Antioxidant Supplementation Reduces Skeletal Muscle Mitochondrial Biogenesis

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ABSTRACT

STROBEL, N. A., J. M. PEAKE, A. MATSUMOTO, S. A. MARSH, J. S. COOMBES, and G. D. WADLEY. Antioxidant Supplementation Reduces Skeletal Muscle Mitochondrial Biogenesis. Med. Sci. Sports Exerc., Vol. 43, No. 6, pp. 1017–1024, 2011. Purpose: Exercise increases the production of reactive oxygen species (ROS) in skeletal muscle, and athletes often consume antioxidant supplements in the belief they will attenuate ROS-related muscle damage and fatigue during exercise. However, exercise-induced ROS may regulate beneficial skeletal muscle adaptations, such as increased mitochondrial biogenesis. We therefore investigated the effects of long-term antioxidant supplementation with vitamin E and α-lipoic acid on markers of mitochondrial biogenesis in the skeletal muscle of exercise-trained and sedentary rats. Methods: Male Wistar rats were divided into four groups: 1) sedentary control diet, 2) sedentary antioxidant diet, 3) exercise control diet, and 4) exercise antioxidant diet. Animals ran on a treadmill 4 d wk⁻¹ at ~70%VO₂max, up to 90 min d⁻¹ for 14 wk. Results: Consistent with the augmentation of skeletal muscle mitochondrial biogenesis and antioxidant defenses, after training there were significant increases in peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) messenger RNA (mRNA) and protein, cytochrome C oxidase subunit IV (COX IV) and cytochrome C protein abundance, citrate synthase activity, Nrf2, and SOD2 protein (P < 0.05). Antioxidant supplementation reduced PGC-1α mRNA, PGC-1α and COX IV protein, and citrate synthase enzyme activity (P < 0.05) in both sedentary and exercise-trained rats. Conclusions: Vitamin E and α-lipoic acid supplementation suppresses skeletal muscle mitochondrial biogenesis, regardless of training status. Key Words: α-LIPOIC ACID, EXERCISE TRAINING, MITOCHONDRIA, Nrf2, PGC-1α, VITAMIN E

A growing body of evidence highlights an important role for exercise-induced reactive oxygen species (ROS) in regulating cell signaling processes that contribute to adaptations in skeletal muscle after exercise training (1,10,14,30). One such process is mitochondrial biogenesis, a complex pathway resulting in an increase in mitochondrial content and density. This process is one of the main adaptations that occur in skeletal muscle after endurance exercise training. A central factor regulating mitochondrial biogenesis is peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) (13,28). PGC-1α coactivates the transcription factors nuclear respiratory factors 1 and 2 (NRF-1 and -2), which regulate nuclear encoded genes within the mitochondria. NRF-1 regulates mitochondrial transcrip-

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of PGC-1α and Tfam, and mitochondrial proteins such as cytochrome C (10). This reduction in markers of mitochondrial biogenesis is associated with a decrease in endurance capacity (10). In humans, supplementation with vitamins C and E for 4 wk also reduced exercise training–induced increases in the messenger RNA (mRNA) expression of mitochondrial biogenesis markers PGC-1α and PGC-1β (30). Importantly, on a functional level, the decrease in mitochondrial biogenesis that occurs after antioxidant supplementation translates to an attenuation in exercise training–induced improvements in insulin sensitivity (30).

Although previous rodent and human studies have provided compelling evidence that antioxidant supplementation prevents the increase in mitochondrial biogenesis with endurance training (10,30), it is possible that these findings have been somewhat misinterpreted because of the absence of an untrained antioxidant supplemented group in the study design. Indeed, it is possible that many of the effects previously observed with antioxidants preventing increased mitochondrial biogenesis with training (10,30) were not actually due to an attenuation of the exercise training response but to an overall down-regulation of basal mitochondrial biogenesis. To clarify this issue in greater detail, it is necessary to investigate potential changes in markers of mitochondrial biogenesis after long-term antioxidant supplementation in both sedentary and exercise-trained muscle.

ROS also activate the antioxidant response element (ARE). Nuclear factor E2–related factor 2, which is called Nfe212 (alternatively called Nrf2), is a transcription factor for the ARE and is considered an important protective factor against oxidative stress (15). ARE controls both basal and activated expressions of the antioxidant enzymes, mitochondrial superoxide dismutase (SOD2) and glutathione peroxidase (GPx), and the phase 2 detoxifying enzymes glutathione S-transferase (GST), NAD(P)H:quinone oxidoreductase 1, and heme oxygenase 1 (HO-1) (17). Currently, to our knowledge, there are no studies investigating the effects of antioxidant supplementation and/or exercise training on skeletal muscle Nfe212.

Therefore, the aim of this study was to investigate whether the effects of antioxidant supplementation on mitochondrial biogenesis and antioxidant defenses in skeletal muscle after endurance training are exercise dependent. To achieve this aim, we assessed the effects of antioxidant supplementation on changes in markers of mitochondrial biogenesis (PGC-1α and Tfam), mitochondrial proteins (cytochrome C, COX IV, citrate synthase, β-HAD), the activity of antioxidant enzymes (SOD2 and GPx), and abundance of the antioxidant transcription factor Nfe212 in skeletal muscle of both exercise-trained and sedentary rats.

METHODS

This experiment was approved by The University of Queensland Animal Ethics Committee in accordance with National Health and Medical Research Council guidelines of Australia and the animal care standards of the American College of Sports Medicine.

Animals. Male Wistar rats (n = 48) aged 10 wk and weighing on average 450 g were purchased from the Central Animal Breeding House (The University of Queensland, Australia). Rats were assigned to one of four groups: sedentary control diet (n = 12), sedentary antioxidant diet (n = 12), exercise control diet (n = 12), and exercise antioxidant diet (n = 12).

Antioxidant supplementation. Rats were fed powdered standard rat chow (Specialty Feeds, Western Australia, Australia) with or without 1000 IU of vitamin E·kg⁻¹ diet (α-tocopheryl succinate, Covitol 1185; Cognis, Melbourne, Australia) and 1.6 g of α-lipoic acid per kilogram diet (Lipoep; Cognis) for 14 wk. Rats were housed two to three rats per cage, maintained on a 12-h/12-h light–dark cycle, and provided with rat chow and tap water ad libitum. The standard rat chow contained a small amount of α-tocopherol (vitamin E), approximately 67 IU·kg⁻¹ diet and no α-lipoic acid. Vitamin E is a potent lipid-soluble antioxidant that scavenges free radicals and is capable of breaking the chain reactions of lipid peroxidation (5). α-lipoic acid is a water-soluble antioxidant that scavenges hydroxyl radicals, hypochlorous acid, and singlet oxygen and also recycles vitamin E (25). Together, these two antioxidants are recognized as a potent antioxidant combination (6,24).

Training protocol. All rats were familiarized with treadmill running for 5 d before placement within groups. Those rats that were willing to run were placed into the exercise groups; previous research has shown that this selection process is considered appropriate because health status and muscle physiology properties do not differ between those rats with a willingness to run and those animals reluctant to exercise (4). Exercising rats were trained 4 d·wk⁻¹ for 14 wk, which has previously been described (24). Exercise intensity was gradually increased until the rats could maintain a relative work rate of 70%VO₂max for 90 min. To encourage the rats to run, mild electric shocks and air compression were used sparingly.

All animals were euthanized at 62 ± 2 h (mean ± SEM) after the final training session to avoid the effects of acute exercise. Animals were weighed and killed with sodium pentobarbital (100 mg·kg⁻¹) using intraperitoneal injection. Red gastrocnemius muscle samples were excised and snap frozen in liquid nitrogen. Blood samples were collected into EDTA vacutainer tubes using aortic puncture and placed on ice. Samples were then centrifuged at 1500g for 10 min at 4°C and aliquotted. Plasma and muscle samples were stored at −80°C until analysis.

Preparation of tissue. Total RNA was extracted from frozen muscle by use of the Micro-to-Midi Total RNA Purification System kit and DNase on-column digestion (Invitrogen, Carlsbad, CA). For immunoblotting and mitochondrial enzyme activity, frozen muscle (10:1 buffer per milligram of muscle) was homogenized as previously described (37) in freshly prepared ice-cold buffer (50 mM Tris...
First-strand complementary DNA (cDNA) was generated from 0.5 μg of RNA using AMV Reverse Transcriptase (Promega, Madison, WI) (39). After reverse transcription, the remaining RNA was degraded by treatment with RNase H (Invitrogen, Australia) for 20 min at 37°C. The amount of single stranded DNA was then determined in each sample and compared against an oligonucleotide standard using OligoGreen reagent (Invitrogen, Australia), which was incubated in the dark at 80°C for 5 min before the measurement of fluorescence (29,38). The primer sequences were obtained from gene sequences from GenBank: PGC-1α, AY237127; Tfam, AB014089; Citrate Synthase, NM_130755; SOD2, NM_017051.2; and GPx1, NM_030826.3 (Table 1).

Real-time polymerase chain reaction using SYBR Green chemistry was performed using the sequence detector software (Rotor-Gene v6; Corbett Research, Sydney, Australia), as previously described (37). Samples were subjected to a heat dissociation protocol after the final cycle of polymerase chain reaction to ensure that only one product was detected. The mRNA of each gene was normalized to the cDNA chain reaction to ensure that only one product was detected.

Protein concentration was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL) with BSA as the standard.

**Reverse transcription–polymerase chain reaction analysis.** First-strand complementary DNA (cDNA) was generated from 0.5 μg of RNA using AMV Reverse Transcriptase (Promega, Madison, WI) (39). After reverse transcription, the remaining RNA was degraded by treatment with RNase H (Invitrogen, Australia) for 20 min at 37°C. The amount of single stranded DNA was then determined in each sample and compared against an oligonucleotide standard using OligoGreen reagent (Invitrogen, Australia), which was incubated in the dark at 80°C for 5 min before the measurement of fluorescence (29,38). The primer sequences were obtained from gene sequences from GenBank: PGC-1α, AY237127; Tfam, AB014089; Citrate Synthase, NM_130755; SOD2, NM_017051.2; and GPx1, NM_030826.3 (Table 1).

Real-time polymerase chain reaction using SYBR Green chemistry was performed using the sequence detector software (Rotor-Gene v6; Corbett Research, Sydney, Australia), as previously described (37). Samples were subjected to a heat dissociation protocol after the final cycle of polymerase chain reaction to ensure that only one product was detected. The mRNA of each gene was normalized to the cDNA content in each sample using the OligoGreen assay, as described above. This is a robust and suitable method of normalization that avoids the many problems associated with “housekeeping genes” (20,38).

**Western blot analysis.** Total lysates for measuring the protein content of PGC-1α, COX IV, cytochrome C, SOD2, and Nfe2l2 were solubilized in Laemmli sample buffer. Equal amounts of total protein were separated by sodium dodecyl sulfate - poly acrylamide gel electrophoresis (SDS-PAGE) and were electrotransferred from the gel to polyvinylidene fluoride (PVDF) membranes. Blots were probed with antibodies against PGC-1α rabbit polyclonal (Chemicon, Temecula, CA), cytochrome C mouse monoclonal (Invitrogen, Carlsbad, CA), COX IV mouse monoclonal (Invitrogen, Carlsbad, CA), SOD2 rabbit polyclonal (Abcam, Cambridge, MA), and Nfe2l2 rabbit polyclonal (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Binding was detected with an IRDye 800-conjugated antirabbit IgG secondary antibody (Rockland, Gilbertsville, PA) or an IRDye 680-conjugated antimouse IgG secondary antibody (Invitrogen, Carlsbad, CA). As a loading control, blots were then stripped and reprobed with anti-α-tubulin mouse monoclonal antibody (Sigma). Data were normalized to the level of α-tubulin, and all data are expressed as integrated intensity after infrared detection (Odyssey Imaging system; LI-COR Biosciences, Lincoln, NE).

**Antioxidants.** Plasma vitamin E concentration was analyzed using a modified high performance liquid chromatography (HPLC) method described by Katsanidis and Addis (16). Plasma samples were mixed with ethanol/butylated hydroxytoluene (BHT) (200 mg L⁻¹) and vortexed for 30 s. Hexane was added, and samples were gently mixed for 10 min and centrifuged at 4000g for 10 min. The hexane layer was removed, dried, and reconstituted with mobile phase containing hexane and isopropanol (99:1). Vitamin E concentrations were measured at 295 nm using HPLC (Shimadzu, Queensland, Australia) with a LiChrospher C18 column (250 × 4 mm, 5 μm, 1 mL min⁻¹ flow rate, 9-MPa back pressure; Merek, Rockland, MA).

Plasma α-lipoic acid concentration was not measured, as it is rapidly converted to various metabolites (36). Our group and others have found that α-lipoic acid is undetectable in the plasma of supplemented animals (23,24).

**Lipid peroxidation.** Malondialdehyde (MDA) content in muscle as a marker of lipid peroxidation was measured by HPLC using the methods of Sim et al. (32). Muscle homogenates (450 μg mL⁻¹) were hydrolyzed with 1.3 M NaOH. Samples were incubated at 60°C in a water bath for 60 min and then cooled on ice for 5 min. Proteins were then precipitated with 35% perchloric acid, cooled for a further 5 min, and centrifuged for 5 min at 3500g. The supernatant was removed, and 2,3-dinitrophenylhydrazine was added before incubation for 10 min in a dark room. The aqueous phase was extracted with hexane, evaporated, and reconstituted with mobile phase containing 45% acetonitrile and 0.2% glacial acetic acid. MDA content was measured

**Enzyme activities.** The activities of antioxidant enzymes GPx and SOD2 and of the pro-oxidant enzyme xanthine oxidase were measured in skeletal muscle. GPx was measured using a modified method for the Cobas Mira spectrophotometric analyzer (Roche Diagnostics, Basel, Switzerland) (2,40). The method used to measure SOD2 activity was modified from Oyanagui (26). Xanthine oxidase activity was measured using a modified version of the Amplex Red Xanthine/Xanthine Oxidase Assay Kit (Invitrogen, Carlsbad, CA). The activities of β-hydroxyacyl-CoA dehydrogenase (β-HAD) (37) and citrate synthase (33) were measured spectrophotometrically.

**Table 1. Primers for mRNA analyses.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGC-1α</td>
<td>ACCACACAGGATAGAACAAACC</td>
<td>GACAAATGCTTGTATTGTC</td>
</tr>
<tr>
<td>Tfam</td>
<td>AGCCCATGGGAGGAGTCTT</td>
<td>TTGTTACACCTCCACTGCTTTA</td>
</tr>
<tr>
<td>Citrate</td>
<td>GGCTCTGGATGAGTACCAA</td>
<td>TTGCGAGTCTTCTCTGATCT</td>
</tr>
<tr>
<td>SOD2</td>
<td>TGGACAACACTGAGCCCCTAA</td>
<td>GACCAAGATCGACCTGATA</td>
</tr>
<tr>
<td>GPx1</td>
<td>CGACACTGACACCCGATAGA</td>
<td>ATGGCTAGGGTGGTACAGG</td>
</tr>
</tbody>
</table>
at 310 nm using HPLC (Shimazu) with a LiChrospher C18 column (150 × 4.6 mm, 3 μm, 1 mL min⁻¹ flow rate, 9.8-MPa back pressure; Merck).

**Statistical analyses.** Data were checked for normality and log-transformed when required. All data were then normally distributed and analyzed using parametric techniques. Two-way ANOVA was then performed to determine the main effects of training and/or antioxidant supplementation. A finding for a main effect for antioxidants indicates that animals in both antioxidant groups, regardless of training status, were significantly different from control diet animals. A main effect for exercise also shows a significant change due to the training intervention, regardless of antioxidant supplementation. Significance was set at $P < 0.05$. Normalized data are presented as mean ± SE, whereas log-transformed data are presented as geometric mean ± 95% confidence intervals.

**RESULTS**

Antioxidant supplementation reduced the expression of PGC-1α mRNA and protein, COX IV protein, and citrate synthase activity in both exercise and sedentary groups ($P < 0.05$; main effect for antioxidant; Figs. 1 and 2). Exercise training significantly increased markers of mitochondrial biogenesis: PGC-1α mRNA and protein (Fig. 1),

![Figure 1](image1.png)

**FIGURE 1**—Effects of antioxidant supplementation and exercise training on PGC-1α mRNA (A) and PGC-1α protein content (B) in the skeletal muscle. Western blots are representative from one rat from each group. Values for PGC-1α mRNA and protein are geometric mean (95% confidence interval); sample sizes for each variable ranged from $n = 7$ to 10 for all groups. *$P < 0.05$, main effect for exercise. †$P < 0.05$, main effect for antioxidant.

![Figure 2](image2.png)

**FIGURE 2**—Effects of antioxidant supplementation and exercise training on citrate synthase mRNA (A) and citrate synthase activity (B) and COX IV (C) and Cyt C (D) protein content. Western blots are representative from one rat from each group. Values are mean ± SE for citrate synthase mRNA and activity, COX IV, and Cyt C protein; sample sizes for each variable ranged from $n = 7$ to 10 for all groups. *$P < 0.05$, main effect for exercise. †$P < 0.05$, main effect for antioxidant.
mitochondrial proteins, COX IV and cytochrome C protein expressions, citrate synthase activity (Fig. 2), and ARE transcription factor Nfe2l2 protein (Fig. 3; \( P < 0.05 \); main effect for training). Neither exercise training nor antioxidant supplementation altered Tfam (1.0 ± 0.8 arbitrary units normalized to cDNA for sedentary control diet vs 1.0 ± 0.8 for exercise control diet vs 1.0 ± 1.2 for sedentary antioxidant diet vs 1.0 ± 0.7 for exercise antioxidant diet). Lastly, there were no differences between groups for citrate synthase mRNA expression (Fig. 2). There were no significant interaction effects between exercise and antioxidants.

Antioxidant supplementation significantly increased plasma vitamin E concentration in both sedentary and trained animals (\( P < 0.05 \); main effect for antioxidant; Table 2). Exercise training significantly increased the muscle MDA content in both exercise groups (\( P < 0.05 \); main effect for exercise; Table 2), and antioxidant treatment did not attenuate this increase in MDA. For \( \beta \)-HAD activity, there were no significant changes (125 ± 14 \( \mu \)mol min \(^{-1} \) g protein \(^{-1} \) for sedentary control diet vs 145 ± 14 for exercise control diet vs 101 ± 15 for sedentary antioxidant diet vs 136 ± 18 for exercise antioxidant diet). There were no significant interaction effects between exercise and antioxidants for \( \beta \)-HAD activity.

The mRNA expression of GPx and SOD2 did not change significantly in any group, but antioxidant supplementation tended to reduce SOD2 (\( P = 0.08 \)) and GPx mRNA (\( P = 0.10 \); Figs. 3 and 4). Exercise training significantly increased SOD2 protein content, whereas it reduced SOD2 enzyme activity in both supplemented and nonsupplemented animals (\( P < 0.05 \); main effect for exercise; Fig. 4). GPx and XO activity remained unchanged in all groups (Table 2).

There were no significant interaction effects between exercise and antioxidants for GPx and SOD2 mRNA, SOD2 protein content and enzyme activity, and GPx and XO activity.

**DISCUSSION**

The major findings of this study were that vitamin E and \( \alpha \)-lipoic acid supplementation did not prevent the exercise-induced increase in skeletal muscle markers of mitochondrial biogenesis and mitochondrial proteins. However, importantly, consistent with a reduction in the basal regulation of mitochondrial biogenesis, the antioxidant supplementation lowered the overall levels of mitochondrial biogenesis markers, including PGC-1\( \alpha \) mRNA and protein, and mitochondrial proteins, such as COX IV protein and citrate synthase activity from both sedentary and exercise trained rats. Mitochondria have a critical role in the regulation of skeletal muscle metabolism and antioxidant defenses. The measurement of mitochondrial proteins (i.e., cytochrome C, COX IV, citrate synthase, \( \beta \)-HAD) and markers of mitochondrial biogenesis (i.e., PGC-1\( \alpha \), and Tfam) is widely used to indicate changes in the regulation of mitochondrial biogenesis and mitochondrial content after prolonged endurance training (10, 28, 35). Furthermore, changes in mitochondrial proteins such as COX or citrate synthase after 10–12 wk of exercise training are highly indicative of changes in mitochondrial mass/content and improvements in exercise capacity (7, 8, 12). Therefore, our findings suggest that prolonged antioxidant supplementation could potentially impair the endogenous metabolic and redox status of skeletal muscle in sedentary people and prevent some of the beneficial adaptations to

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**TABLE 2. Effects of exercise and antioxidant supplementation on skeletal muscle antioxidant enzymes, lipid peroxidation, and plasma vitamin E concentration.**

<table>
<thead>
<tr>
<th></th>
<th>Control Diet</th>
<th>Exercise</th>
<th>Antioxidant</th>
<th>Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPx activity (U mg (^{-1} ) protein)</td>
<td>1.85 ± 0.26</td>
<td>1.83 ± 0.24</td>
<td>1.70 ± 0.28</td>
<td>1.58 ± 0.24</td>
</tr>
<tr>
<td>Xanthine oxidase (U mg (^{-1} ) protein)</td>
<td>0.07 (0.05–0.1)</td>
<td>0.1 (0.07–0.2)</td>
<td>0.08 (0.04–0.2)</td>
<td>0.07 (0.04–0.1)</td>
</tr>
<tr>
<td>MDA (( \mu )M-450 ( \mu )g protein (^{-1} ))</td>
<td>4.8 ± 0.5</td>
<td>5.9 ± 0.6*</td>
<td>4.8 ± 0.3</td>
<td>5.7 ± 0.4*</td>
</tr>
<tr>
<td>Plasma vitamin E (( \mu )M)</td>
<td>19 (17–21)</td>
<td>19 (16–22)</td>
<td>24 (21–27)**</td>
<td>22 (19–25)**</td>
</tr>
</tbody>
</table>

Values are mean ± SE or geometric mean (95% confidence interval; lower–upper); sample sizes for each variable ranged from \( n = 8 \) to 12 for all.

* \( P < 0.05 \), main effect for exercise.

** \( P < 0.05 \), main effect for antioxidant.

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**ANTIOXIDANTS AND MITOCHONDRIAL BIOGENESIS**

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exercise training. Furthermore, for the first time, we can report that exercise training increased protein abundance of the ARE transcription factor, Nfe2l2, in skeletal muscle and that this change was not affected by antioxidant supplementation.

Our findings that the antioxidant combination vitamin E and α-lipoic acid reduce basal levels, independent of the exercise-induced increase in PGC-1α mRNA and protein, COX IV protein, and citrate synthase activity are the first to be reported. Only one previous human study, using a much shorter period of antioxidant supplementation, reported no effect of supplementation on basal levels of skeletal muscle mitochondrial biogenesis (11,30). Hellsten et al. (11) found that supplementation with vitamin C, coenzyme Q10, and vitamin E for 8 d did not alter PGC-1α gene expression in healthy males. Other previous studies reporting that antioxidant supplementation attenuated the exercise increase in mitochondrial biogenesis did not include a sedentary antioxidant treatment group (10,30). Including a sedentary antioxidant treatment group allows us to assess the effects of antioxidant treatment independently of the effects of exercise and therefore provides alternative insights into how ROS regulate mitochondrial biogenesis and antioxidant systems in skeletal muscle. Although exercise training adaptations still seem intact after 14 wk of training, the inclusion of the sedentary antioxidant treatment group suggests that antioxidants attenuate mitochondrial biogenesis in the long-term, in particular PGC-1α mRNA and protein, COX IV protein, and citrate synthase activity possibly through an alternate, unknown mechanism. Given that we did not find a reduction in exercise-induced oxidative stress (MDA), it suggests that this may be a nonantioxidant effect. However, the oxidant effect cannot be ruled out because the measurement of MDA levels may not be a particularly sensitive measure of oxidative stress. Nevertheless, this effect could be due to the reduction of other molecular mechanisms independent of oxidation that are regulating basal mitochondrial biogenesis through an effect on cell signaling. Our findings of similar changes in mitochondrial biogenesis after antioxidant supplementation in both sedentary and exercise-trained animals highlight the need to reinterpret the results of previous research on this topic.

The disparity between the human study described above and our data may also relate to the period of supplementation and the types of antioxidants used (11). The lack of effect of vitamins E and C on basal levels of mitochondrial biogenesis in the study by Hellsten et al. (11) may be due, in addition to the species differences, to the shorter 8-d period of supplementation, compared with 14 wk in our study. Vitamin C concentration is rapidly increased in the plasma (19) but importantly, is poorly taken up into skeletal muscle (27). The lipid-soluble vitamin E is more likely to accumulate in tissues, but vitamin E concentrations in skeletal muscle only plateau after 14 wk of supplementation (21). Therefore, in the study by Hellsten et al. (11), the 8-d supplementation protocol was probably not long enough to increase vitamin E concentration in skeletal muscle. Collectively, these data suggest that at least a few weeks of antioxidant supplementation are required to reduce basal PGC-1α expression in skeletal muscle. Regardless of the different antioxidants used in the present training study compared with those in previous studies (10,11,30), our findings suggest that correct interpretations are not possible without including an antioxidant sedentary control group.

A recent in vitro study has shown that PGC-1α is involved in the regulation of endogenous antioxidants (34). It has
been demonstrated that, when skeletal muscle cells are incubated with H₂O₂, PGC-1α gene expression increases, as does the expression of the antioxidant enzymes SOD1 (cytosolic), SOD2, GPx, and catalase. Furthermore, these increases are abolished when a PGC-1α gene silencer is applied (34). Consistent with a role for PGC-1α in the regulation of antioxidant enzymes, and our observation of reduced PGC-1α mRNA and protein after supplementation, we found a tendency for antioxidant supplements to also reduce the endogenous antioxidants SOD2 gene and protein and GPx1 gene and enzyme activity. Furthermore, a significant reduction in SOD2 activity in skeletal muscle resulting from antioxidant supplementation is consistent with previous findings showing reduced SOD2 activity with long-term vitamin E supplementation in the myocardium of sedentary and exercise-trained muscle (18). Therefore, although speculative, the tendency for reduced endogenous antioxidant enzymes in the present study was probably due to antioxidant supplementation reducing PGC-1α levels, although further studies are now required to confirm this.

A novel finding of the present study was that Nfe2l2 protein in skeletal muscle increased after exercise training. Previous studies have reported that Nfe2l2 increases in the nuclear fraction of the kidney after 6 and 12 wk of aerobic training in aged animals (3,9). Interestingly, in our study, these findings occurred regardless of antioxidant supplementation. Nfe2l2 is confined within the cytoplasm by a cytosolic inhibitor Keap1. When oxidized, Keap1 releases Nfe2l2, which moves into the nucleus to activate ARE genes (15). Evidence from knock-out mice has shown that Nfe2l2 is involved in the regulation of antioxidant enzymes such as SOD and phase 2 detoxifying enzymes including GST, and NAD(P)H:quinone oxidoreductase-1 in macrophages and cardiac fibroblasts (41). From our results, it is currently unclear whether Nfe2l2 signaling in skeletal muscle controls the ARE antioxidants, SOD2 and GPx, because although SOD2 protein expression increased with exercise training, there was no concomitant increase in the gene expression and enzyme activity of SOD2 or GPx. It is possible that Nfe2l2 is more critical to the regulation of phase 2 detoxifying enzymes than antioxidant enzymes in skeletal muscle. Indeed, supplementing rats with sulforaphane to chemically increase Nfe2l2 protein levels in skeletal muscle also upregulates GST and NAD(P)H:quinone oxidoreductase-1 protein levels and attenuates damage caused by exhaustive exercise (22). Further studies are now required to investigate the involvement of Nfe2l2 in the regulation of phase 2 detoxifying enzymes in skeletal muscle after endurance training.

In summary, we found that long-term antioxidant supplementation reduced markers of mitochondrial biogenesis in skeletal muscle independent of training status. These findings add to the growing body of evidence indicating potential negative effects of long-term antioxidant therapy on skeletal muscle. Further research is warranted to investigate the functional implications of long-term antioxidant supplementation not only in healthy people but also in people with long-term diseases that alter the metabolic and redox status of skeletal muscle.

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The authors have no conflicts of interest to declare.

The results of the present study do not constitute endorsement by the American College of Sports Medicine.

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