Vitamin E isoform-specific inhibition of the exercise-induced heat shock protein 72 expression in humans

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Fischer, Christian P., Natalie J. Hiscock, Samar Basu, Bengt Vessby, Anders Kallner, Lars-Börje Sjöberg, Mark A. Febbraio, and Bente K. Pedersen. Vitamin E isoform-specific inhibition of the exercise-induced heat shock protein 72 expression in humans. J Appl Physiol 100: 1679–1687, 2006. First published December 29, 2005; doi:10.1152/japplphysiol.00421.2005.—Increased levels of reactive oxygen and nitrogen species, as seen in response to exercise, challenge the cellular integrity. Important protective adaptive changes include induction of heat shock proteins (HSPs). We hypothesized that supplementation with antioxidant vitamins C (ascorbic acid) and E (tocopherol) would attenuate the exercise-induced increase of HSP72 in the skeletal muscle and in the circulation. Using randomization, we allocated 21 young men into three groups receiving one of the following oral supplementations: RRR-α-tocopherol 400 IU/day + ascorbic acid (AA) 500 mg/day (CE), RRR-α-tocopherol 290 IU/day + RRR-γ-tocopherol 130 IU/day + AA 500 mg/day (CEγ), or placebo (Control). After 28 days of supplementation, the subjects performed 3 h of knee extensor exercise at 50% of the maximal power output. HSP72 mRNA and protein content was determined in muscle biopsies obtained from vastus lateralis at rest (0 h), postexercise (3 h), and after a 3-h recovery (6 h). In addition, blood was sampled for serum HSP72 content. In the Control group, skeletal muscle HSP72 content was not different from that of Rest (0 h) or from the 3-h recovery (6 h). In contrast, CEγ supplementation reduced the exercise-induced increase of HSP72 mRNA, HSP72 protein, and serum HSP72 content to the levels of Rest. CE supplementation partially blunted the exercise-induced increase of HSP72 mRNA, HSP72 protein, and serum HSP72 content. RRR-α-tocopherol completely blunted the exercise-induced increase of HSP72 mRNA, HSP72 protein, and serum HSP72 content. In summary, we demonstrated that supplementation with antioxidant vitamins C and E is effective in reducing exercise-induced increase of skeletal muscle HSP72 content in humans.

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cise-induced increase of skeletal muscle HSP72 while at the same time increasing baseline levels of HSP72, superoxide dismutase, and catalase. The effect of antioxidants on circulating levels of HSP72 has not been investigated previously.

Because the combination of ascorbic acid and α-tocopherol comprises a powerful antioxidant, from both an electrochemical (11) and an experimental (29, 34) point of view, we hypothesized that the combined effect of the two would cause a marked attenuation of the exercise-induced HSP72 mRNA and protein expression in skeletal muscle as well as circulating HSP72. However, because α-tocopherol supplementation suppresses plasma levels of γ-tocopherol, we also examined whether a combination of ascorbic acid, α-tocopherol, and γ-tocopherol would be a more potent inhibitor of the exercise-induced elevation of HSP72 compared with ascorbic acid and α-tocopherol alone. Of note, γ-tocopherol, in contrast to α-tocopherol, is in vitro a potent scavenger of RNS, including peroxynitrite (13, 15), and an inhibitor of cyclooxygenase activity (33), thus possessing potential anti-inflammatory qualities.

Noteworthy, the effect of γ-tocopherol on the response to exercise has not been reported previously.

As a marker of lipid peroxidation, 8-iso-prostaglandin F2α (8-iso-PGF2α) was employed. Of note, 8-iso-PGF2α, is a major isoform of the F2 isoprostanes (50), which are formed by a nonenzymatic interaction between ROS and arachidonic acid (49), thus highly suitable as a marker of lipid peroxidation.

MATERIALS AND METHODS

Participants. Twenty-one healthy men volunteered to participate in this study. All subjects were physically active nonathletes. Group characteristics are listed in Table 1. A general medical examination in combination with blood test screening was performed before inclusion. Exclusion criteria were history of febrile illness within 3 wk before the examination, indication of ongoing disease, recent vaccination, use of any medication, or use of supplementation with known antioxidative properties.

The purpose of the study and possible risks and discomforts were explained to the subjects before obtaining written consent. The study protocol was approved by the local Ethical Committee of Copenhagen and Frederiksborg Communities (no. KF11-131/01) and was performed in accordance with the Declaration of Helsinki.

Supplementation. A single-blind placebo-controlled design with randomization was used to acquire three groups of equal size (CEα, CEγ, and Control). Subjects in the CEα group (n = 7) received oral supplementation with ascorbic acid 500 mg/day and RRR-α-tocopherol 400 IU/day. This dose was based on a previous exercise experiment demonstrating attenuated oxidative stress in response to prolonged exercise (56). Subjects in the CEγ group (n = 7) received oral supplementation with ascorbic acid 500 mg/day, RRR-α-tocopherol 292 IU/day, and RRR-γ-tocopherol 130 IU/day. Accordingly, the supplementation employed in CEα and CEγ, contained virtually equal total vitamin E biological activity. Subjects in the Control group (n = 7) received placebo with similar appearance. The supplementation was ingested once a day together with breakfast. For all three groups, the length of the supplementation period before the final exercise experiment was 28 days. The duration of the supplementation was chosen from a previous study (47) showing that plasma tocopherol concentration reaches new steady state after at least 14 days of oral tocopherol supplementation.

Preexperimental protocol. Three weeks before the exercise experiment, the individual maximal power output, Pmax, of the subjects was determined during two-legged dynamic knee extensor exercise. The test was carried out using a modified ergometer described previously (3). In brief, the subjects performed a 5-min warm-up session consisting of 60 knee extensions with passive repositions per minute at 60 W after becoming accustomed to the exercise model. This was followed by an incremental increase of the workload by 10 W every 2 min until volitional exhaustion. The highest workload that was maintained for at least 1 min was defined as Pmax.

Experimental protocol. All exercise experiments were done between July and November. On day 28 of the supplementation period, the subjects reported to the laboratory at 0800. Subjects had fasted overnight and were asked to consume the supplement at 0700. Furthermore, the subjects were instructed to refrain from exercise for at least 24 h before the exercise. Upon arrival, subjects changed into hospital attire and rested in a supine position. After sterile conditions and with use of local anesthesia (lidocaine, 20 mg/ml, SAD), an indwelling catheter (Arrow) was placed in the femoral artery of the right leg by guide-wire (Seldinger) technique. The femoral artery was cannulated ~2 cm below the inguinal ligament, and a 20-gauge catheter was advanced ~10 cm in the proximal direction.

Before the onset of exercise, subjects in all three groups ingested two boiled eggs to ensure maximal uptake of the supplementation ingested earlier the same morning.

The exercise bout consisted of 3 h of two-legged dynamic knee extensor exercise at 60 extensions per minute at 50% of the individual Pmax.

After the exercise period, the subjects remained supine in the laboratory for a 3-h recovery period. Water was consumed ad libitum throughout the exercise and recovery period, but food was not permitted until the cessation of the recovery period.

Blood samples. Blood samples were drawn from the catheter at rest (0 h), immediately before cessation of the exercise (3 h), and at the end of the recovery period (6 h). Plasma was obtained by drawing blood into precooled EDTA-containing glass tubes, which were immediately centrifuged at 2,200 g for 15 min at 4°C. Plasma for vitamin C analysis was stabilized with 5% metaphosphoric acid. Blood samples for measurement of epinephrine and norepinephrine concentrations were drawn into ice-cold glass tubes containing glutathione and EGTA and spun immediately. Serum for measurement of HSP72 concentration was obtained by drawing blood into glass tubes con-

Table 1. Group characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CEα</th>
<th>CEγ</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Age, yr</td>
<td>25.6 (24.8–26.4) ns</td>
<td>22.3 (21.7–22.9) ns</td>
<td>24.1 (23.3–25.0)</td>
</tr>
<tr>
<td>Height, cm</td>
<td>177 (176–179) ns</td>
<td>179 (178–181) ns</td>
<td>181 (179–182)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.5 (22.9–24.0) ns</td>
<td>24.6 (24.1–25.1) ns</td>
<td>25.9 (25.3–26.4)</td>
</tr>
<tr>
<td>Pmax, W</td>
<td>150 (144–156) ns</td>
<td>153 (142–164) ns</td>
<td>140 (134–146)</td>
</tr>
</tbody>
</table>

Values are means with 95% confidence intervals in parentheses. BMI, body mass index; Pmax, maximal power output (during dynamic knee-extensor exercise); ns, not significant from Control. CEα, ascorbic acid and RRR-α-tocopherol supplementation; CEγ, ascorbic acid, RRR-α-tocopherol, and RRR-γ-tocopherol supplementation; control, placebo.
taining a clot-inducing plug. The tubes were subsequently stored on ice for 30 min before they were centrifuged at 2,200 g for 15 min at 4°C. All samples were stored at −80°C until further analysis.

Plasma concentration of the catecholamines epinephrine and nor-epinephrine was measured by HPLC, and plasma glucose concentration was determined by using an automatic analyzer (Cobas Fara, Roche), both methods described elsewhere (60).

Cortisol was measured using a RIA (no. DSL-10-2000, Diagnostic Laboratories, Webster, Texas), intra-assay coefficient of variability (CV) ∼5%.

In addition, blood samples drawn at 0, 3, and 6 h were analyzed for hemoglobin concentration and hematocrit by automated standard laboratory procedures to correct for possible changes in plasma volume according to the method described by Dill and Costill (18).

In brief, the assay involved the enzymatic oxidation of ascorbic acid was measured in plasma by a modified fluorometric method (17, 62).

The concentration of the F₂-isoprostane 8-iso-PGF₂α was determined by using 8-iso-PGF₂α antibody containing a clot-inducing plug. The tubes were subsequently stored on ice for 30 min before they were centrifuged at 2,200 g for 15 min at 4°C, and the supernatant was collected. The samples were subsequently stored on −80°C for 30 min before they were centrifuged at 2,200 g for 15 min at 4°C. All samples were stored at −80°C until further analysis.

Plasma ascorbic acid concentration. Ascorbic acid concentration was measured in plasma by a modified fluorometric method (17, 62).

Plasma α-tocopherol and γ-tocopherol concentrations were measured by using HPLC with fluorescence detection (53). In brief, 500 µl of plasma were extracted with 500 µl of ethanol containing 0.05 g of butylated hydroxytoluene and 2 ml of hexane. The supernatant (20 µl) was injected into a HPLC column (LiChrospher 100 NH2 250 × 4 mm), and fluorescence was measured with an excitation wavelength of 337 nm and an emission wavelength of 430 nm. Intra-assay CV was ∼4%.

Plasma α-tocopherol and γ-tocopherol concentrations were measured by using HPLC with fluorescence detection (53). In brief, 500 µl of plasma were extracted with 500 µl of ethanol containing 0.05 g of butylated hydroxytoluene and 2 ml of hexane. The supernatant (20 µl) was injected into a HPLC column (LiChrospher 100 NH2 250 × 4 mm), and fluorescence was measured with an excitation wavelength of 337 nm and an emission wavelength of 430 nm. Intra-assay CV was ∼4%.

Plasma 8-iso-PGF₂α concentration. The concentration of the F₂-isoprostane 8-iso-PGF₂α was determined by a RIA using an antibody raised in rabbits by immunization with 8-iso-PGF₂α coupled to bovine serum albumin at the carboxylic acid by the 1,19-carbonyldiimidazole method (6). Sensitivity was ∼23 pmol/l, and intra-assay CV was ∼14.5%.

Serum HSP72. Serum HSP72 concentration was measured using an ELISA kit (no. EKS-700, StressGen Biotechnologies) as described previously (64). Sensitivity was ∼0.2 ng/ml, with intra-assay CV <6%.

On each ELISA plate, samples from all three groups and all samples from single subject were represented. The samples were run in duplicates, and the mean was calculated. Values below the detection limit were assigned to the detection limit.

Muscle biopsies. Muscle biopsies were obtained from the vastus lateralis using the percutaneous needle method (Bergström) with suction (8). Before each biopsy, local anesthesia (lidozaine, 20 mg/ml, SAD) was applied to the skin and fascia superficial of the biopsy site. For each biopsy, a new incision at least 3 cm apart from any previous incisions was made. Time points for the biopsies were preexercise (0 h), immediately postexercise (3 h), and end recovery (6 h). Visible connective tissue and blood contamination was carefully removed before each biopsy was frozen in liquid nitrogen and subsequently stored at −80°C until further analysis.

Skeletal muscle HSP72 mRNA expression. Total RNA was extracted from the muscle tissue using TRIzol according to the manufacturer’s directions (Invitrogen, Grand Island, NY). The resulting RNA pellet was dissolved in diethylpyrocarbonate DEPC-treated water. Reverse transcription reactions were performed on 1 µg of RNA (Applied Biosystems) in a reaction volume of 50 µl. The resulting cDNA products were stored at −20°C until further analysis.

The forward primer 5′-ACCAAGCAGACCGGATCCTC-3′, the reverse primer 5′-GGCCCTCGTACCTGGATACTA-3′, and the Taq-Man fluorescent probe FAM-5′-CTACTCGGACCAACACACC-CCGGG-3′-TMARA (Applied Biosystems) were designed (Primer Express version 1.0, Applied Biosystems) from the gene sequences for human HSP72 (19). 18S rRNA (Applied Biosystems) was used as endogenous reference. The skeletal muscle expression of HSP72 mRNA and 18S rRNA was determined using multiplex conditions: 50 ng cDNA was mixed with 1× TaqMan universal PCR master mix, 900 nM HSP72 forward primer, 900 nM HSP72 reverse primer, 100 nM of Taqman HSP72 probe, and primer-limited 1× 18S. The final reaction volume of 25 µl was obtained by addition of RNase-free water. All samples were run in duplicates on an ABI PRISM 7700 sequence detector (Applied Biosystems) following standard conditions using 40 cycles.

The HSP72 mRNA expression was quantified using the comparative critical threshold (C_T) method, and fold changes from resting conditions were calculated using the 2−ΔΔC_T method (42).

**Skeletal muscle HSP72 protein expression.** Relative HSP72 content was determined by semiquantitative Western blotting technique: The frozen muscle samples were homogenized in ice-cold homogenization buffer (8 µl/mg tissue) containing 50 mM HEPES (no. S5461, Sigma), 150 mM NaCl (no. 106404, Merck), 100 mM NaF (no. S6521, Sigma), 10 mM sodium pyrophosphate (no. S9515, Sigma), 5 mM EDTA (no. E8884, Sigma), 0.5% Triton X-100 (no. 108603, Merck), 2 µg/ml leupeptin (no. L2023, Sigma), 2 µg/ml aprotinin (no. A1153, Sigma), 100 µg/ml PMSF (no. P7626, Sigma), 10 µg/ml pepstatin A (no. P4265, Sigma), and 1 mM Pervanadate. The latter was obtained by adding 30% 3H2O to dissolved sodium orthovanadate (no. S6508, Sigma) for 15 min at room temperature. The buffer was adjusted to pH 7.5 before use. After homogenization, the samples were centrifuged at 1,000 g for 10 min at 4°C and the supernatant was stored at −20°C until further analysis. The total protein concentration of the supernatant was determined with the Bradford technique (9) using a predeveloped protein staining assay (no. 500-0006, Bio-Rad Laboratories, Hercules, CA).

For each sample, 100 µg of protein were mixed with Laemmli buffer (38), boiled for 5 min at 100°C, and loaded onto an 8% Tris-glycine SDS-PAGE along with a prestained protein marker (no. LC5925, Invitrogen). After separation (30 mA per gel for 2 h), the proteins were transferred by electrophoresis (200 mA for 45 min) to nitrocellulose membrane. The proteins were visualized by using 0.1% Ponceau S for verification of correct loading, separation, and blotting. Nonspecific binding was blocked with 5% skimmed milk for 1 h at room temperature before overnight incubation at 4°C with the primary mouse anti-HSP70 monoclonal antibody 0.1 µg/ml (no. SPA-810, StressGen Biotechnologies, Victoria, British Columbia, Canada). The membranes were then washed for 4 × 15 min in wash buffer (PBS containing 0.05% Tween) and incubated with a HRP-conjugated anti-mouse secondary antibody 1:5,000 (no. P0847, DAKO, Glostrup, Denmark) for 1 h at room temperature. After a 4 × 15-min wash, the immune complexes were visualized on X-ray film by using enhanced chemiluminescence (no. RPN 2109, Amersham-Pharmacia) and the optical density of the bands was quantified (Scion Image Software, Scion, Frederick, MD). The final result was expressed relative to rest (0 h). By running control experiments with purified human HSP70 (no. NSP-555, StressGen Biotechnologies), we estimated the detection limit of the HSP72 Western blotting to ∼1 ng/lane and the intra-assay CV to <10%.

**Statistical analysis.** For all data, the distribution of the data was compared with the normal distribution before further statistical analysis. Age, body mass index (BMI), P_max, plasma [ascorbic acid], plasma [α-tocopherol], plasma [γ-tocopherol], plasma 8-iso-PGF₂α, levels, plasma [glucose], plasma [cortisol], plasma [epinephrine], plasma [norepinephrine], and skeletal muscle HSP72 protein expression were normally distributed without transformation (brackets denote concentration). Skeletal muscle HSP72 mRNA expression and serum [HSP70] were normally distributed after log-transformation. Regarding age, BMI, and P_max, the data were tested for differences between groups by using unpaired t-tests. For plasma vitamins and serum HSP72 concentrations, repeated-measures analysis was performed by using a mixed model assuming a first-order autoregressive
covariance structure among the repeated measurements. Significant changes from rest (0 h) were analyzed by Bonferroni-adjusted paired t-tests. For results expressed as fold changes from rest (plasma 8-iso-PGF2α, skeletal muscle HSP72 mRNA, and protein), overall differences between groups were tested by comparing the area under the curve with unpaired t-tests, followed by Bonferroni-adjusted unpaired t-tests at individual time points, and changes from rest within each group were tested by Bonferroni-adjusted one-sample t-tests.

The statistical analysis was performed by use of SAS statistical software (release 8.02, SAS Institute, Cary, NC). Throughout the statistical analysis, a P value lower than 0.05 was set as level of significance.

RESULTS

The three groups were comparable with regard to age, height, BMI and maximal power output (Table 1).

Plasma vitamin C and E concentrations. On day 28 of the supplementation, the three groups were markedly different with regard to plasma vitamin levels: At rest, plasma [ascorbic acid] (Fig. 1A) was approximately twofold higher in both CEα and CEαγ (overall effect of treatment P < 0.01, in both groups) relative to Control. Plasma [ascorbic acid] remained significantly elevated in CEα and CEαγ relative to Control in response to exercise at 3 h and 6 h, although a minor decrease in plasma [ascorbic acid] during the exercise experiment occurred in both CEα and CEαγ (overall effect of time P > 0.05 in both groups), but not in Control. Compared with Control, plasma [α-tocopherol] (Fig. 1B) was ~30% higher in both CEα and CEαγ (overall effect of treatment P < 0.05 in both CEα and CEαγ). No significant changes in plasma [α-tocopherol] from 0 h to 3 h and 6 h were observed in any of the groups. In CEα, plasma [γ-tocopherol] (Fig. 1C) was at all time points threefold lower than in Control (overall effect of treatment P < 0.001) and even decreased over time (overall effect of time P < 0.01). In contrast, plasma [γ-tocopherol] in CEαγ at all time points was more than fivefold higher than in Control (overall effect of treatment P < 0.001) with a borderline-significant increase over time (overall effect of time P = 0.054). In Control, plasma [γ-tocopherol] did not change in response to exercise.

Plasma lipid peroxidation. The plasma 8-iso-PGF2α level (Fig. 1D) increased from 0 h to 3 h in all groups (P < 0.05 in all groups), but compared with Control the increase was markedly attenuated (P < 0.05) in CEα, whereas the plasma 8-iso-PGF2α level in CEαγ increased at least as much as in Control. At 6 h, the plasma 8-iso-PGF2α levels had returned to baseline values in all groups.

Skeletal muscle expression of HSP72. In Control, HSP72 mRNA expression (Fig. 2A) in the contracting skeletal muscle increased ~2.5-fold (P < 0.05) from rest (0 h) to immediately postexercise (3 h) and returned to baseline level after the 3-h recovery period (6 h). In CEα, no significant change in HSP72 mRNA expression from rest to 3 h or 6 h was detected. Also, there was no difference between CEα and Control at any time point. In contrast, the HSP72 mRNA expression at 3 h and 6 h was marked lower (P < 0.05 at both time points) in CEαγ compared with Control. Of note, a borderline-significant decrease (Bonferroni-adjusted P = 0.082) of HSP72 mRNA expression from 0 h to 6 h was observed in CEαγ.
Skeletal muscle HSP72 protein (Fig. 2, B and C) did not change significantly in response to the 3-h bout of knee extensor exercise in any of the three groups, although a trend for an increase was present in Control ($P = 0.066$). However, the skeletal muscle HSP72 protein expression was lower ($P = 0.05$) in CE/H9251/H9253 relative to Control immediately after exercise (3 h).

A scatterplot of the skeletal muscle HSP72 mRNA and protein expression at the end of the exercise (Fig. 2D) showed a positive linear correlation (Pearson correlation: $R^2 = 0.43$, $P < 0.001$) between mRNA and protein. Thus increased HSP72 mRNA was associated with increased HSP72 protein and vice versa. However, fold changes in HSP72 mRNA expression appeared to be much larger than the corresponding fold changes in HSP72 protein.

Circulating levels of HSP72. The basal (0 h) serum HSP72 concentration (Fig. 2E) was not different between groups. In response to exercise, increased serum HSP72 levels were observed in CE, and Control (overall effect of time $P < 0.05$ in both groups), although post hoc analysis only detected a significant increase ($P < 0.05$) from 0 h to 3 h in Control. However, no change in serum HSP72 in response to acute exercise was observed CE/H9251. In fact, five of seven subjects in CE/H9251 had serum HSP72 concentrations below the detection limit of the assay at all time points.

The serum HSP72 concentration at the end of the exercise (3 h) correlated negatively with the plasma γ-tocopherol level (Fig. 2F). Within each group, the highest serum HSP72 concentration at 3 h was associated with the lowest plasma γ-tocopherol level, and vice versa, although the correlation...
was significant only when comparing all subjects irrespective of group.

**Plasma glucose.** No difference in plasma [glucose] (Table 2) at rest (0 h) was observed when comparing groups. In response to exercise, plasma [glucose] decreased in all three groups (overall effect of time \(P<0.05\), in all groups). At the end of the recovery period (6 h), plasma [glucose] was still lower than at rest only in Control, although not significantly different from plasma [glucose] in CE\(_{\alpha}\) and CE\(_{\alpha\gamma}\).

**Plasma cortisol.** At rest (0 h), there was no difference in plasma [cortisol] (Table 2) between groups. However, plasma [cortisol] increased in response to exercise only in Control (overall effect of time \(P<0.05\), whereas no effect of exercise on plasma [cortisol] was observed in CE\(_{\alpha}\) and CE\(_{\alpha\gamma}\). In Control, plasma [cortisol] at 6 h was markedly elevated \((P<0.05)\) from rest and different \((P<0.05)\) from the level observed in CE\(_{\alpha\gamma}\) at the same time point.

**Plasma epinephrine and norepinephrine.** At all time points, there were no differences in plasma [epinephrine] and [norepinephrine] (Table 2) between groups. At the end of the 3-h knee extensor exercise period, there was a marked elevation \((P<0.05)\) from rest in all groups. At the end of the 3-h knee extensor exercise period, there was a marked elevation \((P<0.05)\) from rest and different \((P<0.05)\) from the level observed in CE\(_{\alpha\gamma}\) at the same time point.

**DISCUSSION**

The main finding of the present study was that oral supplementation with vitamins C and E inhibited the exercise-induced increase of HSP72 in contracting skeletal muscle and in the circulation, but only when the supplementation included the vitamin E isoform \(\alpha\)-tocopherol.

Using the dynamic knee extensor exercise model associated with no or minimal muscle damage (21), we observed a 2.5-fold increase in the skeletal muscle HSP72 mRNA in the placebo-treated group. In all three groups, no significant change of the skeletal muscle HSP72 protein level was observed, but a borderline increase was present in the control group \((P=0.066)\). Because of the positive correlation between skeletal muscle HSP72 mRNA and protein postexercise (Fig. 2C), it is very likely that a significant increase in skeletal muscle HSP72 protein would have been detected in the control group, if the interindividual variation had been smaller or more subjects had been included. Accordingly, a type 2 error regarding the skeletal muscle HSP72 protein data should not be excluded. Another limitation is the possible effect of the supplementation on the basal expression of HSP72 mRNA and protein in skeletal muscle, because the aim was to determine differences in inducibility. Although alterations in the basal expression of HSP72 mRNA and protein in skeletal muscle in response to the supplementation may have occurred, the basal levels of serum HSP72 were not different between groups (Fig. 2E). Importantly, the present results regarding the exercise-induced skeletal muscle HSP72 expression in the placebo-treated group are very similar to previous studies investigating the effect of running for 30 min \((58)\) or bicycling for \(\sim3\ h\) \((19)\). A previous study performed in our laboratory \((21)\) using a lower workload \((40\%\ of\ P_{\text{max}})\) only showed a significant increase in skeletal muscle HSP72 mRNA and protein after knee extensor exercise in the leg, which had performed glyco-
gen-depleting exercise the day before the experiment. Otherwise, increased HSP72 protein levels in skeletal muscle have been reported in studies using eccentric exercise \((61)\) or prolonged running \((43, 64)\). In the present study, a fourfold increase of HSP72 in the circulation immediately after exercise was observed. An up to 10-fold increase of serum HSP72 has been reported in two studies using 60 min of running \((64)\) or 120 min of bicycling as exercise \((20)\). Of note, the latter study also showed that the circulating HSP72 comes from the hepa-
tosplanchnic region and not from the contracting muscle, at least in response to concentric exercise. Altogether, the previous studies and the present results indicate that exercise generally increases skeletal muscle mRNA, whereas HSP72 protein levels only increase markedly after more intense exercise or in response to exercise with an eccentric component associated with muscle damage.

Although we found no effect of supplementation with ascorbic acid and \(\alpha\)-tocopherol on HSP72 in response to exercise, the combination of ascorbic acid, \(\alpha\)-tocopherol, and \(\gamma\)-tocopherol clearly inhibited the exercise-induced increase of HSP72 mRNA and protein, as well as HSP72 in the circulation. Importantly, it is the first time that the effect of antioxidants on circulating HSP72 has been reported. In the study by Khassaf et al. \((35)\), vitamin C supplementation alone attenuated the exercise-induced increase of skeletal muscle HSP72, whereas basal HSP72 expression increased, but whether this could be explained by an antioxidative effect of vitamin C was not established. Of note, supplementation with antioxidants may in some instances increase rather than decrease oxidative stress \((12)\). Here, the exercise-induced lipid peroxidation was attenuated in the CE\(_{\alpha}\) group, indicating that the employed supple-
mentation attenuated oxidative stress. However, only the \( \gamma \)-tocopherol-containing supplementation appeared to inhibit the exercise-induced increase of HSP72 within the contracting muscle as well as the release of HSP72 from the hepatosplanchic region.

Two explanations for the differential effect of the two combinations of antioxidants should be considered: either the depletion of \( \gamma \)-tocopherol when providing supplementation with \( \alpha \)-tocopherol partly counteracts the overall antioxidant effect indicated by decreased lipid peroxidation or, alternatively, the effect of surplus \( \gamma \)-tocopherol may specifically inhibit HSP72 in response to exercise. Although not conclusive, the association between high plasma \( \gamma \)-tocopherol levels and low serum HSP72 levels (Fig. 2F) supports the latter point of view. In vitro, a combination of \( \alpha \)-, \( \delta \)-, and \( \gamma \)-tocopherol is a more efficient inhibitor of lipid peroxidation in erythrocytes compared with \( \alpha \)-tocopherol alone (40). The combination containing \( \gamma \)-tocopherol, however, did not appear to be a more efficient antioxidant than the combination without \( \gamma \)-tocopherol, at least when using the F2-isoprostane 8-iso-PGF\(_2\alpha\), as marker of lipid peroxidation. Interestingly, \( \gamma \)-tocopherol, but not \( \alpha \)-tocopherol, is a scavenger of peroxynitrite (13, 66), which at least in vitro is a highly potent stimulus of HSP synthesis in monocytes (1). Moreover, recent data suggest that \( \gamma \)-tocopherol has important anti-inflammatory and antineoplastic properties not related to the antioxidative capacity (24, 28, 31, 33). In the present study, concentric exercise was employed to avoid muscle damage associated with inflammation. Of note, no increase of creatine kinase from rest to 3 h postexercise was observed in any of the groups (not shown). Accordingly, an anti-inflammatory effect of \( \gamma \)-tocopherol is not likely to be involved in the modulation of HSP72 in response to nondamaging exercise. However, specific data demonstrating the differences between \( \alpha \)- and \( \gamma \)-tocopherol in vivo are yet insufficient.

Importantly, although oxidative stress is one important stimulus for increased HSP synthesis (63), several other exercise-related stressors of the cellular homeostasis have been shown to induce HSPs either in vivo or in vitro: elevated temperature (48), decreased glucose availability (7), increased intracellular calcium levels (65), increased adrenergic stimulation (54), ischemia (44), and hypoxia (25). In the present study, we did not observe changes in plasma glucose or catecholamines that indirectly could explain the effect of \( \gamma \)-tocopherol. Of note, the exercise-induced increase of plasma cortisol observed in the placebo-treated group was blunted in both of the antioxidant-treated groups, which is in accordance with previous studies (51, 55). However, because the effect of either antioxidant combination on plasma cortisol was similar, neither cortisol appears to be an important link between antioxidants with \( \gamma \)-tocopherol and the effect on HSP72. Furthermore, because the workload was comparable between groups, it is not likely that perfusion, intramuscular temperature, or intracellular calcium would differ between groups to a point that could explain the effect of supplementation containing \( \gamma \)-tocopherol on HSP72. Accordingly, it is likely that the effect of ascorbic acid, \( \alpha \)-tocopherol, and \( \gamma \)-tocopherol on HSP72 was mediated through an effect on ROS and RNS generated in response to exercise. Of note, the positive correlation between skeletal muscle HSP72 mRNA and protein in all three groups indicates that the effect of the \( \gamma \)-tocopherol-containing supplementation on HSP72 is exerted at the level of gene transcription, although the exact mechanism by which \( \gamma \)-tocopherol may inhibit HSP72 in vivo warrants further research. Moreover, the effect of \( \gamma \)-tocopherol alone was not tested in the present study, because \( \gamma \)-tocopherol was employed to counteract the effect of \( \alpha \)-tocopherol on plasma vitamin E concentrations. However, the present findings indicate that future studies using supplementation with purified \( \gamma \)-tocopherol, with or without ascorbic acid, may provide interesting data.

Because extracellular HSP72 may mediate an inflammatory response via binding to the CD14-receptor located on monocytes (4), it could be speculated that antioxidants containing \( \gamma \)-tocopherol may be used as an anti-inflammatory compound: in vitro, \( \gamma \)-tocopherol is a more potent inhibitor of cyclooxygenase activity in macrophages when compared with \( \alpha \)-tocopherol (33). Also, low plasma concentrations of \( \gamma \)-tocopherol have been associated with increased cardiovascular mortality (46) as well as increased risk of development of prostate cancer (27). In this context, supplementation with \( \gamma \)-tocopherol may be relevant. On the other hand, a suppression of exercise-induced increase of HSP72 might be disadvantageous: whereas regular exercise training increases the basal skeletal muscle HSP72 content (41), low skeletal muscle HSP72 is associated with insulin resistance (37) and Type 2 diabetes (10). Regardless, our results support the opinion that the two vitamin E isoforms, \( \alpha \)-tocopherol and \( \gamma \)-tocopherol, have important different qualities, which should be considered in future studies (32).

In summary, our results show that 28 days of oral supplementation with ascorbic acid, \( \alpha \)-tocopherol, and \( \gamma \)-tocopherol inhibits the increase of HSP72, in skeletal muscle and in the circulation, in response to acute exercise.

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