL, Floras JS. Simvastatin reduces sympathetic outflow and augments endothelium-independent dilation in non-hyperlipidaemic primary hypertension. Heart. 2013; 99:240–246. [PubMed: 23257173]

72. Trebicka J, Hennenberg M, Laleman W, Shelest N, Biecker E, Schepke M, Nevens F, Sauerbruch T, Heller J. Atorvastatin lowers portal pressure in cirrhotic rats by inhibition of RhoA/Rho-kinase and activation of endothelial nitric oxide synthase. Hepatology. 2007; 46:242–253. [PubMed: 17596891]

73. Barretto ACP, Santos AC, Munhoz R, Rondon MUPB, Franco FG, Trombetta IC, Roveda F, de Matos LNJ, Braga AMW, Middlekauff HR, Negrão CE. Increased muscle sympathetic nerve activity predicts mortality in heart failure patients. Int J Cardiol. 2009; 135:302–307. [PubMed: 18582965]

74. Brunner-La Rocca HP, Esler MD, Jennings GL, Kaye DM. Effect of cardiac sympathetic nervous activity on mode of death in congestive heart failure. Eur Heart J. 2001; 22:1136–1143. [PubMed: 11428854]

75. Cohn JN, Levine TB, Olivari MT, Garberg V, Lura D, Francis GS, Simon AB, Rector T. Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. N Engl J Med. 1984; 311:819–823. [PubMed: 6382011]

76. Zucker IH, Hackley JF, Cornish KG, Hiser BA, Anderson NR, Kieval R, Irwin ED, Serdar DJ, Peuler JD, Rossing MA. Chronic baroreceptor activation enhances survival in dogs with pacing-induced heart failure. Hypertension. 2007; 50:904–910. [PubMed: 17846349]

77. Li M, Zheng C, Sato T, Kawada T, Sugimachi M, Sunagawa K. Vagal nerve stimulation markedly improves long-term survival after chronic heart failure in rats. Circulation. 2004; 109:120–124. [PubMed: 14662714]

## PERSPECTIVES

Activation of the RAAS and autonomic dysfunction are pathophysiological hallmarks of cardiovascular disease. The present study shows that the pro-hypertensive, sympatho-excitatory, and baroreflex-perturbing effects of AngII in the brain are mediated by the Rho kinase activation. These data indicate that inhibition of the central Rho kinase pathway may act as a therapeutic brake on the positive feedback between central RAAS activation and sympathetic outflow in many diseases characterized by sympatho-excitation.

**NOVELTY AND SIGNIFICANCE**

**What is New?**

- Angiotensin II in the brain raises blood pressure and increases cardiac and global sympathetic outflow by a Rho kinase-dependent mechanism.

- Direct recordings of sympathetic nerve activity indicate that Rho kinase inhibition prevents the chronic renal sympatho-excitation and baroreflex dysfunction caused by central angiotensin.

**What is Relevant?**

- Because of the link between autonomic dysfunction and mortality in cardiovascular disease, Rho kinase inhibition may be a promising therapy for hypertensive patients.

- Statins reduce Rho kinase activity and sympathetic outflow, and this study suggests that Rho kinase inhibition may be an important LDL-independent protective mechanism for the >30 million Americans currently taking statins.

**Summary**

- The Rho kinase pathway is a crucial mediator of the deleterious autonomic effects of angiotensin II and may be an important therapeutic target in cardiovascular disease.
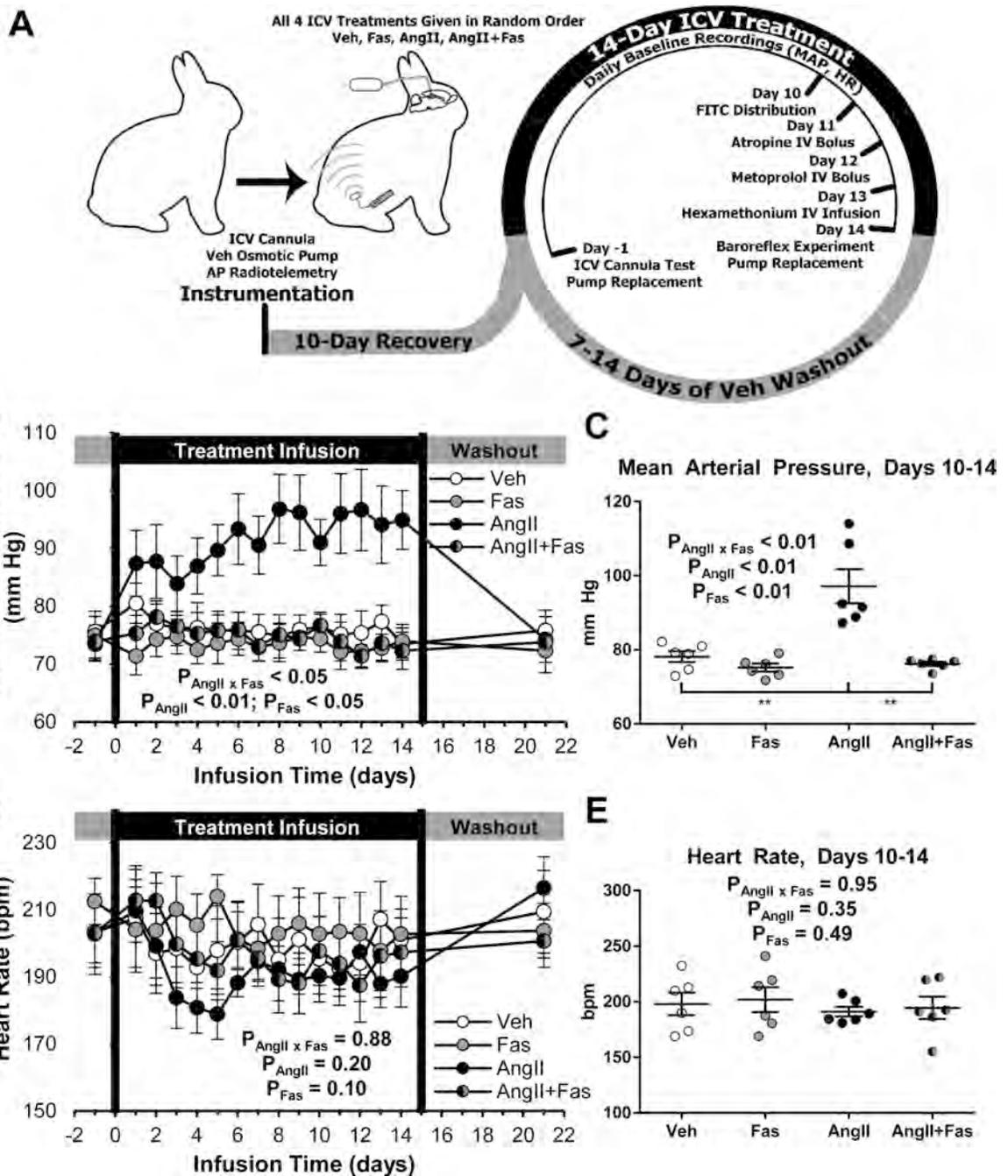
**Figure 1.**
Baseline Hemodynamics from Within-rabbits Cohort. (A) Within-rabbits study design illustrating basic experimental paradigm. (B) Average MAP over the course of each ICV treatment infusion and (C) the average MAP for each rabbit over days 10 through 14 of each ICV treatment. (D) Average HR over the course of each ICV treatment infusion and (E) the average HR for each rabbit over days 10 through 14 of each treatment infusion. *, $P < 0.025$; **, $P < 0.01$
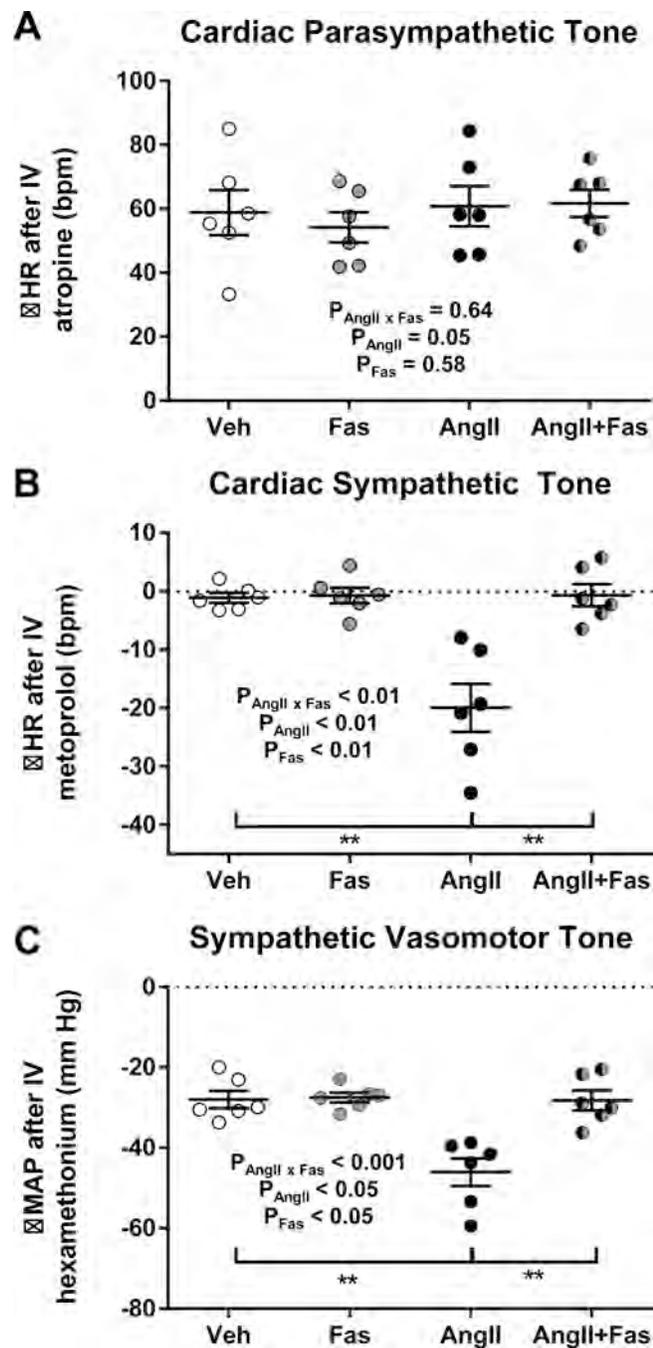
**Figure 2.**
Autonomic Blockade Experiments. (A) Cardiac parasympathetic tone assessed by the HR response to atropine. (B) Cardiac sympathetic tone assessed by the HR response to metoprolol. (C) Global sympathetic vasomotor tone assessed by the MAP response to hexamethonium. *, $P < 0.025$; **, $P < 0.01$
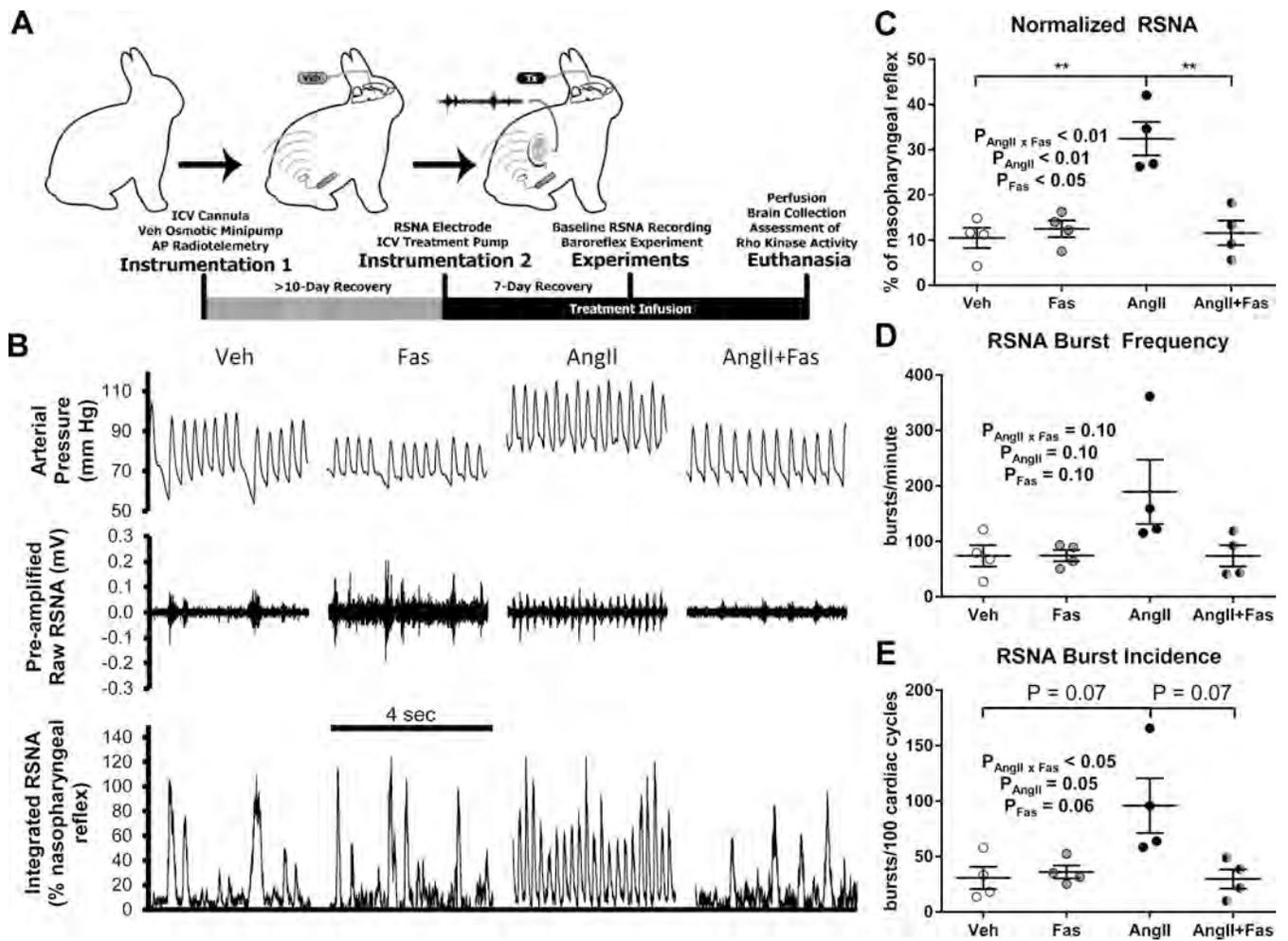
**Figure 3.**
Baseline RSNA from Between-rabbits Cohort. (A) Between-rabbits cohort study design illustrating basic experimental paradigm. (B) Representative tracings of pulsatile arterial pressure, raw RSNA, and integrated RSNA for one rabbit receiving each ICV treatment. (C) RSNA normalized to the nasopharyngeal reflex. (D) Baseline RSNA quantified as burst frequency. (E) Baseline RSNA quantified as burst incidence. **, P < 0.01
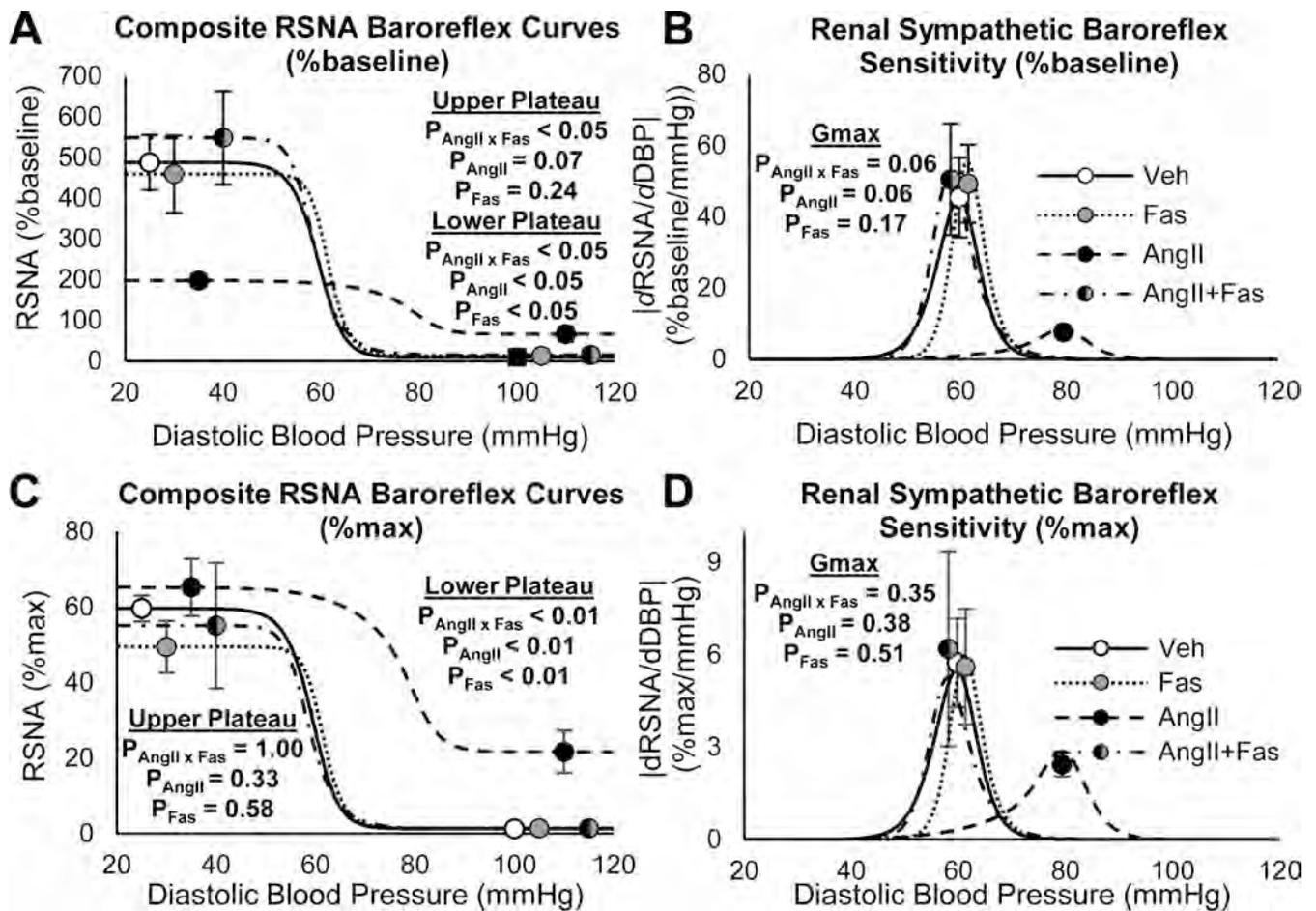
**Figure 4.**
Baroreflex Control of RSNA. (A) Composite RSNA baroreflex curves normalized to baseline RSNA. (B) Composite RSNA baroreflex gain curves normalized to baseline RSNA. (C) Composite RSNA baroreflex curves normalized to the nasopharyngeal reflex. (D) Composite RSNA baroreflex gain curves normalized to the nasopharyngeal reflex.
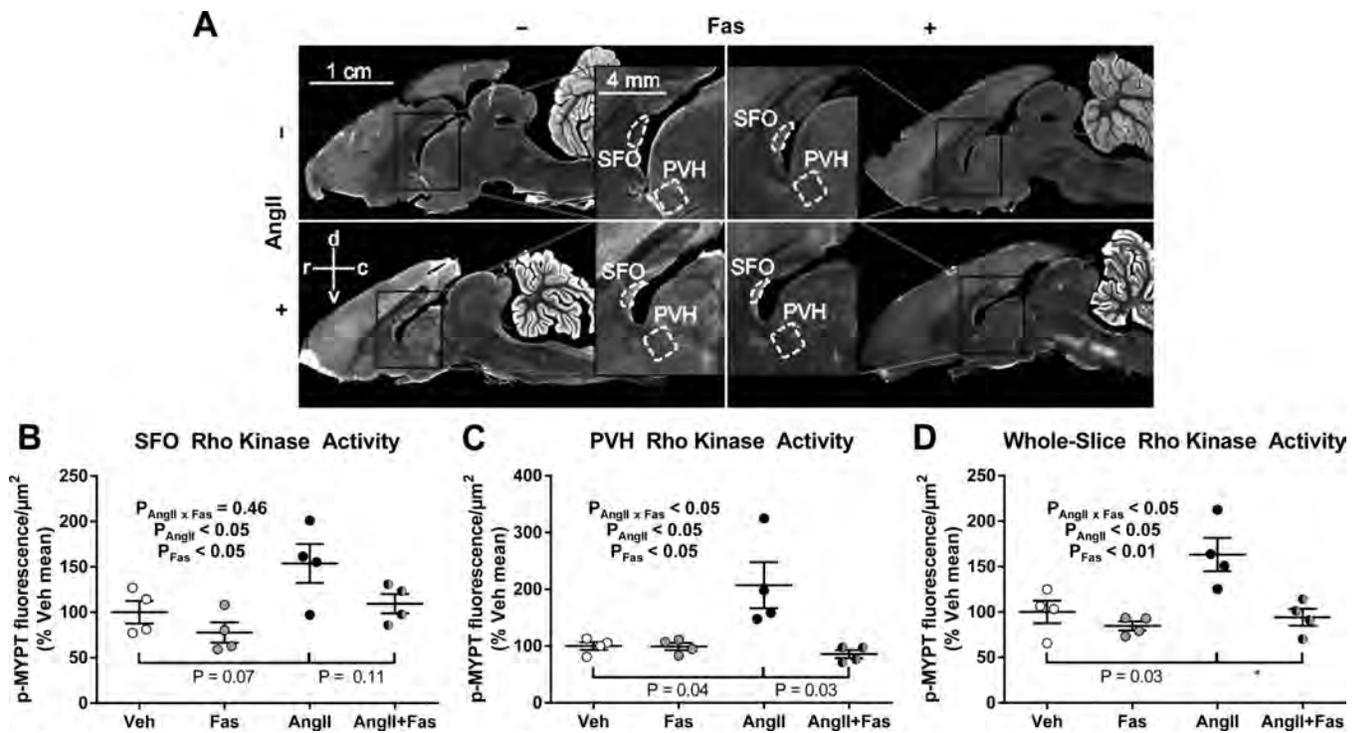
**Figure 5.**
Assessment of Rho Kinase Activity. (A) Representative saggital brain slices stained with antibodies for the Rho kinase target p-MYPT from rabbits infused with each ICV treatment. (B) Mean SFO p-MYPT fluorescence. (C) Mean PVH p-MYPT fluorescence. (D) Mean whole-slice p-MYPT fluorescence.

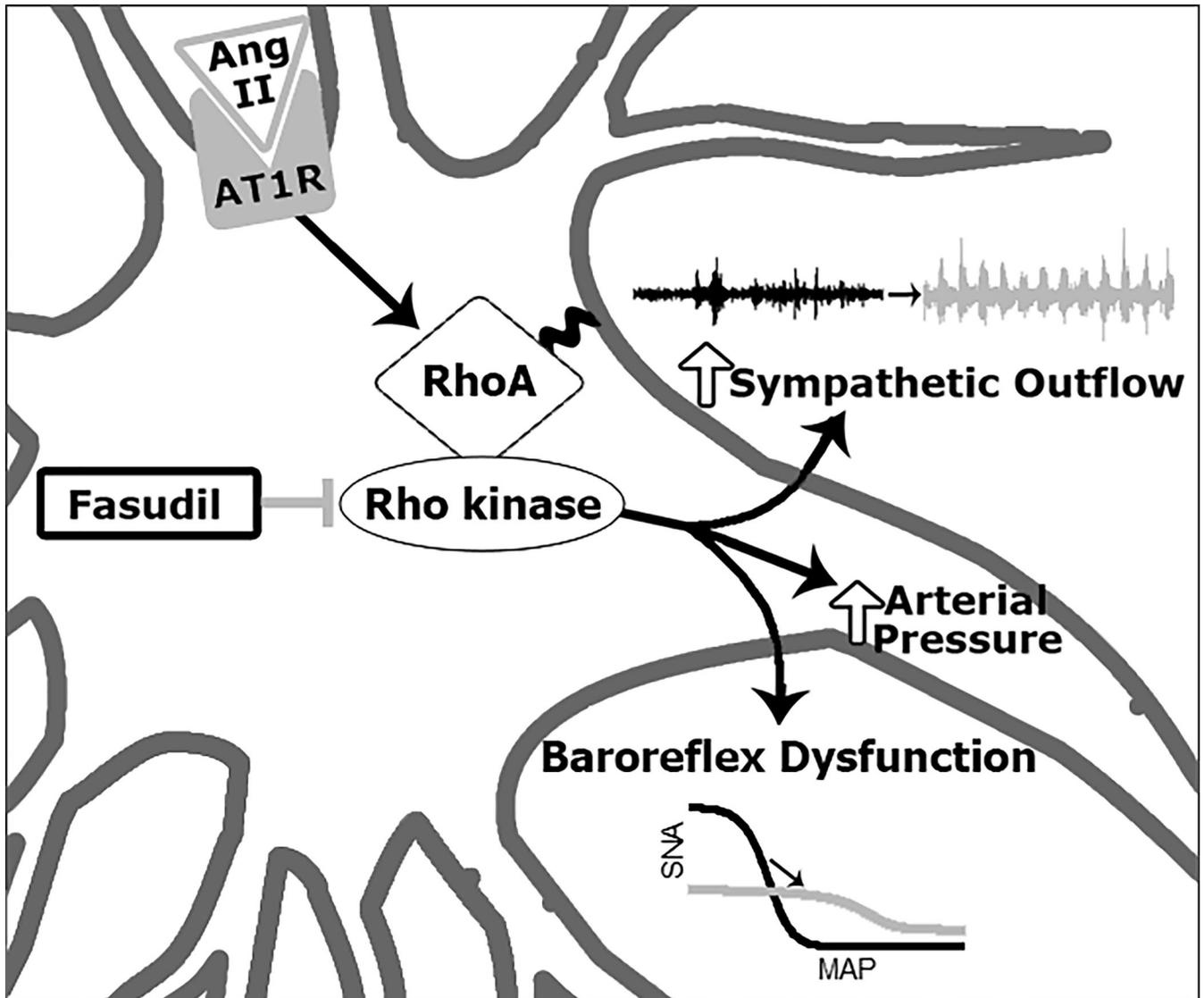d, dorsal direction; v, ventral direction; r, rostral direction; c, caudal direction; *, $P < 0.025$.

**Figure 6.**
Overview Summary. Central AngII activates Rho kinase signaling downstream of AT1R, leading to baroreflex dysfunction, sympatho-excitation, and hypertension.