Normal adaptations to exercise despite protection against oxidative stress

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Submitted 30 November 2010; accepted in final form 17 June 2011

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Normal adaptations to exercise despite protection against oxidative stress. Am J Physiol Endocrinol Metab 301: E779–E784, 2011. First published July 12, 2011; doi:10.1152/ajpendo.00655.2010.—It has been reported that supplementation with the antioxidant vitamins C and E prevents the adaptive increases in mitochondrial biogenesis and GLUT4 expression induced by endurance exercise. We reevaluated the effects of these antioxidants on the adaptive responses of rat skeletal muscle to swimming in a short-term study consisting of 9 days of vitamins C and E with exercise during the last 3 days and a longer-term study consisting of 8 wk of antioxidant vitamins with exercise during the last 3 wk. The rats in the antioxidant groups were given 750 mg·kg body wt⁻¹·day⁻¹ vitamin C and 150 mg·kg body wt⁻¹·day⁻¹ vitamin E. In rats euthanized immediately after exercise, plasma TBARs were elevated in the control rats but not in the antioxidant-supplemented rats, providing evidence for an antioxidant effect. In rats euthanized 18 h after exercise there were large increases in insulin responsiveness of glucose transport in epitrochlearis muscles mediated by an approximately twofold increase in GLUT4 expression in both the short- and long-term treatment groups. The protein levels of a number of mitochondrial marker enzymes were also increased about twofold. Superoxide dismutases (SOD) 1 and 2 were increased about twofold in triceps muscle after 3 days of exercise, but only SOD2 was increased after 3 wk of exercise. There were no differences in the magnitudes of any of these adaptive responses between the control and antioxidant groups. These results show that very large doses of antioxidant vitamins do not prevent the exercise-induced adaptive responses of muscle mitochondria, GLUT4, and insulin action to exercise and have no effect on the level of these proteins in sedentary rats.

ascorbic acid; α-tocopherol; peroxisome proliferator-activated receptor-γ coactivator-1α; mitochondria; superoxide dismutase

Ristow et al. (23) interpreted their findings as evidence that antioxidant vitamins prevent exercise training-induced increases in insulin sensitivity and in skeletal muscle peroxisome proliferator-activated receptor-γ coactivator-1α, PGC-1α, PPARγ, and superoxide dismutases (SOD), which they referred to as “the health-promoting effects of physical exercise.” More recently, Strobel et al. (30) reported that oral administration of the antioxidants vitamin E and α-lipoic acid for 14 wk had no effect on the adaptive increases in mitochondrial proteins in response to exercise training but lowered the baseline levels of mitochondrial enzymes.

A large proportion of endurance athletes, including elite athletes, take vitamin supplements, often in large amounts (28). If taking ascorbic acid and α-tocopherol markedly reduced the effects of training on VO₂max and endurance (6) and prevented the adaptations in skeletal muscle that mediate increased endurance and the enhancement of insulin action responsible for glycogen supercompensation (6, 23), taking antioxidant vitamins would make competitive athletes noncompetitive. It seems unlikely that a phenomenon of this magnitude would not have been noticed over the long period that these vitamins have been available. It also seems improbable that an effect of this magnitude would not have been recognized in the numerous training studies that have been reported. Training studies conducted to determine whether antioxidant vitamins improve exercise performance and/or enhance the effects of training on VO₂max and exercise capacity have generally not shown an improvement in performance (4, 14, 18). However, they have also not shown any reduction of the adaptive response, which argues against the conclusions of Gomez-Cabrera et al. (6), Ristow et al. (23), and Strobel et al. (30). Antioxidant vitamins do have a protective effect against exercise-induced oxidative stress (27), and therefore, it is possible that antioxidant vitamins could have a protective effect against cumulative effects of strenuous exercise-induced free radical damage to heart and skeletal muscle.

In this context, we performed the present study to evaluate the claim that “antioxidants prevent health-promoting effects of physical exercise” (23). Our study was conducted on rats. However, while it was in progress, Yfantl et al. (36) had an article published on the effect of antioxidant vitamins on adaptations to endurance training in humans. Taken together, the results of the study by Yfantl et al. (36) and the present study provide evidence that taking antioxidant vitamins has no effect on the adaptive responses to endurance exercise training.

METHODS

Animals and Exercise Program

This protocol was approved by the Animal Studies Committee of Washington University School of Medicine. Male Wistar rats weighing 170–190 g were obtained from Charles River (Wilmington, MA) and maintained on a diet of powdered Purina Chow and water. The animals were assigned to four groups: antioxidant sedentary (AO-
Sed), antioxidant exercise (AO-EX), control-sedentary (Control-Sed), and control exercise (Control-Ex). The rats in the antioxidant groups were given 750 mg/kg ascorbic acid and 150 mg/kg vitamin E by feeding tube at 8 AM daily for 9 days, whereas the control group was given the vehicle (200 mg of triolein and 20 mg of Tween in 1 ml of saline) by feeding tube (24). In the short-term study, rats were given the antioxidants for 9 days. On the 5th and 6th days, the rats were accustomed to swimming for 15 min/day. For the last 3 days of antioxidant treatment, the animals in the exercise groups were exercised by means of a swimming program consisting of 2-3 h bouts of swimming separated by 45 min of rest (21) starting at 10 AM. In the longer study, rats were given the ascorbic acid and vitamin E for 8 wk and exercised by means of the swimming program for 6 days/wk for the last 3 wk of antioxidant treatment.

**Indicators of Oxidative Stress**

Rats were lightly anesthetized with isoflurane, and blood samples were obtained from the tail vein immediately after the last exercise bout for measurement of thiobarbituric acid-reactive substances (TBARS). Plasma TBARS were measured fluorometrically using a Quantichrom TBARS Assay Kit (Bioassay System).

**Measurement of Glucose Transport Activity**

Eighteen hours after the last exercise bout, and after an overnight fast, rats were anesthetized with 5 mg/100 g body wt of pentobarbital sodium, and the epitrochlearis and triceps muscles were dissected out. The epitrochlearis was used for measurement of insulin responsiveness of glucose transport, and the triceps muscles were frozen. The epitrochlearis muscles were incubated with shaking for 60 min at 30°C in 2 ml of oxygenated Krebs-Henseleit buffer (KHB) in Erlenmeyer flasks gassed continuously with 95% O2-5% CO2. The epitrochlearis is a small, thin muscle of the forelimb that in rats of the size used in this study is suitable for measurement of glucose transport in vitro (11). The KHB was supplemented with 8 mM glucose, 32 mM mannitol, and 0.1% radioimmunoassay-grade BSA in the presence or absence of 2 μM purified porcine insulin. The epitrochlearis is a small, thin muscle of the forelimb that in rats of the size used in this study is suitable for measurement of glucose transport in vitro (11). The KHB was supplemented with 8 mM glucose, 32 mM mannitol, and 0.1% radioimmunoassay-grade BSA in the presence or absence of 2 μM purified porcine insulin. This concentration of insulin maximally activates glucose transport in this muscle preparation. To remove glucose, muscles were then washed for 10 min at 30°C in KHB containing 40 mM mannitol and 0.1% BSA, plus insulin if it was present in the previous incubation, and used for measurement of glucose transport activity. Glucose transport activity was measured using the glucose analog 2-deoxyglucose, as described previously (9, 38). After the wash, epitrochlearis muscles were incubated at 30°C for 20 min in 1.0 ml of KHB containing 4 mM [1,2-3H]2-DG (1.5 μCi/ml), 36 mM [14C]mannitol (0.2 μCi/ml), 0.1% BSA, and insulin if present in previous incubations. Extracellular space and intracellular 2-DG concentration were determined as described previously (38).

**Western Blotting**

Muscles were homogenized in ice-cold buffer containing the following: 50 mM Tris HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA (pH 7.4), 1 mM each of Pefabloc (Roche), EDTA, and NaF. 1 μg/ml each of aprotinin, leupeptin, and pepstatin, 0.1 mM bpV(phen), and 2 mg/ml β-glycerophosphate. Homogenates were sonicated on ice for 15 s and then centrifuged for 10 min at 1,500 g at 4°C. Protein concentration was determined by using the Lowry method (17). Aliquots were solubilized in Laemmli buffer, subjected to SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature in Tris-buffered saline with 0.1% Tween containing 5% nonfat dry milk. Membranes were incubated overnight at 4°C with the following antibodies: cytochrome oxidase (COX) subunit I (monoclonal), COX-IV (monoclonal), succinate-ubiquinone oxidoreductase 30-kDa subunit (SUO; monoclonal), NADH-ubiquinone oxidoreductase (NADH-UO; monoclonal; Invitrogen, Carlsbad, CA), PGC-1α (monoclonal; EMD Chemicals), CuZnSOD (SOD1, monoclonal; Santa Cruz Biotechnology), and MnSOD (SOD2, monoclonal; Cell Signaling Technology). The GLUT4 antibody (polyclonal) was a gift from Mike Mueckler (Washington University School of Medicine), the long-chain acyl-CoA dehydrogenase (LCAD; polyclonal) was a gift from Dan Kelly (Washington University School of Medicine), and β-actin (monoclonal) was purchased from Abcam (Cambridge, UK). The blots were then incubated with the appropriate horseradish peroxidase-conjugated anti-IgG antibody. Antibody-bound protein was detected using enhanced chemiluminescence and quantified by densitometry.

**Statistical Analysis**

Values are expressed as means ± SE. Analysis of differences between groups was performed with two-way analysis of variance and Bonferroni post hoc test for multiple comparisons when appropriate. A P value of <0.05 was considered significant. The statistical software SigmaStat (Systat Software, San Jose, CA) was used.

**RESULTS**

**Nine-Day Study**

**Effect of antioxidant supplements on exercise-induced oxidative stress.** The last exercise bout resulted in an 80% increase in plasma TBARS, which were used as an indicator of oxidative stress (Fig. 1) (16). The increase in TBARS was prevented in the rats given ascorbic acid and α-tocopherol, providing evidence that this antioxidant treatment was effective in protecting against the exercise-generated oxidative stress.

**Adaptive response of SOD to the exercise.** Expression of both SOD1 and SOD2 in skeletal muscle (triceps) increased in response to the 3 days of exercise (Fig. 2A). The antioxidant supplement had no effect on the adaptive increase in SOD proteins induced by exercise.

**Effects of exercise on expression of PGC-1α and mitochondrial proteins.** The expression of PGC-1α (Fig. 2B), five proteins of the mitochondrial electron transport chain, and long-chain acyl-CoA dehydrogenase (Fig. 2C), a marker for the mitochondrial fatty acid oxidation pathway, increased approximately two- to threefold in response to the exercise with no effect of antioxidant treatment. This finding provides evidence that inhibition of oxidative stress does not affect the increase in mitochondrial biogenesis induced by exercise.

**Fig. 1. The antioxidant vitamin supplementation prevented an exercise-induced increase in thiobarbituric acid-reactive substances (TBARSs) in plasma. Values are means ± SE for 3 rats in each of the sedentary (Sed) groups and for 6 rats in each of the exercised (Ex) groups. *P < 0.05 vs. other groups.**
Adaptive increases in the GLUT4 glucose transporter and insulin responsiveness of glucose transport in muscle. As shown in Fig. 3A, there was a more than twofold increase in GLUT4 protein in triceps muscle in response to the 3 days of exercise training. The antioxidant supplements had no influence on this adaptive response. Insulin responsiveness of the glucose transport process increased to about the same extent as GLUT4 in the exercise groups, with no effect of the antioxidant supplements (Fig. 3B).

Eight-Week Study

Adaptive responses of SOD and mitochondrial proteins to the exercise training. In contrast to 3 days of exercise, which resulted in increased expression of both SOD1 and SOD2, only SOD2 expression was increased in triceps muscle after 3 wk of training (Fig. 4A). Expression of five mitochondrial respiratory chain proteins, citrate synthase, and long-chain acyl-CoA dehydrogenase was increased two- to threefold in triceps muscles after 3 wk of swimming training, providing evidence of an increase in mitochondrial biogenesis (Fig. 4B).

GLUT4 adaptive increases and insulin responsiveness. The exercise-induced increases in GLUT4 expression and insulin-stimulated glucose transport activity were unaffected by the antioxidant vitamin treatment (Fig. 5).

DISCUSSION

Two major adaptive responses of skeletal muscle to endurance exercise are an increase in mitochondria and an increase in GLUT4 expression. As shown in Fig. 3A, there was a more than twofold increase in GLUT4 protein in triceps muscle in response to the 3 days of exercise training. The antioxidant supplements had no influence on this adaptive response. Insulin responsiveness of the glucose transport process increased to about the same extent as GLUT4 in the exercise groups, with no effect of the antioxidant supplements (Fig. 3B).

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in expression of the GLUT4 glucose transporter (13, 22). An important consequence of the increase in GLUT4 is a proportional increase in insulin responsiveness of the glucose transport process in skeletal muscle (22). Both the increase in mitochondrial biogenesis and the increase in GLUT4 expression induced by exercise are mediated by PGC-1α protein, which increases rapidly in response to a bout of exercise (1, 2, 26, 31). PGC-1α binds to and activates the transcription factors that regulate transcription of genes that encode mitochondrial proteins and GLUT4 (2, 19, 34, 35). As shown by both our short- and longer-term studies, large doses of antioxidant vitamins do not have any effect on the increase in insulin responsiveness that is mediated by the exercise-induced increase in GLUT4.

Gomez-Cabrera et al. (6) reported that giving men 1.0 g/day ascorbic acid markedly blunted the increase in $V\dot{O}_2$ max in response to an 8-wk exercise program (with a 22% increase in controls and an 11% increase in the ascorbic acid group). They also reported that giving rats 500 mg·kg body wt$^{-1}$·day$^{-1}$ ascorbic acid powerfully prevented the adaptive response to a program of treadmill running, with a 2.8-fold increase in running endurance in the controls and only a 26% increase in the ascorbic acid group. The ascorbic acid completely blocked exercise training-induced increases in MnSOD, PGC-1α, and the transcription factors NRF1 and TFAM, which showed highly significant increases in the control rats. The only measurement of mitochondrial biogenesis made by Gomez-Cabrera et al. (6) was cytochrome $c$, and this finding is difficult to interpret, because they said that they measured "cytosolic cytochrome $c$" and because they reported that cytochrome $c$ increased 2.5-fold in the controls but not significantly in vitamin C group, although their Fig. 4 shows an approximately twofold increase in the vitamin C group.
Our results disagree with those of Gomez-Cabrera et al. (6). A dose of ascorbic acid modestly higher in terms of body weight than that used by Gomez-Cabrera et al. (6) plus α-tocopherol prevented the increase in plasma TBARs that we used as a marker of oxidative stress. However, the antioxidant treatment had absolutely no effect on the large, exercise-induced increases in SOD1, SOD2, PGC-1α, or a number of mitochondrial marker proteins. While this study was in progress, Wadley and McConnell (33) reported that giving rats the same dose of vitamin C used by Gomez-Cabrera et al. (6) had no effect on the early adaptive response of skeletal muscle mitochondrial biogenesis to a single bout of treadmill running. The variables measured immediately after exercise included phosphorylation/activation of p38 MAPK, AMP-activated protein kinase, and activating transcription factor 2, which have been shown to mediate PGC-1α activation and increased expression. The ascorbic acid treatment also did not prevent increases in mRNA levels of SOD1 and SOD2, PGC-1α, NRF1, and NRF2 measured 4 h after exercise. These findings fit well with ours. However, in a more recent study, Strobel et al. (30) reported that 14 wk of oral administration of large doses of the antioxidants vitamin E and α-lipoic acid lowered mitochondrial enzyme levels in sedentary rats but did not prevent an exercise-training induced increase in mitochondrial enzymes.

The study by Gomez-Cabrera et al. (6) was followed by publication of a study on healthy young men by Ristow et al. (23) in which adaptive responses to 4 wk of exercise training were compared in groups of men taking either a placebo or 1.0 g ascorbic acid and 400 IU/day vitamin E, a dose that has been found to be optimal for preventing an exercise-training induced increase in mitochondrial enzyme levels in sedentary rats but did not prevent an exercise-training induced increase in mitochondrial enzymes. The study by Gomez-Cabrera et al. (6) was followed by publication of a study on healthy young men by Ristow et al. (23) in which adaptive responses to 4 wk of exercise training were compared in groups of men taking either a placebo or 1.0 g ascorbic acid and 400 IU/day vitamin E, a dose that has been found to be optimal for preventing an exercise-training induced increase in mitochondrial enzyme levels in sedentary rats but did not prevent an exercise-training induced increase in mitochondrial enzymes.

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ACKNOWLEDGMENTS

We are grateful to Victoria Reckamp for help with preparation of this manuscript.

GRANTS

This research was supported by National Institute on Aging Grant AG-00425. K. Higashida was supported by a Research Fellow of the Japan Society for the Promotion of Science Award. S. H. Kim was supported by a Keimyung University Postdoctoral Fellowship.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES