Inhibition of Oxidative Stress by Antioxidant Supplementation Does Not Limit Muscle Mitochondrial Biogenesis or Endurance Capacity in Rats

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Summary The objective of the present study was to analyze the activation and expression patterns of upstream and downstream factors of PGC-1 α to determine whether antioxidant (AO) supplementation inhibits mitochondrial biogenesis in skeletal muscles as an adaptation to endurance training, as well as to analyze changes in endurance capacity based on such findings. For this objective, 24 male Sprague-Dawley (SD) rats were allocated into 4 groups (vehicle-sedentary, V-Sed; vehicle-exercise, V-EX; antioxidant-sedentary, AO-Sed; antioxidant-exercise, AO-EX) of 6 rats each. The rats were then treated with vitamin C (500 mg·kg⁻¹ body weight·d⁻¹) or a placebo for 8 wk, and a swimming program was implemented in some rats during the last 4 wk of this period. Immediately after the last training session, blood was collected from the tail of each rat, and TBARS was measured to test the effect of vitamin C as an AO. As a result, increased oxidative stress from exercise was inhibited by vitamin C supplementation. Analysis of whether reduced oxidative stress by vitamin C supplementation also inhibited mitochondrial biogenesis within skeletal muscles showed that phosphorylation of p38 MAPK and AMPK, along with levels of PGC-1 α , NRF-1, mtTFA, and mitochondrial electron transport enzymes, increased after endurance training in spite of vitamin C supplementation. Moreover, running time, distance, and total work increased significantly in the exercise group as compared to those in the sedentary group, regardless of vitamin C supplementation. These results indicate that mitochondrial biogenesis and endurance capacity increase as a result of endurance training, regardless of AO supplementation.

Key Words exercise, antioxidants, mitochondria, oxidative stress

Endurance training promotes not only mitochondrial biogenesis (1–3), but also various adaptive responses within skeletal muscles, such as improved insulin sensitivity (4–6), fatty acid oxidation (7, 8), and antioxidant (AO) defense system efficiency (9, 10). Such adaptive responses occur owing to increased expression or activation of peroxisome proliferator-activated receptor γ (PPAR- γ) coactivator-1 α (PGC-1 α) through Ca²⁺-dependent pathways, AMP-activated protein kinase (AMPK), and p38 mitogen-activated protein kinases (MAPK) (11, 12). However, aside from these positive adaptive responses, oxidative stress due to reactive oxygen species (ROS) also increases within skeletal muscles during muscle contractions (13–15). Increased oxidative stress, or increased ROS, is known to play a harmful role in the human body, but recent studies have reported its positive effects, such as being involved in mitochondrial biogenesis (13, 16–18) as well. Because of this, there have been reports from recent studies that have examined the effects of AO supplementation, such as with vitamins, on adaptive responses from endurance training in skeletal muscles. However, these study results conflict with claims that skeletal muscle adaptations from endurance

training are decreased by AO supplementation.

According to Yfanti et al. (19), Wadley and McConell (20), Higashida et al. (2), and Yada and Matoba (21), mitochondrial biogenesis markers that increase following acute exercise or endurance training, including PGC-1 α , are unaffected by AO supplementation. Contrary to this claim, Strobel et al. (22) reported that while the effects of endurance exercise related to mitochondrial biogenesis were not inhibited by AO supplementation, the basal level decreased, while Gomez-Cabrera et al. (23), Ristow et al. (24), Meier et al. (25), and Abadi et al. (26) reported that the effects of endurance training, such as mitochondrial biogenesis, were inhibited by AO supplementation. The basis for this is that the expression of PGC-1 α and a few mitochondrial enzymes that play a key role in mitochondrial biogenesis after endurance training is reduced by AO supplementation. These results imply that various upstream factors (11, 12) responsible for increasing PGC-1 α , such as AMPK and p38 MAPK, can also be inhibited by ROS. However, these studies measured mostly PGC-1 α and its downstream factors, while upstream factors such as AMPK and p38 MAPK were not measured and endurance capacity based on the level of mitochondrial biogenesis was not tested. Accordingly, the present study aimed to analyze the expression patterns of upstream and down-

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stream factors of PGC-1 α to determine whether mitochondrial biogenesis as an adaptation within skeletal muscles from endurance training can be inhibited by AO supplementation. We also sought to analyze effects on endurance capacity based on these findings.

MATERIALS AND METHODS

Animals. In the present study, 24 male Sprague-Dawley (SD) rats, weighing approximately 100–120 g were obtained from Central Lab. Animal Inc. (Seoul, Korea) and allowed to acclimate for 1 wk. Afterwards, the rats were randomly allocated into 4 groups of 6 rats each: vehicle-sedentary (V-Sed), vehicle-exercise (V-Ex), antioxidant-sedentary (AO-Sed), and antioxidant-exercise (AO-Ex). The temperature in the rearing room was maintained at 21°C, and water and feed (carbohydrate 58.9%, fat 12.4%, protein 28.7%; Purina Corp., St. Louis, MO) were given ad libitum. Light (06:00–18:00) and dark periods were adjusted to 12 h each.

Skeletal muscles were extracted after anesthesia using sodium pentobarbital (50 mg/kg body weight; Fort Dodge Animal Health, Overland Park, KS) at 18 h after the final training session. Extracted triceps muscles were rapid-frozen and stored at –80°C until analysis. Moreover, plasma thiobarbituric acid-reactive substance (TBARS) was measured by collecting about 200 μ L of blood from the tail artery immediately after completion of the final training session due to its very short half-life (27). The present study received approval from the Institutional Animal Care and Use Committee in Chonbuk National University (IACUC approval No. CBNU-2016-0029).

Antioxidant supplementation. Rats in the antioxidant groups were given 500 mg/kg ascorbic acid (Sigma, St. Louis, MO) by feeding tube at 8 AM daily for 8 wk and were exercised by swimming program for 5 d/wk for the last 4 wk of antioxidant treatment. The control group was given vehicle [200 mg of triolein (Sigma) and 20 mg of Tween 80 (Sigma) in 1 mL of saline] by feeding tube (28).

Exercise program. The swimming protocol consisted of 4 wk (5 d/wk) of exercise, which was based on partial revisions to the methods by Ploug et al. (29) and Garcia-Roves et al. (30). Prior to the swimming exercise, training for acclimatization to water was conducted for 3 d, for 10 min on each of the first 2 d and 3 h on the third day. For the experiment, rats swam a total of 6 h divided into two 3-h sessions starting at 9 AM. After the first 3-h swim session, a 45-min rest was given, during which time the rats were allowed water and feed ad libitum.

Three rats were placed simultaneously in a cylindrical water tank (80 cm $\times$ 120 cm, water depth 50–60 cm) and allowed to swim freely. To minimize any stress from a change in body temperature, the water temperature was kept constant at 35°C. In addition, during the rest period and after completion of the exercise, a towel was used to remove moisture and a hot air fan was used to maintain body temperature (31).

Indicators of oxidative stress. Plasma and muscle TBARS concentrations were measured via fluoromet-

ric assay using a Quantichrom TBARS Assay kit (Bio-Assay System, Hayward, CA). Freeze-clamped muscle samples were homogenized in ice-cold 50 mM sodium phosphate, pH 7.0, and homogenates were centrifuged at 13,000 $\times g$ for 10 min at 4°C. The supernatants were utilized for the determination of muscle TBARS. After addition and stirring 50 μ L of plasma and supernatants in 50 μ L of 35% TCA, the mixture was added to 50 μ L Tris-HCl buffer (50 mM, pH 7.4), and stirred again. After being cultured at room temperature for 10 min, 1 mL of 0.75% TBA (containing 2 mol/L Na₂SO₄) was admixed and heated for 45 min at 100°C. Next, 1 mL of 70% TCA was admixed and centrifuged for 10 min at 950 $\times g$, after which measurements were taken using a fluorescence microplate reader (BioTek, Winooski, VT).

Endurance capacity. For each rat, after 4 wk of exercise treatment, endurance capacity was measured according to an exercise tolerance protocol using a treadmill. The protocol for the progressive exercise test used a treadmill with a fixed incline of 15°. The exercise started at a speed of 10 m/min for the first 5 min and the speed was then increased by 2 m/min every minute. If the rat was unable to continue running and stayed on the electrical shock bar (no current running), then the rat's rear legs were stimulated by high-pressure air to encourage the rat to continue running. However, if the rat stayed on the electrical shock bar for at least 3 s despite the high-pressure air stimulation, the test was terminated (32), and the total running time and distance were calculated. Total work was calculated using the formula by Park et al. (33): Work (J)=Force [body weight (kg)] $\times$ Vertical distance [$\sin(15^\circ)$ $\times$ speed (m/min) $\times$ time (min)].

Western blotting. Muscles were homogenized at 4°C using an ice-cold buffer [50 mmol/L Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L NaF, 1 μ g/mL aprotinin, 1 μ g/mL leupeptin, 1 μ g/mL pepstatin, 0.1 mmol/L bpV(phen), and 2 mg/mL β -glycerophosphate]. The homogenized samples underwent 3 repetitions of freezing/thawing to completely decompose the mitochondria, after which they were centrifuged for 10 min (1,500 $\times g$, 4°C) and only the supernatant was subsequently used. Quantitative analysis of protein concentration was performed using the method of Lowry et al. (34). Quantified proteins were separated by electrophoresis using SDS-polyacrylamide gel and were transferred to a nitrocellulose membrane. After transfer, the primary antibodies used for immunoblotting were as follows: P38 MAPK, phospho-p38 MAPK, AMPK, phospho-AMPK (Cell Signaling Technology, Danvers, MA); PGC-1 α (monoclonal; EMD Chemicals), mtTEA (Santa Cruz Biotechnology, Dallas, TX); NRF-1, NADH ubiquinone oxidoreductase (NADH-UO), succinate-ubiquinone oxidoreductase 3 kDa subunit (SUO), cytochrome c (cyto c), cytochrome oxidase (COX) subunit-I, COX-IV, ATP synthase (Invitrogen, Waltham, MA); and β -actin (Sigma). ECL (Amersham, Little Chalfont, UK) was used for band visualization, while relative band intensity was analyzed using Sigma-

Fig. 1. The antioxidant supplementation did not affect body weight. Antioxidant supplementation groups were treated once daily with $500 \text{ mg} \cdot \text{kg}^{-1} \text{ body weight} \cdot \text{d}^{-1}$ ascorbic acid for 8 wk. Data are presented as means $\pm$ SE for 6 rats in each of the groups. Data was analyzed using two-way ANOVA with repeated measures and Bonferroni's post hoc test for multiple comparisons when appropriate. * $p < 0.05$ vs. V-Sed. + $p < 0.05$ vs. AO-Sed. V, vehicle; AO, antioxidant; Sed, sedentary; Ex, exercise.

Gel (Jandel Scientific Corp., Erkrath, Germany).

Statistical analysis. The data are presented as mean $\pm$ standard error of the mean (SE). The increases in body weight were analyzed by two-way ANOVA with repeated measures. Two-way ANOVA for the comparison of TBARS level, mitochondrial biogenesis markers and endurance capacity was used to compare the differences among groups. Bonferroni's post hoc test was conducted to determine the significance when appropriate. The significance level was set at $p < 0.05$. The statistical software Sigma Stat (Systat Software, San Jose, CA) was used.

RESULTS

AO treatment did not affect body weight

Changes in body weight during the 8-wk experimental period are shown in Fig. 1. At the start of the experiment, there were no significant differences in body weight among groups, and even after 4 wk of AO supplementation, there were no significant differences. However, the exercise group showed a decreasing trend in body weight as compared to that in the sedentary group, with significant decreases seen at 3 wk and 4 wk after exercise treatment ($p < 0.05$). However, there were no differences in body weight between the AO supplementation and non-supplementation groups, regardless of whether exercise was performed or not.

AO supplementation prevented exercise-induced oxidative stress

To determine changes in oxidative stress following 4 wk of AO supplementation plus 1 (for muscle) and 4 (for plasma) additional weeks of AO supplementation and endurance exercise treatment, plasma and muscle TBARS was analyzed immediately after the final exercise session. The exercise group without AO supplementation

Fig. 2. A: The antioxidant supplementation of $500 \text{ mg} \cdot \text{kg}^{-1} \text{ body weight} \cdot \text{d}^{-1}$ ascorbic acid prevented an exercise-induced increase in thiobarbituric acid-reactive substance (TBARS). Blood samples for plasma TBARS measurement were obtained from the tail vein immediately after the last exercise bout. B: The TBARS level in the triceps muscle after 1 wk of exercise showed the same tendency as the TBARS level in the plasma after 4 wk of exercise. Data are presented as means $\pm$ SE for 6 rats in each group. Data was analyzed by two-way ANOVA and Bonferroni's post hoc test for multiple comparisons when appropriate. * $p < 0.05$ vs. V-Sed. # $p < 0.05$ vs. AO-Ex. V, vehicle; AO, antioxidant; Sed, sedentary; Ex, exercise.

(V-Ex group) showed a significant increase in TBARS as compared to that in the sedentary group (V-Sed group) ($p < 0.05$; Fig. 2). This increase in oxidative stress caused by 4 wk of endurance training was completely inhibited by AO supplementation.

AO supplementation did not prevent mitochondrial biogenesis

Phosphorylation and expression of PGC-1 α and upstream (p38 MAPK and AMPK) and downstream (NRF-1 and mtTFA) factors of PGC-1 α , which plays a key role in mitochondrial biogenesis, significantly increased after 4 wk of endurance training ($p < 0.05$; Fig. 3). AO supplementation had no effect on the phosphorylation or expression of PGC-1 α , upstream and downstream factors of PGC-1 α from endurance training. In addition, AO supplementation had no effect on the increase in

Fig. 3. The effects of AO supplementation for 8 wk and swimming exercise training during the last 4 wk of this period on markers of mitochondrial biogenesis in the triceps muscle. Bottom: PGC-1 α upstream factors, p38 MAPK (A) and AMPK (B) phosphorylation is increased to the same extent in the AO and V group. The expression of PGC-1 α (C) increased to the same extent in the AO and V group. PGC-1 α downstream factors, NRF-1 (D) and mtTFA (E) protein, were increased to the same extent in the AO and V group. Top: Western blots are representative of one rat from each group. Data are presented as means $\pm$ SE for 6 rats in each group. Data was analyzed by two-way ANOVA and Bonferroni's post hoc test for multiple comparisons when appropriate. * $p < 0.05$ vs. V-Sed. # $p < 0.05$ vs. AO-Ex. V, vehicle; AO, antioxidant; Sed, sedentary; Ex, exercise. p38 MAPK, p38 mitogen-activated protein kinase; AMPK, AMP kinase; PGC-1 α , peroxisome proliferator-activated receptor γ coactivator-1 α ; NRF-1, nuclear respiratory factor-1; mtTFA, mitochondrial transcription factor A.

expression of mitochondrial electron transport enzymes from endurance training ($p < 0.05$; Fig. 4).

Effect of AO supplementation on endurance capacity

The results of the endurance capacity test after 4 wk of endurance training showed that exercise time and distance, as well as total work, increased significantly ($p < 0.05$) in the exercise groups, as compared to the sedentary groups, regardless of AO supplementation (Fig. 5).

DISCUSSION

The present study investigated whether inhibition of oxidative stress from AO supplementation during endurance training also inhibits the phosphorylation or protein expression of upstream and downstream factors of PGC-1 α , which plays a key role in mitochondrial biogenesis and expression of mitochondrial enzymes. We also investigated effects of AO supplementation on endurance capacity. For this, plasma TBARS levels were analyzed to determine whether oxidative stress increased as a result of endurance exercise, and if so, whether this increase in oxidative stress can be inhibited by AO supplementation. The plasma TBARS concentration has a very short half-life (27), and as such, blood was col-

lected from the tail immediately after completion of the final session of the 4-wk training period. The TBARS concentration in the skeletal muscle after 1 wk of exercise showed the same tendency as the TBARS concentration in the plasma after 4 wk of exercise (Fig. 2B). The results showed that TBARS increased from endurance training and was reduced to basal levels by vitamin C treatment (Fig. 2), indicating that the vitamin C used in the present study is an effective AO for inhibiting the increase in oxidative stress from endurance training.

The fact that endurance training increases mitochondrial biogenesis within skeletal muscles was discovered in the 1960s (35), and its mechanism was elucidated through the discovery of NRF-1 and NRF-2 by Evans and Scarpulla (36) and Virbasius et al. (37, 38), in the 1990s, and the subsequent discovery of PGC-1 α was by Puigserver et al. (39) and Wu et al. (40). PGC-1 α not only increases the expression of intranuclear transcription factors such as NRF-1 (40, 41), but it also increases the expression of mtTFA, a factor that regulates mitochondrial respiratory chain subunits and mitochondrial DNA replication/transcription by translocating into mitochondria to induce transcription and mtDNA

Fig. 4. Effects of antioxidant supplementation and 4 wk of swimming exercise training on mitochondrial electron transport enzymes in the skeletal muscle. Bottom: The mitochondrial electron transport enzymes increased to the same extent in the vehicle and AO supplemented group. Top: Western blots are representative of one rat from each group. Data are presented as means $\pm$ SE for 6 rats in each group. Data was analyzed by two-way ANOVA and Bonferroni's post hoc test for multiple comparisons when appropriate. * p < 0.05 vs. V-Sed. # p < 0.05 vs. AO-Ex. V, vehicle; AO, antioxidant; Sed, sedentary; Ex, exercise. NADH-UO, NADH-ubiquinone oxidoreductase; SUO, succinate-ubiquinone oxidoreductase; Cyto C, cytochrome c; COX-1 and -4, cytochrome oxidase subunits 1 and 4; ATPs, ATP synthase.

replication (40). The present study also showed similar results, with increased expression of NRF-1, mtTFA, and mitochondrial electron transfer enzymes after endurance training. In addition, although oxidative stress caused by exercise was inhibited by AO supplementation as shown in Fig. 2, there were no differences between the exercise groups regardless of AO supplementation (Fig. 3). However, according to Gomez-Cabrera et al. (23), Ristow et al. (24), and Meier et al. (25), when an increase in ROS from exercise is inhibited by AO supplementation, the expression of PGC-1 α is inhibited, which also inhibits mitochondrial biogenesis. This claim implies that various types of signaling for the activation or expression of PGC-1 α are all regulated by ROS.

The activation or expression of PGC-1 α is regulated by mutually adjusting the signaling networks of various upstream factors, including the p38 MAPK (42–44), AMPK (45–47), and Ca²⁺-dependent pathways (48, 49). Elevated calcium concentrations within skeletal muscles during endurance exercise activate calcium/calmodulin-dependent protein kinase II (CAMKII) to increase the activation of p38 MAPK (43, 49). Increased p38

Fig. 5. The AO supplementation did not affect endurance capacity. Endurance capacity was measured using a treadmill after 4 wk of swimming exercise training. Data are presented as means $\pm$ SE for 6 rats in each group. A: total running time on exercise tolerance test. B: total running distance on exercise tolerance test. C: total work on exercise tolerance test. Data was analyzed by two-way ANOVA and Bonferroni's post hoc test for multiple comparisons when appropriate. * p < 0.05 vs. V-Sed. # p < 0.05 vs. AO-Ex. V, vehicle; AO, antioxidant; Sed, sedentary; Ex, exercise.

MAPK activates MEF2 (50) and ATF2 (51) to increase transcription of PPARGC1A, the gene that encodes PGC-1 α (3, 49, 51, 52). Moreover, chronic AMPK activation within skeletal muscles by pharmacological activators facilitates mitochondrial biogenesis through PGC-1 α and NRF-1 (45), while AMPK activation by exercise also increases PPARGC1A mRNA expression (46). Therefore, AMPK, which regulates intracellular energy metabolism, is also a major regulator of mitochondrial biogenesis (44). In addition, recent studies have shown that AMPK and p38 MAPK play a key role in ROS-mediated increases in PGC-1 α levels for mitochondrial biogenesis. Elevation of ROS by H₂O₂ treatment decreases the cellular ATP level, and as a result, activated AMPK increases

PPARGC1A mRNA expression (16). Moreover, pharmacological inhibition of ROS regulates PGC-1 α expression by inhibiting exercise-mediated activation of p38 MAPK (13). Therefore, in order for mitochondrial biogenesis from endurance training to be inhibited by AO supplementation, activation of AMPK and p38 MAPK must be inhibited, leading to regulation of PGC-1 α . However, as shown in Fig. 4, increases in the phosphorylation of AMPK and p38 MAPK induced by 4 wk of endurance training were not mitigated by AO supplementation. In other words, mitochondrial biogenesis within skeletal muscles from endurance training was not affected at all by AO supplementation. Such findings were also confirmed through the endurance capacity test results (Fig. 5).

The biggest change in physical capacity from mitochondrial biogenesis within skeletal muscles is improved endurance capacity. Improved endurance is closely associated with increases in skeletal muscle respiratory capacity as a result of increased mitochondrial enzyme levels (1, 35). As shown in Fig. 5, endurance capacity test results showed that running time, distance, and total work increased after 4 wk of endurance training regardless of AO supplementation. Based on the aforementioned results, this study indicates that despite AO supplementation to inhibit oxidative stress induced by endurance training, the expression of upstream and downstream factors of PGC-1 α involved in mitochondrial biogenesis was not inhibited, nor was endurance capacity.

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