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

Peter R. Pellegrino, Alicia M. Schiller, Karla K.V. Haack, and Irving H. Zucker

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 (AngII) in the periphery is clear, but the role of Rho kinase in sympathoexcitation caused by central AngII is not well established. We hypothesized that AngII mediates its effects in the brain by the activation of the RhoA/Rho kinase pathway. Chronically instrumented, conscious rabbits received the following intracerebroventricular infusion treatments for 2 weeks via osmotic minipump: AngII, Rho kinase inhibitor Fasudil, AngII plus Fasudil, or a vehicle control. AngII increased mean arterial pressure over the course of the infusion, and this effect was prevented by the coadministration of Fasudil. AngII increased cardiac and vascular sympathetic outflow as quantified by the heart rate response to metoprolol and the depressor effect of hexamethonium; coadministration of Fasudil abolished both of these effects. Each of these end points showed a statistically significant interaction between AngII and Fasudil. Quantitative immunofluorescence of brain slices confirmed that Rho kinase activity was increased by AngII and decreased by Fasudil. Taken together, these data indicate that hypertension, elevated sympathetic outflow, and baroreflex dysfunction caused by central AngII are mediated by Rho kinase activation and suggest that Rho kinase inhibition may be an important therapeutic target in sympathoexcitatory cardiovascular diseases. (Hypertension. 2016;68:1271-1280. DOI: 10.1161/HYPERTENSIONAHA.116.07792) • Online Data Supplement

Key Words: angiotensin II • baroreflex • cardiovascular diseases • hypertension • intracerebroventricular infusions • RhoA GTP-binding protein • Rho-associated kinases

Activation of the renin–angiotensin–aldosterone system (RAAS) and sympathoexcitation 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, increases sympathetic outflow, and impairs 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

Whereas 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 this study, we hypothesized that AngII mediates its central effects by the 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-only Data supplement. Experiments were performed on male New Zealand white rabbits with weight ranging 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 were performed 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, 6 rabbits were instrumented with arterial pressure (AP) radiotelemetry transducers, and intracerebroventricular cannulae. Each rabbit received intracerebroventricular 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 via osmotic minipump in a randomized order. Mean arterial pressure (MAP) and heart rate (HR) were measured daily, and volume status, cardiac parasympathetic tone, cardiac sympathetic tone, global sympathetic vasomotor tone, and baroreflex function were assessed on days 10 through 14 of treatment by intravenous administration of fluorescein isothiocyanate–sinistrin, autonomic blockers, or vasoactive drugs. This time period (days 10–14 of treatment) is referred to as the late phase of treatment. Between treatment infusions, rabbits received a 7- to 14-day washout with a vehicle minipump.

Between-Rabbits Cohort
To test the hypothesis that Rho kinase activation mediates the renal sympatoexcitatory effect caused by central AngII, rabbits were instrumented with radiotelemetry transducers, intracerebroventricular cannulae, and renal sympathetic nerve electrodes. Seven days after renal nerve electrode surgery, resting renal sympathetic nerve activity (RSNA) and baroreflex control of RSNA were assessed. Not all chronic sympathetic nerve electrode implementations were successful (Figure S4B in the online-only Data Supplement), and rabbits without RSNA bursts 1 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 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 means±SEM. Statistical testing on end points from the within-rabbits study was performed using 2-way or 3-way repeated measures ANOVA with α=0.05. Statistical testing on end points from the between-rabbits study was performed using 2-way ANOVA with α=0.05. The P values for the AngII, Fas, and AngII×Fas terms from the ANOVA are reported throughout the article. Crucial to the central hypothesis of this article, $P_{AngII\times Fas} < \alpha$ indicates that the response to AngII is significantly modified by Fas, ie, that there is a nonadditive 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 (ie, $P_{AngII\times Fas} \gg \alpha$), $P_{AngII} < \alpha$ indicates that AngII significantly affects the end point 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 vehicle versus AngII and AngII versus AngII+Fas (α=0.025). These t tests were chosen as they most closely relate to the central hypothesis of the study, whereas other t tests (eg, vehicle versus Fas) were foregone 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 intracerebroventricular treatments (Figure 1A). Power analysis and treatment order for this cohort can be found in Figure S1. Compared with vehicle treatment, intracerebroventricular infusion of AngII significantly increased MAP in all rabbits (Figure 1B and 1C), and this increase in MAP was abolished by the coadministration of Fas. The statistically significant interaction between AngII and Fas indicates that intracerebroventricular 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 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 intracerebroventricular treatment (Figure 2A; Table S1 in the online-only Data Supplement). Cardiac sympathetic tone assessed by the HR response to metoprolol was significantly increased by intracerebroventricular infusion of AngII, and this cardiac sympatoexcitatory effect was blocked by Fas coadministration (Figure 2B; Table S2). The statistically significant interaction between AngII and Fas indicates that the AngII-mediated cardiac sympatoexcitatory effect depends on Rho kinase activation.

Global sympathetic vasomotor tone assessed by the MAP response to hexamethonium was significantly elevated by intracerebroventricular infusion of AngII, and again the coadministration of Fas blocked this elevation despite Fas alone having little effect (Figure 2C; 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 intracerebroventricular 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 (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 intracerebroventricular AngII and AngII did not affect the other 2-compartment model parameters (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 Figure S4. Baseline hemodynamics are shown in Table S6; notably, AngII-treated rabbits in this cohort were hypertensive, whereas HR did not significantly differ between the treatment groups.

Figure 1. Baseline hemodynamics from within-rabbits cohort. A, Within-rabbits study design illustrating basic experimental paradigm. B, Average mean arterial pressure (MAP) over the course of each intracerebroventricular treatment infusion and (C) the average MAP for each rabbit over days 10 through 14 of each intracerebroventricular treatment. D, Average HR over the course of each intracerebroventricular 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.

Rabbits receiving intracerebroventricular 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
Baroreflex Control of Renal Sympathetic Nerve Activity

Intracerebroventricular AngII impaired baroreflex control of RSNA compared with vehicle treatment, and Fas coadministration prevented this baroreflex dysfunction (Figure 4A and 4B). When RSNA was expressed relative to basal RSNA, rabbits receiving AngII showed decreased RSNA baroreflex range (Table S7), with the upper plateau significantly lower ($P<0.025$ versus vehicle) and the lower plateau significantly higher ($P<0.025$ versus vehicle). These effects depended on Rho kinase activation ($P_{\text{AngII} \times \text{Fas}} < 0.05$). Similarly, AngII treatment lowered baroreflex gain when expressed as a percentage of basal RSNA ($P<0.025$ versus vehicle) 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 with the vehicle group ($P<0.025$), and this increase depended on Rho kinase activation ($P_{\text{AngII} \times \text{Fas}} < 0.01$). Thus, central AngII acts via Rho kinase activation to cause deficits in the capacity for baroreflex-induced sympathoinhibition regardless of how RSNA was normalized.

Assessment of Rho Kinase Activity

Rho kinase phosphorylates MYPT in the brain, and this phosphorylation was used as a marker for Rho kinase activity. AngII treatment increased Rho kinase activity in the SFO, PVH, and the entire slice (Figure 5A through 5D). 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 this study, we show that central infusion of AngII increases blood pressure, elevates sympathetic outflow, and blunts baroreflex function by the downstream activation of Rho kinase (Figure 6). This study builds on 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. These data are also consistent with other studies in hypertensive rat models, which have described the central activation of Rho kinase and shown benefits from central Rho kinase inhibition. Ito et al showed that transfection of the nucleus tractus solitarius of both spontaneously hypertensive and Wistar–Kyoto rats with a dominant-negative Rho kinase results in decreases in MAP, HR, and urinary norepinephrine excretion. These effects were greater in the spontaneously hypertensive rats, which also had greater Rho kinase activity in the nucleus tractus solitarius, than in the 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. 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, and measurements of sympathetic nerve activity or baroreflex function were not 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 AP.36–42 This gives weight to the possibility that Rho kinase activation mediates sympathetic baroreflex dysfunction which underlies the sympathoexcitation 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, was 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 whether 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 sympathoinhibition, 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 ($P_{\text{AngII} \times \text{Fas}} = 0.46$). Conversely, in the PVH, which is an important preautonomic integration center, and in the whole
sagittal 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, whereas 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 sympathoexcitation, and hypertension\textsuperscript{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 sagittal 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.

Although solid evidence indicates that central AngII stimulates Rho kinase via AT1R activation,\textsuperscript{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\textsuperscript{19} and p63RhoGEF,\textsuperscript{22} which activate RhoA/Rho kinase, and inhibits the RhoGAP p190A,\textsuperscript{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 sympathoexcitatory superoxide signaling in the preautonomic centers of the brain.\textsuperscript{12,18} The RhoA/Rho kinase pathway is directly activated by reactive oxygen species\textsuperscript{50,51} and stimulates the formation of superoxide by NADPH (nicotinamide adenine dinucleotide phosphate) oxidase.\textsuperscript{17,52,53} Closely intertwined is the effect of Rho kinase to decrease the production of sympathoinhibitory nitric oxide, which rapidly reacts with superoxide.\textsuperscript{54} These free radicals affect neuronal excitability by altering K\textsuperscript{+} and Ca\textsuperscript{2+} currents.\textsuperscript{10,55} Additionally, Rho kinase is known to play a fundamental role in neurotransmitter release and dendritic spine formation,\textsuperscript{28} and thus Rho kinase inhibition might prevent the release of sympathoexcitatory neurotransmitters and impair neuroplastic conversion to a sympathoexcitatory phenotype.

The 2 Rho kinase isoforms (ROCK1 and ROCK2) share $\approx 90\%$ homology in their kinase domains; however, despite this structural similarity, they are differentially distributed and perform distinct functions.\textsuperscript{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.\textsuperscript{57} ROCK2 is more highly expressed in the brain, and induction of heart failure in rabbits by rapid ventricular pacing increases

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**Figure 4.** Baroreflex control of renal sympathetic nerve activity (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.
ROCK2 levels in the rostral ventrolateral medulla, an important brain stem autonomic center. Fasudil inhibits both isoforms with approximately equal affinity, and thus this study is unable to address the contributions of each isoform to AngII-mediated sympathoexcitation.

At present, Fasudil is used clinically in Japan after acute ischemic stroke and for the prevention of vasospasm after surgery for subarachnoid hemorrhage. Another Rho kinase inhibitor, ripasudil, is also approved in Japan for the treatment of glaucoma and ocular hypertension. 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.

Although the clinical use of specific Rho kinase inhibitors is limited, >30 million Americans take statins for their cholesterol- and cardiovascular risk–reducing effects. Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A (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, 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 a low density lipoprotein–independent manner. Our laboratory has similarly found that statin treatment improves autonomic function in experimental and clinical heart failure independent of low density lipoprotein–lowering effects, 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. Given the strong associative and experimental 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.

Figure 5. Assessment of Rho kinase activity. A, Representative sagittal brain slices stained with antibodies for the Rho kinase target myosin phosphatase targeting protein (p-MYPT) from rabbits infused with each intracerebroventricular treatment. B, Mean subfornical organ (SFO) p-MYPT fluorescence. C, Mean paraventricular regions of the hypothalamus (PVH) p-MYPT fluorescence. D, Mean whole-slice p-MYPT fluorescence. AngII indicates angiotensin-II; and Fas indicates Fasudil. c indicates caudal direction; d, dorsal direction; r, rostral direction; v, ventral direction; and * P<0.025.

Figure 6. Overview summary. Central AngII activates Rho kinase signaling downstream of AT1R, leading to baroreflex dysfunction, sympathoexcitation, and hypertension. AngII indicates angiotensin-II; and AT1R, AngII type 1 receptor.
We acknowledge that this study has several limitations. First, it is unclear whether the dose of AngII falls within the limits of pathophysiology or whether this is a strictly pharmacological dose. Similarly, we do not know whether brain tissue levels of Fas are achievable by systemic administration of Fas in humans. Moreover, treatments were administered intracerebroventricularly 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 (eg, SFO). Finally, the precise mechanisms by which Rho kinase is activated by AngII and the downstream mechanisms by which Rho kinase induces sympathoexcitement remain to be elucidated.

**Perspectives**

Activation of the RAAS and autonomic dysfunction are pathological hallmarks of cardiovascular disease. This study shows that the prohypertensive, sympathoexcitatory, and baroreflex-perturbing effects of AngII in the brain are mediated by the Rho kinase activation. These data indicate that the 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 sympathoexcitation.

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**Disclosures**

None.

**References**

26. Haack KK, Gao L, Schiller AM, Curry PL, Pellegrino PR, Zucker H. Central Rho kinase inhibition restores baroreflex sensitivity and angiotensin II type 1 receptor protein imbalance in conscious rabbits with...


### 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 sympathoexcitation 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 low density lipoprotein–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.
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ONLINE MATERIALS AND METHODS

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

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Animals

Rabbits were housed in individual cages in a temperature-controlled room (23°C) with a 12:12 hour light-dark cycle (lights on at 07:00, lights off at 19:00). Rabbits were given ad libitum access to reverse-osmosis purified water and high-fiber rabbit chow (Teklad 2031, Envigo RMS, Indianapolis, IN).

Chronic Instrumentation

Each rabbit was chronically instrumented with an arterial pressure (AP) radiotelemetry and an ICV cannula connected to an osmotic minipump using sterile surgical technique. Following induction with an intramuscular bolus of ketamine (35 mg/kg) and xylazine (5.8 mg/kg), rabbits were intubated, and anesthesia was maintained with 1-2% isoflurane. After a femoral cut down, the tip of an AP telemetry catheter (model PA-C40, Data Sciences International, New Brighton, MN) was advanced into the abdominal aorta via the right femoral artery and secured. A scalp incision was made, the skull was cleared of connective tissue, and an ICV cannula was implanted 3 mm lateral to bregma. A periscapular incision was made, and a catheter was threaded subcutaneously from the scalp to the periscapular subcutaneous pocket. The cranial end of the catheter was connected to the ICV cannula, and the caudal end was connected to a vehicle-filled osmotic minipump (Alzet 2ML2, DURECT Corporation, Cupertino, CA). On the day of surgery, rabbits received pre-surgical enrofloxacin (22.7 mg subcutaneous, Bayer HealthCare LLC, Shawnee Mission, KS) and buprenorphine for pain control upon emergence from anesthesia. Rabbits received a three-day postoperative course of enrofloxacin and carprofen (4 mg/kg subcutaneous, Zoetis, Lincoln, NE) respectively. Each rabbit was allowed to recover for at least ten days, during which time the rabbit was acclimated to the procedure room and trained to rest calmly in a Plexiglas box. Rabbits in the between-rabbits cohort underwent a subsequent surgery in which a bipolar electrode was implanted on the left renal nerve. The left kidney was approached retroperitoneally via a flank incision, the largest renal nerve bundle was carefully dissected, and two platinum electrodes were wrapped around the dissected nerve section and fixed using Kwik-Sil adhesive (World Precision Instruments, Inc., Sarasota, FL). A ground loop electrode was secured to nearby tissue with 6-0 Prolene. Each electrode was electrically coupled to a Teflon-coated stainless steel wire, which was fixed to the renal artery with 6-0 Prolene, and all three leads were exteriorized via a common Silastic tube. The ICV vehicle infusion osmotic minipump was replaced with a treatment osmotic minipump, as described below, during the same surgery. Rabbits were given the same perioperative antibiotic regimen as above, but the analgesic regimen was supplemented with a 72-hour fentanyl patch (25 mcg/hr transdermal). Rabbits were allowed to recover seven days from RSNA surgery before experiments were begun.

Replacement of Osmotic Minipump

Osmotic minipumps were replaced using sterile technique under general and local anesthesia with AP and heart rate (HR) monitoring. Rabbits were anesthetized with an intramuscular bolus of ketamine (17.5 mg/kg) and xylazine (2.9 mg/kg) and a
local periscapular infiltration of bupivacaine (0.25 mg). An incision was made into the periscapular subcutaneous pocket containing the osmotic minipump to be replaced, and the pump was removed and the catheter connecting it to the ICV cannula was cut. A new osmotic minipump was connected to the catheter and implanted. The incision was closed with interrupted, external Prolene sutures. The content of the osmotic minipump was aspirated, and the aspirated volumes were always within 2 standard deviations of the manufacturer tolerances for pump volume and pump rate. When treatment pumps were placed, the function of the catheter and ICV cannula was first tested by giving a 100 nmol AngII bolus, which robustly raises MAP when given ICV but has no effect on if given subcutaneously (e.g., if the catheter is disconnected from the ICV cannula). Rabbits received the same antibiotic regimen as for instrumentation and a one-time post-operative dose of buprenorphine (0.02 mg/kg subcutaneously) for pain control.

**Dose-Finding Study**

Three rabbits were used in a pilot study to identify doses of Angiotensin-II which raised blood pressure when administered ICV. Four ICV doses (10 ng/min, 20 ng/min, 30 ng/min, and 40 ng/min) were attempted, with only the highest showing a pressor effect. Three ICV doses (87.5 ng/min, 175 ng/min, and 350 ng/min) of Fas were co-infused with AngII. The two rabbits who initially received the lowest dose of Fas did not show any attenuation of the pressor effect of AngII. The rabbit who received the highest dose suffered acute neurological symptoms approximately 18 hours after implantation of the minipump and died quickly thereafter. It is unclear if this was related to central Rho kinase inhibition or an off-target mechanism. The 175 ng/min dose of Fas blocked the pressor effect of central AngII, but, clearly, the therapeutic window for central Fas to block the pressor effect of central AngII in rabbits is relatively narrow. The results for the two rabbits who completed the dose-finding study were used for power analysis.

**Baseline Recordings from Within-rabbits Study**

The rabbits rested in a Plexiglas box in a dimly lit procedure room for at least 30 minutes at approximately the same time each morning. Pulsatile AP was digitized at a sampling frequency of 1 kHz via a 16-channel PowerLab system (ADInstruments, Inc., Colorado Springs, CO). Heart rate was derived from the pulse rate of the AP signal.

**Pharmacological Assessment of Autonomic Tone**

Intravenous (IV) administration of autonomic blockers in the afternoon of days 11 through 13 was used to assess the effects of ICV treatments on autonomic tone. After local infiltration with bupivacaine, an IV catheter was inserted into a marginal ear vein to allow IV administration of cardiac autonomic blockers. Each rabbit was allowed to rest for ten minutes after the placement of the IV catheter, at which point a 1 mL bolus of saline was given and recording of pulsatile arterial pressure was initiated. Fifteen minutes later, atropine methyl bromide (0.2 mg/kg bolus; Sigma-Aldrich Corp., St. Louis, MO), metoprolol bitartrate (1 mg/kg bolus; Sigma-Aldrich Corp., St. Louis, MO), or hexamethonium bromide (3 mg/kg/min infusion for ten minutes; Sigma-Aldrich Corp., St. Louis, MO) was administered IV. Atropine, a muscarinic antagonist, blocks the effects
of vagal control of sinoatrial node rhythmicity, and thus the increase in HR after atropine is a surrogate for resting cardiac parasympathetic tone. Metoprolol, a beta1-adrenergic antagonist, blocks the effects of sympathetic control of sinoatrial node rhythmicity, and thus the decrease in HR after metoprolol administration is a surrogate for resting cardiac sympathetic tone. Hexamethonium, a ganglionic blocker, causes hypotension mainly by blocking sympathetic vasoconstrictor tone, and thus the maximal decrease in AP during the course of the hexamethonium infusion is a surrogate for global resting sympathetic vasomotor tone.

**Assessment of Baroreflex Control of Heart Rate**

On the afternoon of day 14, baroreflex control of HR was assessed by infusion of vasoactive drugs. After IV access was obtained as above, an intravenous infusion of sodium nitroprusside (100 µg/kg/min) was initiated and AP was driven down until HR reached a plateau. After the rabbit recovered completely, an infusion of phenylephrine (40 µg/kg/min) was initiated, and AP was driven up until HR reached a plateau. The HR and systolic blood pressure (SBP) for each beat were paired, and these pairs were then separated based on SBP into 2 mmHg wide bins. The median SBP and HR for each bin were fitted to a five-parameter logistic equation.

**Volume Status Experiment**

Four of the six rabbits underwent a volume status study on day 10 of ICV treatment using a previously described protocol in order to assess effects of the ICV treatment on volume regulation. This was a technique that we had not perfected when the first two rabbits were enrolled, hence their exclusion. Briefly, the marginal ear vein and central ear artery of the conscious rabbit were catheterized after local lidocaine infiltration. A bolus of FITC-sinistrin (Fresenius Kabi, Linz, Austria) was given IV and quickly flushed. Blood samples (3 mL) from the arterial catheter were collected at 3, 7, 10, 15, 45, 75 and 105 minutes after the FITC-sinistrin bolus. The plasma fluorescence was measured and compared to a standard curve in order to derive the FITC-sinistrin plasma concentration. The FITC-sinistrin plasma concentration was fitted to a two-exponential decay equation, and the physiological parameters of the fitted open two-compartment pharmacokinetic model were derived. Specifically, glomerular filtration rate, the volumes of the high- and low-perfusion compartments ($V_{\text{high-perfusion}}$ and $V_{\text{low-perfusion}}$, respectively), and the first-order kinetic constants between the compartments $k_{\text{high} \rightarrow \text{low}}$ and $k_{\text{low} \rightarrow \text{high}}$ and out of the system $k_{\text{elim}}$ (i.e. GFR/$V_{\text{high-perfusion}}$) are reported. The sum of the high- and low-perfusion compartments equals the extracellular fluid volume (ECFV).

**Recording of RSNA**

RSNA was differentially pre-amplified 10,000-fold, band-pass filtered from 100 to 1000 Hz (Grass P55, Natus Medical Inc., Warwick, RI), digitized at 1 kHz, rectified, and integrated with a 20-ms time constant. Baseline RSNA was quantified as a percent of the nasopharyngeal reflex, burst frequency, and burst incidence. The nasopharyngeal reflex was elicited by drawing thick cigarette smoke into a 60-mL syringe and emptying
the syringe into the face of the rabbit over 10 seconds. At the end of the experiment, the electrical zero (i.e. noise) was determined by administering a 30 mg/kg IV bolus of hexamethonium, which abolished RSNA. The baseline integrated nerve activity minus the electrical zero was expressed as a percent of the maximal five-second integrated RSNA during elicitation of the nasopharyngeal reflex minus the electrical zero (i.e. \( \frac{\text{baseline} - \text{electrical zero}}{\text{nasopharyngeal maximum} - \text{electrical zero}} \times 100\% \)). Burst frequency was determined by manually setting a burst threshold above the noise floor of an artifact-free section of baseline with a retrigger delay of 100 msec and expressed as bursts/min. Burst incidence, the number of bursts per 100 heart beats, was determined by normalizing the burst frequency to the HR. Baroreflex control of RSNA was assessed just as performed as described above, except the rate of phenylephrine infusion was decreased from 40 µg/kg/min to 10 µg/kg/min. Beat-averaged integrated RSNA was paired with the preceding DBP, and these pairs were then separated based on DBP into 2 mmHg wide bins. The median DBP and integrated RSNA for each bin were fitted to a five-parameter logistic equation, but because baroreflex control of RSNA in the rabbit shows hypo- and hypertensive reversal, the lower plateau and the range of the logistic curve were fixed to match the DBP bins with the lowest and highest RSNA, respectively.

**Tissue Collection**

Rabbits were anti-coagulated with heparin (1000 international unit IV bolus), euthanized with 3 mL Fatal-Plus (Vortech Pharmaceuticals, Dearborn Michigan) IV bolus, and an intracardiac perfusion was performed with Tris-buffered saline (TBS) followed by 4% paraformaldehyde.

**Tissue Slice Preparation**

The tissue was quickly removed and post-fixed for 36 hours in 4% paraformaldehyde and dehydrated in 3% sucrose prepared with TBS at 4°C. Next, a midline saggital cut was made and one half of the brain was blocked on an aluminum plate in Tissue Tek OCT Compound (Sakura Finetek, Torrance, CA) medium and stored at – 80°C until further processing. On the day of processing, brain tissue was acclimated to -20°C and the tissue was sliced into 150 µm sections starting at the midline and placed in TBS.

**Tissue Immunofluorescence**

Tissue slices were placed in TBS-based blocking buffer for 1 hour at room temperature with gentle shaking (LiCor, Lincoln, NE). Following this, the slices were exposed to a solution of 1:1000 p-MYPT mouse primary antibodies (sc-377531, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C with gentle shaking. The following morning, slices were rinsed with moderate shaking 4x for 15 minutes in 0.1% TBS-T. Slices were then exposed to a 1:10,000 dilution of donkey anti-mouse secondary antibody (925-68022, LiCor Biosciences, Lincoln, NE) while protected from light with gentle shaking at room temperature. After incubation the tissues were rinsed with moderating shaking 4x for 15 minutes at room temperature, while protected from light. Slices were then placed into TBS until mounted. Tissues were removed from the TBS and mounted using a Versatool Kit (Elegant Instruments, Omaha, NE), placed on a
glass slide, briefly rinsed in water and allowed to dry at room temperature protected from light for an allowed to completely dry before scanning.

**Brain Slice Scanning and Processing**

All brains were scanned together at 42 µm resolution using a LiCor Odyssey Scanner. The resulting fluorescence image was then exported to MATLAB for image processing. The fluorescence intensity histogram was clearly bimodal, with the low-intensity background pixels composing one peak and the high-intensity brain tissue pixels composing another peak. The fluorescence intensity histogram was used to manually identify the maximum background intensity for the entire fluorescent image by selecting an intensity value that was between both peaks. Then, each brain slice was coarsely traced, and that region was then thresholded to exclude pixels whose intensity was below the maximum background intensity (indicating there was no brain, e.g., pixels in the third ventricle). This brain mask was then used to quantify the mean intensity (after subtracting the maximum background intensity) for each slice as well as for the quantification of the mean p-MYPT fluorescence intensity in the SFO and PVH of each brain. Within each brain mask, the SFO was carefully traced, and the difference between the mean fluorescence intensity the maximum background intensity was used as the Rho kinase activity level. In a sagittal slice, it is impossible to know the exact borders of the paraventricular hypothalamic nucleus (PVN), so we have adopted the nomenclature PVH in acquiescence of this fact. Uniformly sized and oriented boxes (see Figure 5A) were placed in the PVH, and the mean p-MYPT fluorescence intensity above the background was used as an assessment of Rho kinase activity in the PVH.

**Power Analysis and Sample Size Justification**

The within-rabbits cohort was powered to detect an AngII x Fas interaction on MAP via two-way ANOVA (Online Figure S1A). Data from the dose-finding study were used for power analysis. The mean and standard deviation of MAP for each treatment were calculated and used to calculate the AngII x Fas and within-treatment sum-square error using a simple between-rabbits two-way ANOVA approach, obviating known difficulties with multi-factor RM ANOVA power analysis. This yields a more conservative estimate of sample size as the within-rabbits design increases power. Since we did not have data for Fas alone infusion and had previously observed no effect on MAP from ICV Fas infusion (albeit a different dose) in healthy rabbits, the Veh mean and standard deviation were used for power analysis. The AngII x Fas and within-treatment sum of squares were used to calculate the effect size $\eta^2$, and GPower was used to compute the required sample size for $\beta > 0.80$. This showed that we needed 5 rabbits per treatment; we enrolled 6 rabbits due to the likelihood of instrumentation failure over the course of such a long study. All rabbits completed all four treatments.

The between-rabbits cohort was powered to detect an AngII x Fas interaction on baseline RSNA via two-way ANOVA using data from the literature (Online Figure S4A). A study of 15 normal rabbits showed that RSNA when normalized to the nasopharyngeal reflex was 7.0% with a standard deviation of 4.6%. Another previous study showed that a seven-day infusion of a non-pressor dose of ICV AngII caused a
2.6-fold increase in baseline RSNA normalized to the nasopharyngeal reflex with a similar increase in the standard deviation of baseline RSNA, thus 18.4% was estimated as the mean and 12.0% the standard deviation for AngII-infused rabbits. We assumed that Fas alone would not affect RSNA and that coadministration of Fas would completely block the AngII-mediated renal sympatho-excitation, mirroring what we had observed with global sympathetic vasomotor tone in the within-rabbits study (i.e. $\mu_{\text{Fas}} = \mu_{\text{AngII+Fas}} = \mu_{\text{Veh}}$ and $\sigma_{\text{Fas}} = \sigma_{\text{AngII+Fas}} = \sigma_{\text{Veh}}$). This was used to compute an effect size $\eta^2$, and GPower was used to compute the required sample size for $\beta > 0.80$.

**Expanded Statistical Analysis**

Group data for Figures 1C, 1E, 2A-C, 3C-E, 5B-D, and Online Figure S3A-C are shown as dot plots with each dot representing one rabbit and horizontal lines indicating the group mean ± SEM. All other data is displayed as group mean ± SEM. Statistical testing on endpoints from the within-rabbits study was performed using full-factorial two-way or three-way repeated measure (RM) analysis of variance (ANOVA) with AngII, Fas, and, where applicable (i.e. Figure 1B, Figure 1D), time as within-subject factors. Neither the factor time nor its interactions with treatment factors ever reached statistical significance after Greenhouse-Geisser corrections for non-sphericity and thus are not reported in the manuscript. RM-ANOVAs for which a within-rabbits factor or interaction term reached statistical significance were followed with paired t-tests of Veh versus AngII and AngII versus AngII+Fas, which were Bonferroni-corrected for two comparisons. Statistical testing on endpoints from the between-rabbits study was performed using a two-way ANOVA with AngII and Fas as fixed factors. Two-way ANOVAs for which the corrected model reached statistical significance were followed with unpaired t-tests of Veh versus AngII and AngII versus AngII+Fas, which were Bonferroni-corrected for these two comparisons. All statistical testing was performed with IBM SPSS Statistics 22.0 (IBM Corporation, Armonk, NY).
ONLINE REFERENCES


## SUPPLEMENTAL TABLES AND FIGURES

### Table S1. HR from atropine experiments.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Veh</th>
<th>Fas</th>
<th>AngII</th>
<th>AngII+Fas</th>
<th>$P_{\text{AngII} \times \text{Veh}}$</th>
<th>$P_{\text{AngII}}$</th>
<th>$P_{\text{Fas}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR before atropine (bpm)</td>
<td>196 ± 10</td>
<td>205 ± 9</td>
<td>199 ± 5</td>
<td>197 ± 14</td>
<td>0.55</td>
<td>0.63</td>
<td>0.67</td>
</tr>
<tr>
<td>HR after atropine (bpm)</td>
<td>255 ± 17</td>
<td>259 ± 13</td>
<td>260 ± 10</td>
<td>259 ± 16</td>
<td>0.83</td>
<td>0.65</td>
<td>0.83</td>
</tr>
</tbody>
</table>

HR before and after administration of atropine IV bolus, n = 6 per treatment.

### Table S2. HR from metoprolol experiments.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Veh</th>
<th>Fas</th>
<th>AngII</th>
<th>AngII+Fas</th>
<th>$P_{\text{AngII} \times \text{Veh}}$</th>
<th>$P_{\text{AngII}}$</th>
<th>$P_{\text{Fas}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR before metoprolol (bpm)</td>
<td>204 ± 15</td>
<td>193 ± 13</td>
<td>205 ± 7</td>
<td>192 ± 15</td>
<td>0.88</td>
<td>0.94</td>
<td>0.06</td>
</tr>
<tr>
<td>HR after metoprolol (bpm)</td>
<td>203 ± 14</td>
<td>193 ± 12</td>
<td>185 ± 3</td>
<td>191 ± 14</td>
<td>0.41</td>
<td>0.29</td>
<td>0.66</td>
</tr>
</tbody>
</table>

HR before and after administration of metoprolol IV bolus, n = 6 per treatment.

### Table S3. MAP from hexamethonium experiments.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Veh</th>
<th>Fas</th>
<th>AngII</th>
<th>AngII+Fas</th>
<th>$P_{\text{AngII} \times \text{Veh}}$</th>
<th>$P_{\text{AngII}}$</th>
<th>$P_{\text{Fas}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP before hexamethonium (mm Hg)</td>
<td>82 ± 2</td>
<td>79 ± 2</td>
<td>101 ± 4</td>
<td>78 ± 2</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>MAP after hexamethonium (mm Hg)</td>
<td>53 ± 2</td>
<td>52 ± 2</td>
<td>55 ± 5</td>
<td>50 ± 2</td>
<td>0.56</td>
<td>0.96</td>
<td>0.23</td>
</tr>
</tbody>
</table>

MAP before and after hexamethonium IV infusion, *, $P < 0.025$ vs AngII; ††, $P < 0.01$ vs. AngII+Fas, n = 6 per treatment.
Table S4. Baroreflex control of HR fitted to five-parameter logistic equation for each treatment.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Veh</th>
<th>Fas</th>
<th>AngII</th>
<th>AngII+Fas</th>
<th>P_{AngII} x Veh</th>
<th>P_{AngII}</th>
<th>P_{Fas}</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2, Range (bpm)</td>
<td>268 ± 19</td>
<td>245 ± 17</td>
<td>267 ± 37</td>
<td>247 ± 23</td>
<td>0.27</td>
<td>0.54</td>
<td>0.63</td>
</tr>
<tr>
<td>P3, Lower Curvature</td>
<td>-0.06 ± 0.01</td>
<td>-0.11 ± 0.02</td>
<td>-0.10 ± 0.02</td>
<td>-0.14 ± 0.06</td>
<td>0.53</td>
<td>0.20</td>
<td>0.34</td>
</tr>
<tr>
<td>P5, Upper Curvature</td>
<td>-0.10 ± 0.04</td>
<td>-0.05 ± 0.01</td>
<td>-0.03 ± 0.01</td>
<td>-0.06 ± 0.01</td>
<td>0.13</td>
<td>0.10</td>
<td>0.71</td>
</tr>
<tr>
<td>P4, BP_{50} (mmHg)</td>
<td>97 ± 3</td>
<td>100 ± 4</td>
<td>119 ± 7</td>
<td>100 ± 5</td>
<td>0.09</td>
<td>&lt; 0.05</td>
<td>0.07</td>
</tr>
<tr>
<td>Asymmetry</td>
<td>0.02 ± 0.51</td>
<td>0.74 ± 0.07</td>
<td>1.18 ± 0.27</td>
<td>0.61 ± 0.17</td>
<td>0.06</td>
<td>0.19</td>
<td>0.54</td>
</tr>
<tr>
<td>Curvature</td>
<td>0.05 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.08 ± 0.02</td>
<td>0.10</td>
<td>0.97</td>
<td>0.09</td>
</tr>
</tbody>
</table>

P2, the range parameter; P3, the lower curvature parameter; P5, the upper curvature parameter; P4, the systolic blood pressure at which half of the range in heart rate was attained from the fitted 5-parameter logistic function, n = 6 for each treatment.

Table S5. Parameters from two-compartment FITC-Sinistrin pharmacokinetics for each treatment.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Veh</th>
<th>Fas</th>
<th>AngII</th>
<th>AngII+Fas</th>
<th>P_{AngII} x Veh</th>
<th>P_{AngII}</th>
<th>P_{Fas}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular Filtration Rate (mL/min)</td>
<td>11.2 ± 0.6</td>
<td>11.2 ± 1.7</td>
<td>12.1 ± 1.2</td>
<td>10.0 ± 1.0</td>
<td>0.50</td>
<td>0.93</td>
<td>0.13</td>
</tr>
<tr>
<td>k_{elim} (min^{-1})</td>
<td>0.042 ± 0.002</td>
<td>0.073 ± 0.011</td>
<td>0.055 ± 0.012</td>
<td>0.060 ± 0.010</td>
<td>0.18</td>
<td>0.97</td>
<td>0.23</td>
</tr>
<tr>
<td>k_{high→low} (min^{-1})</td>
<td>0.079 ± 0.008</td>
<td>0.143 ± 0.033</td>
<td>0.138 ± 0.057</td>
<td>0.160 ± 0.039</td>
<td>0.09</td>
<td>&lt; 0.05</td>
<td>0.07</td>
</tr>
<tr>
<td>k_{low→high} (min^{-1})</td>
<td>0.057 ± 0.004</td>
<td>0.076 ± 0.016</td>
<td>0.074 ± 0.017</td>
<td>0.075 ± 0.17</td>
<td>0.53</td>
<td>0.36</td>
<td>0.60</td>
</tr>
</tbody>
</table>

k_{elim}, plasma elimination kinetic constant; k_{high→low}, kinetic constant for flux from high-perfusion to low-perfusion compartment; k_{low→high}, kinetic constant for flux from low-perfusion to low-perfusion compartment, n = 4 for each treatment.
Table S6. Resting hemodynamics for between-rabbits study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Veh</th>
<th>Fas</th>
<th>AngII</th>
<th>AngII+Fas</th>
<th>$P_{AngII \times Veh}$</th>
<th>$P_{AngII}$</th>
<th>$P_{Fas}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mm Hg)</td>
<td>75 ± 3</td>
<td>78 ± 2</td>
<td>91 ± 4*</td>
<td>74 ± 3†</td>
<td>&lt; 0.01</td>
<td>0.05</td>
<td>0.08</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>203 ± 5</td>
<td>205 ± 6</td>
<td>197 ± 10</td>
<td>205 ± 12</td>
<td>0.70</td>
<td>0.71</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Resting mean arterial pressure (MAP) and heart rate (HR) 7 days after RSNA electrode implantation. *, P < 0.025 vs AngII; †, P < 0.025 vs. AngII+Fas, n = 4 for each treatment.

Table S7. Baroreflex control of RSNA fitted to five-parameter logistic equation for each group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Veh</th>
<th>Fas</th>
<th>AngII</th>
<th>AngII+Fas</th>
<th>$P_{AngII \times Veh}$</th>
<th>$P_{AngII}$</th>
<th>$P_{Fas}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2, Range (%baseline)</td>
<td>479 ± 72</td>
<td>447 ± 93</td>
<td>132 ± 27*</td>
<td>533 ± 111†</td>
<td>&lt; 0.05</td>
<td>&lt; 0.05</td>
<td>0.14</td>
</tr>
<tr>
<td>P2, Range (%max)</td>
<td>58.3 ± 4.1</td>
<td>48.0 ± 7.0</td>
<td>43.5 ± 9.5</td>
<td>53.7 ± 16.4</td>
<td>0.34</td>
<td>1.00</td>
<td>0.67</td>
</tr>
<tr>
<td>P3, Lower Curvature</td>
<td>-0.35 ± 0.20</td>
<td>-0.56 ± 0.14</td>
<td>-0.15 ± 0.08</td>
<td>-0.56 ± 0.11</td>
<td>0.33</td>
<td>0.09</td>
<td>0.34</td>
</tr>
<tr>
<td>P5, Upper Curvature</td>
<td>-0.40 ± 0.23</td>
<td>-0.43 ± 0.23</td>
<td>-0.34 ± 0.17</td>
<td>-0.21 ± 0.08</td>
<td>0.69</td>
<td>0.79</td>
<td>0.46</td>
</tr>
<tr>
<td>P4, BP$_{50}$ (mmHg)</td>
<td>56.4 ± 2.6</td>
<td>61.5 ± 3.2</td>
<td>77.6 ± 5.3*</td>
<td>58.7 ± 1.5†</td>
<td>&lt; 0.05</td>
<td>0.05</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Asymmetry</td>
<td>0.24 ± 0.46</td>
<td>0.50 ± 0.58</td>
<td>-0.70 ± 0.88</td>
<td>1.08 ± 0.31</td>
<td>0.22</td>
<td>0.11</td>
<td>0.77</td>
</tr>
<tr>
<td>Curvature</td>
<td>0.30 ± 0.06</td>
<td>0.34 ± 0.10</td>
<td>0.11 ± 0.08</td>
<td>0.29 ± 0.11</td>
<td>0.45</td>
<td>0.22</td>
<td>0.20</td>
</tr>
</tbody>
</table>

P2, the range parameter; P3, the lower curvature parameter; P5, the upper curvature parameter; P4, the systolic blood pressure at which half of the range in RSNA was attained from the fitted 5-parameter logistic function. *, P < 0.025 vs. Veh, †, P < 0.025 vs. AngII.
**Figure S1.** Study Design and Flow Chart for the Within-rabbits Cohort. (A) Power analysis for MAP based on dose-finding study. (B) Each rabbit received all four ICV treatments in random order.

**Figure S2.** Baroreflex Control of HR. (A) Composite HR baroreflex curves. (B) Composite RSNA baroreflex gain curves, n = 6 per treatment.
Figure S3. Assessment of Volume Status by FITC-Sinistrin Distribution. (A) Extracellular fluid volume. (B) Volume of the Low-Perfusion Compartment. (C) Volume of the High-Perfusion Compartment, n = 4 per treatment.

Figure S4. Study Design and Flow Chart for the Between-rabbits Cohort. (A) Power analysis for baseline RSNA based on literature values. (B) Each rabbit received one ICV treatment, rabbits without RSNA bursts after a one-week recovery from RSNA surgery were excluded from analysis.