Angiotensin II-induced reduction in exercise capacity is associated with increased oxidative stress in skeletal muscle

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THE LIMITED EXERCISE CAPACITY represents a major symptom and an important prognostic factor in patients with cardiovascular disease and with risk factor for cardiovascular disease (19, 24). The exercise capacity is generally believed to be limited in the presence of abnormalities in skeletal muscle, which include atrophy, transition of fiber type, and abnormal energy metabolism (6, 26, 30). The activation of renin–angiotensin system, particularly the increase in angiotensin II (ANG II), has been well established to play an important role in the pathogenesis and progression of various cardiovascular diseases (7, 15). Moreover, angiotensin-converting enzyme inhibitors have been shown to improve exercise capacity in patients with cardiovascular disease and in spontaneously hypertensive rats (5, 22, 34). These results suggest that ANG II may directly impair skeletal muscle function and reduce exercise capacity. However, the mechanisms by which ANG II induces skeletal muscle dysfunction remain unknown.

ANG II leads to the production of superoxide (O$_2^-$), the major reactive oxygen species (ROS), via NAD(P)H oxidase activation (2, 10). The production of O$_2^-$ in skeletal muscle has been shown to be increased in the mouse model after myocardial infarction (31). In addition, our laboratory reported that the exercise capacity was decreased in heterozygous man-ganese superoxide dismutase knockout mice, and that this decrease resulted from the impaired skeletal muscle energy metabolism induced by O$_2^-$ (16). These findings suggest that the mechanisms whereby ANG II reduces exercise capacity may involve increased O$_2^-$ production. However, it remains unclear whether ANG II has any direct adverse effects on skeletal muscle function or exercise capacity.

The objective of the present study was thus to investigate whether the infusion of ANG II reduces exercise capacity in mice, and whether skeletal muscle energy metabolism is impaired. In addition, it was evaluated whether an inhibition of O$_2^-$ production could ameliorate these effects. It has been reported that ANG II (500 ng·kg$^{-1}$·min$^{-1}$) at high dose induced hypertension and skeletal muscle atrophy in mice in association with apoptosis and protein degradation (28). Therefore, to exclude the effects of hypertension and skeletal muscle atrophy, a low dose of ANG II (50 ng·kg$^{-1}$·min$^{-1}$) that affects neither blood pressure nor skeletal muscle atrophy was used in the present study.

METHODS

Experimental animals. All procedures and animal care were approved by our institutional animal research committee and conformed to the animal care guideline for the Care and Use of Laboratory Animals in Hokkaido University Graduate School of Medicine. Male C57BL/6J mice (8–12 wk of age) were housed in an animal room under controlled condition on a 12:12-h light-dark cycle. An osmotic minipump (Alzet model 1002) was implanted into the peri- toneal cavity to infuse ANG II (50 ng·kg$^{-1}$·min$^{-1}$) continuously for 1 wk. Saline was used as vehicle. Each group of mice was randomly divided into two groups, with or without 10 mmol/l apocynin (Sigma-Aldrich), an inhibitor of NAD(P)H oxidase activation, in drinking water. The concentration of apocynin was chosen on the basis of our laboratory’s previous studies (21, 35). Thus the present study was performed in the following four groups of mice: I) vehicle (n = 9), II) vehicle + apocynin (n = 9), III) ANG II (n = 9), and IV) ANG II + apocynin (n = 9). These assignment procedures were performed using numeric codes to identify the animals.

One week after treatment, exercise tests were performed. After that, the measurements of blood pressure and echocardiographic studies were performed. All mice were killed, and organ weight was measured. Because the amount of skeletal muscle samples was limited, they were divided into the experiments for mitochondrial oxygen (O$_2$)
consumption ($n = 9$), complex activity ($n = 7$), the histological analysis ($n = 4$), and those for the biochemical assay, including the immunoblotting for uncoupling protein (UCP)-3 ($n = 5$), the measurements of $O_2^–$ production ($n = 5$), and NAD(P)H oxidase activity ($n = 4$).

**Blood pressure and echocardiographic measurements.** Systemic blood pressure was measured using tail-cuff method (BP-98A, Softon) without anesthesia. Echocardiographic studies were performed under light anesthesia with tribromoethanol/amylene hydrate (2.5% wt/vol, 8 μg/g body wt) intraperitoneally and spontaneous respiration, as previously reported. A commercially available echocardiography system (EUB-8000, Hitachi, Tokyo) was utilized with dynamically focused 13-MHz linear array transducer using a depth setting of 2.0 cm. Two-dimensional targeted M-mode tracing were recorded at a paper speed of 50 mm/s (21, 35).

**Treadmill testing and exercise capacity.** Mice were treadmill tested to measure indexes, defining exercise capacity as described previously (16, 35). At the time of treadmill testing, each mouse was placed on a treadmill enclosed by a metabolic chamber through which air flow of constant speed (1 l/min) is passing (Oxymax 2, Columbus Instruments). $O_2$ and carbon dioxide (CO$_2$) gas fractions were monitored at both the inlet and output ports of the metabolic chamber. Basal measurements were obtained over a period of 10 min. Then mice were provided with a 10-min warm-up period at 6 m/min at 0°. After the animals warmed up, the angle was fixed at 10°, and the speed was incrementally increased by 2 m/min every 2 min until the mouse reached exhaustion. This protocol was designed so that the mice could quickly attain a plateau, reaching their maximal $O_2$ uptake (V$_{O2}$) before exhaustion. When the work-V$_{O2}$ relationship was plotted, V$_{O2}$ was increased linearly with the work. Exhaustion was defined as spending time (10 s) on the shocker plate without attempting to reengage the treadmill. Whole body V$_{O2}$ and CO$_2$ production (V$_{CO2}$) were automatically calculated every 10 s by taking the difference between the inlet and output gas flow. Respiratory exchange ratio was calculated as V$_{CO2}$/V$_{O2}$. The work was defined as the product of the vertical running distance to exhaustion and body weight.

Treadmill testing was performed one time at the end of treatment. Because the treatment protocol was short-term for 7 days, we did not observe time course of the changes in exercise capacity. Our laboratory previously reported that there was no difference in indexes of exercise capacity between the first run and second run, performed after 7 days of first run (16, 35). Therefore, our treadmill testing was well reproducible.

**Organ weight.** After treadmill testing, mice were excised under deep anesthesia with an intraperitoneal injection of tribromoethanol/amylene hydrate (2.5% wt/vol, 10 μl/g body wt). Heart and hindlimb skeletal muscle were rapidly removed and weighed.

**Histology in heart and skeletal muscle and apoptosis.** Heart and skeletal muscle were excised, fixed in 4% paraformaldehyde, and embedded in paraffin for histological analysis. Samples were cut in 5-μm thick sections and stained using Masson’s trichrome and picrosirius red. Morphological analysis of myocyte cross-sectional area and interstitial fibrosis was performed in at least 100 cells from each mouse (21, 35). To detect apoptosis, skeletal muscle tissue sections were stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining. The number of TUNEL-positive skeletal muscle myocyte nuclei was counted, and the data were normalized per 10$^5$ total nuclei identified by hematoxylin-positive staining in the same sections (21). Furthermore, it was examined whether apoptosis was present by the more sensitive ligation-mediated PCR fragmentation assays (Maxim Biotechnology, Rockville, MD) (21).

**Mitochondrial $O_2$ consumption in skeletal muscle.** Hindlimb skeletal muscle tissues were quickly harvested, and mitochondria were isolated as previously described. Before the measurement of $O_2$ consumption, the isolated mitochondria protein concentration was measured by the BCA Protein Assay (Pierce, Rockford, IL). $O_2$ consumption by the isolated mitochondria was measured polarographically using an O$_2$ electrode (Yellow Springs Instruments, Yellow Springs, OH) in a closed and magnetically stirred glass chamber at 28°C, according to the methods described previously (35). After a 1-min equilibration period, mitochondrial respiration was initiated by the addition of 2.5 mmol/l glutamate and malate as substrates. ADP-stimulated (state 3) respiration was determined after adding ADP (40 μmol/l). Non-ADP-stimulated (state 4) respiration was measured in the absence of ADP phosphorylation and validated by oligomycin (2 μg/ml), an ATPase inhibitor. Respiratory control index (RCI) was calculated as the ratio of state 3 to state 4 respiration, indicating overall mitochondrial respiratory activity.

**Mitochondrial complex activity in skeletal muscle.** The specific enzymatic activity of mitochondrial electron transport chain (ETC) complex I (rotenone-sensitive NADH-ubiquinone oxidoreductase), complex II (succinate-ubiquinone oxidoreductase), complex III (ubiquinol-cytochrome-c oxidoreductase), and complex IV (cytochrome-c oxidase) were measured in the mitochondria isolated from skeletal muscle, as described previously (35).

**Enzymatic activities of substrate metabolism in skeletal muscle.** The enzymatic activities of hexokinase (HK; a key enzyme of glucose utilization), β-hydroxyacyl CoA dehydrogenase (β-HAD; a key enzyme of β-oxidation of fatty acids), and citrate synthase (CS; a key enzyme of tricarboxylic acid cycle) were spectrophotometrically determined in the tissue homogenate from skeletal muscle sample, as described previously (39).

**ATP production.** ATP production in skeletal muscle was measured using a luciferase/luciferin assay kit (Toyo Ink, Tokyo, Japan) per the manufacturer’s protocol. The luciferase luminescence was recorded with a luminometer (AccuFLEX Lumi 400; Aloka, Tokyo, Japan).

**Immunoblotting for UCP-3 in skeletal muscle.** Equal amounts of protein extracted from skeletal muscle tissues were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with antibodies against UCP-3 (Abcam). Specific bands were labeled with the enhanced chemiluminescence and visualized by exposure of the membranes to films. After exposure, the nitrocellulose membranes were blot-stripped using Re-Blot Plus Western Blot Recycling Kit (CHMICON) and reblotted with antibodies against GAPDH.

$O_2^–$ production, NAD(P)H oxidase activity, and nitrotyrosine protein level in skeletal muscle. The chemiluminescence elicited by $O_2^–$ in the presence of lucigenin (5 μmol/l) was measured in skeletal muscle using a luminometer (AccuFLEX Lumi 400; ALOKA, Tokyo, Japan), as previously described (21, 35). To validate that the chemiluminescence signals were derived from $O_2^–$, the measurements were also performed in the presence of tiron (20 mmol/l), a cell-permeant, nonenzymatic scavenger of $O_2^–$. NAD(P)H oxidase activity was measured in the homogenates isolated from hindlimb skeletal muscle by the lucigenin assay after the addition of NAD(P)H (300 μmol/l), as previously described (21, 35).

In separate experiments, the source of $O_2^–$ production and the role of other enzyme activity for $O_2^–$ production in isolated skeletal muscle from ANG II mice were determined. $O_2^–$ production was performed in the absence or presence of various inhibitors, such as rotenone (100 μmol/l), oxyrinol (100 μmol/l), N$^o$-nitro-L-arginine methyl ester (100 μmol/l), or apocynin (100 μmol/l). Chemiluminescence was also measured in the homogenates isolated from skeletal muscle in ANG II mice by the lucigenin assay after the addition of succinate (5 mmol/l), xanthine (100 μmol/l), L-arginine (1 mmol/l), or NAD(P)H (100 μmol/l).

Skeletal muscle tissues were immunostained with antibody against mouse nitrotyrosine, followed by counterstaining with hematoxylin.

**Statistical analysis.** Data are expressed as means ± SE. Two-group comparison of means was performed by an unpaired Student’s t-test. Multiple-group comparison of means was performed by one-way ANOVA followed by t-tests. Theoretically, the comparison between vehicle + apocynin and ANG II or ANG II + apocynin groups makes no sense in the present study. Therefore, we established four of six null hypotheses among four groups as family of subset null hypotheses in multiple-comparison procedure. The Bonferroni correction
was applied for multiple comparisons of means, and the significance level of each comparison was adjusted as 0.05/4. In multiple-group comparison of means between presence and absence of inhibitor or substrate, Dunnett correction was applied. A value of $P < 0.05$ was considered to be statistically significant.

**RESULTS**

**Animal characteristics, echocardiography, and cardiac histology.** Table 1 shows animal characteristics in each group. Body weight, the changes in body weight, and blood pressure were comparable among the four groups. There were no significant differences among the four groups in heart rate, left ventricle (LV) diameters, fractional shortening, LV ejection fraction, cardiac output, and LV wall thickness measured by echocardiography. LV weight/body weight and histological measurements of myocyte size and interstitial fibrosis did not differ, indicating that ANG II did not induce cardiac abnormalities at the dose used in the present study.

**Exercise capacity.** The running distance (Fig. 1A) and work (Fig. 1B) to exhaustion were significantly decreased in ANG II compared with vehicle and were significantly improved in ANG II + apocynin. Coincident with the limited exercise capacity, peak $V\dot{O}_2$ and $V\dot{C}_O_2$ were significantly decreased in ANG II compared with vehicle, and this decrease was ameliorated in ANG II + apocynin (Table 2). In contrast, apocynin did not affect the exercise capacity in vehicle mice (Fig. 1 and Table 2). Importantly, the peak respiratory exchange ratio at the endpoint of exercise judged by “exhaustion” was >1.0 in all groups and did not differ among groups (Table 2), confirming that the mice were adequately exercised and had reached the same level of “exhaustion”.

**Histomorphometry and apoptosis.** Histomorphometric analysis of skeletal muscle by Mason’s trichrome and picrosirius red staining showed that myocyte cross-sectional area and interstitial fibrosis were comparable between vehicle and ANG II (Fig. 2, A–D). There were rare TUNEL-positive nuclei in skeletal muscle from both vehicle and ANG II, and there was no difference in its number between vehicle and ANG II (Fig. 2E). In addition, the intensity of the DNA ladder indicated that the infusion of ANG II did not cause apoptosis in skeletal muscle (Fig. 2F).

**Mitochondrial respiration.** There was no difference in state 3 respiration among vehicle, vehicle + apocynin, ANG II, and ANG II + apocynin (Fig. 3A). In contrast, state 4 respiration was increased in ANG II compared with vehicle (Fig. 3B). As a result, RCI was significantly decreased in ANG II compared with vehicle (Fig. 3C). ANG II + apocynin had significantly improved state 4 respiration compared with ANG II. RCI tended to be increased in ANG II + apocynin compared with ANG II, which, however, did not reach statistical significance. Apocynin did not affect state 4 respiration and RCI in vehicle mice.

**Mitochondrial complex activity.** Coincident with impaired mitochondrial respiratory activity in ANG II, mitochondrial ETC complex I and III activities were significantly decreased in ANG II compared with vehicle, which was completely normalized by apocynin (Fig. 4, A and C). Apocynin did not affect complex I and III activities in vehicle mice. There were no significant differences in complex II and IV activities.

**Table 2. Expired gas analysis**

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<th>Vehicle</th>
<th>Vehicle + Apocynin</th>
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<th>ANG II + Apocynin</th>
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<td>$n$</td>
<td>9</td>
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<tr>
<td>Peak $V\dot{O}_2$/BW, ml·kg$^{-1}$·min$^{-1}$</td>
<td>127 ± 3</td>
<td>123 ± 3</td>
<td>116 ± 3*</td>
<td>126 ± 2†</td>
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<tr>
<td>Peak $V\dot{C}_O_2$/BW, ml·kg$^{-1}$·min$^{-1}$</td>
<td>128 ± 3</td>
<td>124 ± 4</td>
<td>117 ± 5</td>
<td>121 ± 2</td>
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<tr>
<td>Respiratory exchange ratio</td>
<td>1.03 ± 0.02</td>
<td>1.03 ± 0.02</td>
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Values are means ± SE; $n$, no. of animals. $V\dot{O}_2$, $O_2$ uptake; $V\dot{C}_O_2$, $CO_2$ production. *$P < 0.05$ vs. vehicle. †$P < 0.05$ vs. ANG II.
ANGIOTENSIN II AND EXERCISE CAPACITY

Fig. 1. The summary data of the running distance (A) and the work to exhaustion (B) in vehicle (n = 9), vehicle + apocynin (n = 9), angiotensin (ANG) II (n = 9), and ANG II + apocynin (n = 9). Values are means ± SE. *P < 0.05 vs. vehicle. †P < 0.05 vs. ANG II.

Among vehicle, vehicle + apocynin, ANG II, and ANG II + apocynin (Fig. 4, B and D).

Enzymatic activities of substrate metabolism. There was no difference in HK activity among groups (Fig. 5A). β-HAD activity and CS activity were significantly decreased in ANG II, which was completely inhibited by apocynin (Fig. 5, B and C).

ATP production. ATP production tended to be decreased in ANG II compared with vehicle and attenuated by apocynin (Fig. 5D). However, these changes did not reach statistically significance.

UCP-3 protein level. UCP-3 protein level was significantly increased in ANG II compared with vehicle, and this increase was ameliorated in ANG II + apocynin (Fig. 6).

O$_2^-$ production. NAD(P)H oxidase activity, and nitrotyrosine protein level. O$_2^-$ production was significantly increased in skeletal muscle from ANG II compared with vehicle, and this change was completely inhibited by apocynin (Fig. 7B).

The increased O$_2^-$ production in ANG II mice was inhibited in the presence of apocynin, and it was not affected in the presence of other inhibitors (Fig. 7C). Chemiluminescence signal in the homogenates isolated from skeletal muscle in ANG II mice was enhanced by the addition of NAD(P)H and was not affected by other substrates (Fig. 7D). Therefore, these results suggested that the increased O$_2^-$ production in ANG II mice was due to the increased NAD(P)H oxidase activity, but not mitochondrial dysfunction, xanthine oxidase activity, or nitric oxide synthase activity.

Nitrotyrosine protein was slightly increased in skeletal muscle myocyte from ANG II compared with vehicle, and this was attenuated in ANG II + apocynin (Fig. 7E). In contrast, it was not detected in skeletal muscle vasculature from vehicle and ANG II (Fig. 7E).

Effects of exercise test on mitochondrial respiration, O$_2^-$ production, and NAD(P)H oxidase activity. To exclude the effect of exercise test on biochemical alterations in skeletal muscle, mitochondrial respiration, O$_2^-$ production, and NAD(P)H oxidase activity were measured in the skeletal muscle from sedentary mice, which did not perform exercise test. These experiments were performed in an additional four groups of mice as follows: vehicle + exercise test (n = 5), ANG II + exercise test (n = 5), vehicle + sedentary (n = 5), and ANG II + sedentary (n = 5). There were no differences in each parameter between exercise test group and sedentary group (Fig. 8), indicating that the biochemical changes in the skeletal muscle were due to the ANG II infusion and not due to treadmill exercise protocol.

DISCUSSION

The most important finding in the present study was a significant reduction in exercise capacity in mice after infusion of ANG II for 1 wk compared with vehicle mice. This was accompanied by a significant decrease in peak VO$_2$ as measured by expired gas analysis. Coincident with these altera-
tions, mitochondrial state 4 respiration was increased, and ETC complex III activity was decreased in skeletal muscle from ANG II mice. Moreover, ANG II-induced reductions in exercise capacity and mitochondrial dysfunction were reversed by the treatment of mice with apocynin. Concurrently, in the skeletal muscle of ANG II mice, increased O$_2^·$/H$_2$O$_2$ production and NAD(P)H oxidase activity were inhibited by apocynin. These results suggest that NAD(P)H oxidase-derived O$_2^·$/H$_2$O$_2$ induces mitochondrial dysfunction and plays an important role in reducing exercise capacity in ANG II-infused mice.

Reduction in exercise capacity by ANG II. Exercise capacity and peak V˙O$_2$ were decreased in ANG II mice (Fig. 1 and Table 2). In the present study, ANG II at an infusion rate of 50 ng·kg$^{-1}$·min$^{-1}$ for 1 wk did not increase blood pressure or cause LV hypertrophy (Table 1). These results are consistent with the previous study with slightly higher doses of ANG II at 200 ng·kg$^{-1}$·min$^{-1}$ for 4 wk, in which increased blood pressure or LV hypertrophy was not observed (13). The absence of hypertension or LV hypertrophy is due to the lower doses of ANG II in our study compared with those used in previous studies (1.4–2.0 μg·kg$^{-1}$·min$^{-1}$) (8, 20). In contrast, Zhong et al. showed that the infusion of subpressor dose of ANG II (0.15 mg·kg$^{-1}$·day$^{-1}$ = 100 ng·kg$^{-1}$·min$^{-1}$) for 14 days induced mild LV hypertrophy and fibrosis with diastolic dysfunction (37). In contrast, LV wall thickness did not differ significantly between vehicle and ANG II mice, and histopathology of the myocardium showed no myocyte hypertrophy or increase in interstitial fibrosis in ANG II mice in the present study (Table 1). These differences might be due to differences in the dose of ANG II (100 vs. 50 ng·kg$^{-1}$·min$^{-1}$) and the duration of infusion (14 vs. 7 days). Based on these results, the reduced exercise capacity in mice with ANG II infusion was not due to the abnormalities in cardiac structure or function.

The present study also demonstrated that body weight or skeletal muscle weight did not differ between vehicle and ANG II mice (Table 1). These findings are not consistent with those previously reported by Song et al. (28), in which ANG II caused skeletal muscle atrophy. They used high dose of ANG II (500 ng·kg$^{-1}$·min$^{-1}$, 10-fold higher than that in our study), with resulting increase in blood pressure and decrease in both skeletal muscle weight and body weight. The decrease in skeletal muscle

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**Fig. 3.** The summary data of state 3 respiration (A), state 4 respiration (B), and respiratory control index (C) in vehicle, ANG II, and ANG II + apocynin (n = 9 for each group). Values are means ± SE. *P < 0.05 vs. vehicle. ††P < 0.05 vs. ANG II.

**Fig. 4.** Mitochondrial electron transport chain (ETC) complex I (A), II (B), III (C), and IV (D) enzymatic activities in skeletal muscle from vehicle, ANG II, and ANG II + apocynin (n = 7 for each). Values are means ± SE. *P < 0.05 vs. ANG II. †P < 0.05 vs. ANG II + apocynin.
weight was due to muscle cell atrophy via protein degradation and apoptosis induced by ANG II. Their studies have suggested that high dose of ANG II is not suitable to evaluate the direct effects of ANG II on skeletal muscle and exercise capacity. We thus employed the low dose of ANG II on exercise capacity and skeletal muscle energy metabolism, which does not cause skeletal muscle atrophy and fibrosis. In fact, skeletal muscle in mice infused with ANG II for 1 wk did not show any atrophic changes assessed by myocyte cross-sectional area, apoptosis by TUNEL staining and DNA ladder, and interstitial fibrosis (Fig. 2). The present study model was thus able to exclude the effects of ANG II on skeletal muscle atrophy.

Relationship between reduced exercise capacity and skeletal muscle mitochondrial energy metabolism. VO$_2$ during exercise, as measured by expired gas analysis, reflects O$_2$ consumption in exercising muscle (17). In other words, this reflects mitochondrial O$_2$ consumption and is an important parameter of energy metabolism in skeletal muscle. In addition, long-term exercise capacity depends on oxidative phosphorylation in exercising muscle mitochondria (33). Therefore, to clarify the mechanism by which exercise capacity was reduced in ANG II mice, we measured mitochondrial O$_2$ consumption isolated from hindlimb skeletal muscle using NADH as a substrate. In ANG II mice, the rate of ADP-independent O$_2$ consumption (state 4) was significantly increased (Fig. 3B). This increase in state 4 signifies an increase in O$_2$ consumption not involved in energy production, meaning that O$_2$ is wasted. Therefore, even if O$_2$ measured by expired gas analysis during exercise was not altered, energy production in skeletal muscle and exercise capacity could be reduced. In contrast, the rate of ADP-dependent O$_2$ consumption (state 3) was unchanged (Fig. 3A). This increase in state 4 signifies an increase in O$_2$ consumption not involved in energy production, meaning that O$_2$ is wasted. Therefore, even if O$_2$ measured by expired gas analysis during exercise was not altered, energy production in skeletal muscle and exercise capacity could be reduced. In contrast, the rate of ADP-dependent O$_2$ consumption (state 3) was unchanged (Fig. 3A). State 3 is affected by state 4 in the measurement of mitochondrial respiratory capacity. Thus RCI was calculated as an overall parameter of mitochondrial oxidative phosphorylation and was significantly decreased in ANG II mice (Fig. 3C). Moreover, mitochondrial complex III activity was also decreased (Fig. 4C). In accordance with these results, mitochondrial ATP production tended to be decreased in ANG II mice (Fig. 5D). These results support slight, but significant, decrease in peak VO$_2$ in ANG II mice (Table 2).

CS activity was decreased in ANG II mice (Fig. 5C), suggesting that the supply of substrate to ETC was also impaired. Furthermore, δ-HAD activity was decreased and HK activity was not altered in ANG II mice (Fig. 5, A and B), suggesting that the utilization of fatty acid, but not glucose, was impaired.

Role of oxidative stress in skeletal muscle. Another important finding in the present study was the involvement of NAD(P)H oxidase-derived O$_2^-$ in reduced exercise capacity and skeletal...
muscle mitochondrial dysfunction in ANG II mice. ANG II stimulates NAD(P)H oxidase activity and O$_2$•\(^{-}\) production in a variety of cells, including vascular endothelial cells (18), smooth muscle cells (10), and myocardial cells (29). NAD(P)H oxidase consists of cytoplasmic and membrane components and is activated by transition of the cytoplasmic components to the cell membrane to form a complex with the membrane component in response to ANG II type 1 receptor stimulation (23). Apocynin inhibits NAD(P)H oxidase activity by specifically blocking this complex formation. NAD(P)H oxidase has been shown also in skeletal muscle cells (12). In ANG II mice treated with apocynin, O$_2$•\(^{-}\) production and NAD(P)H oxidase activity in skeletal muscle were completely inhibited, and exercise capacity and mitochondrial function improved. In the vehicle mice, treatment with apocynin had slight, but no significant, O$_2$•\(^{-}\) production (Fig. 7A). It has been reported that apocynin stimulates ROS production in cultured rat vascular fibroblasts (32). Therefore, apocynin may produce ROS under normal conditions in vivo. However, insignificant O$_2$•\(^{-}\) production in vehicle + apocynin did not affect exercise capacity in the present study (Fig. 1). Thus apocynin itself did not affect exercise capacity, but rather improved exercise capacity via the inhibition of NAD(P)H oxidase activity induced by ANG II.

The mechanisms by which NAD(P)H oxidase-derived O$_2$•\(^{-}\) damages mitochondrial function are unknown. In the present study, the abnormality in mitochondrial function observed in ANG II mice was an increase in state 4. The initiation of state 4 requires the involvement of UCP-3 and mitochondria permeability transition (3, 9, 14). UCP-3 protein level was significantly increased in skeletal muscle from ANG II compared with vehicle, and this increase was ameliorated in ANG II+apocynin (Fig. 6). ROS have been shown to increase UCP-3 gene expression in cultured rat skeletal muscle cells, and this can be inhibited by antioxidant enzymes (27). Therefore, O$_2$•\(^{-}\) may well be involved in the induction of UCP-3 and the increase in state 4 in ANG II mice. In addition, various oxidants and ROS can induce mitochondria permeability transition pore opening (38). Interestingly, the opening of mitochondria permeability transition pore is an important mechanism responsible for the induction of apoptotic cell death (4). In our model, increased apoptosis of skeletal muscle cells was not observed (Fig. 2, C and D). The impaired energy metabolism in skeletal muscle thus precedes skeletal muscle atrophy due to apoptosis and, indeed, may be a regulating factor in skeletal muscle atrophy.

RCI and ETC complex III activity were also decreased in skeletal muscle from ANG II mice in the present study (Fig. 4). These findings suggest that ROS may damage the ETC via their direct effects on mitochondria. The ETC complex contains an iron-sulfur center that is susceptible to attack by ROS. However, the detailed mechanisms by which NAD(P)H oxidase-derived O$_2$•\(^{-}\) damages mitochondria remain unknown, and further research is clearly needed.

Fig. 7. Superoxide (O$_2$•\(^{-}\)) production (n = 5 for each group; A) and NAD(P)H oxidase activity (n = 4 for each group; B) in skeletal muscle from vehicle, vehicle + apocynin, ANG II, and ANG II + apocynin. Effects of various inhibitors on O$_2$•\(^{-}\) production (n = 5 for each group; C) and effects of various substrates on enzymatic activities (n = 5 for each group; D) in skeletal muscle from ANG II are shown. Values are means ± SE. *P < 0.05 vs. vehicle. †P < 0.05 vs. ANG II. ‡P < 0.05 vs. absence of inhibitor or substrate. E. representative photomicrographs of immunohistochemical staining with nitrotyrosine in skeletal muscle from vehicle (a), vehicle + apocynin (b), ANG II (c), and ANG II + apocynin (d), and their high-power photomicrographs in skeletal muscle from vehicle (e) and ANG II (f). Scale bar, 10 µm. RLU, relative light unit; Rot, rotenone; Oxy, oxyprinol; L-N, N$^\text{G}$-nitro-L-arginine methyl ester; Suc, succinate; Xan, xanthine; L-arg, L-arginine.
Limitations of the study. There are several limitations that should be acknowledged in the present study. First, even though there were no significant differences in cardiac function at rest among groups, we could not completely exclude the contribution of abnormal function during exercise. Nevertheless, we were unable to assess these changes in mice during exercise due to technical difficulty. Second, O$_2^-$ could impair nitric oxide-dependent vascular relaxation, which may decrease skeletal muscle blood flow, especially during exercise, and limit the exercise capacity. Nitrotyrosine protein was not detected in vasculature form ANG II (Fig. 7E), indicating that the formation of peroxinitrite did not occur by ANG II in vasculature in our model. Furthermore, Ojaimi et al. (25) demonstrated that peak V\text{O}_2 was not decreased in endothelial nitric oxide synthase knockout mice during maximal exercise, indicating that O$_2$ delivery to limb skeletal muscle was preserved, even in the condition in which nitric oxide-dependent vascular relaxation was impaired. Therefore, skeletal muscle blood flow could hardly affect the exercise capacity in mice. Finally, we could not completely exclude the effects of other sources of O$_2^-$ on the exercise capacity. The specificity of apocynin for inhibiting NAD(P)H oxidase activation is a matter of debate (1, 36). Apocynin has been widely used as an inhibitor of NAD(P)H oxidase activation, in both tissue preparations and in vivo (10, 21, 35). However, a recent report using the cultured cells indicated that apocynin might predominantly act as an antioxidant (11). In their in vitro study, the inhibitory action of apocynin for NAD(P)H oxidase activation was restricted to myeloperoxidase-expressing leukocytes and not demonstrated in myeloperoxidase-free vascular cells. However, apocynin could inhibit the activation of NAD(P)H oxidase even in myeloperoxidase-free skeletal muscle in in vivo situations. Further studies, such as those through gene manipulation, are needed to evaluate the exact contribution of NAD(P)H oxidase and obtain the direct evidence for a role of this ROS production in this setting. Assuming that apocynin might act as an antioxidant, our results would support the concept that the enhanced oxidative stress in skeletal muscle contributed to the limited exercise capacity in ANG II mice.

Conclusion. This study provides the first direct evidence that ANG II-induced oxidative stress in skeletal muscle limits exercise capacity and leads to skeletal muscle mitochondrial dysfunction. Thus oxidative stress may be a key mediator of the reduced exercise capacity in cardiovascular disease, where ANG II plays a major role. These findings suggest that the treatment to inhibit the action of ANG II and production of O$_2^-$ in skeletal muscle can be effective to improve exercise capacity in these state.

ACKNOWLEDGMENTS

We thank Kaoruko Kawai for technical assistance in the experiments.

GRANTS

This study was supported, in part, by grants from the Ministry of Education, Science, and Culture (17390223, 18790487, 20590854, 20117004, and 21390236) and Japan Heart Foundation Research.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
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AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00534.2011 • www.ajpheart.org