Central Rho Kinase Inhibition Restores Baroreflex Sensitivity and Angiotensin II Type 1 Receptor Protein Imbalance in Conscious Rabbits With Chronic Heart Failure

Karla K.V. Haack, Lie Gao, Alicia M. Schiller, Pamela L. Curry, Peter R. Pellegrino, Irving H. Zucker

Abstract—The small GTPase RhoA and its associated kinase ROCKII are involved in vascular smooth muscle cell contraction and endothelial NO synthase mRNA destabilization. Overactivation of the RhoA/ROCKII pathway is implicated in several pathologies, including chronic heart failure (CHF), and may contribute to the enhanced sympathetic outflow seen in CHF as a result of decreased NO availability. Thus, we hypothesized that central ROCKII blockade would improve the sympathovagal imbalance in a pacing rabbit model of CHF in an NO-dependent manner. CHF was induced by rapid ventricular pacing and characterized by an ejection fraction of ≤45%. Animals were implanted with an intracerebroventricular cannula and osmotic minipump (rate, 1 µL/h) containing sterile saline, 1.5 µg/kg per day fasudil (Fas, a ROCKII inhibitor) for 4 days or Fas+100 µg/kg per day No-Nitro-l-arginine methyl ester hydrochloride, a NO synthase inhibitor. Arterial baroreflex control was assessed by intravenous infusion of sodium nitroprusside and phenylephrine. Fas infusion significantly lowered resting heart rate by decreasing sympathetic and increasing vagal tone. Furthermore, Fas improved baroreflex gain in CHF in an NO-dependent manner. In CHF Fas animals, the decrease in heart rate in response to intravenous metoprolol was similar to Sham and was reversed by No-Nitro-l-arginine methyl ester hydrochloride. Fas decreased angiotensin II type 1 receptor and phospho-ERM protein expression and increased endothelial NO synthase expression in the brain stem of CHF animals. These data strongly suggest that central ROCKII activation contributes to cardiac sympathoexcitation in the setting of CHF and that central Fas restores vagal and sympathetic tone in an NO-dependent manner. ROCKII may be a new central therapeutic target in the setting of CHF. (Hypertension. 2013;61:723-729.)

Key Words: angiotensin AT1 receptor ■ autonomic nervous system ■ heart failure ■ NO synthase ■ rho-associated kinase

Hallmarks of chronic heart failure (CHF) include increased circulating angiotensin II, upregulation of the angiotensin II type 1 receptor (AT1R), and an imbalance in the autonomic nervous system.1 In CHF, there is heightened sympathetic outflow and a decrease in vagal tone, two driving forces in the progression of the disease.2 This sympathovagal imbalance may be mediated, in part, by decreased NO bioavailability. Synthesized by endothelial NO synthase (eNOS), NO can diffuse to nearby cells to activate guanylyl cyclase to increase intracellular cyclic guanosine monophosphate and activate downstream signaling cascades leading to vasodilation and other antiinflammatory and antiproliferative effects.3

The sympathovagal imbalance seen in CHF may also reflect a blunted baroreflex, an integral mechanism in maintaining cardiovascular homeostasis.4,5 A decrease in baroreceptor sensitivity may participate in hyperactivity of cardiovascular control centers of the brain, including the major center of presympathetic neurons that project to the spinal cord, the rostral ventrolateral medulla (RVLM).6,7

Activation of the RhoA/RhoA associated kinase (ROCK) II pathway can occur as a downstream consequence of the activation of Gα protein subunits via G-protein–coupled receptor signaling. For example, angiotensin II signaling through AT1R has been shown to indirectly activate RhoA/ROCKII pathway in cardiac myocytes.8–10 RhoA/ROCKII pathway hyperactivity is seen in several pathophysiological states, including stroke, hypertension, and CHF.3,11,12 After cysteine modification by superoxide, RhoA protein can exchange GDP for GTP and can subsequently bind ROCKII.8–10 RhoA/ROCKII signaling cascade leads to upregulation of nuclear factor-kB, increased myosin light chain kinase activation, and phosphorylation of ezrin/radixin/moesin proteins,11,14 leading to actin cytoskeletal rearrangement and reduction in dendritic spine formation,15 and a shift toward a proinflammatory state.8

Although the role of RhoA/ROCKII in peripheral vasoconstriction and eNOS activation has been well established,16–18 there is still less of an understanding of how central RhoA/ROCKII exerts its effects under both physiological and pathophysiological conditions. The RhoA/ROCKII pathway has been shown to be hyperactive in the nucleus tractus solitarius (NTS) of spontaneously hypertensive rats, and...
that the RhoA/ROCKII pathway in the NTS contributes to neurogenic hypertension in part by chronic NOS inhibition.\(^{19,20}\) Given its ability to disrupt tight junction proteins, central RhoA/ROCKII pathway activation has also been implicated in blood–brain barrier dysfunction in experimental models of multiple sclerosis and in some animal stroke models.\(^{3,21}\) However, the contribution of the central RhoA/ROCKII pathway to the blunted baroreflex and sympathovagal balance seen in CHF has yet to be determined. Therefore, the goal of this study was to determine the role of the central Rho Kinase pathway in the sympathovagal imbalance seen in CHF: The RhoA/ROCK II pathway destabilizes eNOS mRNA, thereby reducing NO availability. This decrease in NO prevents the inhibition of sympathetic neurons in the RVLM, which contributes to the increase in sympathetic outflow in CHF and ultimately leads to a decrease in baroreflex sensitivity. We, therefore, hypothesized that blockade of the RhoA/ROCKII pathway with the specific ROCKII inhibitor fasudil (Fas) would restore cardiac sympathovagal balance and baroreflex sensitivity in CHF rabbits in an NO-dependent manner.

**Methods**

**Animals**

Experiments were carried out on a total of 36 New Zealand white male rabbits weighing 3.0 to 4.5 kg (Charles River Laboratories, Wilmington, MA). All experiments were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Animals were randomly placed in 1 of 5 experimental groups: Sham Vehicle (Sham Veh), Sham Fasudil (Sham Fas), Chronic Heart Failure Vehicle (CHF Veh), Chronic Heart Failure Fasudil (CHF Fas), or Chronic Heart Failure Fas+No-Nitro-l-arginine methyl ester hydrochloride (l-NAME) (CHF Fas+l-NAME).

**Surgical Instrumentation**

Rabbits were instrumented as described previously.\(^ {22}\) In brief, animals were given a preanesthetic cocktail of 35 mg/kg ketamine, 5.8 mg/kg xylazine, and 0.01 mg/kg atropine in 1 cc of lactated Ringer’s solution. After intubation, anesthesia was maintained using a low level of isoflurane (0.5%–1.0%). A radiotelemetry transducer (Data Sciences International, MN) was implanted in the right femoral artery to monitor mean arterial blood pressure (MAP) and heart rate (HR) using the Data Sciences International telemetry and ADInstruments (Colorado Springs, CO) Powerlab data acquisition systems. During the same surgical procedure, a left thoracotomy was carried out and a reference electrode was attached to the left atrium and a platinum pacing electrode was sutured to the left ventricle. Pacing wires were tunneled beneath the skin and exited in the midscapular area.

At least 2 weeks after thoracic surgery, an intracerebroventricular (icv) cannula was inserted and reinforced with dental cement to the surface of the skull. An osmotic minipump (Alzet 2001, rate: 1 µL/h, Cupertino, CA) containing sterile normal saline, 1.5 µg/kg per day Fas (Tocris, Minneapolis, MN), or 1.5 µg/kg per day Fas plus 100 µg/kg per day l-NAME (Sigma Aldrich, St. Louis, MO) was attached to the brain cannula using a Microrenathane (.020/.060, Norton, Akron, OH) catheter. On the basis of rat studies reported in the literature we scaled the dose of l-NAME to be used in rabbits.\(^ {23}\)

**Induction of CHF**

As previously described, CHF was induced by chronic ventricular pacing.\(^ {23}\) Please see the expanded methods in the online-only Data Supplement.

**Cardiac Baroreflex Analysis and Autonomic Blockade**

Animals were allowed to acclimate by sitting in a Plexiglass box in a dimly lit laboratory for 20 minutes with the pacemaker turned off for CHF rabbits before experimentation. After 5 minutes of baseline MAP and HR recording, the maximal changes in HR and MAP were assessed by the response to nasopharyngeal stimulation with 60 mL of cigarette smoke. This maneuver has been used previously in our laboratory as a means of measuring vagal tone.\(^ {24,25}\) After HR and MAP returned to baseline, baroreflex curves were generated by intravenous infusion (marginal ear vein) of 100 µg/kg sodium nitroprusside and 80 µg/kg phenylephrine at a rate of 0.5 mL/min. Sodium nitroprusside was infused until MAP was decreased to ±40 to 45 mm Hg, at which point the sodium nitroprusside infusion was replaced with phenylephrine until MAP reached ±100 mm Hg. After generation of a control baroreflex curve, a second curve was constructed after intravenous bolus administration of 0.2 mg/kg atropine methylbromide or 1 mg/kg metoprolol bitartrate. After 10 minutes, a second 10-minute baseline was recorded, and baroreflex experiments were repeated. Atropine and metoprolol experiments were done on separate days.

In CHF vehicle-treated rabbits, 24 hours after completion of all baroreflex experiments, the saline-containing pump was replaced with a pump filled with Fas or Fas+l-NAME. Three days after pump implantation, baseline recordings and baroreflex assessments were repeated.

**Arterial Baroreflex Analysis**

Arterial baroreflex curves were constructed as described previously.\(^ {25}\) Briefly, curves were constructed by sampling data points for HR every 5 seconds from the lowest to highest MAP after sodium nitroprusside and phenylephrine infusions. Individual logistic regression curves were fit to the data points as described previously.\(^ {25}\)

**Brain Micropunch and Western Blot Analyses**

RVLM micropunches were isolated and processed as described previously.\(^ {26}\) Additional details can be found in the online-only Data Supplement.

**Statistical Analyses**

Data are expressed as mean±SEM. All statistical analyses were performed with GraphPad Prism Software (GraphPad, La Jolla, CA). Differences between groups was assessed with 1-way ANOVA with a Bonferroni correction when appropriate. P<0.05 was considered statistically significant.

**Results**

Echocardiographic profiles of pre- and postpace animals are shown in Table S1 in the online-only Data Supplement. Pacing animals had a significantly lower ejection fraction and fractional shortening, and significantly higher left ventricular systolic volume and left ventricular end systolic diameter compared with prepace animals. The Table summarizes resting HR and MAP. Baseline HR was higher in CHF Veh animals compared with Sham groups, and HR was significantly lowered by icv Fas treatment in CHF rabbits. The decrease in HR seen in CHF Fas animals was reversed by coadministration of icv l-NAME. MAP was unchanged between groups.

To determine whether icv Fas had an effect on autonomic balance, we recorded baseline HR before and after intravenous bolus administration of atropine, a muscarinic receptor antagonist, or metoprolol, a β-1 adrenergic receptor antagonist (Figure 1). The tachycardia seen in response to atropine is indicative of the degree of vagal tone; conversely, the extent of bradycardia seen on metoprolol administration reflects sympathetic tone. Fas restored the blunted tachycardia in response to atropine seen in CHF Veh animals (Figure 1A). Simultaneous icv administration of Fas+l-NAME prevented the improvement in vagal tone. Similarly, Fas also normalized the exaggerated bradycardic response to metoprolol in CHF
Veh animals (Figure 1B). This protective effect seen in CHF Fas animals was also reversed by l-NAME, suggesting that the Fas-mediated restoration of the sympathovagal imbalance may be attributable, in part, to increased NO production via NOS.

The bradycardic response to nasopharyngeal smoke is vagally mediated; thus, we examined the change in resting HR after administration of smoke across all groups of animals (Figure 2). Given that Fas normalized the tachycardic response to atropine in CHF animals, we hypothesized that the response to smoke in CHF Fas animals would be as robust as Sham controls. Indeed, there was a blunted response to nasopharyngeal smoke in CHF Veh animals as compared with Sham, and this response was restored with icv Fas treatment. l-NAME administration partially reversed the improved vagal tone in the CHF Fas animals.

To further assess the protective effect of icv Fas on the autonomic profile in CHF animals, we constructed baroreflex curves in all 5 groups of animals (Figure 3). As expected, in CHF, there was a significantly blunted baroreflex curve compared with controls. CHF Fas animals exhibited significantly improved baroreflex sensitivity as compared with CHF Veh. The normalized baroreflex in CHF Fas animals was prevented by icv Fas. Animals receiving CHF Fas+l-NAME showed a blunted response compared with Sham animals, further suggesting that Fas is restoring autonomic balance in an NO-dependent manner.

Table. Baseline Hemodynamics in Vehicle, Fas-Treated, and Fas-Treated Plus l-NAME Normal and CHF Rabbits.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham Veh (n=8)</th>
<th>Sham Fas (n=6)</th>
<th>CHF Veh (n=9)</th>
<th>CHF Fas (n=6)</th>
<th>CHF Fas+l-NAME (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline MAP, mm Hg</td>
<td>63±3</td>
<td>68±1</td>
<td>66±1</td>
<td>67±4</td>
<td>70±3</td>
</tr>
<tr>
<td>Baseline HR, bpm</td>
<td>210±7</td>
<td>197±17</td>
<td>238±4*</td>
<td>210±7†</td>
<td>231±7*</td>
</tr>
</tbody>
</table>

CHF indicates chronic heart failure; Fas, fasudil; HR, heart rate; l-NAME, N- Nitro-l-arginine methyl ester hydrochloride; MAP, mean arterial blood pressure; and Veh, vehicle.

* P<0.05 vs Sham, † P<0.05 vs CHF Veh and CHF Fas+l-NAME.

To assess whether the protective effect of Fas in CHF also correlated with a change in protein expression, we performed Western blot analysis of RVLM micropunches (Figure 5). There was a significant decrease in eNOS protein expression in CHF Veh compared with Sham animals (Figure 5A), which was normalized by Fas treatment. The Fas-mediated increase in eNOS was prevented by l-NAME. Conversely, a hallmark of CHF is an upregulation in AT R. CHF Veh animals had significantly higher AT R compared with Sham animals (Figure 5B). Fas treatment dramatically decreased AT R protein in CHF animals to below Sham levels. CHF Fas+l-NAME–treated animals maintained an increase in AT R compared with Sham. Finally, to confirm that Fas had an effect on downstream RhoA/ROCKII targets, we examined changes in ROCKII and phosphorylated ezrin/radixin/moesin proteins, a readout of RhoA/ROCKII activity (Figure 5C and 5D). There was an increase in both of these proteins in CHF Veh compared with control, and these values were normalized with Fas treatment, confirming that the Fas indeed did have an effect RhoA/ROCKII targets. Interestingly, l-NAME did not fully reverse the increase in CHF Veh animals compared with CHF Fas. This may imply that the hyperactivation of the RhoA/ROCKII pathway in CHF has both NO-dependent and NO-independent components.

Figure 1. Fasudil (Fas) treatment restores sympathetic and vagal imbalance in chronic heart failure (CHF) as determined by the change in resting heart rate (HR) after intravenous bolus of atropine (A) or metoprolol (B). A. The tachycardic response after atropine was blunted in CHF vehicle (Veh) animals. The tachycardia was restored by intracerebroventricular (icv) Fas but prevented when l-NAME was given simultaneously. B. The exaggerated bradycardia in response to autonomic blockade in CHF Veh animals was prevented by icv Fas. Animals receiving CHF Fas+l-NAME also had an increased bradycardia in response to metoprolol compared with Sham groups. n=5 to 9 per group. * P<0.05 vs Sham, † P<0.05 vs CHF Veh, ‡ P<0.05 vs CHF Veh and CHF Fas+l-NAME.
The key findings of this study are that central Fas treatment restores baroreflex sensitivity, eNOS, and \( \text{AT}_{1} \)R protein imbalance in an NO-dependent manner in a rapid ventricular pacing rabbit model of CHF. To our knowledge this is the first demonstration that central ROCKII blockade has been shown to alter autonomic function in the setting of CHF. Furthermore, these data show a correlation between \( \text{AT}_{1} \)R and eNOS expression in one potent cardiovascular regulatory area, the RVLM. Previous work from this laboratory has shown that both peripheral and central administration of chronic simvastatin to conscious animals with CHF reduces renal sympathetic nerve activity and restores both cardiac and peripheral baroreflex function.27,28 In a recent study carried out in humans with CHF, one month of simvastatin therapy reduced muscle sympathetic nerve activity and was correlated with a reduction in global oxidative stress.29 Although statin therapy primarily blocks 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase activity, clearly shown that both statins and ROCKII inhibitors given directly into the NTS in spontaneously hypertensive rats and Wistar Kyoto rats restored glutamate sensitivity, reduced blood pressure, and in some cases, increased NOS expression and no changes in ROCKII expression.16,17,20,36 However, in the current study, ROCKII protein expression was increased in the periphery and in the central nervous system, one pathway that has been implicated in abnormal NOS signaling is the \( \text{RhoA/ROCKII} \) pathway.30,31 Most of the previous work on the central \( \text{RhoA/ROCKII} \) pathway relates to changes in neuronal plasticity, axonal and dentritic spine formation, and so on.15,36 There is little evidence to suggest that this pathway directly alters neuronal activity. The implications of the current work suggest that inhibition of this pathway in cardiovascular disease reduces autonomic outflow and may constitute a new and novel therapeutic target in those disease states characterized by increased sympathetic outflow and decreased vagal outflow. Indeed, studies by Hirooka and coworkers have clearly shown that both statins and ROCKII inhibitors given directly into the NTS in spontaneously hypertensive rats and Wistar Kyoto rats restored glutamate sensitivity, reduced blood pressure, and in some cases, increased NOS expression and no changes in ROCKII expression.16,17,20,36 However, in the current study, ROCKII protein expression was increased in the RVLM of CHF animals, and the decrease in ROCK II protein seen in CHF Fas-treated animals was only partially increased by \( \text{L-NAME} \). Although most of the data in this study suggest that the beneficial effects of Fas are largely NO dependent,
it is certainly possible that there are other mediators, such as angiotensin II, that can lead to the hyperactivity of the RhoA/ROCKII pathway in CHF. Several studies have now shown that arterial baroreflex function can be impacted by the balance between NO generation and angiotensin II signaling. The finding in the current study that Fas evokes a decrease in AT1R expression in the RVLM may suggest a mechanism by which this pathway regulates both baroreflex function and global autonomic outflow. Indeed, it is well established that activation of the RhoA/ROCKII pathway can upregulate the transcription factor, nuclear factor-kB; similarly, angiotensin II activation also increases both nuclear factor-kB and RhoA/ROCKII. Several areas of the central nervous system have been shown to exhibit increases in oxidative stress in the CHF state. In this regard, angiotensin II signaling would contribute to oxidative stress. Inhibition of the RhoA/ROCKII pathway can contribute to a reduction in central oxidative stress by lowering AT1R expression and by increasing the bioavailability of NO. In the current study, we only examined the effects of Fas on eNOS protein. Previous studies have shown that eNOS upregulation is an important mechanism in the protective effects of statin therapy in a murine stroke model and that this is dependent on the RhoA/ROCKII pathway. However, the connection between the RhoA/ROCKII pathway and other NOS isoforms has not been well defined. It will be necessary in future work to establish the interaction between the RhoA/ROCKII pathway, central AT1R, and reactive oxygen species.

Arterial baroreflex function has been shown to be depressed in several cardiovascular diseases, including CHF and hypertension. The regulation of cardiac baroreflex sensitivity is mediated by both sympathetic and vagal outflow. The results of the current study suggest that central administration of Fas impacts both arms of autonomic outflow to the heart. Because Fas was chronically administered by the icv route it is not possible to determine the precise location of its action. Icv Fas administration would have immediate access to several areas that have been shown to be integral in angiotensin II and oxidative stress signaling. One such area is the subfornical organ, which is well endowed with AT1Rs. This area of the midbrain is also accessible by the systemic route, thus promoting the possibility that RhoA/ROCKII inhibition, globally, may have effects on autonomic outflow. The current study focused on proteins in the RVLM; given that there was a pronounced effect on vagal tone it is possible that both the nucleus ambiguus and the dorsal motor nucleus of the vagus may also be a target for RhoA/ROCKII inhibition. Additionally, future studies addressing the effects of systemic Fas on sympathetic and vagal outflow as well as changes in both the peripheral and central RhoA/ROCKII pathways would be of interest.
It is of interest that although baroreflex function was improved, we did not examine cardiac function after Fas treatment. There is limited evidence that central manipulation of sympathetic outflow in CHF impacts cardiac function per se. One study carried out in a mouse myocardial infarction model suggests, however, that reducing oxidative stress in the subfornical organ does indeed increase myocardial performance. However, global reduction in sympathetic outflow and increases in vagal outflow are antiarrhythmic and decreasing eNOS expression that is a hallmark of CHF.

Perspectives

This study demonstrates that central blockade of the RhoA/ROCKII pathway with Fas can restore blunted baroreflex sensitivity seen in CHF animals in an NO-dependent manner. Furthermore, central Fas can also restore the increase in AT1R and decreased eNOS expression that is a hallmark of CHF. Taken together, the RhoA/ROCKII pathway may be a novel and potential therapeutic target for the treatment of CHF.

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Disclosures

None.

References


Novelty and Significance

What Is New?

- Central blockade of the RhoA/RhoA associated kinase II pathway with fasudil restores baroreflex sensitivity in an NO-dependent manner in a rabbit pacing model of chronic heart failure (CHF).
- Central fasudil normalizes angiotensin II type 1 receptor and endothelial NO synthase protein imbalances in the rostral ventrolateral medulla of CHF rabbits in an NO-dependent manner.

What Is Relevant?

- This study points to a novel central mechanism that may regulate the interaction between multiple regulators of sympathetic and vagal outflow and thereby impact the course of the CHF state.

- Targeting RhoA/RhoA associated kinase II may be a potential therapeutic for the treatment of CHF.

Summary

We conclude that chronic inhibition of central RhoA/RhoA associated kinase II plays an important role in autonomic regulation in the setting of CHF. The mechanisms responsible for this effect are dependent on the activation of endothelial NO synthase and potentially inhibition of angiotensin II type 1 receptor expression.
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CENTRAL RHO KINASE INHIBITION RESTORES BAROREFLEX SENSITIVITY AND AT1R PROTEIN IMBALANCE IN CONSCIOUS RABBITS WITH CHRONIC HEART FAILURE

Karla K.V. Haack, Lie Gao, Alicia M. Schiller, Pamela L. Curry, Peter R. Pellegrino and Irving H. Zucker
Methods

Induction of CHF Left ventricular pacing at a rate of 380 beats per minute (BPM) was performed for 1-2 weeks prior to experimentation. Cardiac function was assessed weekly by echocardiography (Accuson Sequoia 512 C; Siemens, Malvern, PA), with heart failure characterized as a decrease in ejection fraction to between 40-45% or lower. Experiments were performed at least 20 minutes after pacemakers were turned off.

Brain Micropunch and Western Blot Analyses Following euthanization, brains were removed and quickly frozen on dry ice. Coronal sections were cut through the medulla at the level of the RVLM using a cryostat and, following the Palkovits technique \(^1\), the nuclei were punched bilaterally using a diethylpyrocarbonate (DEPC)-treated blunt 18-gauge needle attached to a syringe. Punches were lysed in 200 µL of RIPA buffer with fresh protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and fresh Halt phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL). Total protein concentration was measured using a BCA Assay kit according to manufacturer instructions (Thermo Scientific). RVLM micropunch samples were adjusted to contain the same concentration of total protein, and equal volumes of 2X 4% SDS sample buffer were added. The samples were boiled for 3 min and then loaded onto a 7.5% SDS-PAGE gel (35 µg of protein per well). Gels were subjected to electrophoresis at 120 V/gel for 60 min. The fractionated proteins on the gel were electrophoretically transferred to a PVDF membrane (Millipore, Billerica, MA) at 50 V for 90 min. Following 30 minutes of incubation in Li-Cor Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE), the membrane was probed with any of the primary antibodies overnight diluted in 0.05% Tween in PBS (PBS-T): rabbit anti- Myosin Light Chain Kinase, rabbit anti-pRhoA, goat anti-AT1R, rabbit anti-NOS3 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-α-tubulin (1:1000, Santa Cruz), mouse anti-ROCK II (1:1000, BD Transduction Laboratories, San Jose, CA) and/or rabbit anti-phospho-ezrin/radixin/moesin (1:1000, Cell Signaling, Danvers, MA). Following thorough washes with PBS-T, the samples were then probed with the appropriate secondary antibodies (1:15,000, Li-Cor Biosciences) diluted in PBS-T+ 0.01% SDS. Blots were developed using a Li-Cor Odyssey scanner and quantitative analysis of band densitometry was performed using the Li-Cor Odyssey software. The relative abundance of each protein was calculated as the ratio of intensity of the band relative to the intensity of α-tubulin. Graphs summarizing individual experiments are shown as a fold change compared to the Sham Veh animals.
References

Table S1. Echocardiographic data before and after ventricular pacing. LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; LVd Vol, left ventricle volume during diastole; LVs Vol, left ventricle volume during systole; FS%, fractional shortening; EF%, ejection fraction. *p<0.05 vs pre-pace.

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Body Weight (kg)</th>
<th>LVEDD (mm)</th>
<th>LVESD (mm)</th>
<th>LVd Vol (mL)</th>
<th>LVs Vol (mL)</th>
<th>FS%</th>
<th>EF%</th>
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<tbody>
<tr>
<td>pre-pace (n=14)</td>
<td>3.7 ± 0.1</td>
<td>15.6 ± 0.4</td>
<td>10.9 ± 0.3</td>
<td>6.8 ± 0.4</td>
<td>2.4 ± 0.2</td>
<td>33.0 ± 0.9</td>
<td>65.1 ± 1.2</td>
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<tr>
<td>post-pace (n=15)</td>
<td>3.7 ± 0.1</td>
<td>16.1 ± 0.5</td>
<td>13.0 ± 0.4*</td>
<td>7.3 ± 0.6</td>
<td>5.2 ± 0.9*</td>
<td>19.1 ± 0.6*</td>
<td>42.6 ± 1.0*</td>
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<tr>
<td>Group</td>
<td>Range</td>
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<td>BP&lt;sub&gt;50&lt;/sub&gt; (mm Hg)</td>
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<tr>
<td><strong>Sham Veh</strong></td>
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<tr>
<td>control (n=8)</td>
<td>216.9 ± 22.9</td>
<td>127.4 ± 19.2</td>
<td>75.2 ± 5.2</td>
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<td>32.5 ± 9.1</td>
<td>271.1 ± 15.8</td>
<td>66.9 ± 3.6</td>
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<tr>
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<td>141.8 ± 24.8</td>
<td>82.2 ± 2.2</td>
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<td>102.1 ± 28.0</td>
<td>77.9 ± 6.2</td>
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<td>atropine (n=6)</td>
<td>65.3 ± 12.8</td>
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<td>70.5 ± 8.1</td>
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<td>metoprolol (n=6)</td>
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<td><strong>CHF Veh</strong></td>
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<tr>
<td>control (n=9)</td>
<td>127.7 ± 8.5*</td>
<td>192.4 ± 11.6*</td>
<td>84.6 ± 4.0</td>
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<tr>
<td>atropine (n=5)</td>
<td>33.4 ± 5.2</td>
<td>271.1 ± 9.4</td>
<td>67.8 ± 7.2</td>
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<tr>
<td>metoprolol (n=5)</td>
<td>21.1 ± 6.1*</td>
<td>210.4 ± 10.9*</td>
<td>84.4 ± 5.2</td>
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<tr>
<td><strong>CHF Fas</strong></td>
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<tr>
<td>control (n=6)</td>
<td>184.6 ± 30.3†</td>
<td>108.5 ± 30.6†</td>
<td>86.1 ± 8.4</td>
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<tr>
<td>atropine (n=6)</td>
<td>39.3 ± 20.4</td>
<td>277.1 ± 12.8</td>
<td>61.6 ± 7.6</td>
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<tr>
<td>metoprolol (n=6)</td>
<td>151.9 ± 38.1†</td>
<td>129.4 ± 17.1†</td>
<td>90.2 ± 6.6</td>
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<tr>
<td><strong>CHF Fas + L-NAME</strong></td>
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<tr>
<td>control (n=5)</td>
<td>63.8 ± 12.4*</td>
<td>192.2 ± 19.0*</td>
<td>78.3 ± 3.2</td>
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<tr>
<td>atropine (n=5)</td>
<td>30.2 ± 18.4</td>
<td>280.3 ± 11.9</td>
<td>71.3 ± 7.6</td>
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<tr>
<td>metoprolol (n=5)</td>
<td>67.3 ± 17.5</td>
<td>166.4 ± 26.7</td>
<td>90.4 ± 9.4</td>
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</tbody>
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Table S2. Analysis of Baroreflex curve data. Min HR, minimum heart rate. *p<0.05 vs Sham, †p<0.05 vs CHF Veh.