Effect of antioxidant supplementation on insulin sensitivity in response to endurance exercise training

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1Centre of Inflammation and Metabolism, Department of Infectious Diseases and CMRC, Rigshospitalet, Faculty of Health Sciences; 2Molecular Physiology Group, Department of Exercise and Sport Sciences, Section of Human Physiology; and 3Section of Biomedicine, Department of Disease Biology, Faculty of Life Sciences, University of Copenhagen, Copenhagen, Denmark

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Yfantì C, Nielsen AR, Åkerström T, Nielsen S, Rose AJ, Richter EA, Lykkesfeldt J, Fischer CP, Pedersen BK. Effect of antioxidant supplementation on insulin sensitivity in response to endurance exercise training. Am J Physiol Endocrinol Metab 300: E761–E770, 2011. First published February 15, 2011; doi:10.1152/ajpendo.00207.2010.—While production of reactive oxygen and nitrogen species (RONS) is associated with some of the beneficial adaptations to regular physical exercise, it is not established whether RONS play a role in the improved insulin-stimulated glucose uptake in skeletal muscle obtained by endurance training. To assess the effect of antioxidant supplementation during endurance training on insulin-stimulated glucose uptake, 21 young healthy (age 29 ± 1 yr, BMI 25 ± 3 kg/m2) men were randomly assigned to either an antioxidant [AO; 500 mg vitamin C and 400 IU vitamin E (α-tocopherol) daily] or a placebo (PL) group that both underwent a supervised intense endurance-training program 5 times/wk for 12 wk. A 3-h euglycemic-hyperinsulinemic clamp, a maximal oxygen consumption (V̇O2max) and maximal power output (Pmax) test, and body composition measurements (fat mass, fat-free mass) were performed before and after the training. Muscle biopsies were obtained for determination of the concentration and activity of proteins regulating glucose metabolism. Although plasma levels of vitamin C (P < 0.05) and α-tocopherol (P < 0.05) increased markedly in the AO group, insulin-stimulated glucose uptake increased similarly in both the AO (17.2%, P < 0.05) and the PL (18.9%, P < 0.05) group in response to training. V̇O2max and Pmax also increased similarly in both groups (time effect, P < 0.0001 for both) as well as protein content of GLUT4, hexokinase II, and total Akt (time effect, P ≤ 0.05 for all). Our results indicate that administration of antioxidants during strenuous endurance training has no effect on the training-induced increase in insulin sensitivity in healthy individuals.

vitamin C; vitamin E; glucose uptake

Recently, it was shown that a high-fat diet can induce insulin resistance by increasing the skeletal muscle mitochondrial production of hydrogen peroxide (1). Conversely, shifting the balance back toward antioxidant defense by dietary administration of antioxidants partially improves health indexes including insulin-stimulated glucose disposal in individuals with type 2 diabetes (20), and, in particular, combined vitamin E and C supplementation may improve insulin-stimulated glucose metabolism in insulin-resistant, elderly individuals (41).

On the other hand, endurance exercise is also associated with the generation of reactive oxygen and nitrogen species (RONs) by the mitochondria as by-products of oxidative metabolism. Despite this, regular physical exercise promotes numerous health benefits, protects against all caused mortality (4), and plays a critical role in the treatment of a number of chronic diseases, such as type 2 diabetes (34). Accordingly, endurance exercise training has been shown to improve insulin resistance in patients with type 2 diabetes (15, 28) and enhances both insulin-stimulated and non-insulin-mediated glucose uptake in skeletal muscle (15, 16). The enhanced insulin-stimulated glucose disposal by skeletal muscle occurs as a result of increased protein expression of hexokinase II (HKII), glucose transporter 4 (GLUT4) (21, 39, 52) and increased expression or activity of several other molecules involved in the insulin cascade (21, 39, 52).

There has been considerable evidence that exercise-induced RONS production plays an important role in the regulation of signaling pathways (13, 37). In particular, both in vitro (2) and in vivo (11) experiments have shown that nitric oxide (NO), essential for the formation of RNS, acts as a stimulus for exercise-mediated skeletal muscle glucose uptake, indicating a possible mechanism of the enhanced insulin sensitivity in response to exercise training.

The RONS-dependent expression (44) of the transcription factor peroxisome proliferator-activated receptor-γ (PPARγ) and its coactivator-1α (PGC-1α), which increase the expression of genes involved in mitochondrial biogenesis and oxidative phosphorylation (10), has been shown to be reduced in type 2 diabetes patients (33). In contrast, endurance training has been suggested to increase the expression of both PPARγ (45) and PGC-1α (42, 48), although there have been some controversial results (35, 49). Furthermore, overexpression of skeletal muscle PGC-1α increases insulin-stimulated glucose disposal both in insulin-resistant (6) and in healthy (7) rats.

Recently, the hypothesis that antioxidant supplementation during endurance training may attenuate some of the beneficial effects of endurance exercise has been suggested. For instance, Gomez-Cabrera et al. (23) found that vitamin C supplementation in rodents attenuates training-induced mitochondrial biogenesis and endurance capacity. Another recent study showed that antioxidant supplementation with vitamins C and E inhibits the beneficial effects of 4 wk of training on insulin sensitivity and other markers of metabolic training adaptation (40). Moreover, when a 6-wk aerobic exercise training program is applied in patients with hypertension, supplementation with antioxidants (vitamins C and E, and α-lipoic acid) is associated with elevated blood pressure and inhibition of exercise-induced flow-mediated vasodilatation (50). In support of this hypothesis, experi-
ments in mice have shown that when the antioxidant enzyme glutathione peroxidase (GPX) is overexpressed, the animals become obese and develop insulin resistance (32), whereas in case of lacking this enzyme, they are protected from high-fat diet-induced insulin resistance (45), both indicating the importance of RONS on insulin signaling and the deleterious effects of blocking their action. Thus, even though antioxidant treatment may improve glucose metabolism in patients with type 2 diabetes, it may also inhibit other positive health benefits of regular physical activity.

To examine whether antioxidant treatment affects the adaptive increase in insulin sensitivity with regular physical activity, we conducted a training study using a highly demanding training protocol in order to achieve the maximum possible increase in insulin sensitivity. We hypothesized that administration of vitamins C and E during training would attenuate the expected increases in insulin sensitivity in healthy young individuals.

MATERIALS AND METHODS

Ethical approval. The study was approved by the local Ethics Committee of Copenhagen and Frederiksberg (KF 01 289434) and was in accordance with the Declaration of Helsinki. The purpose of the study and possible risks and discomforts were explained to the participants before written consent was obtained.

Participants. Twenty-one young, healthy, physically active men (age 18–40 yr) participated in the study. Participants’ characteristics are listed in Table 1. Before inclusion in the study, participants underwent a medical examination with blood test screening, an oral glucose tolerance test (OGTT), and a maximal power (Pmax) test using an electrically braked cadence-independent cycle ergometer (Monark 839E; Monark, Varberg, Sweden). Exclusion criteria included physical exercise more than 2–3 times/wk, body mass index (BMI) > 30 kg/m², smoking, impaired glucose tolerance, use of medication, and supplementation with antioxidants.

Supplementation. A double-blinded design was employed, and the participants were allocated to either the Antioxidant (AO) (n = 11) or the Placebo (PL) (n = 10) group by using a minimization model (47) according to age, BMI, and Pmax. Participants in the AO group received oral supplementation with vitamin C (ascorbic acid, 500 mg daily) and vitamin E (RRR-α-tocopheryl succinate, 400 IU daily) for 16 wk, while the PL group received placebo tablets. The employed supplement dosages of vitamin C and vitamin E were ~5 and ~15 times higher, respectively, than the recommended dietary allowances. Of note, this combination of vitamins C and E has previously been shown to attenuate oxidative stress following acute exercise (19). All participants were instructed to take the supplementation once a day before a breakfast. The supplementation started 4 wk before onset of the training and continued through the whole training period.

Pmax and VO₂max. VO₂max was measured during an incremental exercise test on an electrically braked cadence-independent cycle ergometer (Monark 839E) using an indirect calorimetry system (Quark b2; CosMed, Rome, Italy). After a 5-min warm-up at 40% of VO₂max, the workload increased every minute by 1/10 of 60% of expected VO₂max (12). The expected VO₂max was calculated on the basis of the Pmax obtained from the screening test. The test was terminated when the participant was unable to maintain a cadence of 60 rpm for more than 15 s despite verbal encouragement. Expired oxygen and carbon dioxide were recorded online. VO₂max tests were performed before and after the training period.

The Pmax test was performed in the same way as the VO₂max test without recording of expired gases. A Pmax test was performed at the prescreening for allocation of the subjects to the two groups and at the beginning of each training week to determine the intensity of the training for the following days of the week.

Training protocol. The mode of exercise selected for training was cycling, and the training frequency was five times a week for 12 wk. A Pmax test was performed every Monday to determine the intensity of the training for the following days of the week. Every Tuesday and Thursday, the training consisted of intervals (intensity 75–91% Pmax and duration 60–80 min) and every Wednesday and Friday of continuous biking (intensity 55–66% Pmax and duration 85–155 min). The participants were allowed to miss only 5% of the total amount of training, which was equal to 3 training days. In case they had to refrain from training for more than 3 days, the missing training was performed during the weekend.

Euglycemic-hyperinsulinemic clamp. The participants reported at the laboratory at 8:00 AM after an overnight fast. An intravenous catheter was placed in an antecubital vein of one arm for infusion of insulin and glucose. A second intravenous catheter was placed in a dorsal hand vein of the contralateral arm for blood sampling. The catheterized hand was wrapped in a heating blanket to obtain arterialized venous blood samples for measurement of glucose and potassium concentrations during the clamp. After baseline blood samples had been obtained, infusion of insulin (100 IU/ml Actrapid; Novo Nordisk Insulin, Copenhagen, Denmark) started at a constant rate of 80.0 mU·min⁻¹·m⁻² body surface area. Euglycemia (5 mM) was achieved by coinfusion of glucose (200 g/l·1,000 ml) at a variable rate. To maintain potassium at normal value, isotonic saline with potassium (51 meq/l) was coinfused if needed. Arterialized blood was analyzed for plasma glucose and potassium concentrations every 10 min. Euglycemic-hyperinsulinemic clamps were performed before and >72 h after the last training bout of the training period.

Blood samples. Blood samples were drawn at baseline and at time points 60, 120, and 180 min after initiation of the clamp. The blood samples were drawn into EDTA-containing glass tubes, and they were immediately centrifuged at 3,500 g for 15 min at 4°C. Plasma samples were then stored in −80°C until analyzed.

Muscle biopsies. Muscle biopsies from vastus lateralis were taken at time points 0 and 180 min during the insulin clamp, before and after the training period. All tissue samples were obtained using the percutaneous needle method with suction (8) under local anesthesia of the skin and fascia, using 3–5 ml of 20 mg/ml Lidocaine (SAD Nordisk Insulin, Copenhagen, Denmark) started at a constant rate of 80.0 mU·min⁻¹·m⁻² body surface area. Muscle tissue was immediately frozen in liquid nitrogen and stored at −80°C until further analysis.

Body composition. Whole body fat and fat-free tissue mass were estimated using a dual-energy X-ray absorptiometry (DEXA) scanner with software version 8.8 (Lunar Prodigy; GE Medical Systems).

Laboratory analysis: plasma. Plasma insulin was measured with a singleplex assay for human insulin using electrochemiluminescence (product no. K151BZC; Meso Scale Discovery) according to the manufacturer’s instructions. The lower limit of detection is 6.0 pg/ml. The mean coefficient of variance was <10% between duplicates.

Plasma concentration of free fatty acids (FFA) was determined in duplicates using an automatic analyzer (Cobas fara, Roche; FFA: NEFA C; Wako Chemicals, Neuss, Germany). High-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides (TG) were determined using standard laboratory procedures in the routine chemistry laboratory.
procedures (Department of Clinical Biochemistry, Rigshospitalet University Hospital, Copenhagen, Denmark).

**RNA isolation and quantitative real-time PCR.** Skeletal muscle biopsies were homogenized in TRIzol Reagent (Invitrogen, Carlsbad, CA) using a motor-driven homogenizer (Polytron, Kinematica, Newark, NJ), and total RNA was isolated according to the manufacturer’s protocol. Total RNA was dissolved in RNase-free water, quantified spectrophotometrically at 260 nm, and reverse-transcribed using reverse transcription reagents (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. Random hexamers were used for first-strand cDNA synthesis. The mRNA levels of PPARγ and PGC-1α, and the endogenous control 18S RNA were determined by real-time RT-PCR using an ABI PRISM 7900HT sequence detector (Applied Biosystems).

Primers and MGB probes were designed using Primer Express software (Applied Biosystems) or obtained using the Universal Probe Library (Roche Applied Science).

Primers and probes were premixed with TaqMan Universal Master Mix or SYBR Green PCR Master Mix (Applied Biosystems) and distributed into 384-well MicroAmp optical plates (Applied Biosystems). cDNA aliquots of 3 μl were added in triplicates. The amplification of genomic DNA typically amounted to a maximum of <1% of the target gene when the TRIzol protocol was used. A twofold dilution series was made from a pooled sample of a small part of all the samples. This was run on each plate together with the samples and used to construct a standard curve from which the mRNA content of the unknown samples. The relative expressions of PPARγ and PGC-1α were determined after normalization to the endogenous control 18S.

**Muscle lysate preparation.** Twenty-five milligrams of skeletal muscle was used for protein extraction. Muscle lysates were prepared adding 500 μl of ice-cold homogenization buffer [50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM PMSF, 1 mM diethiothreitol, 0.5% (vol/vol) protease inhibitor cocktail and 1% (vol/vol) Nonidet P-40] to muscle tissue. The muscle tissue was then homogenized using cooled racks in a TissueLyser (Qiagen) for 1 min at 20 Hz followed by a 15-min incubation on ice. The procedure was repeated twice and, if there were still visible particles of the tissue, a third time. Homogenates were then rotated end over end for 1 h at 4°C and centrifuged at 13,000 g at 4°C for 15 min. The supernatant was taken and stored at −80°C. An aliquot of 10 μl of each lysate was taken and diluted for protein concentration determination prior to storage.

**Western blotting.** Equal amounts of denatured proteins (27) from the tissue homogenates were separated by gel electrophoresis using a NuPage 8% Bis-Tris gel (Invitrogen, Taasstrup, Denmark), followed by immunoblotting to PVDF membranes (Hybond-P, GE Healthcare). Membranes were then incubated in blocking buffer (skimmed milk) for 1 h at room temperature to reduce background signal. Subsequently, the membranes were incubated with primary and secondary antibodies for optimized times and concentrations and washed with Tris-buffered saline containing 0.1% Tween-20. Primary antibodies used were anti-Akt substrate of 160 kDa (AS160; catalog no. 2447, Cell Signaling Technology), anti-phospho-Ser473-Akt (catalog no. 9611, Cell Signaling Technology), anti-Akt (catalog no. 9272, Cell Signaling Technology), anti-GLUT4 (catalog no. PA1-1065, Affinity Bioreagents), and anti-HKII (catalog no. 6521, Santa Cruz Biotechnology). Secondary antibodies were from DakoCytomation (Denmark). Protein bands were detected using Supersignal West Femto (Pierce) and quantified using a CCD image sensor (ChemidocXRS, Bio-Rad) and software (Quantity One, Bio-Rad).

Preliminary experiments demonstrated that the amounts of protein loaded were within the dynamic range for the conditions used and the

Table 2. Changes in vitamin plasma concentration over time

<table>
<thead>
<tr>
<th></th>
<th>Antioxidant Group (n = 11)</th>
<th>Placebo Group (n = 10)</th>
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<tbody>
<tr>
<td></td>
<td>Presupplementation</td>
<td>Pretraining</td>
</tr>
<tr>
<td>Vitamin C (μmol/l)</td>
<td>53.8 (42.2–65.4)</td>
<td>82.0 (68.6–95.4)*</td>
</tr>
<tr>
<td>Vitamin E (μmol/l)</td>
<td>17.0 (15.1–19.1)</td>
<td>25.4 (22.0–28.0)*</td>
</tr>
</tbody>
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Values are presented as means ± 95% CI in μmol/l. *P < 0.05 vs. presupplementation values.

Table 3. Changes before and after 12 wk of endurance training

<table>
<thead>
<tr>
<th></th>
<th>Antioxidant Group (n = 11)</th>
<th>Placebo Group (n = 10)</th>
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<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Fitness V̇O₂max, ml kg⁻¹ min⁻¹</td>
<td>50.4 (45.8–55.0)</td>
<td>58.9 (53.8–64.0)*</td>
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<tr>
<td>Body composition</td>
<td></td>
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<tr>
<td>Fat mass, kg</td>
<td>15.2 (9.1–21.3)</td>
<td>13.9 (8.8–18.9)</td>
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<tr>
<td>Fat-free mass, kg</td>
<td>60.2 (55.0–65.3)</td>
<td>61.5 (57.4–65.7)</td>
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<tr>
<td>Body weight, kg</td>
<td>79.7 (71.5–87.9)</td>
<td>78.2 (70.8–85.7)</td>
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<tr>
<td>Metabolic parameters</td>
<td></td>
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<tr>
<td>HDL, mmol/l</td>
<td>1.4 (1.3–1.6)</td>
<td>1.7 (1.5–1.9)*</td>
</tr>
<tr>
<td>LDL, mmol/l</td>
<td>2.5 (2.1–2.9)</td>
<td>2.6 (2.3–3.0)</td>
</tr>
<tr>
<td>TG, mmol/l</td>
<td>0.9 (0.7–1.2)</td>
<td>1.0 (0.7–1.3)</td>
</tr>
<tr>
<td>FFA, μmol/l</td>
<td>414.7 (280.8–541.5)</td>
<td>339.4 (204.3–474.5)</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>5.3 (5.0–5.7)</td>
<td>5.4 (5.1–5.8)</td>
</tr>
</tbody>
</table>

Values are presented as means ± 95% CI. TG, triglycerides; FFA, free fatty acids. *P < 0.05, †P < 0.0001 vs. pre training values.
results obtained (data not shown), and immunoreactive bands migrated at expected relative mobilities. Reactive Brown (RB) protein stain (51) was used as a loading and transfer control.

Analysis of vitamin C and α-tocopherol. Plasma samples for vitamin C measurements were mixed with an equal amount of 10% metaphosphoric acid containing 2 mM disodium-EDTA and frozen immediately until analysis. The concentration of ascorbate was measured by reversed-phase HPLC with colorimetric detection (31). The concentration of α-tocopherol in plasma samples was measured by HPLC with amperometric detection (18).

Statistical analysis. All data were tested for normality of distribution before further analysis by use of histograms and probability plots. The data that were not normally distributed a priori were subjected to logarithmic transformation to obtain normal distribution.

A general linear mixed model was used to analyze the effect of time and supplementation. An interaction between the two was also tested. A random subject-specific component was introduced to adjust for interindividual variations. The fit of the model was evaluated by testing the residuals for normal distribution and variance homogeneity. In the post hoc analysis, possible effects of time and group were tested by Student’s paired t-tests. The level of significance was set at \( P < 0.05 \).

The statistical analysis was performed using SAS statistical software (9.1, SAS Institute, Cary, NC). Results are presented as means ± SE or geometric means ± 95% confidence interval (CI) for the log-transformed data.

RESULTS

The two groups did not differ at baseline regarding \( \dot{V}O_{2\text{max}} \) and anthropometrical measurements (Table 1). Plasma insulin concentrations during the glucose clamp did not differ between groups and did not change with exercise training (969 ± 57 to 990 ± 85 pmol/l pre- vs. posttraining for AO, 1,004 ± 76 to 982 ± 82 pmol/l pre- vs. posttraining for PL).

Vitamins C and E concentration in plasma. Vitamin concentrations were measured at baseline, after 4 wk of supple-

![Fig. 1. Glucose infusion rate (GIR) measured during a 180-min hyperinsulinemic-euglycemic clamp for the Antioxidant group (A) and the Placebo group (B). GIR calculated as area under the curve (AUC) for the time 60–180 min during hyperinsulinemic-euglycemic clamp for both groups before and after training. GIR AUC is expressed as mg/kg body wt. Open bars, Antioxidant group (n = 11); filled bars, Placebo group (n = 10). Values are means ± SE. *P < 0.05 vs. pretraining values.](http://ajpendo.physiology.org/)
mentation without training, and at the end of 12 wk of training. The two groups did not differ at baseline. Plasma ascorbic acid and vitamin E (α-tocopherol) concentrations increased ($P < 0.05$) in the AO group after 4 wk of supplementation and remained elevated ($P < 0.05$) during the whole training period (Table 2).

$\dot{V}O_{2\max}$ and $P_{\text{max}}$. $\dot{V}O_{2\max}$ (Table 3) increased by $\sim 17\%$ ($P < 0.0001$) in the AO group and $20\%$ in the PL ($P < 0.0001$) group. $P_{\text{max}}$ (see Fig. 4) increased gradually every week in a similar way for both groups (time effect: $P < 0.0001$). $P_{\text{max}}$ was $24\%$ higher the AO ($P < 0.0001$) and $20\%$ higher in the PL ($P < 0.0001$) group after the training period. $\dot{V}O_{2\max}$ and $P_{\text{max}}$ were similar when AO and PL were compared before as well as after training.

Insulin-stimulated glucose uptake. Insulin-stimulated glucose uptake was increased $\sim 15\%$ after the 12-wk training period (Fig. 1) in both the AO ($P < 0.05$) and the PL ($P < 0.05$) groups. There were no differences between the two groups ($P = 0.68$).
Protein content of insulin signaling molecules. The protein contents of the insulin signaling molecules IR, Akt, and AS160, total and phosphorylated, are presented in Fig. 2. Protein content of the total IR, Akt, and AS160 was measured before and after the training period. Phosphorylation level of the same proteins was measured during insulin stimulation and before and after the training period. Both the total and the phosphorylated proteins were normalized to the loading control Reactive Brown protein stain. Noteworthy, using normalization of the phosphorylated proteins to the total proteins rather than the protein stain did not change the outcome of the data analysis.

Total protein content of Akt was significantly increased after 12 wk of training in both groups (P < 0.05 for both groups). There was also a tendency for increase after training of IR (time effect: P = 0.08). There was no difference between groups for total protein content of AS160 (P = 0.45) or for any of the phosphorylated proteins (p-IR: P = 0.40; p-Akt: P = 0.10; p-AS160: P = 0.68).

Protein contents of GLUT4 and HKII. Protein content of HKII increased significantly in response to training in both the AO group (P < 0.05) and the PL group (P < 0.005; Fig. 3). With regard to GLUT4 (Fig. 3), the increase in response to training was, however, borderline significant (P = 0.05). There was no difference between groups for either HKII (P = 0.70) or GLUT4 (P = 0.11).

Skeletal muscle PPARγ and PGC-1α mRNA expression. There was an overall increase in PPARγ basal mRNA expression (overall training effect: P < 0.001) in response to 12 wk of endurance training that was localized in the AO group. More specifically, PPARγ basal mRNA expression increased by 77% in the AO group (P < 0.005) and by 26% in the PL group (P > 0.05) but with no difference between the groups (P = 0.87). There was no effect of either the training or the supplementation on the basal mRNA expression of PGC-1α (Fig. 4).

Body composition and metabolic parameters. Total body fat mass decreased and total body fat-free mass increased after 12 wk of training; however, the post-values were not significantly different from the pre-values. There were no changes in total body weight (Table 3).

Plasma HDL increased in response to training by ~21% (P < 0.05) in the AO group and 17% in the PL (P < 0.05) group. There were no changes in plasma LDL, plasma TG, plasma FFA, and plasma glucose concentration before and after the training period (Table 3).

DISCUSSION

The aim of the present study was to test whether antioxidant supplementation in healthy individuals would reduce training-induced insulin sensitivity. In contrast to our hypothesis, the present study showed that combined supplementation with vitamins C and E before and during 12 wk of supervised strenuous bicycle exercise training had no effect on insulin-stimulated glucose uptake in skeletal muscle.

An intense endurance training protocol containing progressive increases in both duration and intensity of the training sessions was employed in the present study to obtain a robust training effect whereby possible effects of the supplementation would be easier to detect. There was, however, no indication that the supplementation with antioxidants had any effect on performance or insulin-stimulated glucose uptake. Despite the limited number of participants in the present study, the highly controlled design (e.g., supervised training sessions 5 times/wk) and absence of P values anywhere near statistical significance when the two groups were compared indicate that the risk of a type 2 error is low.

In the present study, we observed a very clear increase in \(\dot{V}O_2\text{max}\) and insulin-stimulated glucose uptake in response to the training. Clearly, there are currently very few studies that have examined the interaction of antioxidant supplementation and exercise training on insulin sensitivity. Supplementation with the antioxidant \(\alpha\)-lipoic acid during endurance training has no effect on insulin action in rodents (43), however, a recent study in humans reports that antioxidant supplementation with 400 IU of vitamin E and 1,000 mg of vitamin C daily may inhibit the insulin-sensitizing effect of training (40). Accordingly, our results are in contrast to previous findings.

Importantly, we also found that the increased insulin sensitivity was associated with corresponding increases in total Akt, GLUT4, and HKII in skeletal muscle, which is in agreement with previous training studies (16, 21). Previous studies have demonstrated a significant increase of GLUT4 protein content in response to endurance training (15, 21, 26, 29), although the
increase just failed to reach statistical significance ($P = 0.05$) in the present study. However, it should be noted that the participants in our study were young, healthy, and physically active men with mean V\(\dot{O}_2\)max $> 50$ ml·kg$^{-1}$·min$^{-1}$ even before training, thus a priori well above normal average V\(\dot{O}_2\)max for young, healthy, Scandinavian men (17). Therefore, it could be that the protein content of GLUT4 was already high before the participants started training, whereby a further increase in response to the employed training may have been limited. Basal expression of the transcription factor PPAR\(\gamma\) was increased after the training period, which is in accord with results from previous studies (40, 45). The increase, however, reached statistical significance in the post hoc analysis only in the AO group. The biological relevance of this finding appears, however, to be limited, since the two groups otherwise produced very similar responses to the training. Nevertheless, the result is in contrast with the results from a previous study that has examined the effect of vitamins C and E supplementation during 4 wk of training on PPAR\(\gamma\) basal mRNA expression (40), showing that the antioxidant supplementation blunts the expression. Recent studies have suggested a mechanistic link between oxidative stress and PPAR\(\gamma\). Thus, PPAR\(\gamma\) activation reduces oxidative stress in adipocytes (30), whereas in a skeletal muscle cell model of insulin resistance PPAR\(\gamma\) activation exerts an antioxidant effect that prevents insulin resistance (14). On the other hand, oxidative stress may reduce PPAR\(\gamma\) mRNA expression and activity in endothelial cells (9), whereas the use of antioxidant vitamins prevents this reduction. Considering the above, the increased levels on PPAR\(\gamma\) mRNA content in the AO group in our study could be the result of the additive effect of the exercise training and the antioxidant supplementation. However, since the exact mechanisms of the relation among oxidative stress, exercise, and PPAR\(\gamma\) activation are not clarified yet, it is difficult to draw further conclusions.

Regarding PGC-1\(\alpha\), basal mRNA expression did not change in response to either endurance training or antioxidant supplementation. Results from a rodent study (24) have demonstrated a 163% increase in mRNA content after 7 days of swimming training, but results from human studies are controversial: Pilegaard et al. (35) found no change in PGC-1\(\alpha\) mRNA content after 4 wk of knee extensor training. Tunstall et al. (49) demonstrated the same result after 9 days of bicycling training, whereas in another human study (42), PGC-1\(\alpha\) mRNA content increased after 6 wk of endurance running training. The reason for the disparate results among the above studies, including ours, may be related to differences in training mode, exercise intensity, and duration.

Of note, the number of studies investigating the effect of antioxidant supplementation during endurance training on the expression of the transcription factor PPAR\(\gamma\) and its coactivator PGC-1\(\alpha\) is limited. The contraction-induced RONS may act as a signal for the upregulation of PGC-1\(\alpha\) (25, 44), and experiments in muscle cells (44) have demonstrated that antioxidant supplementation suppresses this effect. Supplementation with 1,000 mg of vitamin C daily during endurance training in rodents blunted the upregulation of PGC-1\(\alpha\) protein expression.
content (23). Human data (40) demonstrate that the combination of 1,000 mg of vitamin C and 400 IU of vitamin E daily during 4 wk of training also prevents the increase in PPARγ and PGC-1α mRNA content.

The discrepancy between our results and previous findings may have different explanations. It could be speculated that the continuous increases in the duration and intensity of the training sessions resulted in a progressively higher amount of RONS produced during each exercise session. In support of this hypothesis, it has been shown both in animals (22) and in humans (46) that the higher the exercise intensity the higher the amount of RONS produced. Consequently, even if the antioxidant vitamins prevented some of the RONS produced, it could be speculated that the exercise-induced RONS might exceed the antioxidative capacity of the vitamins and thus still be capable of activating the various redox-sensitive signaling pathways (46). In addition, some oxidants have insulin-mimicking effects, which activate insulin-signaling pathways (3).

Given the above, it could be speculated that amount of RONS generated during each exercise session in the AO group could have been adequate to trigger the insulin-signaling pathways despite increased antioxidative capacity obtained by the supplementation. On the other hand, regular exercise seems to lower both the basal levels of RONS and the levels of RONS produced during exercise of the same initial intensity (38). Accordingly, if the amount of antioxidant supplements remains the same, the latter would probably be more effective in blunting the small amount of RONS produced during exercise at the end of a training program, thereby preventing RONS-required adaptations.

Taking into account the long duration of the training period in the present study, it could also be speculated that the vitamins C and E supplementation might have had an effect during the first week of the training period, at the initial phase of the training adaptation. Weekly measurements of $P_{\text{max}}$ did not, however, indicate such transient effects of the antioxidants as $P_{\text{max}}$ was increasing every week in a similar way in both groups (Fig. 5).

It is worth noticing that in the human study by Ristow et al. (40) the duration of the individual training sessions was constant during the training period, yet there is no information about the intensity of the exercise. Furthermore, the duration of the training period was only 4 wk compared with 12 wk in our training study. Taken together, the two training protocols differ with regard to duration, intensity, and total amount of training. Each of these factors may explain the different results between the two studies.

We investigated the effect of vitamins C and E supplementation on insulin sensitivity, assessed by differences in the glucose infusion rate during insulin clamps, and differences in the protein content of the insulin signaling cascade molecules. We did not find any differences in the phosphorylation level of the proteins p-IR, Akt Ser473, or p-AS160 during the insulin clamps before or after training. However, we do not know whether the antioxidant supplementation perhaps changed the kinetics of the phosphorylation level of the proteins between the PL and the AO groups. It has been shown, for example, that TNF-α infusion decreases insulin-induced phosphorylation of AS160 after 1 h, whereas this effect is lost after 3 h (36). A marked change in phosphorylation kinetics is, however, unlikely, since the glucose infusion rate profiles were similar in the two groups (Fig. 1, A and B).

In conclusion, the present study has demonstrated a marked effect of 12 wk of strenuous endurance training on insulin sensitivity in healthy young males and thereby underscores the fact that there is room for further improvement in glucose metabolism, even in healthy, moderately- to well-trained people. In addition, supplementation with vitamins C and E during strenuous endurance training did not affect the training-induced improvement in insulin sensitivity in healthy, young individuals. Although the results from the present study do not indicate a harmful effect of the antioxidant supplementation during training, it is clear that, at least in this population, the antioxidant vitamins do not offer any additional beneficial effect to the endurance training. Considering the extent of usage of antioxidant supplements within the sports world, individuals who are healthy, follow a balanced diet, and are engaged in a regular training program to improve their fitness, should probably be more critical toward antioxidant vitamin supplementation.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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