Abstract—Sympathetic vasoconstriction is attenuated in exercising muscle by locally generated vasodilators, including NO. Skeletal muscle also produces reactive oxygen species (ROS), such as superoxide ($O_2^-$), which inactivates NO. We, therefore, hypothesized that excessive ROS production would result in enhanced sympathetic vasoconstriction in exercising muscle. To increase $O_2^-$ by activating NADPH oxidase, rats underwent chronic infusion of angiotensin II (Ang II) or unilateral renal artery stenosis (2K1C) to increase endogenous Ang II. At rest, sympathetic nerve stimulation (range: 1 to 5 Hz) evoked similar graded decreases in femoral vascular conductance (range, −34% to −66%) in rats infused with vehicle, Ang II, or norepinephrine and in 2K1C or sham-operated rats. These sympathetically mediated decreases in femoral vascular conductance were markedly attenuated during hindlimb contraction in the vehicle, norepinephrine, and sham rats (range, −3% to −26%) and to a lesser degree in the Ang II (range, −16% to −47%) and 2K1C (range, −16% to −45%) rats. In muscles from Ang II and 2K1C rats, ROS were elevated and the NADPH oxidase subunit gp91phox was upregulated. The $O_2^-$ scavenger tempol restored the normal attenuation of sympathetic vasoconstriction in the contracting hindlimbs of the Ang II and 2K1C rats, but this effect was prevented by pretreatment with an NO synthase inhibitor. Taken together, these data indicate that chronically elevated Ang II increases muscle ROS, which disrupts the normal NO-dependent attenuation of sympathetic vasoconstriction. These findings may have implications for muscle oxidative stress and sympathetic vasoregulation when the renin–angiotensin system is chronically activated. (Hypertension. 2006;48:637–643.)

Key Words: angiotensin ■ free radicals ■ sympathetic nervous system ■ vasoconstriction ■ muscles

The sympathetic nervous system plays an essential role in the cardiovascular response to exercise by increasing cardiac contractility and rate, augmenting venous return, and increasing vascular resistances in the viscera and inactive muscles. These adjustments increase cardiac output and redirect it to the active muscles. At the same time, the vasoconstrictor response to sympathetic nerve discharge in the active muscles is attenuated in part by the metabolic consequences of contraction.1–9 Such attenuation, termed functional sympatholysis,5 is attenuated in part by the metabolic consequences of contraction. At the same time, the vasoconstrictor role of the microcirculation,1,9 which may help to optimize intramuscular blood flow distribution while preserving sympathetic control of systemic vascular resistance and blood pressure.

NO is proposed to be one of the factors mediating functional sympatholysis,2,3,8,10,11 although it may not always play an essential role.2,4 Skeletal muscle, which expresses high levels of the neuronal isoform of NO synthase (NOS; nNOS) and lower levels of endothelial NOS,12 produces NO at a low basal rate that increases during contraction.13–15 We and others have shown that α-adrenergic vasoconstriction is enhanced in contracting muscle by NOS inhibition, either alone or in combination with cyclooxygenase inhibition,2,3,8,10,11 and by genetic mutations that decrease muscle nNOS expression.6,7 Thus, in these studies, functional sympatholysis was impaired in NO-deficient muscle.

Muscle NO deficiency also could result from enhanced NO metabolism. NO is susceptible to scavenging by reactive oxygen species (ROS), including superoxide anions ($O_2^-$), which rapidly react with NO when the 2 are produced in concert.16,17 Like NO, ROS are produced in skeletal muscle at a low basal rate, which increases with contractile activity.15,18–20 ROS also are reported to be chronically elevated in many common cardiovascular conditions, such as hypertension, atherosclerosis, and heart failure.16,21 One of the triggers for ROS overproduction in these conditions may be angiotensin II (Ang II), which is a potent activator of the nonphagocytic reduced nicotinamide-adenine dinucleotide phosphate (NAPDH) oxidases that were first described in vascular tissues but have now been identified in other tissues, including skeletal muscle.22–25 Whether Ang II stimulates excessive ROS production in muscle and whether such production mediates vascular and other target tissue injury and dysfunction remains to be determined.
oxidative stress would interfere with NO signaling in contracting muscle and impair functional sympatholysis are not known.

Therefore, the goal of this study was to test the hypothesis that chronic elevation of Ang II increases muscle ROS, which reduces the NO-dependent attenuation of sympathetic vasoconstriction. This effect of oxidative stress to impair sympathetic vasoregulation would be predicted to occur mainly during contraction, when muscle NO is normally increased.

To test this hypothesis, we performed experiments in rats in which Ang II levels were increased by infusion of exogenous Ang II or by stimulation of endogenous Ang II production in response to unilateral renal artery stenosis.

**Methods**

All of the procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center.

**Animal Models**

Sprague–Dawley rats (200 to 300 g) were randomly assigned to 1 of 5 groups. Groups 1 to 3 were anesthetized with 2.5% to 3% isoflurane and implanted with minipumps (Alzet model 2002, Alza Corporation) to deliver Ang II (0.7 mg/kg per day; n = 19), norepinephrine ([NE] 2.8 mg/kg per day; n = 12), or vehicle (n = 12) subcutaneously for 14 days. Groups 4 to 5 were anesthetized with sodium methohexital (60 mg/kg, IP) and underwent a sham operation (n = 8) or placement of a U-shaped silver clip with ID of 0.2 mm around the left renal artery (2K1C; n = 12). Experiments were performed in these rats 3 to 4 weeks later.

**In Vivo Experiments**

Anesthesia was initiated with ketamine (80 mg/kg, IP) and maintained with α-chloralose (30 mg/kg per hour, IV). Rats were given atropine sulfate (0.5 mg/kg, SC) to reduce tracheal secretions and were mechanically ventilated with room air and supplemental O2. Catheters were inserted in a jugular vein and carotid artery, and a Doppler flow probe (Crystal Biotech) was placed around the left femoral artery. Stimulating electrodes were affixed to the left lumbar sympathetic chain and to the left sciatic nerve. The left hindlimb was connected to a force transducer (FT-10, Grass Instruments) via the calcaneal tendon.

After a 20-minute stabilization period, arterial pressure and femoral blood flow velocity (FVBV) responses to lumbar sympathetic nerve stimulation (1-ms pulses of 5 V at randomized frequencies of 1, 2.5, or 5 Hz for 1 minute) were measured at rest and during contraction of the left hindlimb once the muscles had fatigued to 50% of peak force (vehicle, NE, and 2K1C, n = 8 each; Ang II, n = 15; sham, n = 5). Contractions were produced by sciatic nerve stimulation at 2 to 3 times the motor threshold voltage with 100-ms trains of pulses at a rate of 30 trains/min. After a 20-minute recovery, a continuous infusion of the O2 scavenger 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl (tempol) 180 μmol/kg per hour, IV) was begun. Thirty minutes later, sympathetic vasoconstrictor responses were reevaluated in resting and contracting hindlimbs. In a subset of Ang II–infused rats (n = 7), the NOS inhibitor N-nitro-L-arginine methyl ester ([l-NNAME] 5 mg/kg, bolus IV followed by 5 mg/kg per hour IV) was infused before tempol. Rats were killed with an overdose of sodium pentobarbital (150 mg/kg, IV), and gastrocnemius muscle samples were immediately excised, frozen in liquid nitrogen, and stored at −80°C.

**Detection of In Situ ROS Generation**

Rats (vehicle, Ang II, NE, and 2K1C, n = 4 each; sham, n = 3) were anesthetized, and the left hindlimbs were prepared as described above. After 15 minutes of contraction, the plantaris muscles were excised, embedded in OCT compound (Sakura Finetek), frozen in liquid nitrogen, and stored at −80°C. Contracting muscles were removed first, within 90 to 120 s, followed by resting muscles.

**Immunoblotting**

Muscles were homogenized in 10 volumes of buffer containing 50 mmol/L of Tris HCl (pH 7.5), 1 mmol/L of EDTA, 20 mmol/L of 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, 1 mmol/L of dithiothreitol, 0.5 mmol/L of diisopropyl fluorophosphate (DFP), and 0.25 mmol/L of E-64. Samples (100 to 200 μg) were resolved by SDS-PAGE on a 7.5% gel and transferred to nitrocellulose. Membranes were incubated overnight at 4°C with a rabbit polyclonal anti-gp91phox (1:500, Upstate Biotechnology), washed, and incubated for 1 hour at room temperature with horse-radish peroxidase–conjugated goat anti-rabbit antibodies (1:3000). Immunoreactivity was detected by enhanced chemiluminescence and quantified by densitometry. Protein concentrations were determined using a Bio-Rad Protein Assay kit. Equal loading was assured by visual inspection of stained protein bands using MemCode Reversible Protein Stain kit (Ferace Biotechnology).

**Data Analysis**

Hemodynamic and hindlimb force data were acquired and analyzed using PowerLab hardware and Chart software (ADInstruments). Femoral vascular conductance ([FVC] kHz/mm Hg) was calculated online as the mean Doppler shift (kHz) divided by mean arterial pressure ([MAP] mm Hg). Statistical analyses were performed by 1-way ANOVA or 2-way repeated-measures ANOVA followed by Bonferroni post hoc tests. Values of P < 0.05 were considered statistically significant. Data are presented as mean ± SEM.

**Results**

**Sympathetic Vasoregulation Is Impaired in the Hindlimbs of Ang II–Infused Rats**

MAP was significantly elevated in Ang II and NE rats versus vehicle rats (Table 1). Resting FBFVs and FVCs were similar in all 3 of the groups (Table 1), as were the decreases in FBFV and FVC evoked by sympathetic stimulation (Figures 1 and 2).

During contraction, peak hindlimb forces were similar among vehicle (1.66 ± 0.07 kg), Ang II (1.79 ± 0.07 kg), and NE (1.75 ± 0.06 kg) rats (P > 0.05), as were the hyperemic

<table>
<thead>
<tr>
<th>Group</th>
<th>MAP, mm Hg</th>
<th>FBFV, kHz</th>
<th>FVC, kHz/mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>Vehicle</td>
<td>86 ± 5</td>
<td>1.23 ± 0.26</td>
</tr>
<tr>
<td>Ang II</td>
<td>113 ± 7†</td>
<td>1.04 ± 0.17</td>
<td>0.009 ± 0.001</td>
</tr>
<tr>
<td>NE</td>
<td>113 ± 3†</td>
<td>1.01 ± 0.22</td>
<td>0.010 ± 0.003</td>
</tr>
<tr>
<td>Contraction</td>
<td>Vehicle</td>
<td>96 ± 4†</td>
<td>4.26 ± 0.43†</td>
</tr>
<tr>
<td>Ang II</td>
<td>115 ± 4†</td>
<td>3.79 ± 0.39†</td>
<td>0.032 ± 0.003†</td>
</tr>
<tr>
<td>NE</td>
<td>115 ± 6†</td>
<td>3.69 ± 0.57</td>
<td>0.031 ± 0.004†</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. Vehicle, n = 8; Ang II, n = 15; NE, n = 8.

*P < 0.05 vs vehicle; †P < 0.05 vs rest.
responses (Table 1). Sympathetic stimulation evoked relative decreases in FBFV and FVC that were attenuated in contracting compared with resting muscle (Figures 1 and 2). However, the degree of attenuation was significantly less in Ang II rats compared with vehicle or NE rats.

**Skeletal Muscle ROS Are Elevated in Ang II–Infused Rats**

In resting muscle sections, the number of ethidium-positive nuclei was greater in Ang II rats (147±4) compared with vehicle (102±10) or NE rats (110±2; Figure 3A, 3C, 3E, and 3G). The number of ethidium-positive nuclei was increased in contracting versus resting muscle in all 3 of the groups but remained greater in Ang II rats (185±7) than in vehicle (136±4) or NE rats (140±2; Figure 3B, 3D, 3F, and 3H). Immunoreactivity to gp91phox was increased in muscle from Ang II rats versus vehicle or NE rats (Figure 3H).

**Tempol Normalizes Sympathetic Vasoregulation in the Hindlimbs of Ang II–Infused Rats**

Tempol had no effect on hemodynamics at rest or during contraction, except for an increase in resting FBFV (2.26±0.18 kHz) and FVC (0.019±0.002 kHz/mm Hg) in Ang II rats (P<0.05 versus before tempol). Tempol had minimal effects on sympathetic vasconstriction at rest, only slightly reducing the FVC response to 1-Hz stimulation in vehicle rats (Figure 4A to 4C). In contrast, tempol further attenuated sympathetic vasoconstriction in the contracting hindlimbs of Ang II rats but had no additional effect in vehicle and NE rats (Figure 4A to 4C).

To determine whether the effect of tempol in the Ang II rats was mediated by increased NO bioactivity, studies were performed in Ang II rats treated with l-NAME. MAP increased from 105±5 mm Hg to 142±5 mm Hg after l-NAME (P<0.05), with no significant changes in FBFV or FVC. l-NAME did not alter the vasoconstrctor responses to 1-, 2.5-, or 5-Hz sympathetic stimulation in resting hindlimb (ΔFVC, −37±6%, −55±6%, and −66±5% before l-NAME versus −42±6%, −58±6%, and −71±5% after l-NAME; P>0.05) or in contracting hindlimb (ΔFVC, −24±5%, −36±7%, and −56±8% before l-NAME versus −21±4%, −43±6%, and −62±7% after l-NAME; P>0.05). Thus, l-NAME alone did not further impair sympathetic vasoregulation in Ang II rats. Subsequent infusion of tempol did not affect basal hemodynamics or sympathetic vasoconstrictor responses at rest or during hindlimb contraction (Figure 4D).

**ROS Also Impair Sympathetic Vasoregulation in the Hindlimbs of 2K1C Rats**

Renal artery stenosis resulted in significantly elevated MAP (Table 2) and decreased ratios of left to right kidney masses (2K1C, 0.71±0.03 versus sham, 0.95±0.02; P<0.05). In resting muscle of 2K1C versus sham rats, the number of ethidium-positive nuclei was greater (136±4 versus 93±10; P<0.05), and gp91phox immunoreactivity was increased (Figure 5A and 5B).

Sympathetic vasoconstrictor responses at rest were similar in sham and 2K1C rats. These responses were attenuated in the contracting hindlimbs of both groups, but the degree of attenuation was less in 2K1C rats (Figure 5C and 5D). Tempol had no significant effect on hemodynamics at rest or during contraction in either group, except for a decrease in resting MAP (−9±4 mm Hg) in the 2K1C rats (P<0.05 versus before tempol). Tempol restored the normal attenuation of sympathetic vasoconstriction in the contracting hindlimbs of 2K1C rats but had no effect in sham rats (Figure 5C and 5D).

**Discussion**

The novel findings of our study are that muscle ROS are elevated in Ang II–dependent hypertension and that these pathophysiological levels of ROS interfere with sympathetic vasoregulation. We observed that the robust attenuation of sympathetic vasoconstriction that normally occurs in contracting.
tracting muscles (ie, functional sympatholysis) was impaired in both Ang II rats and 2K1C rats. This impairment was normalized by infusion of the $O_2^-$ scavenger tempol alone but not during concomitant infusion of tempol and the NOS inhibitor L-NAME. In contrast, muscle ROS were not elevated, and functional sympatholysis was not impaired in rats with NE-dependent hypertension. These findings suggest that Ang II can induce oxidative stress in skeletal muscle, thereby upsetting the balance between ROS and NO, which seems to be a key factor that regulates sympathetic vasoconstrictor responsiveness in contracting muscle.

Previous studies have shown that ROS are generated in resting muscle and that production increases in contracting muscle.15,18–20 Our results using DHE as an oxidative probe to detect ROS are consistent with those previous reports. Ethidium, the fluorescent product of DHE oxidation by $O_2^-$, is a polar compound that does not cross cell membranes and, therefore, reflects intracellular ROS production.20 Ethidium fluorescence is not affected by physiological concentrations of various enzymes, metals, or antioxidants or by increases in $H^+$, $Ca^{2+}$, phosphate, or temperature, making this probe amenable for use in contracting muscle.20 Our approach using muscle cryosections likely underestimates in vivo ROS production during contraction; however, ethidium fluorescence was consistently increased in sections of the contracting muscles compared with those of the contralateral resting muscles.

The increased ethidium fluorescence that we observed in muscles from the Ang II and 2K1C rats indicates that Ang II may be an important stimulus for ROS production in muscle. The mechanism by which Ang II increases ROS in skeletal muscle remains speculative. In endothelial and smooth muscle cells, Ang II binding to the AT1 receptor subtype activates

Figure 3. ROS and gp91phox in muscle. Basal ethidium fluorescence in resting muscle (A, C, and E) was further increased by contraction (B, D, and F). Scale bar=40 μm. G, Ethidium+ nuclei were increased at rest and during contraction in Ang II rats versus vehicle or NE rats. *$P<0.05$ vs rest; †$P<0.05$ vs vehicle; $n=4$ each group. H, Western blot analysis showing enhanced gp91phox immunoreactivity in muscle from Ang II rats. *$P<0.05$ vs vehicle; $n=8$ each group.
membrane-bound NADPH oxidases, which are thought to be the most significant source of $\text{O}_2^-$ in vascular tissue. A similar mechanism may be operative in skeletal muscle cells, which are reported to express AT1 receptors, as well as NADPH oxidases. Our data showing increased protein expression of the NADPH oxidase subunit gp91phox in muscles from Ang II and 2K1C rats suggest that NADPH oxidases in muscle may be sensitive to induction by Ang II.

Increased muscle ROS in the Ang II and 2K1C rats seemed to underlie the impaired functional sympatholysis in these animals, as shown by the effect of acute infusion of the $\text{O}_2^-$ scavenger tempol to normalize sympatholysis. This effect of acute tempol also excludes a mechanistic role for NO.

**TABLE 2. Hemodynamics in Sham-Operated and 2K1C Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>MAP, mm Hg</th>
<th>FBFV, kHz</th>
<th>FVC, kHz/mm Hg</th>
</tr>
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<tbody>
<tr>
<td>Rest</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>92±7</td>
<td>1.04±0.06</td>
<td>0.012±0.001</td>
</tr>
<tr>
<td>2K1C</td>
<td>128±7*</td>
<td>1.37±0.18</td>
<td>0.011±0.001</td>
</tr>
<tr>
<td>Contraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>101±5</td>
<td>4.76±0.65</td>
<td>0.047±0.006†</td>
</tr>
<tr>
<td>2K1C</td>
<td>141±6†</td>
<td>5.27±0.77</td>
<td>0.037±0.005†</td>
</tr>
</tbody>
</table>

Data are mean±SEM. Sham, n=5; 2K1C, n=8. *P<0.05 vs Sham; †P<0.05 vs rest.

The effect of ROS to impair functional sympatholysis may be mediated by a decrease in bioactive NO. Skeletal muscle cells express both endothelial NOS and nNOS, the latter of which localizes to the sarclemma and is thought to be an important source of NO in contracting muscle. Although the mechanistic significance of NO in functional sympatholysis remains unresolved, we have shown in previous studies that sympatholysis is impaired when muscle NO production is reduced by pharmacological or genetic strategies. The present study suggests that ROS may have a similar effect to create a relative NO deficiency, perhaps by directly scavenging NO. This is supported by the finding that the effect of tempol to restore functional sympatholysis in the Ang II rats is prevented by NOS inhibition. This finding also excludes the possibility that tempol restores sympatholysis by alleviating effects of ROS to directly constrict vascular smooth muscle or to release endothelial-derived constrictors, because these effects would not be prevented by NOS inhibition.

In addition, our results might be explained by ROS-dependent uncoupling of NOS resulting in decreased NO production and increased $\text{O}_2^-$ production, decreased expression of soluble guanylyl cyclase resulting in reduced cellular responsiveness to NO, or inhibition of dimethylarginine dimethylaminohydrolase leading to accumulation of the endogenous NOS inhibitor asymmetrical dimethylarginine. However, the latter 2 mechanisms are unlikely to explain the impaired functional sympatholysis in our study, given that acute (45 to 60 minutes) treatment with tempol normalized sympatholysis. This temporal pattern is more consistent with an effect of ROS to reversibly disrupt cellular signaling pathways than to alter expression of redox-sensitive genes or irreversibly damage cellular constituents.

The effect of tempol to attenuate sympathetic vasoconstriction in the contracting but not in the resting hindlimbs of the Ang II and 2K1C rats suggests that the disequilibrium between ROS and NO is exacerbated in contracting muscle. In resting muscle, the low basal NO concentration does not favor the reaction with $\text{O}_2^-$, which will instead be metabolized by superoxide dismutase (SOD). Further reducing $\text{O}_2^-$ levels with the SOD mimetic tempol would, therefore, have minimal impact on NO bioactivity and sympathetic vasoconstriction. However, in contracting muscle, local NO concentrations may increase sufficiently to compete with SOD and react with $\text{O}_2^-$, resulting in a relative NO deficiency and enhanced sympathetic vasoconstriction that can be reversed by tempol. Interestingly, tempol had no effect in the vehicle and NE rats, despite elevated ROS in the contracting muscles.
This suggests that the increase in $O_2^-$ that occurs during brief bouts of contraction does not interfere with the NO-dependent attenuation of sympathetic vasoconstriction, which seems to require excessive increases in muscle $O_2^-$. Alternatively, differences in the sources and sites of ROS production may differentially affect NO signaling. Sources of ROS that have been implicated in skeletal muscle include the mitochondrial electron transport chain, xanthine oxidase, phospholipase A$_2$, lipoxygenase, cyclooxygenase, cytochrome P450, and, more recently, NADPH oxidases. The latter have been localized to the sarcolemma and, based on our data showing increased gp91phox in muscle from Ang II rats, may be regulated by circulating Ang II. It is tempting to speculate that NADPH oxidase may be in close proximity to sarcolemmal nNOS, providing a spatial localization that could favor the interaction between $O_2^-$ and NO. However, we cannot draw any conclusions about specific sites of ROS production from our data, because ethidium intercalates into DNA, which localizes the fluorescent signal to the nucleus. A better understanding of the roles played by ROS in skeletal muscle will continue to evolve as more sensitive and selective techniques to monitor individual ROS and their sources are developed.

**Perspectives**

Our data indicate that skeletal muscle is a potential site of ROS production in some forms of Ang II–dependent hypertension. Muscle ROS also are reported to increase in aging and in heart failure. Similar to our findings in hypertensive rats, sympathetic vasoconstriction is enhanced in the exercising muscles of older humans and of heart failure rats. We speculate that a ROS/NO disequilibrium may be a common factor underlying the increased sympathetic vasoconstrictor responsiveness in these conditions, resulting in reduced muscle perfusion during exercise. Excessive sympathetic vasoconstriction also may cause an exaggerated rise in blood pressure during exercise, which is often seen with aging and in hypertension and heart failure and is predictive of future cardiovascular risk. Perhaps some of the clinical benefits derived from exercise training or inhibition of the renin–angiotensin system in these conditions might be attributed to decreased muscle oxidative stress and improved sympathetic vasoregulation.

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Disclosures

None.

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Reactive Oxygen Species Impair Sympathetic Vasoregulation in Skeletal Muscle in Angiotensin II–Dependent Hypertension

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