Role of vitamin C and E supplementation on IL-6 in response to training

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1The Centre of Inflammation and Metabolism, Department of Infectious Diseases, and Copenhagen Muscle Research Centre, Rigshospitalet, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark; 2Department of Biochemistry and Biotechnology, University of Thessaly, Larissa, Greece; and 3Department of Disease Biology, Section of Biomedicine, Faculty of Life Sciences, and 4Department of Biology, Section of Molecular, Integrative Physiology, University of Copenhagen, Copenhagen, Denmark

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Yfanti C, Fischer CP, Nielsen S, Åkerström T, Nielsen AR, Veskoukis AS, Kouretas D, Lykkesfeldt J, Pilegaard H, Pedersen BK. Role of vitamin C and E supplementation on IL-6 in response to training. J Appl Physiol 112: 990–1000, 2012. First published December 29, 2011; doi:10.1152/japplphysiol.01027.2010.—Vitamin C and E supplementation has been shown to attenuate the acute exercise-induced increase in plasma interleukin-6 (IL-6) concentration. Here, we studied the effect of antioxidant vitamins on the regulation of IL-6 expression in muscle and the circulation in response to acute exercise before and after high-intensity endurance exercise training. Twenty-one young healthy men were allocated into either a vitamin (VT; vitamin C and E, n = 11) or a placebo (PL, n = 10) group. A 1-h acute bicycling exercise trial at 65% of maximal power output was performed before and after 12 wk of progressive endurance exercise training. In response to training, the acute exercise-induced IL-6 response was attenuated in PL (P < 0.02), but not in VT (P = 0.82). However, no clear difference between groups was observed (group × training: P = 0.13). Endurance exercise training also attenuated the acute exercise-induced increase in muscle IL-6 mRNA in both groups. Oxidative stress, assessed by plasma protein carbonyls concentration, was overall higher in the VT compared with the PL group (group effect: P < 0.005). This was accompanied by a general increase in skeletal muscle mRNA expression of antioxidative enzymes, including catalase, copper-zinc superoxide dismutase, and glutathione peroxidase 1 mRNA expression in the VT group. However, skeletal muscle protein content of catalase, copper-zinc superoxide dismutase, or glutathione peroxidase 1 was not affected by training or supplementation. In conclusion, our results indicate that, although vitamin C and E supplementation may attenuate exercise-induced increases in plasma IL-6 there is no clear additive effect when combined with endurance training.

PLASMA INTERLEUKIN-6 (IL-6) concentration increases up to 100-fold in response to exercise (11), and one of the main sources of this increase is the skeletal muscle. Previous work from our group has demonstrated that both mRNA (40) and protein (22, 42) levels of IL-6 increase in skeletal muscle in response to exercise, and that IL-6 is released from working muscles into the circulation (47). Data from epidemiological studies have shown that there is an inverse association between physical activity level and basal plasma IL-6 levels (4, 6, 12, 41). In addition, from studies on athletes, it has been shown that IL-6 production during acute exercise is also inversely associated with fitness level (16), suggesting that the higher the fitness level, the lower the increase in exercise-induced plasma IL-6. Previously, our group has shown that 10 wk of exercise training lowered the systemic plasma IL-6 response to 3-h knee-extensor exercise (1), as well as the increase in IL-6 mRNA (14). In accordance, recent data from Croft et al. (8) demonstrate that 6 wk of high-intensity interval training attenuates the exercise-induced increase in plasma IL-6 in response to acute exercise, supporting the idea of an adaptive response of the level of exercise-induced IL-6 after training.

Antioxidant vitamin supplementation before nondamaging exercise has been shown to attenuate the exercise-induced increase in plasma IL-6 and the release of IL-6 from contracting skeletal muscle (13, 50), but not the skeletal muscle IL-6 mRNA response (13). At the same time, such supplementation also prevented the oxidative stress that usually occurs with exercise. However, it has also been shown that murine myotubes express IL-6 when exposed to free radicals (26), indicating that increased radical production may act as a stimulus for the exercise-induced increases in plasma IL-6, potentially via increases in the skeletal muscle IL-6 expression.

Endurance training has been suggested to lower both basal (18) and exercise-induced levels of oxidative stress (2, 18). This is probably achieved through increases in both activity and expression of antioxidative enzymes (21, 39) and heat shock proteins (24) that play an important role in protecting the cells by promoting the correct folding of newly synthesized cellular proteins (36). In addition, antioxidant vitamin supplementation has been suggested to attenuate oxidative stress when administered before acute exercise (35, 37, 48), although results from these studies are not entirely consistent.

Moreover, there is little information on the effects of antioxidant vitamin supplementation during long-term endurance training with regard to changes in cytokines, oxidative stress, and antioxidant defense mechanisms.

The present study was undertaken to investigate the role of supplementation with the antioxidant vitamins C and E on the training-induced regulation of the systemic and skeletal muscle IL-6 responses to exercise. Because chronic antioxidant supplementation during a training period has been suggested to interfere with training adaptations (17), we hypothesized that vitamin C and E administration during 12 wk of endurance training would attenuate the effect of training on the exercise-induced IL-6 plasma and mRNA responses.

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MATERIALS AND METHODS

Ethical Approval

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

Participants

Twenty-one young, healthy, physically active men participated in the study (55). We employed a double-blinded, placebo-controlled design and used a minimization model (49) to randomize the participants into two groups, vitamin (VT) \( (n = 11) \) and placebo (PL) \( (n = 10) \). The participants were randomized according to age, body mass index (BMI), and maximal power output \( (P_{\text{max}}) \) on the bicycle. Accordingly, the two groups were comparable from the beginning regarding anthropometric measurements and maximal oxygen consumption \( (V_{\text{O2max}}) \). The mean age for the VT and the PL group was \( 29 \pm 5 \) (mean \( \pm \) SD) and \( 31 \pm 5 \) yr, the mean weight was \( 80 \pm 12 \) and \( 81 \pm 9 \) kg, and the mean height was \( 1.79 \pm 0.06 \) and \( 1.80 \pm 0.05 \) m, respectively. BMI at baseline was \( 25 \pm 3 \text{ kg/m}^2 \) for the VT group and \( 25 \pm 2 \text{ kg/m}^2 \) for the PL group, and \( V_{\text{O2max}} \) was \( 50 \pm 7 \text{ ml·kg}^{-1}·\text{min}^{-1} \) for the VT group and \( 52 \pm 9 \text{ ml·kg}^{-1}·\text{min}^{-1} \) for the PL group.

Before inclusion in the study, a medical examination with blood test screening, a test for \( P_{\text{max}} \) assessment, and an oral glucose tolerance test were performed. Exclusion criteria included physical exercise less than two and more than four times a week, BMI \(< 25\text{ kg/m}^2\) for the PL group, and \( V_{\text{O2max}} \) was \( 50 \pm 7 \text{ ml·kg}^{-1}·\text{min}^{-1} \) for the VT group and \( 52 \pm 9 \text{ ml·kg}^{-1}·\text{min}^{-1} \) for the PL group.

Supplementation

Participants in the VT group received oral supplementation (tablets) with vitamin C (ascorbic acid, 500 mg daily) and vitamin E (RRR-\( \alpha \)-tocopheryl succinate, 400 IU daily) for 16 wk, while the PL group received placebo tablets. The placebo tablets for vitamin E consisted mainly of microcrystalline cellulose, calcium hydrogen anhydrous, and starch 1500 PT. The placebo tablets for vitamin C consisted mainly of sorbitol. All placebo tablets were masked to look similar to the treatment tablets in color, shape, and taste. The dosages of vitamin C and vitamin E were \( \sim 5 \) and \( \sim 15 \) times higher, respectively, than the recommended dietary allowances. This supplementation protocol has previously been shown to offer adequate antioxidant effect (13, 44).

All participants were instructed to take the supplementation once a day with breakfast. The supplementation started \( 4 \) wk before onset of the training and continued throughout the training period. The participants were instructed to maintain their habitual diet. A schematic presentation of the outline of the study is shown in Fig. 1.

Experimental Procedures

Training protocol. The mode of exercise selected for training was cycling, and the training frequency was \( 5 \) times/wk for \( 12 \) wk. A \( P_{\text{max}} \) test, which was performed the same way as the \( V_{\text{O2max}} \) test (55), was performed at the beginning of each training week to determine the intensity of the training for the following days of the week. On Tuesdays, the training consisted of ten 3-min intervals at \( 85\% \ P_{\text{max}} \), interspersed with 3-min recoveries at \( 40\% \ P_{\text{max}} \). The next day, the training was continuous at \( 60\% \ P_{\text{max}} \) and the duration was \( 60 \) min. On Thursdays, the training consisted of five 8-min intervals at \( 75\% \ P_{\text{max}} \) with 4-min rest at \( 40\% \ P_{\text{max}} \) in between. On Fridays, the training was continuous for \( 120 \) min at \( 55\% \ P_{\text{max}} \). For the first \( 6 \) wk, the duration of each training session was increased by \( 5\% \)/wk, while, within the last \( 6 \) wk, the duration remained stable, and the intensity was increased by \( 1\% \)/wk. The participants were allowed to miss only \( 5\% \) of the total amount of the training, which was equal to \( 3 \) training days. If they had to refrain from training for more than \( 3 \) days, the training was performed during the weekend. This specific intense training protocol was chosen to obtain the maximal possible training effect.

Acute exercise trial. Before and after the training period, the participants performed an acute exercise bout. On the experimental day, participants arrived at the laboratory between 7:30 and 8:00 AM after a 12-h overnight fast. After resting for 10 min on a hospital bed, a catheter was placed in an antecubital vein for blood sampling. The participants rested on a hospital bed for \( 3 \) h. Blood samples and muscle biopsies were taken before and immediately after cessation of exercise and at \( 3 \) h of the recovery period, as previously described (55). The acute exercise trial was performed between \( 72 \) and \( 96 \) h after the last training session. Changes in plasma volume during the acute exercise bouts were calculated as described by Dill and Costill (9). IL-6, IL-1 receptor antagonist (IL-1ra), and cortisol concentrations were corrected for changes in plasma volume.

**Fig. 1.** Schematic representation of the study. Supplementation with vitamins C and E started \( 4 \) wk (week 0) before the onset of training. During weeks 3 and 4 (pretraining [Pre]) and weeks 16 and 17 (posttraining [Post]), the acute exercise trial and the incremental test were performed. The total duration of the training was 12 wk.
IL-6 was measured by ELISA with an intra-assay coefficient of variation for the nosorbent assay (ELISA) (IL-6: no. SS600B, R&D Systems, Minneapolis, MN; IL-1ra: no. SRA008, R&D Systems; cortisol: no. KGE008, R&D Systems). The intra-assay coefficient of variation for ascorbate was measured by reversed-phase HPLC with colorimetric detection (34). The concentration of α-tocopherol in plasma samples was measured by HPLC with amperometric detection (10).

**Enzyme-linked immunosorbent assays.** Plasma IL-6, IL-1ra, and cortisol concentrations were measured using Enzyme-linked immunosorbent assay (ELISA) (IL-6: no. SS600B, R&D Systems, Minneapolis, MN; IL-1ra: no. SRA008, R&D Systems; cortisol: no. KGE008, R&D Systems). The intra-assay coefficient of variation for IL-6 was <5%, for IL-1ra <5%, and for cortisol <7%.

To minimize the effect of interassay variation, samples from the antioxidant and the PL group were equally represented on each ELISA plate. All samples were analyzed in duplicate, and the mean of the duplicates was used for statistical analysis.

**RNA isolation and quantitative real-time PCR.** Skeletal muscle biopsies were homogenized in TRIzol Reagent (Invitrogen) using a motor-driven homogenizer (Polytron, Kinematica, Newark, NY), and total RNA was isolated according to the manufacturer’s protocol. Total RNA was dissolved in RNase-free water and quantified spectrophotometrically at 260 nm. Total RNA was 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. Detection of specific mRNA was performed using an ABI-PRISM 7900 Sequence Detection system (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 triplicate. The amplification of genomic DNA typically amounted to a maximum of <1% of the target gene when using the TRIzol protocol. A twofold dilution series was made from a pooled sample of a small part of all of 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 target genes was calculated from the cycle threshold values of the unknown samples.

**Determination of single-stranded DNA content.** The mRNA content of the measured genes was normalized to the total single-stranded DNA content in each RT sample measured by a fluorescence-based method using OligoGreen reagent (Molecular Probes, Leiden, the Netherlands), as described previously (33).

**Western Blotting.**

**Preparation of muscle lysates.** Muscle tissue was homogenized as previously described (55), and the homogenate was rotated end over end for 1 h at 4°C and centrifuged at 13,000 g at 4°C for 15 min. An aliquot of 10 μl of each lysate was taken and diluted for protein concentration determination before storage using the bicinchoninic acid (Pierce). A maximal coefficient of variation of 5% was accepted between replicates.

**SDS-PAGE and Western blotting.** Equal amounts of denatured proteins (28) from the tissue homogenates were separated by gel electrophoresis using NuPage 4–12% Bis-Tris gels (Invitrogen, Taastrup, Denmark), followed by immunoblotting to polyvinylidene difluoride membranes (hybond-P, GE Healthcare), as previously described (55). Pilot experiments demonstrated that the amounts of protein loaded were within the dynamic range for the conditions used and the results obtained (data not shown), and immunoreactive bands migrated at expected relative mobilities. Reactive Brown protein stain (56) was used as a loading control.

**Malondialdehyde.** Malondialdehyde (MDA) concentration was determined as follows: 50 μl of muscle lysate was mixed with 924 μl of 5% TCA and 576 μl of 0.8% 2,6-di-tert-butyl-4-methyphenol, and vortexed for 30 s. The samples were then centrifuged at 3,000 g for 3 min at 4°C, the supernatant was discarded, and 900 μl of 5% TCA were added to the remaining solution. The samples were vortexed and aliquoted into three new tubes containing 625 μl each. Then 375 μl of 0.8% thiobarbituric acid was added to each sample (including a control sample which was mixed with 625 μl of 5% TCA). The samples were then incubated in a water bath at 70°C for 30 min, and afterwards immediately transferred to an ice bath for 10 min. They were centrifuged at 3,000 g for 3 min at 4°C, and the absorbance was measured spectrophotometrically at 521.5 nm. The MDA concentra-

![Fig. 2. Plasma IL-6 concentration at rest (0), immediately after (1), and 3 h after (4) 1-h bicycling exercise Pre (open background) and Post (shaded background) 12 wk of training. Values are presented as geometric means ± 95% confidence interval (CI).](http://jap.physiology.org/Downloadedfrom)
tion was calculated from a standard curve of known MDA concentrations. The intra-assay coefficient of variation for MDA was <6%.

**Protein carbonyls.** For protein carbonyls, 50 μl of 20% TCA were added to 50 μl of plasma, and this mixture was incubated in an ice bath for 15 min and centrifuged at 15,000 g for 5 min at 4°C. The supernatant was discarded, and 500 μl of 10 mM 2,4-dinitrophenyl-hydrazine (in 2.5 N HCl) for the sample, or 500 μl of 2.5 N HCl for the blank, were added to the pellet. The samples were incubated in the dark at room temperature for 1 h, with intermittent vortexing every 15 min, and were centrifuged at 15,000 g for 5 min at 4°C. The supernatant was discarded, and 1 ml of 10% TCA was added. The samples were then vortexed and centrifuged at 15,000 g for 5 min at 4°C. The supernatant was again discarded, and 1 ml of ethanol-ethyl acetate (1:1 vol/vol) was added. The samples were then vortexed and centrifuged at 15,000 g for 5 min at 4°C. The washing step was repeated two more times. The supernatant was again discarded, and 1 ml of 5 M urea (pH 2.3) was added. The samples were again vortexed and incubated at 37°C for 15 min. The samples were finally centrifuged at 15,000 g for 3 min at 4°C, and the absorbance was read at 375 nm.

**Statistical Analyses**

All data were tested for normality of distribution before further analysis using histograms and probability plots. If the data were not normally distributed, log-transformation was applied to achieve normality.

A three-way general linear mixed model (Proc Mixed) with repeated measures was used to analyze the effects of supplementation (VT vs. PL), time (0, 1, 4 h), and training (pre vs. post) for plasma cytokines, skeletal muscle IL-6 mRNA levels, MDA, protein carbonyls, and antioxidant enzymes mRNA levels. A two-way general linear model (Proc Mixed) was used to analyze the effect of supplementation (VT vs. PL) and training (pre vs. post) for plasma vitamin concentrations, VO2max, Pmax, body mass, and antioxidant enzyme protein levels. All possible interactions between the main effects were included in the models. The fit of the model was evaluated by testing the residuals for normal distribution and variance homogeneity.

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 mean ± 95% confidence interval (CI) for the log-transformed data.

**RESULTS**

**Vitamin C and E Concentration in Plasma**

Vitamin concentrations in plasma were measured at baseline, after 4 wk of supplementation without training, and at the end of 12 wk of training with supplementation. The VT and PL groups did not differ at baseline. Plasma ascorbic acid increased (P < 0.05) in the VT from 53.8 μmol/l (CI 42.2–65.4...
μmol/l) at baseline to 82.0 μmol/l (CI 68.6–95.4 μmol/l) after 4 wk of supplementation and remained elevated (P < 0.05) (75.0 μmol/l, CI 60.7–89.2 μmol/l) compared with presupplementation levels throughout the training period. Likewise, plasma α-tocopherol concentrations increased (P < 0.05) in the VT group from 17.0 μmol/l (CI 15.1–19.1 μmol/l) at baseline to 25.4 μmol/l (CI 22.0–28.0 μmol/l) after 4 wk of supplementation and remained elevated (P < 0.05) until the end of the training period (22.5 μmol/l, CI 20.2–25.0 μmol/l) compared with presupplementation levels. In the PL group, plasma ascorbic acid and α-tocopherol concentrations at baseline were 64.7 μmol/l (CI 56.3–73.2 μmol/l) and 20.8 μmol/l (CI 17.8–24.2 μmol/l), respectively, and did not change throughout the training period.

\[ \dot{V}O_{2\text{max}}, \ P_{\text{max}}, \ Body \ Mass \]

\[ \dot{V}O_{2\text{max}} \text{ increased in the VT group from } 50 \pm 7 \text{ ml·kg}^{-1}·\text{min}^{-1} (or 4.0 \pm 0.2 \text{ l/min}) \text{ before training to } 59 \pm 8 \text{ ml·kg}^{-1}·\text{min}^{-1} (or 4.6 \pm 0.2 \text{ l/min}) \text{ after 12 wk of training (P < 0.0001 for both), and in the PL group from } 52 \pm 9 \text{ ml·kg}^{-1}·\text{min}^{-1} (or 4.2 \pm 0.2 \text{ l/min}) \text{ to } 62 \pm 10 \text{ ml·kg}^{-1}·\text{min}^{-1} (or 4.9 \pm 0.2 \text{ l/min}) (P < 0.0001 for both, with no difference between groups). \]

In addition, \( P_{\text{max}} \) increased in the VT group from 314 ± 88 W pretraining to 387 ± 103 W posttraining (P < 0.0001 for both, with no difference between groups). Similarly, in the PL group, \( P_{\text{max}} \) increased from 328 ± 113 W pretraining to 392 ± 114 W posttraining (P < 0.0001). Although there was a decrease in body weight for both groups, the difference was not statistically significant.

**Plasma IL-6**

Plasma IL-6 (Fig. 2) increased immediately after exercise and remained elevated during the recovery period, both before and after the 12 wk of training (effect of time: P < 0.0001). In response to training, the acute exercise-induced IL-6 response was attenuated in PL (P < 0.02), but not in VT (P = 0.81). There was, however, no overall group effect (P = 0.89) or interaction between group and training (P = 0.13).

**Plasma IL-1ra and Cortisol**

Plasma IL-1ra (Fig. 3A) increased 3 h after cessation of exercise in both groups before and after the training period (effect of time: P < 0.0001). A time × training interaction (P < 0.01) was detected with the IL-1ra levels at the recovery period being lower compared with before training. Plasma cortisol concentration (Fig. 3B) increased immediately after cessation of exercise and returned to baseline values in both groups before and after the training period (effect of time: P < 0.0001). Furthermore, a group × time (P < 0.005) interaction was detected with the plasma cortisol levels being higher immediately after exercise in the VT group compared with PL.

**Skeletal Muscle IL-6 mRNA Expression**

Skeletal muscle IL-6 mRNA expression (Fig. 4) increased immediately after cessation of exercise and remained elevated for 3 h post exercise (effect of time: P < 0.0001) in both groups. Twelve weeks of endurance training reduced the exercise-induced skeletal muscle IL-6 mRNA expression in both groups (effect of training: P < 0.0005). In addition, a time × training interaction (P < 0.05) was present as the increase of IL-6 mRNA levels during recovery from 1-h exercise was attenuated after 12 wk of endurance training. When comparing groups, there was no difference.

**Skeletal Muscle MDA**

Skeletal muscle MDA levels (Fig. 5) increased in response to acute exercise (effect of time: P < 0.01) in both groups. In addition, MDA levels were overall higher posttraining (effect of training: P < 0.0001).

**Plasma Protein Carbonyls**

Plasma protein carbonyl concentration (Fig. 6) was higher overall in the VT than in the PL group (group effect: P < 0.005). In addition, a main effect of protein carbonyls being increased in response to

![Fig. 4. Skeletal muscle IL-6 mRNA content at rest (0), immediately after (1), and 3 h after (4) 1-h bicycling exercise Pre (open background) and Post (shaded background) 12 wk of endurance training. Values are geometric means ± 95% CI.](image-url)
exercise. Furthermore, 12 wk of training increased protein carbonyl concentration in plasma (effect of training: $P < 0.01$).

**Skeletal Muscle Antioxidant Enzyme mRNA Expression**

Main effects of group and time were detected for the expression of the antioxidant enzymes in the skeletal muscle (Fig. 7). More specifically, mRNA levels of SOD1 ($P < 0.001$; Fig. 7B) and glutathione peroxidase 1 (GPX1; $P = 0.05$; Fig. 7C) were higher overall in the VT group compared with PL. Furthermore, mRNA expression increased immediately after 1 h of exercise and remained elevated at 3 h postexercise [effect of time: $P < 0.005$ for catalase (CAT), $P < 0.05$ for copper-zinc superoxide dismutase (CuZnSOD), and $P < 0.01$ for GPX1].

**Skeletal Muscle Basal Protein Content of Antioxidant Enzymes**

There was a tendency (group effect: $P = 0.09$) for an overall higher protein content of CAT in the antioxidant group than in the PL group (Fig. 8A). There was no difference in protein content of CuZnSOD (Fig. 8B) or GPX1 (Fig. 8C) between the VT and the PL group, either before or after the 12 wk of training. In addition, there was no change with training in the protein content for any of the antioxidant enzymes.

**Discussion**

The present study demonstrates that the exercise-induced increase in plasma IL-6, as well as the exercise-induced increase in skeletal muscle IL-6 mRNA, is attenuated as a consequence of 12 wk of endurance training. A clear decrease in the plasma IL-6 response was observed only in the placebo group. When comparing groups, however, the acute IL-6 response was not different, either measured as plasma concentration or as mRNA expression in skeletal muscle.

The present finding that the acute plasma IL-6 response was modulated as a result of training adaptation is in accordance with previous studies. Accordingly, it has been shown that 10 wk of exercise training lowers the systemic plasma IL-6 response to 3 h of knee-extensor exercise (1). Furthermore, Croft et al. (8) demonstrated that 6 wk of high-intensity interval...
training attenuates the exercise-induced increase in plasma IL-6 in response to acute exercise. Twelve weeks of strenuous endurance training also reduced the increase of IL-6 mRNA in response to acute exercise in the present study. This supports previous work from our group (14), where 10 wk of knee-extensor endurance training reduced the acute exercise-induced increase in skeletal muscle IL-6 mRNA.

The present study investigated the effect of vitamin C and E supplementation during endurance training on the acute exercise-induced IL-6 response. Previous work from our laboratory has demonstrated that vitamin C and E supplementation for 4 wk before acute exercise attenuates the systemic IL-6 response to exercise (13). In this study, the same supplementation protocol, when applied during 12-wk of endurance-training seems to attenuate the expected training-induced reduction of the systemic IL-6 response to acute exercise. Although the statistical analysis did not reveal a significant difference between the groups, a significant training effect was observed within the PL group, but not in the VT group. In addition, when calculating the area under the curve for plasma IL-6 during acute exercise, there was a significant reduction for the PL group after 12 wk of training, while there was no difference in the VT group (Fig. 9). As in the study by Fischer et al. (13), supplementation started 4 wk before training. Although it is possible that the supplementation attenuated the effect of the training, it is also possible that the supplementation attenuated the acute exercise IL-6 response only before training, without any further effect when in combination with aerobic training for 12 wk. An additional acute exercise trial, including biopsies before the start of the supplementation, could perhaps provide information to help answer this question. The number of experimental conditions and sampling time points, however, made us limit the number of acute exercise experiments to just one before and one after the training period.

Based on previous results showing that murine myotubes express IL-6 when exposed to oxidative stress (26), we hypothesized that the exercise-induced increase in free radical production could act as a stimulus for the exercise-induced increase in plasma IL-6, potentially via increases in the skeletal muscle IL-6 expression. However, the skeletal muscle IL-6
mRNA was downregulated similarly in PL and VT groups, while plasma IL-6 was only attenuated by training in the PL group. While this seems to contradict our hypothesis, we believe that it is difficult to draw any definite conclusions, as this discrepancy could very well be attributed to posttranscriptional regulation, as well as to differences in the mRNA and protein turnover rates. For example, micro-RNAs (miRNAs) may have modified the translational process. The exact mechanism(s) is not known; however, recent data show that reactive oxygen species (ROS) regulate the expression of miRNAs in different cell types (27, 31, 54). Therefore, antioxidant vitamin supplementation could have modified the expression of ROS-induced miRNAs and thereby the posttranscriptional regulation of the IL-6 protein. In addition, considering that the mRNA levels represent the total amount measured at that particular time point, we have no information regarding possible changes in the IL-6 mRNA turnover rate that could possibly lead to changes in the protein levels.

Unfortunately, we have no data regarding skeletal muscle IL-6 protein regulation, which would provide new insights regarding the transcriptional regulation within skeletal muscle. So far we have been unable to measure IL-6 protein in the skeletal muscle using quantitative techniques.

Because vitamin C and E supplementation attenuated the decrease in peak plasma IL-6 levels in response to acute exercise that normally occurs after a training period, we further investigated the effect of the vitamin C and E supplementation on the oxidative stress and the skeletal muscle antioxidant defense during the acute exercise. We found an overall increase in plasma protein carbonyls in the VT group compared with the PL group, both before and after the training period. In addition, although there was not a significant difference between the groups, we also found that supplementation with vitamins C and E led to increased levels of skeletal muscle MDA during the acute exercise posttraining, while there was no change within the PL group (Fig. 10). Although the observed increase in oxidative stress in the VT group may seem rather surprising, there are recent data from similar studies which found the same effect: supplementation for 2 wk with a cocktail of vitamins, including vitamin C, vitamin E, β-carotene, and selenium, may increase plasma MDA concentration at rest (30), and the authors attributed the increased lipid peroxidation to the lack of an increase in plasma vitamin C. However, in the present study, the plasma vitamin C levels were increased after supplementation up to the saturation level; therefore, the results cannot be explained by insufficient systemic antioxidant vitamin availability. However, we cannot rule out that a different effect could be found in antioxidant-deficient individuals subjected to the same regimen.

In agreement with the present findings though, Veskoukis et al. (51) reported that acute administration of allopurinol, a potent antioxidant that inhibits xanthine oxidase, a major contributor of free radicals during exercise (52), also increases both lipid and protein oxidation at rest. Moreover, vitamin E supplementation for 2 mo before a competitive triathlon race has been shown to promote lipid peroxidation, assessed by plasma F2-isoprostanes, and inflammation, in response to acute exercise (38). In line with this, oxidative stress was also increased when vitamin C and N-acetyl-cysteine were given after an acute bout of eccentric exercise (5).

Although the mechanism(s) behind this phenomenon remain unknown, it has been speculated that pro-oxidative properties of antioxidant vitamins, and especially vitamin C, may be involved. Vitamin C can efficiently convert Fe$^{3+}$ to Fe$^{2+}$, which subsequently reacts with oxygen or hydrogen peroxide...
and results in formation of superoxide anion and hydroxyl radicals (20, 45). There is an intracellular pool of metals (mostly iron), which is released into the extracellular matrix during cell injury or inflammation (19). Hypothetically, if one assumes that exercise is associated with cellular damage (46), ascorbate and \( \alpha \)-tocopherol may react with free metals and generate \( \text{OH}^\bullet \), which, subsequently, can cause oxidative damage. In addition, increased levels of \( \alpha \)-tocopherol, as a result of increased oxidative stress, may lead to elevated levels of \( \alpha \)-tocopherol radicals, which may in itself subsequently initiate processes of lipid peroxidation (43). However, it is important to stress that prooxidant effects of vitamin C have so far not been shown to occur in vivo.

In line with an increased oxidative stress in the VT group after training, we observed higher mRNA expression of the antioxidant enzymes CAT, CuZnSOD, and GPX1 in the present study. Although regulation of the antioxidant defense mechanisms provides useful information regarding the oxidant-antioxidant homeostasis, interpretation is sometimes difficult. The higher mRNA levels of antioxidant enzymes post-training in the VT group may be the result of increased oxidative stress in that group, as a compensatory mechanism aimed at maintaining oxidant-antioxidant homeostasis. However, the finding that mRNA expression of the antioxidant enzymes was high in the VT group, compared with the PL group, already before the training period, shows that supplementation with vitamin C and E for 4 wk is sufficient for eliciting such changes. Unfortunately, we did not have a skeletal muscle biopsy sample before the onset of supplementation; thus it cannot be ruled out that the difference in the mRNA levels of CuZnSOD and GPX1 before training was due to random group differences. However, it has been shown previously that supplementation with vitamin C for 8 wk increases the basal activity of superoxide dismutase and CAT in lymphocytes, as well as the heat shock protein 70 content in skeletal muscle (25). Moreover, the two groups in our study were comparable before the beginning of training with regard to all other variables measured (e.g., physical characteristics, plasma and skeletal muscle cytokines). Taken together, it is reasonable to believe that the most likely explanation for the differences in the mRNA content of the antioxidant enzymes before training is an actual effect of the antioxidant supplementation.

Despite the increases in the mRNA expression of the antioxidant enzymes in the VT group, changes were not detected at the protein level, either before or after the training period. The reason for this discrepancy remains elusive and may both be ascribed to technical limitations, or be due to the different regulation of mRNA and protein (53). Biologically, it may be attributed to posttranscriptional regulation, as well as to differences in the mRNA and protein turnover rates (7, 23). For example, miRNAs, short RNA molecules that bind to complementary sequences of target mRNAs and inhibit translation from the mRNA, may have modified the translational process (3). In addition, given that PCR is a much more sensitive method than Western blotting, it is possible that a small corresponding change in the protein level was present, but not detected due to technical differences between the employed methods. But still, such potential small changes in antioxidant enzymes appear to have been insufficient to prevent an increase in oxidative stress during antioxidant supplementation.

Taken together, the present results indicate that, while vitamin C and E supplementation might attenuate the acute exercise-induced increase in plasma IL-6, such supplementation does not seem to further decrease IL-6 levels after 12 wk of vitamin supplementation combined with endurance training.
Future mechanistic studies are required to clarify the relationship between exercise, IL-6 production, and redox balance, and to provide a better insight into the role of antioxidant vitamin supplementation on IL-6 regulation in response to exercise. Based on our results, we cannot conclude that there are any beneficial effects of supplementation with vitamins C and E during periods of endurance training.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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