Oxidative stress exaggerates skeletal muscle contraction-evoked reflex sympathoexcitation in rats with hypertension induced by angiotensin II

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Submitted 30 May 2012; accepted in final form 16 October 2012

Koba S, Watanabe R, Kano N, Watanabe T. Oxidative stress exaggerates skeletal muscle contraction-evoked reflex sympathoexcitation in rats with hypertension induced by angiotensin II. Am J Physiol Heart Circ Physiol 304: H142–H153, 2013. First published October 19, 2012; doi:10.1152/ajpheart.00423.2012.—Muscle contraction stimulates thin fiber muscle afferents and evokes reflex sympathoexcitation. In hypertension, this reflex is exaggerated. ANG II, which is elevated in hypertension, has been reported to trigger the production of superoxide and other reactive oxygen species in muscle. In the present study, we tested the hypothesis that increased ANG II in hypertension exaggerates skeletal muscle contraction-evoked reflex sympathoexcitation by inducing oxidative stress in the muscle. In rats, subcutaneous infusion of ANG II at 450 ng·kg⁻¹·min⁻¹ for 14 days significantly (P < 0.05) elevated blood pressure compared with sham-operated (sham) rats. Electrically induced 30-s hindlimb muscle contraction in decerebrate rats with hypertension evoked larger renal sympathoexcitatory and pressor responses [+]1.173 ± 2.124 arbitrary units (AU) and +35 ± 5 mmHg, n = 10] compared with sham normotensive rats (+419 ± 103 AU and +13 ± 2 mmHg, n = 11). Tempol, a SOD mimetic, injected intra-arterially into the hindlimb circulation significantly reduced responses in hypertensive rats, whereas this compound had no effect on responses in sham rats. Tiron, another SOD mimetic, also significantly reduced reflex renal sympathetic and pressor responses in a subset of hypertensive rats (n = 10). Generation of muscle superoxide, as evaluated by dihydroethidium staining, was increased in hypertensive rats. RT-PCR and immunoblot experiments showed that mRNA and protein for gp91phox, a NADPH oxidase subunit, in skeletal muscle tissue were upregulated in hypertensive rats. Taken together, these results suggest that increased ANG II in hypertension induces oxidative stress in skeletal muscle, thereby exaggerating the muscle reflex.

HYPERTENSION is associated with an increased risk of cardiovascular disease (50). Antihypertensive treatments include increased physical activity or exercise (8, 23). It has been noted that in hypertension, the sympathoexcitatory and elevation of blood pressure seen during exercise are excessive (10, 39). This cardiovascular hyperexcitability is potentially dangerous because it can elevate risks for adverse cardiac events such as acute myocardial ischemia, myocardial infarction, left ventricular hypertrophy, or arrhythmia as well as stroke after a bout of exercise (23, 36). Determining the causes underlying the hyperexcitability in this pathological condition is clinically important.

A reflex originating in exercising skeletal muscle is activated as thin fiber muscle afferents (groups III and IV) are stimulated by mechanical deformation of the afferents’ receptive fields as well as by metabolic byproducts during contraction (9, 33). In turn, afferent engagement reflexly activates the sympathetic nervous system by stimulating the medulla. This muscle-based reflex is termed the exercise pressor reflex (35). A series of studies by Smith and colleagues (24, 37, 38, 43) using a decerebrate rat preparation has suggested that in spontaneous hypertensive rats (SHRs), both mechanically and chemically sensitive muscle afferents engaged during skeletal muscle contraction are stimulated excessively compared with those in normotensive rats, thereby evoking exaggerated sympathoexcitatory and pressor responses to contraction. In humans with hypertension, the elevation in muscle sympathetic nerve activity seen during postexercise ischemia, a maneuver that selectively excites chemically sensitive muscle afferents, was higher than that in normotensive subjects (10). These rodent and human studies suggest that the skeletal muscle contraction-mediated reflex sympathetic nerve response is exaggerated in hypertension, thereby contributing to the excess sympathoexcitatory and blood pressure elevation seen during exercise in this pathological condition. However, the mechanisms by which the exercise pressor reflex becomes abnormal in hypertension remain to be determined.

The activity of the renin-angiotensin-aldosterone system (RAAS) is increased in hypertension (12). ANG II, an effector molecule of the RAAS, plays an important role in developing and maintaining hypertension by constricting vessels via its action on smooth muscle cells (5, 14). Moreover, this peptide has been known to activate NADPH oxidases, thereby triggering the production of superoxide and other reactive oxygen species (ROS) in various tissues, including skeletal muscle (13, 41, 51, 55). The development of oxidative stress has also been implicated in the pathogenesis of hypertension (26, 55). Superoxide functions in the neural process for encoding peripheral mechanical and thermal stimuli (18, 25). Koba et al. (19) previously reported that treatment of the hindlimb with tempol, a membrane-permeable compound that mimics the enzymatic activity of SOD, reduced reflex sympathetic nerve and pressor responses to hindlimb skeletal muscle contraction in rats with heart failure, a cardiovascular disease that has elevated ROS and often develops from prolonged hypertension. On the basis of these findings, we hypothesized that, in hypertension, increased RAAS activity exaggerates skeletal muscle contraction-evoked reflex sympathoexcitation by inducing oxidative stress in the muscle. The purpose of the present study was to test this hypothesis. In rats, we chronically stimulated the RAAS by an exogenous infusion of ANG II for 14 days to develop experimental hypertension (16, 27, 29, 55). This model was used to focus on the physiological roles played by increased RAAS activity in the pathological characteristics of hypertension. In the decerebrate rat, we examined renal sympathetic nerve and cardiovascular responses to contraction...
of the hindlimb skeletal muscle before and after tempol injection intra-arterially into the hindlimb circulation and compared the effect of tempol on these responses between rats with experimental hypertension and normotensive control rats. We also examined the effect of tiron, another SOD mimic, on skeletal muscle contraction-mediated responses in hypertensive rats. Moreover, we examined ROS production and mRNA and protein expression of gp91phox, a NADPH oxidase subunit, in the skeletal muscle of rats.

METHODS

All procedures outlined in this study complied with the “Guiding Principles for the Care and Use of Animals in the Fields of Physiological Sciences” published by the Physiological Society of Japan and were approved by the Animal Care Committee of Tottori University (protocol nos. 10-Y-3 and 12-Y-10). Experiments in this study were performed on 86 male Sprague-Dawley rats. Rats were housed in standard rodent cages in a temperature-controlled room (24°C) and regulated on a 12:12-h light-dark schedule. Food and water were made available ad libitum.

Animal models. Rats (8–11 wk, 280–350 g) were anesthetized with 1–4% isoflurane in oxygen and implanted under the shoulder skin with ALZET microosmotic pumps (1002, Durect) to deliver low (150 ng·kg⁻¹·min⁻¹) or high (450 ng·kg⁻¹·min⁻¹) doses of ANG II diluted in normal saline subcutaneously for 14 days [ANGII150 (n = 29) and ANGII450 (n = 28) groups, respectively]. Another subset of rats underwent a sham operation (sham group; n = 24). Huang et al. (16) showed that in male Wistar rats, subcutaneous infusion of ANG II at 150 ng·kg⁻¹·min⁻¹ for 14 days did not significantly change plasma ANG II levels but elevated mean arterial pressure (MAP) with a peak of +20 mmHg under the conscious state and that the infusion of ANG II at 500 ng·kg⁻¹·min⁻¹ caused a fourfold increase in plasma ANG II levels and elevated MAP with a peak of +60 mmHg. In the present experiments, based on these findings, we chose the 150 ng·kg⁻¹·min⁻¹ dose and its threefold dose, 450 ng·kg⁻¹·min⁻¹, for 14 days, as the concentrations and periods for ANG II infusion. In the present experimental preparation, ANG II exogenously infused at 450 but not 150 ng·kg⁻¹·min⁻¹ for 14 days significantly (P < 0.05) elevated baseline arterial pressure compared with sham rats (Table 1). The body weights of rats 14 days after the pump implantation surgery were not different between the groups (means ± SE: 410 ± 11, 399 ± 9, and 388 ± 8 g for sham, ANGII150, and ANGII450 rats, respectively, P > 0.05). Additionally, normal rats without pump implantation (n = 5, 8–12 wk, 354 ± 21 g body wt) were used for another set of experiments to study the acute effects of ANG II infusion on the exercise pressor reflex (as described below).

In vivo experiments to observe exercise pressor reflex responses. Fourteen days after the implantation surgery, the surgery and experiments to observe the exercise pressor reflex responses were conducted. The implanted pump was within the rats throughout the surgery and data collection (as described below). Rats were anesthetized with 1–4% isoflurane in oxygen. The trachea was cannulated, and the lungs were artificially ventilated (SN480-7, Shinano). The left jugular vein and common carotid artery were cannulated to administer drugs and record arterial pressure, respectively. The arterial catheter was attached to a pressure transducer (P23XL, BD). Heart rate (HR) was calculated beat to beat from the arterial pressure pulse. Arterial pH was monitored with a pH meter (B-212, Horiba) and maintained within a normal range (pH 7.4) by the intravenous infusion of a sodium bicarbonate solution (8.4%). Rectal body temperature was monitored with a digital thermometer and adequately maintained at 37.5–38.5°C with an external heating lamp. To measure renal sympathetic nerve activity (RSNA), a bipolar electrode made of a Teflon-insulated stainless steel wire (790600, A-M Systems) was connected to the renal nerve directed to the left kidney, as conducted in our previous experiments (19–22). The RSNA signal was amplified with an alternating current amplifier (P511, Grass Instruments) with a band-pass low-frequency filter of 100 Hz and a high-frequency filter of 3 kHz and made audible. A catheter (PE-10, 10 cm) was inserted into the right femoral artery and then threaded into the right common iliac artery. The catheter tip was stabilized at the junction of the iliac arteries. A reversible ligature was placed around the left common iliac vein. The left Achilles tendon and left triceps surae muscles were isolated by cutting the calcaneus bone and dissecting the tendon and muscles free from the connective tissue that attached to the tibia. The hindlimb was fixed in space with a patellar precision clamp to prevent limb movement. The tension developed by the triceps surae muscles was measured with a force transducer (FTD3, Grass Instruments) connected to the Achilles tendon. Rats were held in a stereotactic head unit with a customized spinal frame (900LS, David Kopf Instruments).

A laminectomy exposed the lower lumbar portions of the spinal cord (L2–L6). The meningeal layers surrounding the cord were cut and reflected laterally. Two nerve bundles obtained from L4 and L5 cord (L2–L6). The meningial layers surrounding the cord were cut and reflected laterally. Two nerve bundles obtained from L4 and L5 ventral roots were carefully isolated and sectioned. The peripheral cut ends of the roots were placed on an insulated bipolar electrode. The exposed neural tissue was immersed in mineral oil and maintained at

Table 1. Baseline values of MAP, HR, and the signal-to-noise ratio of RSNA before and after tempol injection during continuous and intermittent contraction bouts in sham, ANGII150, and ANGII450 rats

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>ANGII150</th>
<th>ANGII450</th>
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<tbody>
<tr>
<td></td>
<td>Before tempol</td>
<td>After tempol</td>
<td>Before tempol</td>
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<tr>
<td></td>
<td>n</td>
<td></td>
<td>n</td>
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<tr>
<td>Continuous contraction</td>
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<tr>
<td>MAP, mmHg</td>
<td>91 ± 4</td>
<td>83 ± 6</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>379 ± 6</td>
<td>357 ± 13</td>
<td>371 ± 9</td>
</tr>
<tr>
<td>Signal-to-noise ratio</td>
<td>6.2 ± 0.5</td>
<td>6.1 ± 0.6</td>
<td>5.0 ± 0.5</td>
</tr>
<tr>
<td>Intermittent contraction</td>
<td></td>
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<tr>
<td>MAP, mmHg</td>
<td>87 ± 5</td>
<td>82 ± 6</td>
<td>91 ± 3</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>377 ± 6</td>
<td>355 ± 12</td>
<td>371 ± 10</td>
</tr>
<tr>
<td>Signal-to-noise ratio</td>
<td>6.6 ± 0.5</td>
<td>6.5 ± 1.1</td>
<td>5.2 ± 0.6</td>
</tr>
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</table>

Values are means ± SE; n, no. of rats/group. Shown are baseline values of mean arterial pressure (MAP), heart rate (HR), and the signal-to-noise ratio for renal sympathetic nerve activity (RSNA) before and after an intra-arterial injection of tempol before 30-s continuous static contraction and 1-min intermittent (1–4 s of stimulation to relaxation) bouts of static contraction of hindlimb muscles from sham-operated (sham), 150 ng·kg⁻¹·min⁻¹ ANG II-treated (ANGII150), and 150 ng·kg⁻¹·min⁻¹ ANG II-treated (ANGII450) rats. Baseline data were obtained from the averaged values for 30 s immediately before each muscle contraction protocol. *P < 0.05 vs. sham and ANGII150 rats.
a temperature of 37°C. A decerebration was performed at the midcollicular level, as conducted in our previous studies (19–22). A recovery period of at least 90 min after the decerebration was allowed before the experimental protocol was begun.

The decerebrate rats were mechanically ventilated (tidal volume: 6.0 ml/kg and frequency: 70 min–1). The triceps surae muscles were lightly stretched to create a baseline tension of 50–100 g. Contraction of the left hindlimb muscles was induced by the excitation of the ventral roots with constant-current electrical stimulation [1.5 × motor threshold (MT), 0.1 ms, 40 Hz]. The minimum current intensity necessary to induce a muscle twitch served as the MT. We used two forms of contraction: 30 s of continuous static contraction and intermittent (1–4 s of stimulation to relaxation) static contraction for 1 min. Continuous contraction stimulates both mechanically and chemically sensitive muscle afferents, whereas a short period (1 s) of contraction during the intermittent contracting manner is considered to predominantly activate the mechanical component of the exercise pressor reflex (1, 17, 19–22, 34, 46). When both maneuvers were tested in the same rat, 15 min was allowed between each maneuver. Subsequently, tempol (10 mg dissolved in 0.2 ml normal saline) was injected slowly over a 2-min period through the catheter placed in the right common iliac artery. Immediately before the injection of tempol, the reversible ligature around the left common iliac vein was tightened for 10 min to trap the injectate in the hindlimb circulation. During the 2-min injection, a string placed around the base of the tail was ligatured for 72 h independent of its antioxidant effects (32) but necessary to induce a muscle twitch served as the MT. We used two sets of ANG II type 1 receptor (AT1R) plays a role in maintaining the hypertensive state seen in ANGII450 rats. In decerebrate rats, we compared the effect of a systemic infusion of losartan (10 mg/kg), an AT1R antagonist, via a jugular vein on the resting arterial pressure between groups.

Another set of experiments to study the acute effects of ANG II infusion on the exercise pressor reflex was also performed. In normal resting arterial pressure between groups.

In situ ROS detection in rat skeletal muscle. In other subsets of sham, ANGII150, and ANGII450 rats, in which neither tempol nor losartan was injected into the hindlimb circulation, intracellular superoxide generation in the resting gastrocnemius muscle was evaluated with dihydroethidium (DHE) staining (11, 55). In isoflurane (<4% in oxygen)-anesthetized rats, the middle parts of gastrocnemius muscle were excised, embedded in OCT compound (Sakura Finetek), rapidly frozen in liquid nitrogen, and stored at −80°C until analysis. Muscles from a set of one sham, one ANGII150, and one ANGII450 rat were processed to the experiment on the same day. Muscles were sectioned transversely (15 μm, CM1900, Leica), and >3 muscle sections/rat were randomly chosen for the following process. Sections were exposed to Hoechst dye (2 μg/ml) and incubated in the dark for 5 min at 37°C to detect nuclei. After a rinse in PBS, sections were counterstained with DHE (2 μmol/l) and incubated in the dark for 30 min at 37°C. Images were then obtained using an epifluorescence microscope system with a camera (DMRB. Leica, exposure duration: 100 ms). Red fluorescent ethidium results from the oxidation of DHE. The numbers of ethidium-positive nuclei and nuclei stained by Hoechst dye were counted. The ratio of ethidium-positive nuclei per total nuclei was then averaged from three images of data collected from three muscle sections to obtain the mean value per rat.

gp91phox expression in rat skeletal muscle. We examined, by RT-PCR, whether gp91phox mRNA is upregulated in the rat skeletal muscle tissue by ANG II. In isoflurane (<4% in oxygen)-anesthetized rats, samples of the gastrocnemius muscle were harvested from the middle part of the resting muscle and rapidly frozen in liquid nitrogen. Total RNA was extracted from the brayed muscle tissues with the use of a commercial RNAeasy Mini kit (Qiagen). Total RNA (0.5 μg) was then reverse transcribed to synthesize cDNA using a First-Strand CDNA Synthesis kit (Fermentas). In the PCR with Taq DNA polymerase (Roche Diagnostics), all transcripts were amplified by means of 35 cycles of annealing (53°C, 30 s), extension (72°C, 30 s), and denaturation (94°C, 1 min). The primer pairs used for rat gp91phox were as follows: forward primer, 5′-TGACCTCGGTGCTGCGC-3′; and reverse primer, 5′-GGCAGGATCGAAGTTAGC-3′ (31). The PCR products were then subjected to 2% agarose gel electrophoresis in buffer [44.5 mM Tris, 44.5 mM boric acid, and 2 mM ethylenediaminetetraacetic acid (pH 8.0)]. The gel was then stained with ethidium bromide. DNA bands were photographed under UV light (UVB BioDoc-It System, Funakoshi). The PCR products, which were expected to be 336 bp (31), were quantified by densitometry with the image-processing program ImageJ (version 1.44, National Institutes of Health).

We also examined, by Western blot analysis, whether gp91phox protein is upregulated in the rat skeletal muscle tissue by ANG II. Frozen gastrocnemius muscle samples (30–100 mg) were homogenized in 10 volumes of RIPA buffer containing protease inhibitor cocktail (2 μg/ml, Sigma). Protein concentrations of the samples were adjusted to 2 mg/ml with a Protein Assay kit (Bio-Rad). Samples (5 μl) were resolved by SDS-PAGE on a 10% gel and transferred to a nitrocellulose membrane (Hybond-C Super, Amersham) overnight at 4°C. The next day, equal loading of the proteins was assayed by the visual inspection of stained protein bands with Ponceau staining (Sigma) of the membrane. The membrane was blocked by 2% BSA, incubated for 1 h at room temperature with rabbit polyclonal anti-gp91phox antibody (1:1,000, ab80508, Abcam), washed in Tris-buff ered saline with 0.1% Tween 20, and incubated for 1 h at room temperature with an anti-rabbit IgG secondary antibody (1:1,000, NA934, GE Healthcare). After the membrane had been washed, blots were developed by an enhanced chemiluminescence system (ECL Plus, Amersham) and visualized by exposure to X-ray film (ECL Hyperfilm, Amersham). Immunoreactivity was quantified by densi-
tometry with ImageJ. In the RT-PCR and Western blot experiments, GAPDH was used to verify equal mRNA/protein loading.

Data acquisition and statistical analyses. In the experiments to observe exercise pressor reflex responses, all measured variables were displayed continuously on a computer monitor and stored on a hard disk at a sampling rate of 1 kHz through an analog-digital interface (Powerlab/8s, AD Instruments). Baseline data were obtained from averaged values for 30 s immediately before each muscle contraction protocol. RSNA and cardiovascular responses to either continuous or intermittent static contraction in rats, as well as tension developed by the triceps surae muscles, were determined as analyzed in our previous studies and those of others (19–22, 37, 38). Briefly, peak responses to muscle contraction and integrated values of the responses during muscle contraction were examined. To quantify RSNA responses to muscle contraction, relative changes in RSNA from the baseline level (considered as 100%) were evaluated.

Data are expressed as means ± SE. To assess significant differences, data were analyzed with a paired \( t \)-test or one-way or two-way repeated-measures ANOVA followed by the appropriate post hoc test (Tukey’s method). The level of significance was set at \( P < 0.05 \).

RESULTS

Continuous contraction. RSNA and cardiovascular responses to 30-s continuous static contraction of the left hindlimb muscles were examined in 11 sham, 15 ANGII150, and 10 ANGII450 rats. Baseline MAP was significantly higher in ANGII450 rats than in sham and ANGII150 rats, whereas baseline HR and the signal-to-noise ratio for RSNA were not significantly different between these rats (Table 1). Continuous static contraction increased arterial pressure, HR, and RSNA in these rats (Figs. 1 and 2). Peak and integrated RSNA and peak pressor responses to continuous contraction in ANGII450 rats were significantly larger than those in sham or ANGII150 rats, despite the fact that triceps surae muscle tension was not significantly different (Fig. 2). Between sham and ANGII150 rats, there were no significant differences in peak and integrated RSNA and peak pressor responses (Fig. 2). The peak HR response to contraction did not significantly differ between these rats (Fig. 2).

The effect of tempol (10 mg) injected intra-arterially into the hindlimb circulation on RSNA and cardiovascular responses to continuous hindlimb muscle contraction was also examined. Tempol did not affect baseline MAP, HR, or the signal-to-noise ratio for RSNA in these rats, and baseline MAP in ANGII450 rats was significantly higher than that in sham and ANGII150 rats (Table 1). In ANGII450 rats, tempol injected intra-arterially significantly reduced RSNA, MAP, and HR responses, despite the fact that this treatment did not affect development of triceps surae muscle tension (Figs. 1 & 2). In sham and ANGII150 rats, tempol had no significant effect on these responses (Fig. 2). These responses after tempol injection in ANGII450 rats were at the equivalent level as those seen in sham or ANGII150 rats before or after tempol injection (Fig. 2).

In a subset of five normal rats, we examined if acute, but not chronic, infusion of ANG II modulates the exercise pressor response.

![Representative recordings from one rat treated with 450 ng·kg⁻¹·min⁻¹ ANG II (ANGII450 group) of tension developed by the left triceps surae muscles, renal sympathetic nerve activity (RSNA; in \( \mu \)V), relative changes in RSNA (in %), arterial pressure (AP), and heart rate [HR; in beats/min (bpm)] during 30-s continuous contraction of the left hindlimb muscles before (left) and after (right) intra-arterial injection of tempol (10 mg) into the hindlimb circulation. In this ANGII450 rat, tempol appeared to reduce RSNA and cardiovascular responses to continuous muscle contraction.](http://ajpheart.physiology.org/)

Fig. 1. Representative recordings from one rat treated with 450 ng·kg⁻¹·min⁻¹ ANG II (ANGII450 group) of tension developed by the left triceps surae muscles, renal sympathetic nerve activity (RSNA; in \( \mu \)V), relative changes in RSNA (in %), arterial pressure (AP), and heart rate [HR; in beats/min (bpm)] during 30-s continuous contraction of the left hindlimb muscles before (left) and after (right) intra-arterial injection of tempol (10 mg) into the hindlimb circulation. In this ANGII450 rat, tempol appeared to reduce RSNA and cardiovascular responses to continuous muscle contraction.
reflex. Infusion of ANG II at 450 ng·kg\(^{-1}\cdot\text{min}^{-1}\) for 20–30 min did not affect baseline MAP, HR, the signal-to-noise ratio for RSNA, or RSNA and cardiovascular responses to continuous contraction of the hindlimb muscles (Table 2). These results suggest that the exaggeration of the exercise pressor reflex in ANGII450 rats is mediated through chronic, but not acute, effects of ANG II infusion.

**Intermittent contraction.** RSNA and cardiovascular responses to 1-min intermittent (1–4 s of stimulation to relaxation) bouts of static muscle contraction were examined in 11 sham, 16 ANGII150, and 9 ANGII450 rats. Baseline MAP was significantly larger in ANGII450 rats than in sham and ANGII150 rats, whereas baseline HR and the signal-to-noise ratio for RSNA were not significantly different between these rats (Table 1). During the 1 min of intermittent contraction in these rats, the RSNA response was synchronized to the increase in tension (Fig. 3). Intermittent contraction had little effect on the averaged changes in MAP from baseline in each rat group because this contraction protocol induced variable patterns in the MAP response across rats. These characteristics of RSNA and arterial pressure dynamics seen during intermittent contraction were consistent with data previously observed in rats and cats (19–22, 46). The RSNA response to intermittent contraction in ANGII450 rats was significantly larger than that seen in sham or ANGII150 rats at the equivalent level of developed muscle tension (Fig. 4). The RSNA response in ANGII150 rats was not significantly different from that in sham rats (Fig. 4).

The effect of tempol (10 mg) injected intra-arterially into the hindlimb circulation on RSNA and cardiovascular responses to intermittent hindlimb muscle contraction was also examined. Tempol did not affect baseline MAP, HR, or the signal-to-noise ratio for RSNA in these rats, and baseline MAP after tempol injection in ANGII450 rats was significantly larger than that in sham and ANGII150 rats (Table 1). In ANGII450 rats, tempol injected intra-arterially significantly reduced the RSNA response to contraction (Figs. 3 and 4) at the equivalent level of developed muscle tension. Tempol did not have this effect on the RSNA response in either sham or ANGII150 rats (Fig. 4). The RSNA response after tempol injection in ANGII450 rats was significantly larger than that seen in sham or ANGII150 rats before or after tempol injection (Fig. 4).

**Effect of tiron on the exercise pressor reflex in the hypertensive rats.** In a subset of 10 ANGII450 rats, we examined if tiron (100 mg) modulates the exercise pressor reflex. As with tempol, tiron intra-arterially injected into the hindlimb circulation significantly reduced RSNA and pressor responses to continuous contraction of the hindlimb muscles in hypertensive rats (Fig. 5). Baseline MAP, HR, and the signal-to-noise ratio for RSNA were 0.05 vs. before tempol. Significant differences were detected by a Tukey post hoc test after two-way repeated-measures ANOVA.

Table 2. Baseline values and responses to 30-s continuous static contraction of hindlimb skeletal muscles in normal control rats before and during acute infusion of ANG II (20–30 min after the onset of infusion)

<table>
<thead>
<tr>
<th></th>
<th>Before ANG II</th>
<th>During Acute Infusion of ANG II</th>
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<tbody>
<tr>
<td><strong>Baseline values</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>87 ± 10</td>
<td>90 ± 13</td>
</tr>
<tr>
<td>HR, beats per min</td>
<td>368 ± 21</td>
<td>370 ± 23</td>
</tr>
<tr>
<td>Signal-to-noise ratio for RSNA</td>
<td>4.4 ± 0.5</td>
<td>4.2 ± 0.3</td>
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<tr>
<td><strong>Responses</strong></td>
<td></td>
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</tr>
<tr>
<td>Peak change in tension, g</td>
<td>+423 ± 31</td>
<td>+435 ± 35</td>
</tr>
<tr>
<td>Tension-time index, g·s</td>
<td>+112 ± 9 (×10(^2))</td>
<td>+110 ± 13 (×10(^2))</td>
</tr>
<tr>
<td>Peak change in RSNA, %</td>
<td>+71 ± 13</td>
<td>+76 ± 24</td>
</tr>
<tr>
<td>Integrated change in RSNA, arbitrary units</td>
<td>+458 ± 164</td>
<td>+495 ± 90</td>
</tr>
<tr>
<td>Peak change in MAP, mmHg</td>
<td>+14 ± 5</td>
<td>+13 ± 5</td>
</tr>
<tr>
<td>Peak change in HR, beats/min</td>
<td>+6 ± 2</td>
<td>+5 ± 1</td>
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</table>

Values are means ± SE; n = 5 rats/group.
for RSNA before and after tiron were 112 ± 2 and 110 ± 4 mmHg, 402 ± 8 and 389 ± 8 beats/min, and 4.5 ± 0.4 and 4.4 ± 0.4, respectively.

Effect of intravenous infusion of losartan on resting arterial pressure. Intravenous infusion of losartan (10 mg/kg) significantly reduced MAP at rest in ANGII450 rats (n = 8; Fig. 6).

In sham (n = 7) and ANGII150 (n = 8) rats, this infusion had no effect.

In situ ROS detection in skeletal muscle. In cryosections of the gastrocnemius muscle, ethidium fluorescence was significantly greater in ANGII450 rats (n = 6) than in sham rats (n = 6; Fig. 7). There were no significant differences in the ethidium
fluorescence of ANGII150 rats \((n = 6)\) compared with sham or ANGII450 rats.

**RT-PCR and Western blot experiments.** RT-PCR experiments showed that mRNA expression for gp91<sup>phox</sup> in the gastrocnemius muscle was enhanced in ANGII450 rats \((n = 6)\) compared with sham \((n = 6)\) and ANGII150 \((n = 8)\) rats (Fig. 8A). Although there was a trend, no significant differences were found in the densitometry intensity between sham and ANGII150 rats.

Western blot experiments showed that protein expression for gp91<sup>phox</sup> in the gastrocnemius muscle was enhanced in ANGII450 \((n = 8)\) rats compared with sham \((n = 8)\) rats (Fig. 8B). Although there were trends, no significant differences were found in the densitometry intensity of ANGII150 rats \((n = 8)\) compared with sham or ANGII450 rats.

**DISCUSSION**

Subcutaneous infusion of ANG II at 450, but not 150, ng·kg<sup>−1</sup>·min<sup>−1</sup> for 14 days elevated baseline MAP compared with sham rats. We found that RSNA and pressor responses to continuous contraction and the RSNA response to intermittent contraction were significantly larger in ANGII450 rats than those in sham rats. We also found that, in ANGII450 rats, intra-arterial injection into the hindlimb circulation of tempol, a membrane-permeable SOD mimetic, reduced RSNA, arterial pressure, and HR responses to continuous contraction as well as the RSNA response to intermittent contraction. Tiron, another SOD mimetic, mirrored the effect of tempol; intra-arterial injection of Tiron into the hindlimb circulation reduced RSNA and pressor responses to continuous contraction of the hindlimb muscles of hypertensive rats. In sham and ANGII150 rats, on the other hand, tempol injected intra-arterially did not modulate RSNA and cardiovascular responses to either continuous or intermittent static contraction. Moreover, DHE staining experiments showed the greater number of ethidium-positive nuclei in the skeletal muscle of ANGII450 rats compared with sham rats, suggesting that RAAS stimulation by exogenous infusion of ANG II increases rat skeletal muscle superoxide, supporting the results of a previous report (55). This result further suggests that oxidative stress induced in the...
muscle of ANGII450 rats underlies the exaggerated exercise pressor reflex, as shown by the effect of acute injection of tempol into the hindlimb circulation. Collectively, these data support our hypothesis that increased RAAS activity in hypertension exaggerates skeletal muscle contraction-evoked reflex sympathoexcitation by inducing oxidative stress in the muscle.

Increased RAAS activity and developed oxidative stress are involved in the pathogenesis of hypertension (12, 26, 55) and are a hallmark in cardiovascular diseases that often develop from prolonged hypertension. In heart failure, we (19) have previously demonstrated that oxidative stress in the muscle plays a role in exaggerating the exercise pressor reflex.

Systemic infusion of losartan, an AT1R blocker, significantly reduced the higher resting blood pressure in ANGII450 rats, whereas in sham and ANGII150 rats, this treatment had no effect. This result suggests that AT1Rs play an important role in maintaining the hypertensive state in ANGII450 rats. It is further suggested that AT1Rs had been chronically stimulated during the periods of ANG II infusion in ANGII450 rats.

In previous experiments to determine the roles played by the exercise pressor reflex in hypertension, the SHR has been used as a hypertension model (24, 37, 38, 43). In our study, we used a well-characterized rat model with experimental hypertension induced by exogenous infusion of ANG II, which allowed us to focus on the physiological roles of increased RAAS activity in pathological characteristics of hypertension (16, 27, 29, 55). The present findings demonstrating that muscle reflex responses are exaggerated in the ANG II-dependent hypertensive rat model comply with the concept proposed by previous experiments using SHRs.

In the present experiments to observe muscle reflex responses, we used two contraction protocols. One was 30-s continuous static contraction, commonly used to study the physiological roles of the exercise pressor reflex (19, 20, 37, 38, 43). Another was 1-min intermittent (1–4 s of stimulation to relaxation) static contraction. A short period of contraction during the intermittent contracting manner is considered to predominantly stimulate mechanically sensitive muscle afferents (1, 19–22, 34, 46), as observed in the present experiment showing that renal sympathoexcitation in response to intermittent contraction was synchronized as muscle tension was developed. Therefore, it is reasonable to consider that ANG II in ANGII450 rats played a role in sensitizing mechanically sensitive muscle afferents engaged during intermittent bouts of

Fig. 7. Representative confocal images of Hoechst-loaded nuclei and dihydroethidium (DHE)-loaded nuclei as well as the merged images in gastrocnemius muscle cryosections of sham, ANGII150, and ANGII450 rats, respectively. Scale bars = 100 μm. Comparison of ethidium-positive nuclei per total nuclei (Hoechst loaded) between sham (n = 6), ANGII150 (n = 6), and ANGII450 (n = 6) rats is also shown. Values are means ± SE. *P < 0.05 vs. sham rats. Significant differences were detected by a Tukey post hoc test after one-way ANOVA.
Values are means representative of gp91phox mRNA obtained from a set of sham, ANGII150, and ANGII450 rats. Comparison of the gp91phox mRNA level with respect to the GAPDH mRNA level (as a percentage of the mean from sham rats) among sham (n = 6), ANGII150 (n = 8), and ANGII450 (n = 8) rats is also shown. B: monochrome images showing representative gp91phox protein immunoblots of the gastrocnemius tissue as well as GAPDH protein obtained from two sets of sham, ANGII150, and ANGII450 rats. Comparison of the gp91phox protein level with respect to the GAPDH protein level (as a percentage of the mean from sham rats) among sham (n = 8), ANGII150 (n = 8), and ANGII450 (n = 8) rats is also shown. Values are means ± SE. *P < 0.05 vs. sham rats; †P < 0.05 vs. ANGII150 rats. Significant differences were detected by a Tukey post hoc test after one-way ANOVA.

The mechanisms by which increased superoxide sensitizes muscle afferents that respond to contraction need to be elucidated. Addressing this issue, several lines of potential clues have been provided from previous studies. In cultured neurons, superoxide has been shown to play a role in inhibiting activities of voltage-gated K⁺ channels (44, 45, 54). Furthermore, superoxide has been shown to increase the intracellular concentration of Ca²⁺ by inducing an influx of extracellular Ca²⁺ through voltage-gated Ca²⁺ channels in cultured neuronal cells (49, 58). Based on those previous studies, superoxide is believed to play a crucial role in increasing the neuronal firing rate of ANGII-stimulated neurons by regulating these ion channels and controlling action potential generation (56). Moreover, Wang et al. (47) recently found that tempol reduced voltage-gated Na⁺ current in rat dorsal root ganglion neurons directed to skeletal muscle, suggesting that superoxide plays a role in controlling the discharge of muscle afferents through an action of Na⁺ channels. In the present study, the ANG II-induced increase in superoxide might modulate functions of voltage-gated K⁺, Ca²⁺, and Na⁺ channels located on the mechanically sensitive muscle afferents.

In sham rats, we found no effects on the exercise pressor reflex of tempol (10 mg) injected infra-arterially into the hindlimb circulation. This result supports previous data reported by Kobayashi et al. (19) and McCord et al. (32) but is in disagreement with a previous study by Wang et al. (48) showing that tempol (10 mg/kg) injected intra-arterially into the hindlimb circulation reduced exercise pressor reflex responses in normal healthy rats. There is the fact that Wang et al. (48) did not trap the injectate within the hindlimb circulation, whereas Kobayashi et al. (19), McCord et al. (32), and we did. Systemic infusion of tempol (10 mg) via a jugular vein has been previously shown to reduce exercise pressor reflex responses in normal healthy rats (19). This suggests that, in the study by Wang et al. (48), the reduced exercise pressor reflex was mediated by a systemic effect of tempol and that Tempol at <10 mg is not capable of modulating the exercise pressor reflex by acting on peripheral muscle afferents in healthy rats.

The DHE staining experiment showing overproduction of superoxide in skeletal muscle tissue of ANGII450 rats supports the results of a previous study (55). Since in vitro and in vivo studies by Griendling et al. (13) and Rajagopalan et al. (41), ANG II has been known to produce superoxide in various tissues by activating NADPH oxidases via stimulation of the AT1R. The upstream signaling mechanisms by which AT1R stimulation activates NADPH oxidases include activation of phospholipase D, PKC, and c-Src tyrosine kinase, which leads to phosphorylation of p47phox (6, 42). Aside from phosphorylation of p47phox, c-Src activation has been suggested to stim-
ulate the transactivation of the EGF receptor, which activates phosphatidylinositol 3-kinase and then stimulates Rac1 (6, 42).

RT-PCR and immunoblot experiments showed that expressions of gp91phox mRNA and protein in rat skeletal muscle were increased in hypertensive ANGII450 rats compared with sham rats, supporting the results of a previous study (55). The previous and present studies suggest that transcriptional up-regulation of NADPH oxidases containing gp91phox by ANG II is, at least in part, the molecular basis for the overproduction of superoxide in skeletal muscle. The possible intracellular signal transduction pathways for this upregulation include activator protein-1 (30) and nuclear factor-κB (3). These factors are activated by stimulation of the AT1R through activation of the small G protein Ras and p38 MAPK pathways (3, 30).

The samples for the RT-PCR and immunoblot experiments were collected from whole skeletal muscle tissue. Therefore, sources of NADPH oxidase are likely localized in multiple cells, such as skeletal muscle, endothelial, and vascular smooth muscle cells. There is evidence demonstrating the expression of gp91phox at message and protein levels in these cells (4).

The present study focused on the roles played by ANG II-induced oxidative stress in skeletal muscle in evoking the exercise pressor reflex responses. The exercise pressor reflex arc includes central cardiovascular pathways. ANG II is suggested to elevate sympathetic nerve activity through its influence on central cardiovascular pathways, which contributes to the development of hypertension (2, 28). Moreover, this peptide has been reported to activate NADPH oxidases in the central nervous system (57). In the present study, it is likely that functions of central cardiovascular pathways in the reflex arc are also mediated by ANG II, thereby contributing to the exaggerated exercise pressor reflex responses in ANGII450 rats. Moreover, skeletal muscle afferent input is suppressed in the central nervous system by peripheral baroreceptor input (40). One-week infusion of ANG II at 50 ng·kg⁻¹·min⁻¹ in rabbits was reported to reduce the gain of the baroreceptor-HR reflex (29). Therefore, it is possible that the exaggerated responses in ANGII450 rats were mediated not only by muscle afferent sensitization but also by suppressed peripheral baroreceptor input. To address these issues, future investigations are needed.

Tempol had large effects on suppressing exercise pressor reflex responses in ANGII450 rats, especially in RSNA responses rather than cardiovascular responses. The RSNA response in ANGII450 rats was reduced after tempol injection by an equivalent level with that in sham rats before Tempol, whereas the pressor response was not. It is unclear why the tempol effect on RSNA responses was more dominant than that on the pressor response in ANGII450 rats. In addition, while the present study focused on the effect of chronically stimulated RAAS by exogenous infusion of ANG II at 450 ng·kg⁻¹·min⁻¹ for 14 days, it is not known if acute administration of ANG II at a higher dose than 450 ng·kg⁻¹·min⁻¹ subcutaneously or at a certain dose intravenously exaggerates the exercise pressor reflex via its action on superoxide production mechanisms. This question arises from the fact that administration of ANG II for 4 h to cultured smooth muscle cells triggered superoxide production (13). To address these issues, future studies are necessary.

Increased RAAS activity, which induces oxidative stress in skeletal muscle, is considered a cause of the cardiovascular hyperactivity in response to acute exercise in hypertension through the exercise pressor reflex arc. As stated, while significant reductions in blood pressure after aerobic exercise therapy programs of mild to moderate intensity for hypertensive individuals have been a consistent finding of many well-controlled studies (23), the abnormal cardiovascular responses to exercise in hypertension are dangerous for affected individuals. The present findings identifying a cause of the cardiovascular hyperactivity in response to acute exercise suggest that antioxidant treatment in skeletal muscle may hold therapeutic potential for hypertension. Whether this approach will suppress the abnormal cardiovascular responses to a bout of exercise in hypertensive individuals remains to be investigated in the future.

In conclusion, the main findings presented in this article were that 1) hindlimb skeletal muscle contraction-mediated sympathetic nerve and pressor responses were exaggerated in rats with hypertension induced by exogenous infusion of ANG II for 14 days; 2) exaggerated muscle reflex responses were reduced by intra-arterial injection into the hindlimb circulation of either tempol or tiron, SOD mimetics; and 3) superoxide production and mRNA and protein expressions for gp91phox were increased by ANG II infusion. We suggest that increased RAAS activity in hypertension exaggerates skeletal muscle contraction- evoked reflex sympathoexcitation by inducing oxidative stress in the muscle. Consequently, increased RAAS activity, which induces oxidative stress in skeletal muscle, may be a cause of the cardiovascular hyperactivity seen during exercise in hypertension through the exercise pressor reflex arc.

ACKNOWLEDGMENTS

The authors thank Dr. Marc Kaufman (Penn State Heart and Vascular Institute) for critical reading of the manuscript. The authors also thank Dr. Michio Miyoshi (Faculty of Medicine, Tottori University) for advice regarding the Western blot experiments.

GRANTS

This work was supported by Japan Society of the Promotion of Science Grant-In-Aid for Young Scientists (B) 22790226 (to S. Koba), a research grant from the Uehara Memorial Foundation Research (to S. Koba), and a research grant from the Nakatomi Foundation (to S. Koba).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: S.K. and R.W. conception and design of research; S.K., R.W., and N.K. performed experiments; S.K., R.W., and N.K. analyzed data; S.K., R.W., and T.W. interpreted results of experiments; S.K. prepared figures; S.K. drafted manuscript; S.K. and T.W. edited and revised manuscript; S.K., R.W., N.K., and T.W. approved final version of manuscript.

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