Enalapril Attenuates the Exaggerated Sympathetic Response to Physical Stress in Prenatally Programmed Hypertensive Rats

Masaki Mizuno, German Lozano, Khurrum Siddique, Michel Baum, Scott A. Smith

Abstract—Adulthood hypertension can be prenatally programmed by maternal dietary protein deprivation. We have shown that the sympathetically mediated pressor response to physical stress is exaggerated in prenatally programmed hypertensive (PPH) rats. The mechanisms underlying this abnormal responsiveness remain undetermined. The renin–angiotensin system is known to affect sympathetic nerve activity. Therefore, the purpose of this study was to determine whether inhibition of the renin–angiotensin system attenuates the enhanced sympathetic and pressor responses to physical stress in PPH rats. Changes in renal sympathetic nerve activity and blood pressure in response to hindlimb contraction, hindlimb stretch, and hindlimb intra-arterial capsaicin administration were assessed in control and PPH rats treated (from age 3 weeks) with either vehicle or the angiotensin-converting enzyme inhibitor enalapril. Conscious resting systolic arterial pressure was significantly greater in PPH rats (142±5 mm Hg) than in control (128±2 mm Hg) after vehicle treatment (P<0.05). Resting systolic pressure was reduced by enalapril treatment in PPH rats (125±2 mm Hg) but had no effect in control (128±2 mm Hg). The pressor and renal sympathetic responses to muscle contraction and stretch were significantly higher in decerebrate PPH rats than in decerebrate control in vehicle-treated groups. Responses to capsaicin were variable. Enalapril significantly attenuated the enhanced contraction-induced elevations in mean pressure (vehicle, 45±6 mm Hg; enalapril, 21±3 mm Hg) and renal sympathetic activity (vehicle, 175±22%; enalapril, 89±23%) in PPH rats. Its effects were similar on responses to stretch in PPH rats but were equivocal during capsaicin administration. The results suggest that the renin–angiotensin system contributes to the enhancement of the renal sympathetic and pressor responses to physical stress in PPH rats. (Hypertension. 2014;63:324-329.) • Online Data Supplement

Key Words: autonomic nervous system • blood pressure • diet, protein-restricted • embryonic and fetal development • exercise • hypertension • renin-angiotensin system

Small-for-gestational-age infants subjected to prenatal insults are at risk for developing hypertension in adulthood.1-4 Animal models using rats have demonstrated that maternal dietary protein deprivation, prenatal administration of glucocorticoids, and uteroplacental insufficiency lead to small-for-gestational-age neonates that develop hypertension in adulthood.5-7 These models have been used to study the pathophysiology of hypertension. However, the mechanism(s) underlying the predisposition of offspring to develop hypertension in adulthood is unclear. Current evidence suggests that the sympathetic nervous system may play a crucial role because renal sympathetic denervation normalizes baseline blood pressure in hypertensive adult offspring of mothers administered glucocorticoids or having reduced uterine perfusion during pregnancy.8-10 Moreover, we have recently demonstrated in adult rats that prenatal programming of hypertension (PPH) induces sympathetic overactivity in response to physical stress.11 The renin–angiotensin system (RAS) has also been implicated in PPH. Despite plasma angiotensin II levels being within normal range in adult offspring after prenatal insult,6,12,13 hypertension that develops as a result of prenatal programming is normalized by either administration of angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor blockers.14-17 Further, it has been demonstrated that intrarenal RAS activity was increased and renal angiotensin II receptor type 1 (AT1) abundance enhanced with prenatal programming.5,12,13 With regard to the latter, AT1 expression has also been shown to be elevated in the brain of adult offspring whose mothers were fed a low-protein diet as compared with control, suggesting that increases in central RAS activity may likewise play a role in PPH.16 Despite the probable roles of RAS and sympathetic nervous system in the development of high blood pressure in PPH, a direct link between the 2 has not been established previously. To better understand the pathophysiology of the disease, it is

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important to determine whether such a link exists. For example, it is possible that an increase in either central or peripheral RAS activity may enhance sympathetic outflow to the kidney, which could affect renal function including renal sodium transport, leading to hypertension. In addition, RAS-mediated elevations in sympathetic nerve activity (SNA) could increase vasoconstrictor tone, contributing to high blood pressure. The purpose of this study was, therefore, to examine the role of RAS in the generation of enhanced sympathetic responsiveness in PPH, which has been shown to occur during acute physical stress. The renal SNA (RSNA) response to physical stress was assessed in controls and PPH rats treated (from 3 weeks of age) with either vehicle or ACE inhibitor enalapril in their drinking water. It was hypothesized that disruption of RAS activity would attenuate the exaggerated RSNA response to acute physical stress in PPH rats.

Materials and Methods
For a complete description of materials and methods, see the online-only Data Supplement.

Animals
Pregnant Sprague–Dawley rats were fed either a control 20% protein diet (Teklad) or an isocaloric 6% protein diet (Teklad) from day 12 of gestation until delivery of their offspring. After weaning (≈3 weeks of age), offspring from the 20% and 6% protein groups received either vehicle (0.4% ethanol) or enalapril (100 mg/L) in their drinking water until the time of experimentation. The procedures outlined were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. All experiments were performed in accordance with the US Department of Health and Human Services/National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Preliminary Measurements
Animals were placed in metabolic cages to measure 24-hour urine output after 3 days of acclimation. Noninvasive blood pressure measurements were obtained in conscious rats at ≈12 weeks of age using a CODA blood pressure system. Plasma angiotensin II levels were assessed using an enzyme immunoassay kit.

Acute Surgical Procedures
Isoflurane-anesthetized rats were instrumented for mechanical ventilation as well as measurement of blood pressure (left common carotid artery), heart rate (HR; ECG), SNA (renal nerve), and hindlimb muscle tension (triceps surae muscles). In addition, the circulation of right hindlimb was isolated for the intra-arterial administration of chemicals. After instrumentation, animals were decerebrated precociously and isoflurane anesthesia discontinued.

Experimental Procedures
Physical stress was induced by activation of exercise pressor reflex (EPR) as well as its individual functional components: muscle mechanoreflex and metaboreflex. These stressors were chosen because they significantly enhance SNA, arterial blood pressure, and HR when preferentially stimulated. EPR was stimulated by contracting the triceps surae muscles. Isolated L₃ and L₄ ventral roots were stimulated for 30 seconds (40 Hz, 0.1 ms at 3 times motor threshold, the minimum current required to produce a muscle twitch). Muscle mechanoreflex was preferentially engaged by stretching the triceps surae muscles of the hindlimb for 30 seconds using a calibrated 9.5-mm rack and pinion system. To selectively activate the muscle metaboreflex, capsaicin was administered into the arterial supply of the hindlimb (0.3 and 1.0 μg/100 μL). In all animals, heart was excised and weighted postmortem. Tibia was harvested and its length measured.

Statistical Analyses
Data were analyzed using 2-way ANOVA with a post hoc Student–Newman–Keuls test when appropriate. In the Table, the factor diet indicates comparisons between control and PPH rats, whereas the factor treatment indicates comparisons between vehicle (ie, ethanol) and enalapril. An unpaired t test was performed to compare plasma angiotensin II concentration between groups. The significance level was set at P≤0.05. All values are expressed as mean±SEM.

Results
Characterization of PPH
In agreement with previous findings, systolic arterial pressure (SAP) in conscious state was significantly higher in

### Table. Morphometric Characteristics and Baseline Hemodynamics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (Ethanol n=10)</th>
<th>Enalapril (n=10)</th>
<th>P (Treatment)</th>
<th>P (Interaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>438±16</td>
<td>427±16</td>
<td>&lt;0.0001</td>
<td>0.8799</td>
</tr>
<tr>
<td>Heart weight/body weight, mg/g</td>
<td>2.70±0.04</td>
<td>2.52±0.06</td>
<td>0.0007</td>
<td>0.6892</td>
</tr>
<tr>
<td>Heart weight/tibial length, mg/mm</td>
<td>28.7±0.7</td>
<td>25.9±1.0</td>
<td>0.0007</td>
<td>0.6892</td>
</tr>
<tr>
<td>Under anesthesia</td>
<td>n=10</td>
<td>n=10</td>
<td>n=10</td>
<td>n=10</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>88±5</td>
<td>97±5</td>
<td>0.3197</td>
<td>0.0874</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>346±5</td>
<td>362±8</td>
<td>0.0961</td>
<td>0.7124</td>
</tr>
<tr>
<td>Baseline signal to noise ratio for RSNA</td>
<td>4.1±0.9</td>
<td>5.0±0.8</td>
<td>0.4267</td>
<td>0.7626</td>
</tr>
<tr>
<td>After decerebration</td>
<td>n=10</td>
<td>n=10</td>
<td>n=10</td>
<td>n=10</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>93±7</td>
<td>74±4</td>
<td>0.0008</td>
<td>0.9691</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>410±18</td>
<td>430±12</td>
<td>0.1776</td>
<td>0.9749</td>
</tr>
<tr>
<td>Baseline signal to noise ratio for RSNA</td>
<td>6.6±1.1</td>
<td>5.7±1.2</td>
<td>0.8519</td>
<td>0.3918</td>
</tr>
</tbody>
</table>

Values are mean±SEM. HR indicates heart rate; MAP, mean arterial pressure; PPH, prenatally programmed hypertensive; and RSNA, renal sympathetic nerve activity.
vehicle-treated PPH rats compared with all other groups (Figure 1). Treatment with enalapril significantly attenuated the increase in SAP in conscious PPH rats but had no effect on controls (Figure 1). The Table summarizes the morphometric characteristics and baseline hemodynamics of animals studied. Body weight was lower in rats whose mothers received a low-protein diet (ie, PPH). Heart weight/body weight ratios were lower in enalapril-treated animals although not significantly so in PPH rats. Heart weight/tibial length ratios were greater in vehicle-treated control animals than in all other groups. Under anesthesia, mean arterial pressure (MAP), HR, and RSNA baseline signal/noise ratio were not significantly different. Similar findings were obtained for these variables after decerebration, although rats treated with enalapril had lower baseline MAP. There was no difference in plasma angiotensin II concentrations between control and PPH rats (7.3±1.8 versus 10.0±1.8 pg/mL, respectively). There was also no difference in daily urine output between control or PPH rats with (12.6±1.8 versus 11.5±1.8 mL/day, respectively) or without (13.2±0.6 versus 9.1±0.7 mL/day, respectively) enalapril treatment.

Effect of Enalapril on Responses to EPR Activation

As previously reported, stimulation of EPR evoked significantly greater increases in MAP, HR, and RSNA in vehicle-treated PPH rats compared with control animals (Figure 2). Enalapril treatment did not affect sympathetic and cardiovascular responses to EPR stimulation in control. In contrast, enalapril treatment significantly attenuated the increases in MAP and RSNA, but not HR, in response to EPR activation in PPH rats. Tension developed during muscle contraction was similar between all groups.

Effect of Enalapril on Responses to Mechanoreflex Activation

Sympathetically mediated cardiovascular responses to stimulation of mechanically sensitive component of the EPR by passive muscle stretch were exaggerated in vehicle-treated PPH rats as compared with control (Figure 3). In PPH rats, but not in control, MAP and RSNA responses to muscle stretch were significantly reduced by enalapril treatment. In contrast, enalapril had no significant effect on HR response to this maneuver. Tension developed during muscle stretch was similar between all groups.

Effect of Enalapril on Responses to Metaboreflex Activation

RSNA response to activation of chemically sensitive afferent fibers in skeletal muscle via the intra-arterial administration of 0.3 μg/100 μL capsaicin was larger in PPH rats compared with control. Enalapril treatment had no effect on this exaggerated response (Figure 4A). MAP and HR responses at this dose of capsaicin were not different between groups and were unaffected by enalapril treatment. In contrast, at higher concentrations of capsaicin (1.0 μg/100 μL), pressor response was significantly greater in vehicle-treated PPH rats as compared with control. The augmented MAP response was significantly attenuated by enalapril treatment (Figure 4B). Similarly, RSNA response was larger in vehicle-treated PPH rats than in control although statistical significance was not reached (P=0.08). HR

Figure 1. Conscious systolic arterial pressure (SAP) measured by tail cuff in control-vehicle (n=10), control-enalapril (n=10), PPH-vehicle (n=10), and PPH-enalapril (n=6) rats. Ena indicates enalapril-treated; PPH, prenatally programmed hypertensive; and Veh, vehicle-treated. *P<0.05 vs control and Ena PPH rats.

Figure 2. Cardiovascular and sympathetic responses to activation of exercise pressor reflex in control-vehicle (n=10), control-enalapril (n=10), PPH-vehicle (n=8), and PPH-enalapril (n=10) rats. Ena indicates enalapril-treated; HR, heart rate; MAP, mean arterial pressure; PPH, prenatally programmed hypertensive; RSNA, renal sympathetic nerve activity; and Veh, vehicle-treated. *P<0.05 vs control and Ena PPH rats; †P<0.05 vs control.

Figure 3. Cardiovascular and sympathetic responses to activation of mechanically sensitive component of the exercise pressor reflex in control-vehicle (n=10), control-enalapril (n=10), PPH-vehicle (n=8), and PPH-enalapril (n=10) rats. Ena indicates enalapril-treated; HR, heart rate; MAP, mean arterial pressure; PPH, prenatally programmed hypertensive; RSNA, renal sympathetic nerve activity; and Veh, vehicle-treated. *P<0.05 vs control and Ena PPH rats; †P<0.05 vs control.
responses were not significantly different between groups at this dose of capsaicin. Neither RSNA nor HR responses to 1.0 μg/100 μL of capsaicin were significantly affected by treatment with enalapril in either control or PPH animals.

**Discussion**

Significant findings of the present investigation were (1) ACE inhibition with enalapril lowered elevated baseline SAP in conscious PPH rats; (2) enalapril significantly attenuated the exaggerated increases in RSNA and MAP in response to activation of the EPR and mechanoreflex as well as the augmented MAP response to stimulation of the metaboreflex (at higher dose of capsaicin) in decerebrate PPH rats; (3) HR responses to stimulation of EPR, mechanoreflex, and metaboreflex in decerebrate PPH rats were largely unaffected by enalapril treatment; and (4) enalapril had no effect on baseline SAP in conscious healthy controls or the sympathetically mediated cardiovascular response to physical stress in decerebrate controls. These findings support the contention that RAS plays a significant role not only in the generation of raised basal blood pressures but also in the development of enhanced renal sympathetic and pressor responses to physical stress in prenatally programmed adult hypertensive rats.

In agreement with the current findings, previous studies have provided evidence that RAS mediates, at least in part, hypertension with prenatal programming. By prenatal programming, it has been demonstrated that both ACE inhibition and angiotensin II receptor blockade normalize baseline blood pressure in conscious PPH rats. In addition, ACE inhibition has been shown to correct abnormal increases in blood pressure variability and alterations in arterial baroreflex control of HR in PPH rats. We previously demonstrated that sympathetically mediated cardiovascular response to physical stress (eg, stimulation of EPR, mechanoreflex, and metaboreflex) is markedly exaggerated in PPH rats. In the present study, inhibition of ACE was shown to normalize, in large part, these responses as well. The results are novel in that they establish, for the first time to our knowledge, a direct link between RAS and altered sympathetic regulation in PPH.

Given this link, the question arises as to how RAS activation evokes exaggerations in RSNA in PPH rats. A definitive answer remains elusive although several possibilities exist. The control of SNA resides in autonomic centers within the brain. When considering the systemic RAS, circulating angiotensin II can cross the blood brain barrier (via fenestrated capillaries in certain areas of the brain) and serve to increase SNA. In humans, for example, systemic infusion of angiotensin II has been shown to enhance SNA. However, plasma angiotensin II levels were found to be comparable between control and PPH animals in the present study. Thus, it is unlikely that higher plasma angiotensin II levels acted centrally to augment SNA. This is similar to others who examined offspring at 28 days of age and found no difference in angiotensin II levels between PPH and control animals. That being stated, Padlys et al have demonstrated greater expression of angiotensin II receptor (AT1) in several areas of the brain, including the vascular organ of lamina terminalis, the subfornical organ, and nucleus tractus solitarii, in PPH rats compared with control rats. Of these areas, nucleus tractus solitarii is a major central site for sympathetically mediated cardiovascular regulation both at rest and during exercise. An increased expression of AT1 within the nucleus tractus solitarii could evoke exaggerated responses in PPH rats via this mechanism despite the lack of difference in circulating angiotensin II compared with controls.

Independent of systemic RAS and circulating angiotensin II, there is a brain RAS system that may also play a significant role in generating stress-induced SNA overactivity in PPH rats. As evidence, intracerebroventricular administration of losartan (an AT1 antagonist) lowers baseline blood pressure in PPH rats but has no effect on control rats. Enalapril may affect this central RAS system independent of its actions on circulating angiotensin II. For example, enalapril has been shown to decrease the expression of mRNA for AT1 in the brain stem as well as the cerebral cortex in stroke-prone spontaneously hypertensive rats. It is possible that enalapril-induced reductions in AT1 expression in the brain stem could account for the attenuation of exaggerated RSNA and MAP responses to physical stress in PPH demonstrated in the present study. It should be noted, however, that some studies suggest that enalapril does not cross the blood brain barrier because oral treatment with ACE inhibitor seems to have no effect on intracerebral RAS in spontaneously hypertensive rats. In contrast, some studies have suggested that inflammation-induced increases in blood brain barrier permeability, which has been shown in diabetic rats, allows passage of enalapril into the brain.
kidney disease have shown that there is an increase in SNA likely because of stimulation of renal afferent nerves because patients with a bilateral nephrectomy have comparable levels of SNA as controls with normal renal function.\textsuperscript{59} Injecting as little as 50 \mu L of 10\% phenol into a rat kidney can increase blood pressure via an increase in SNA.\textsuperscript{30} Moreover, the increase in blood pressure and secretion of norepinephrine from the posterior hypothalamic nuclei with phenol injection is not present in rats that undergo renal denervation.\textsuperscript{50} In patients with chronic kidney disease, the increase in SNA can be abrogated by blockade of the RAS.\textsuperscript{31–33} Adult rat offsprings whose mothers were fed a low-protein diet during pregnancy develop renal injury with glomerulosclerosis and interstitial fibrosis.\textsuperscript{34} Thus, it is possible that the exaggerated increases in SNA observed in the present study, which were largely attenuated by treatment with enalapril, could be mediated by activation of renal afferent nerves.

The effects of enalapril treatment on the function of EPR in this investigation are noteworthy. In our previous study, we found that the EPR and each of its functional components were enhanced by programming.\textsuperscript{11} That being stated, the magnitude to which metaboreflex function was accentuated in programmed rats was far less than that of the mechanoreflex and only manifested at the highest dose of capsaicin administered. Similar results were obtained in the present study. Collectively, the data suggest that prenatal programming may alter mechanoreflex function to a greater degree than the metaboreflex. Given these findings, it is not surprising that, with regard to the metaboreflex, the lone effect of enalapril was to normalize MAP response to the highest dose of capsaicin administered in PPH. In contrast, enalapril treatment effectively normalized mechanoreflex-mediated increases in MAP and RSNA in PPH. Thus, it is logical to suggest that the ability of enalapril to largely correct EPR dysfunction is mediated by its effect on the mechanically sensitive component of the reflex. Of note, urine output (and presumably by extension water intake) was similar between controls and PPH rats, the latter of which had significantly lower body weights. Thus, it is acknowledged that PPH animals may have inadvertently been given more enalapril per kilogram of body weight. This may have contributed to the enalapril-induced reductions in sympathetic and pressor responses to stress reported in PPH rats and the absence of such reductions in control rats. Despite this possibility, the findings clearly demonstrate that chronic administration of enalapril mitigates EPR overactivity in PPH.

Surprisingly, HR responses to activation of EPR were unaffected by enalapril treatment. These findings suggest that enalapril differentially modulates sympathetic outflow to the heart and kidneys. Practically, this is an important point to consider when selecting pharmacological treatment for EPR overactivity in PPH. Clearly, other treatments (eg, \(\beta\)-blockade) would be required to abrogate augmented sympathetic activity to the heart. Regardless, the demonstrated ability of enalapril to largely correct EPR dysfunction in PPH is significant and suggests that it may serve as a viable treatment for EPR overactivity in this form of hypertension. This is a significant finding because the cardiovascular response to exercise in hypertension is exaggerated and mediated, in part, by an overactive EPR.\textsuperscript{11,35,36} This enhanced circulatory responsiveness to physical activity increases the risk for occurrence of an adverse cardiovascular event (eg, myocardial infarction, stroke) during or immediately after a bout of exercise.\textsuperscript{37,38} Thus, effectively treating EPR dysfunction in hypertension, as was demonstrated with enalapril in the present study, may reduce the risks associated with physical activity in this disease.

It should be noted that chronic exposure to enhanced SAP can induce pathological hypertrophic cardiac remodeling leading to heart failure.\textsuperscript{39} It is commonly accepted that EPR is exaggerated in cardiomyopathic rats, although the function of its individual components (mechanoreflex and metaboreflex) remains an area of controversy.\textsuperscript{40} Therefore, it was important to establish in this study that the alterations in EPR, mechanoreflex, and metaboreflex function in PPH and the effects of enalapril on this function were independent of the development of heart failure. To this end, both heart weight to body weight and heart weight to tibial length ratios were greatest in vehicle-treated control animals, suggesting that cardiac hypertrophy did not develop in PPH. As such, the stress-induced changes in RSNA, MAP, and HR in hypertensive animals before and after enalapril treatment were likely not confounded by the presence of heart failure.

Perspectives

These data demonstrate, for the first time to our knowledge, that RAS plays a crucial role in the generation of exaggerated renal sympathetic and pressor responses to physical stress due to prenatal insults. This enhanced responsiveness likely contributes to the pathogenesis of hypertension in PPH. These studies may have clinical relevance for patients who are born small-for-gestational age and who are predisposed to developing hypertension.

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Disclosures

None.

References

Novelty and Significance

What Is New?

- We have shown previously that the sympathetic response to physical stress is exaggerated in adult prenatally programmed hypertensive rats (induced by maternal dietary protein restriction during gestation). The mechanisms underlying this abnormal sympathetic responsiveness to physical stress remain undetermined. The present study assessed a possible role for the renin-angiotensin system (RAS) in the generation of this hyper-responsiveness by directly measuring renal sympathetic activity during physical stress in normotensive and prenatally programmed hypertensive rats treated with or without angiotensin-converting enzyme (ACE) inhibitorenalapril.

What Is Relevant?

- Enalapril decreased resting blood pressure in adult prenatally programmed hypertensive rats. Importantly, sympathetic and pressor responses to physical stress were likewise attenuated by treatment with enalapril in these animals, whereas ACE inhibition had no effect in normotensive rats.

Summary

This investigation has demonstrated a direct link between RAS and altered sympathetic regulation in adult rats subjected to dietary protein restriction during gestation. The results suggest that, in response to physical stress, RAS contributes to the generation of sympathetic overactivity in adult prenatally programmed hypertensive rats. This enhanced sympathetic responsiveness can be normalized by inhibiting RAS with enalapril.
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ENALAPRIL ATTENUATES THE EXAGGERATED SYMPATHETIC RESPONSE TO PHYSICAL STRESS IN PRENATALLY PROGRAMMED HYPERTENSIVE RATS

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Running title: Enalapril and sympathetic dysfunction in programmed hypertension
SUPPLEMENTARY MATERIALS AND METHODS

Animals
Pregnant Sprague Dawley rats were fed either a control 20% protein diet (Teklad) or an isocaloric 6% protein diet (Teklad) from day 12 of gestation until delivery of their offspring. After birth the mothers and the weaned offspring were fed a 20% protein diet. The characteristics of maternal weight gain, food and water intake and the fact that prenatal programming leads to rats that are small at the time of birth has been described by our laboratory and others previously. Only male offspring were used because 1) males are more severely affected by prenatal programming than females and 2) our previous work examining the sympathetic response to stress utilized this sex. With regard to the latter, the use of males allowed comparisons between investigations. Animals were housed on 12-h light-dark cycles and were given food and water ad libitum.

Measurement of Blood Pressure in Conscious Animals
Blood pressure was measured in rats at ~12 weeks of age using a CODA blood pressure system (Kent Scientific, Torrington CT). Rats were placed in Lucite tubes and a blood pressure cuff was inflated several times for the four days prior to the day of study. On the fifth day, there were approximately 10 readings of blood pressure and the mean blood pressure was used as the blood pressure for that rat. The person who trained the rats and measured the blood pressure was not aware of the group from which the blood pressure was measured in mature age matched rats.

Plasma angiotensin II levels
Rats were anesthetized with intraperitoneal ketamine at 100 mg/kg (Sigma Chemical) and xylazine at 10mg/kg (Vedco Inc). A midabdominal incision was performed and blood was collected from the aorta with a syringe that was flushed with heparin and immediately transferred to a cooled tube (4°C) containing EDTA (Gibco) at a final concentration of 5 mmol/l. The tube was centrifuged at 1,500g at 4°C for 30 min and the plasma frozen at −80°C until angiotensin II was extracted and assayed. The angiotensin II measurement was performed on a 96 well plate by an angiotensin II enzyme immunoassay kit (Spi-Bio).

Acute Surgical Procedures
Rats were anesthetized with isoflurane gas (2–4% in 100% oxygen) and intubated for mechanical ventilation (Model 683, Harvard) prior to physical stress testing. A fluid-filled catheter was placed in the left common carotid artery for the measurement of blood pressure (MLT0380/D, ADInstruments) and in the right jugular vein for the administration of solutions. To obtain
electrocardiograph recordings and heart rate (HR) measurements (Animal Bio Amp, ADInstruments), needle electrodes were placed on the back of the animal. A solution (2ml 1M NaHCO3 and 10 ml 5% dextrose in 38 ml Ringer Solution) was continuously administrated through the jugular vein (3–5ml h-1 kg-1) to stabilize fluid balance and maintain baseline arterial blood pressure (ABP). Body temperature was maintained within normal ranges throughout the experiment. The renal nerve was dissected from the surrounding tissues through a left flank incision and attached to a pair of stainless steel wire electrodes (Bioflex wire AS633, Cooner Wire, CA). The nerve and electrodes were covered with silicone glue (Kwik-Sil, World Precision Instruments, Sarasota, FL) to insulate and fix the tissue in place. The pre-amplified nerve signal was band-pass filtered at 150–1000 Hz (Neuro Amp EX, ADInstruments) then full-wave rectified and low-pass filtered with a cutoff frequency of 30 Hz. A laminectomy exposing the lower lumber portions of the spinal cord (L2–L6) was performed to allow contraction of muscle in the hindlimb. The L4 and L5 ventral roots were carefully isolated and sectioned. The triceps surae muscles of the right hindlimb were isolated. The calcaneal bone of the right hindlimb was cut and the Achilles’ tendon connected to a force transducer for the measurement of muscle tension (FT-10, Grass Instruments). A catheter (PE-10, polyethylene tubing) was placed in the left common iliac artery and its tip advanced to the bifurcation of the abdominal aorta to administer chemicals into the arterial supply of the right hindlimb. A reversible vascular occluder was placed around the common iliac vein. Animals were held in a stereotaxic head unit (Kopf Instruments), and a pre-collicular decerebration was performed. Dexamethasone (0.2 mg) was given intravenously to minimize edema before decerebration. Immediately following the decerebrate procedure, gas anesthesia was discontinued. As previously described, a minimum recovery period of 1.25 h was employed after decerebration before beginning any experimental protocol3.

**Experimental protocols**

Physical stress was induced by activation of the exercise pressor reflex (EPR) as well as its individual functional components; the muscle mechanoreflex and metaboreflex. These stressors were chosen as they significantly enhance renal sympathetic nerve activity (RSNA), ABP and HR when preferentially stimulated7. The EPR is a feedback mechanism engaged during contraction of skeletal muscle8. During contraction, sensory signals from muscle are generated by activation of group III (primarily mechanically sensitive fibers associated with the mechanoreflex) and group IV (primarily chemically sensitive fibers associated with the metaboreflex) afferent neurons9. The sympathetically mediated cardiovascular response to activation of the EPR, as well as its individual components, was assessed in control and prenatally programmed hypertensive (PPH) decerebrate rats treated with either vehicle or enalapril. Decerebrate rats were used as the RSNA, ABP and HR responses to activation of skeletal muscle reflexes after this procedure is employed closely resembles those elicited during exercise in the conscious state6. This is an important point as these reflexes cannot be preferentially isolated and stimulated in the conscious state in rats. In contrast, stimulation of these
stressors in rats anesthetized with injectable or inhalant anesthetics elicits either a reduction or no change in RSNA, ABP and HR due to the depressive nature of the drugs. Thus, a decerebrate model, which renders the animal insentient and eliminates the need for inhalant or injectable anesthesia, was necessitated in this investigation. This decerebrate model has been used extensively in our lab and others to study the EPR in rats.

**Stimulation of the EPR**

The EPR was stimulated by contracting the triceps surae muscles. Isolated L4 and L5 ventral roots were stimulated for 30 s (40 Hz, 0.1 ms at 3 times motor threshold: the minimum current required to produce a muscle twitch). Muscles were initially stretched to 70–100 g of tension prior to each perturbation. Both the mechanically and metabolically sensitive components of the EPR are stimulated concomitantly by contracting hindlimb skeletal muscle in this manner.

**Stimulation of the Muscle Mechanoreflex**

The muscle mechanoreflex was preferentially engaged by stretching the triceps surae muscles of the hindlimb for 30s using a calibrated 9.5 mm rack and pinion system (Harvard Apparatus). To evoke a mechanical stimulus similar to that elicited during muscle contraction, care was taken to generate the same pattern and magnitude of tension developed during static contractions as previously shown. Muscles were initially stretched to 70–100 g of tension prior to each perturbation. Passively stretching hindlimb skeletal muscle does not appreciably increase muscle metabolism and, therefore, is commonly employed to selectively activate the mechanically sensitive component of the EPR.

**Stimulation of the Muscle Metaboreflex**

To selectively activate the muscle metaboreflex, capsaicin was administered into the arterial supply of the hindlimb (0.3 and 1.0 µg/100 µL) while a reversible ligature placed around the right common iliac vein was occluded. The maneuver essentially isolated the circulation of the hindlimb preventing capsaicin from entering the general circulation. The capsaicin receptor, transient receptor potential vanilloid 1 (TRPv1), is primarily associated with Group IV afferent fibers in skeletal muscle although the receptor has also been identified in a small number of Group III neurons. As such, stimulation of these receptors predominately activates neurons known to mediate metaboreflex activity and is commonly used for this purpose. By convention, a low and high dose of capsaicin was administered to examine the graded effects of stimulating the metaboreflex in this manner.

The order in which the protocols were conducted was randomized and all trials were separated by a
minimum of 10 minutes. At the conclusion of all experiments, an intravenous infusion of hexamethonium bromide (60 mg/kg) abolished RSNA signals indicating that they were recorded from postganglionic renal sympathetic fibers. RSNA background noise was determined over a 30 min period after the insentient decerebrated animal was humanely killed by intravenous injection of saturated potassium chloride (4M, 2 ml/kg). In all animals, the heart was excised and weighed post mortem. The tibia was harvested and measured. The latter tissues were obtained in order to generate heart weight to body weight and heart weight to tibial length ratios. These ratios are commonly used as surrogate measures or cardiac hypertrophy\textsuperscript{15}.

**Data Acquisition**

All data were recorded with data acquisition software (LabChart, ADInstruments) and stored in a computer hard drive (Dell). Baseline values for mean arterial pressure (MAP, mmHg), HR (bpm) and tension (kg) were determined by averaging 30s of recorded data prior to EPR, mechanoreflex and metaboreflex testing. The peak response of each variable was defined as the greatest change from baseline elicited by muscle contraction, stretch or capsaicin administration. To quantify RSNA responses, basal measurements were obtained by taking the mean value of 30 s of baseline data immediately prior to the maneuver. This mean was considered 100% of basal RSNA. Subsequently, relative changes in RSNA (%) from this baseline were evaluated. The noise signal component, which was defined as the signal recorded postmortem, was subtracted from rectified RSNA.
References


