Vitamin C and E supplementation alters protein signalling after a strength training session, but not muscle growth during 10 weeks of training

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Key points

- Although antioxidant supplements are generally believed to give health benefits, recent experiments show that they may adversely affect adaptations to endurance exercise.
- This study is the first to investigate the effects of high dosages of vitamins C and E on the cellular and physiological adaptations to strength training in humans.
- Here we report that vitamin C and E supplementation interfered with exercise-induced signalling in muscle cells after a session of strength training, by reducing the phosphorylation of p70S6 kinase and mitogen-activated protein kinases p38 and ERK1/2.
- The vitamin C and E supplement did not significantly blunt muscle hypertrophy during 10 weeks of training; however, some measurements of muscle strength revealed lower increases in the supplemented group than the placebo group.
- Even though the cellular events are not clearly reflected in physiological and performance measurements, this study implies that redox signalling is important for inducing skeletal muscle adaptations to strength training and that vitamin C and E supplements in high dosages should be avoided by healthy, young individuals engaged in strength training.

Abstract This study investigated the effects of vitamin C and E supplementation on acute responses and adaptations to strength training. Thirty-two recreationally strength-trained men and women were randomly allocated to receive a vitamin C and E supplement (1000 mg day−1 and 235 mg day−1, respectively), or a placebo, for 10 weeks. During this period the participants’ training involved heavy-load resistance exercise four times per week. Muscle biopsies from m. vastus lateralis were collected, and 1 repetition maximum (1RM) and maximal isometric voluntary contraction force, body composition (dual-energy X-ray absorptiometry), and muscle cross-sectional area (magnetic resonance imaging) were measured before and after the intervention. Furthermore, the cellular responses to a single exercise session were assessed midway in the training period by measurements of muscle protein fractional synthetic rate and phosphorylation of several hypertrophic signalling proteins. Muscle biopsies were obtained from m. vastus lateralis twice before, and 100 and 150 min after, the exercise session (4 × 8RM, leg press and knee-extension). The supplementation did not affect the increase in muscle mass or the acute change in protein synthesis, but it hampered certain strength increases (biceps curl). Moreover, increased phosphorylation of p38 mitogen-activated protein kinase, Extracellular signal-regulated protein kinases 1 and 2 and p70S6 kinase after the exercise session was blunted by vitamin C and E supplementation. The total ubiquitination levels after the exercise session, however, were lower.

Introduction

Effective strategies to increase muscle mass are of interest for athletes and coaches, clinicians prescribing treatment for muscle loss, and individuals training for recreation. Skeletal muscle mass is a determinant for physical performance and its functions are vital for good health (Pedersen & Saltin, 2006; Williams et al. 2007; Phillips & Winett, 2010). Resistance exercise is undisputedly effective for maintaining and increasing muscle mass, but nutrients are prerequisites (Hawley et al. 2011). Interestingly, in the search for types and dosages of various nutrients that will accelerate the effects of exercise, it has become clear that certain allegedly healthy nutrients may both facilitate and hamper cellular adaptations for exercise (Peternelj & Coombes, 2011; Hawley et al. 2011). Indeed, high doses of antioxidants may interfere with the exercise-induced activity of cell signalling pathways, e.g. pathways initiating mitochondrial biogenesis (Ristow & Zarse, 2010; Peternelj & Coombes, 2011). The focus has hitherto been on endurance exercise (Peternelj & Coombes, 2011; Braakhuis, 2012; Paulsen et al. 2014b), and less is known about effects of antioxidant supplementations on physiological and cellular adaptations to resistance exercise, i.e. strength training (Wadley, 2013). However, Makanae et al. (2013) have recently reported that high dosages of vitamin C can attenuate hypertrophy of overloaded muscles in rats. The investigators observed that both phosphorylated Extracellular signal-regulated protein kinases 1 and 2 and p70S6 kinase (p70S6K) were reduced in the vitamin C supplemented rats, and these alterations appeared to be related to diminished muscle growth. That key cellular regulators of muscle hypertrophy are regulated by oxidative stress (reactive oxygen and nitrogen species, RONS) is supported in previous animal models (Wretman et al. 2001; Ito et al. 2013).

Human studies that have looked into the influence of antioxidants on adaptations to strength training are sparse. However, in a study applying high-force eccentric exercise for the knee-extensors, Theodorou et al. (2011) administered 1000 mg vitamin C and 400 IU vitamin E daily to recreationally trained men. In short, the supplementation had no effects on muscle performance after 4 weeks of training (8 sessions), nor on the recovery after a session of eccentric exercise—conducted both before and after the training period. With elderly untrained participants, Bobeuf et al. (2010, 2011) observed tentative evidence that vitamin C (1000 mg day$^{-1}$) and vitamin E (400 IU day$^{-1}$) supplementation facilitated muscle gain during 6 months of strength training. Furthermore, Chuin et al. (2009) reported a protective effect of vitamin C (1000 mg day$^{-1}$) and vitamin E (600 mg day$^{-1}$) supplementation on bone loss in elderly women during a 6 month period. This effect was, however, similar to the effect of resistance exercise training alone; thus, there was no additive effect of the supplementation and exercise. Apparently, these latter studies indicate no negative effects of the supplementation. On the other hand, Ristow et al. (2009) demonstrated a reduced mRNA response for several genes linked to endogenous antioxidant systems (e.g. glutathione peroxidase) and the peroxisome-proliferator-activated receptor γ co-activators (PGC-1α/β) after 4 weeks of combined endurance exercise and circuit training. Similarly, Malm et al. (1997) observed that Coenzyme Q10 supplementation seemed to inhibit performance improvement of high-intensity, anaerobic bicycling training.

Accordingly, it seems clear that antioxidant supplements, such as vitamins C and E, under certain conditions can interfere with cellular adaptations to exercise, but not much can be concluded from previous studies when it comes to strength training. In fact, to date, no studies have investigated the potential interaction between antioxidant supplementation and traditional, heavy-load, strength training in healthy, young adult humans. Therefore, we aimed to investigate the effects of vitamin C and E supplementation on the adaptation to traditional strength training in healthy, recreationally trained men and women. We hypothesized that the antioxidant supplementation would blunt some of the oxidative stress normally generated during resistance exercise and thereby attenuate cellular signalling regulating muscle protein synthesis. As a consequence, muscle growth and strength development would be diminished during 10 weeks of training.

The hypothesis was tested in a double-blind, randomized, controlled design. We combined a prospective study over 10 weeks with acute measurements conducted before
Table 1. Characteristics of participants in the vitamin C and E and the placebo groups

<table>
<thead>
<tr>
<th></th>
<th>Vitamin C + E</th>
<th>Placebo</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>( n = 17: )</td>
<td>( n = 15: )</td>
</tr>
<tr>
<td>5 women and 12 men</td>
<td>6 women and 9 men</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>27 ± 6</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.76 ± 0.08</td>
<td>1.76 ± 0.08</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>76.6 ± 11.9</td>
<td>72.0 ± 14.0</td>
</tr>
</tbody>
</table>

Values are means and standard deviations.

and after a single exercise session in order to assess both acute cellular responses and long-term adaptations.

**Methods**

**Participants**

Thirty-two recreationally strength-trained volunteers completed the study (Table 1). ‘Recreationally strength trained’ was defined as a person that had trained for 1–4 sessions per week during the previous 6 months with strength exercises, including both upper and lower body exercises. Many of the volunteers were also regularly engaged in some kind of aerobic endurance exercise, but during the intervention, only one endurance session per week was allowed.

The participants had to be healthy with no injuries in the musculoskeletal system that would limit the execution of training. All participants underwent a medical screening before entering the study.

The use of any form of dietary supplementation, except the experimental C and E vitamin supplement, was prohibited during the intervention. Participants that did use some sort of supplements stopped this practice 2 weeks before the training intervention at the latest.

Six of the 38 participants entering the study dropped out (Paulsen et al. 2014a). Two participants experienced injuries unrelated to the study, and four withdrew because of a time schedule that was too busy.

The study was approved by the Regional Ethics Committee for Medical and Health Research of South-East Norway and performed in accordance with the Declaration of Helsinki. All participants signed a written informed consent form.

**Study design**

This study was a double-blinded, randomized controlled trial (Paulsen et al. 2014a). Tests and measurements were conducted before and after 10 weeks of heavy-load traditional strength training at four exercise sessions per week (see below).

Enrolled volunteers started on a preparatory strength-training programme during a period with familiarization to tests and pre-tests and measurements (1–4 weeks): a whole body workout programme, three sessions per week, 8–12 repetitions with approximately 15RM loads. The purpose of this preparatory, run-in training programme was to familiarize the participants with the exercises in the intervention programme.

After the pre-tests and measurements, participants were randomized to receive a vitamin C and E supplement or placebo. The randomization process was stratified by sex and maximal strength (1RM).

In order to measure the acute effects of exercise, a subgroup of participants (7 from the vitamin C and E group and 8 from the placebo group) conducted an exercise session midway into the training intervention. The exercise was preceded and followed by muscle biopsies and blood samples (see ‘Acute exercise session’ below).

**Supplements**

The C and E vitamin and placebo pills were produced under Good Manufacturing Practice (GMP) requirements at Petefa AB (Västra Frölunda, Sweden). Each vitamin pill contained 250 mg of ascorbic acid and 58.5 mg DL-α-tocopherol acetate. The placebo pills had the same shape and appearance as the vitamin pills.

Participants ingested two pills (500 mg of vitamin C and 117 mg vitamin E) 1–3 h before every training session and two pills in the hour after training. On non-training days, the participants ingested two pills in the morning and two pills in the evening. The intake of pills was recorded in a training and supplement diary. Thus, the daily dosage was 1000 mg of vitamin C and 235 mg of vitamin E.

The participants were asked to drink no more than two glasses of juice and four cups of coffee or tea per day. Juices especially rich in antioxidants, such as grape juice, were to be avoided.

We aimed to keep the participants in a slight positive energy balance and encouraged the participants to continue their habitual diets, but we gave advice about the intake of a protein-rich meal/drink (e.g. 0.5 l of milk) shortly after training. The participants completed a 4-day weighed food registration diary (Black et al. 1991) at the start and end of the intervention period. Participants used a digital food weighing scale (Vera 67002; Soehnle-Waagen GmbH & Co., Murrhardt, Germany; precision 1 g). The dietary registrations were analysed with a nutrient analysis programme (Mat på data 4.1; Norwegian Nutrition Society, Oslo, Norway). Participants with a protein intake lower than 1.0 g × (kg body weight)−1 at the first recording were recommended to increase their protein intake.
Table 2. Outline of the strength training programme

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Load (RM)</th>
<th>Sets</th>
<th>Inter-set rest (min)</th>
<th>Sessions per week</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–6</td>
<td>9–11</td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>7–10</td>
<td>6–8</td>
<td>3–4</td>
<td>1.5</td>
<td>4</td>
</tr>
</tbody>
</table>

Upper-body exercises Session #1 | Lower-body exercises Sessions #2 | Upper-body exercises Sessions #3 | Lower-body exercises Sessions #4

Bench press | Squat | Incline chest press | Deadlift
Dumbbell flies | Lunge | Pullover | Lunge
Standing shoulder press | Knee-extension | Lateral rise | Leg press
Triceps push-down | Straight leg deadlift | Pull-down (narrow grip) | Knee-flexion
Sitting rowing | Standing calf raise | Standing over-bent rowing | Standing calf raise
Pull-down (wide grip) | Self-elected abdominal exercise | Biceps-curl (Scott curl) | Self-elected abdominal exercise
Self-elected abdominal exercise | Self-elected abdominal exercise

Training programme

The participants followed a traditional strength-training programme with four sessions per week (Table 2). During the first 6 weeks, the loads were 3 × 9–11RM whereas in the last 4 weeks the load was 3–4 × 6–8RM. The inter-set rest periods were short (1–1.5 min). Exercises for all the major muscle groups were included in a 2 day split routine: two upper body and two lower body sessions per week.

The volunteers were supervised during the first sessions, and they had the opportunity to be continuously supervised during training. However, most of the participants trained unsupervised after the initial sessions because they were familiar with strength training and the exercises included in the programme. Importantly, all the participants recorded their training in a diary that was regularly controlled by the investigators.

Acute exercise session

After 4–6 weeks of the intervention, 15 of the participants were tested in an ‘acute’ experiment. The exercise sessions included 4 × 10RM of leg press and knee-extension, with 1 min of rest between sets and 3 min between exercises. Muscle biopsies and blood samples were collected before and after the exercise session (Fig. 1).

Participants ingested the supplements, vitamins C and E or placebo, together with a standardized breakfast (3 g oats × (kg body weight)^-1 boiled in water with 5 g sugar) 2 h before meeting in the laboratory. A new dose of supplements was taken before the post tests. The performance tests were included to monitor the acute changes in muscle function and the recovery (3 and 24 h after exercise). Muscle protein fractional synthetic rate was measured before and after the exercise session (Fig. 1).

Tests and measurements

Maximal strength. Maximal strength was assessed by 1 repetition maximum (1RM) tests and a maximal isometric voluntary contraction (MVC) before and after the training intervention.

1RM was tested in knee-extension, knee-flexion, biceps curl and elbow extension. Each leg and arm was tested (unilateral tests). A specific warm-up was performed with 10 repetitions of loads corresponding to 50% of expected 1RM and then followed by 6, 3 and 1 repetition with increased loads, corresponding to approximately 70%, 80% and 90% of expected 1RM, respectively. Two to five attempts were normally used to find 1RM. The loads could be adjusted with steps as low as 3%. The left and right leg/arm were tested interchangeably so that each muscle rested approximately 2 min between attempts. Range of motion in each exercise was strictly controlled. Individual mean values from the left and right leg/arm were used in the statistical analyses. The coefficient of variation (CV) of this assessment was <5%.

Unilateral MVC was tested for the knee-extensors using a custom-made knee-extension apparatus (Gym2000, Geithus, Norway). The participants were fixed in a chair with belts over chest and hips, 90 deg in the knee and hip joints. Participants were allowed three 5 s attempts to reach their MVC; 1 min of rest was given between attempts. Force was measured with a force transducer (HBM U2AC2, Darmstadt, Germany). Individual mean values from the left and right leg were used in the statistical analyses. The CV of this assessment was <5%.

Specific muscle force was calculated by dividing the MVC values by the individual largest cross-sectional area (measured using magnetic resonance imaging (MRI)) of the quadriceps muscle.
**Dual-energy X-ray absorptiometry.** Body composition was measured by dual-energy X-ray absorptiometry (Hologic Discovery, Waltham, MA, USA) before and after the intervention period. Participants were scanned from head to toe in a supine position, providing values for total bone mineral content, lean tissue and fat mass. The CV of these assessments was <2%.

**MRI of thigh and arm muscles.** Transverse section images were captured of the dominant arm and both thighs (GE Signa 1.5 Tesla Echospeed, GE Medical Systems, Madison, WI, USA) before and after the intervention period. Thigh muscles: joint gaps were used as reference points, and nine images (5 mm) were captured with 35.5 mm inter-image distance. Upper arm muscles: the humerus bone of each participant was sectioned in nine evenly distributed images (5 mm). The images [Digital Imaging and Communications in Medicine (DICOM)] were analysed using OsiriX 3.9.3 (Pixmeo, Bernex, Switzerland), giving the cross-sectional area (CSA) of individual muscles. The CV of these assessments was <2%.

**Biopsy collection and pre-analytical analyses.** Muscle biopsies were collected from the mid portion of m. vastus lateralis before and 2–4 days after the last training session. The biopsies obtained before the acute experiment were preceded by 2–3 days of rest.

The procedure was conducted under local anaesthesia (Xylocaine with adrenaline, 10 mg ml\(^{-1}\) + 5 μg ml\(^{-1}\), AstraZeneca, London, UK). Approximately 200 mg (2–3 × 50–150 mg) of muscle tissue was obtained with a modified Bergström technique. Biopsy samples were quickly washed in physiological saline in order to remove blood; fat and connective tissue were discarded, and the specimens were weighed before being rapidly frozen in isopentane cooled on dry ice or liquid nitrogen. Muscle biopsies intended for morphological analyses (immunohistochemistry) were mounted in Tissue-Tek (4583, Sakura Finetek, Torrance, CA, USA) and rapidly frozen in isopentane cooled on liquid nitrogen. All muscle samples were stored at −80 °C for later treatments/analysis.

About 50 mg of muscle sample was homogenized in 1 ml ice-cold T-PER (Tissue Protein Extraction Reagent, Thermo Scientific, Rockford, IL, USA), 20 μl Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific), and 20 μl EDTA (Thermo Scientific).

Protein concentrations were measured using a commercial kit (BioRad DC protein micro plate assay, Bio-Rad, Hercules, CA, USA), a filter photometer (Expert 96, ASYS Hitech, Cambridge, UK), and the provided software (Kim, ver. 5.45.0.1, Daniel Kittrich, daniel@ok.cz). Protein measurements were run in triplicates (analytical CV was ~5%).

**Protein immunoblot.** Fifteen to thirty micrograms of protein was separated by 4–12% SDS-PAGE gels under denatured conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Immuno-blot, Bio-Rad). Membranes were blocked for 1 h at room temperature in 5% fat-free skimmed milk and 0.05% TBS-t solution [Tris-buffered saline (TBS; Bio-Rad); Tween 20 (VWR International, Radnor, PA, USA); skimmed milk powder (Merck, Germany)] and incubated overnight at 4°C with primary antibodies (see below), followed by incubation with appropriate secondary antibodies for 1 h at room temperature (see below). Membrane was washed in 0.05% TBS-t solution between stages. Protein bands were visualized by luminol-based enhanced chemiluminescence (Super Signal West Dura Extended Duration Substrate, Pierce Biotechnology, Thermo Scientific, Rockford, IL, USA) and quantified using Kodak Image Station 2000R and calculated using Thermo Scientific, Rockford, IL, USA), a filter photometer (Expert 96, ASYS Hitech, Cambridge, UK), and the provided software (Kim, ver. 5.45.0.1, Daniel Kittrich, daniel@ok.cz). Protein measurements were run in triplicates (analytical CV was ~5%).

Phosphorylation states are given as the ratio between phosphorylated and total protein (phosphorylated and non-phosphorylated), except for Proline-rich Akt substrate of 40 kDa, where only phosphorylated states are reported. Ubiquitinated proteins were quantified as described elsewhere (Hulmi et al. 2013).

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**Figure 1. Timeline for the acute experiment**

MVC, maximal voluntary contraction.
Primary and secondary antibodies were diluted in a 1% fat-free skimmed milk and 0.05% TBS-t solution. Antibodies against p70S6K (cat. no. 9202), phospho-p70S6K Thr189 (cat. no. 9205), Ribosomal protein S6 (cat. no. 2217), phospho-RPS6 Ser240/244 (cat. no. 2215), Eukaryotic elongation factor 2 (cat. no. 2332), phospho-eEF-2 Thr56 (cat. no. 2331), p38 mitogen-activated protein kinase (MAPK) (cat. no. 9212), phospho-p38 MAPK Thr180/Tyr182 (cat. no. 9211), ERK1/2 (cat. no. 9102), phospho-ERK1/2 Thr202/Tyr204 (cat. no. 9101), AMP-activated protein kinase (cat. no. 2603), phospho-AMPKα Thr172 (cat. no. 2531S), Eukaryotic initiation factor 4E-binding protein 1 (cat. no. 9644) and phospho-4EBP1 Thr37/46 (cat. no. 2855) were obtained from Cell Signaling Technology (Beverly, MA, USA). Phospho-PRAS40 Thr246 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from MBL (Woburn, MA, USA) and Abcam (Cambridge, UK), respectively. Secondary antibodies were obtained from Cell Signaling Technology. Protein stripping was conducted using Restore Western Blot Stripping Buffer (Pierce Biotechnology, Rockford, IL, USA). Blots are shown in Fig. 2.

Immunohistochemistry. Serial cross sections (8 μm) were cut using a microtome (CM3050; Leica Biosystems GmbH, Wetzlar, Germany) at −20°C and mounted on Superfrost Plus microscope slides (Thermo Scientific), air-dried, and stored at −80°C until further analysis.

The muscle cross sections were blocked for 30 min with 1% bovine serum albumin (Sigma Life Science, St Louis, MO, USA) in a phosphate-buffered saline (PBS) and 0.01% Tween20 solution (PBS-t) (PBS; Calbiochem, EMD Biosciences, San Diego, CA, USA; Tween20; Sigma-Aldrich, St Louis, MO, USA) before incubation with antibodies against myosin heavy chain type 2 (1:500; SC71; gift from Prof. S. Schiaffino, University of Padua, Padua, Italy) and dystrophin (1:1000; Abcam) diluted in the blocking solution overnight at 4°C. The muscle sections were then incubated with appropriate secondary antibodies (Alexa Fluor, A10005 and A11001, Invitrogen, Carlsbad, CA, USA). The muscle sections were washed 2–3 × 10 min in PBS-t between each step and finally mounted with cover glass and visualized with ×20 magnification objectives using a high resolution camera (DP72, Olympus Corp., Tokyo, Japan) attached to an Olympus microscope (BX61) with a fluorescence light source (X-Cite 120PCQ, EXFO Photonic Solutions Inc., Mississauga, ON, Canada). The individual fibre cross-sectional areas and fibre type were analysed using TEMA software (CheckVision, Hadsund, Denmark). Based on the fibre-type staining, values were calculated for type 1 and type 2 fibres, separately. A mean of 534 fibres (range 113–1192) was analysed on each cross section.

Muscle protein fractional synthetic rate (FSR). The procedure is described in detail by Zhang et al. (2002). Briefly, a baseline blood sample was taken for background measurement of amino acid enrichment. Thereafter, a bolus injection of 13C6-phenylalanine was given in a dose of 50 μmol kg−1 (Cambridge Isotope Laboratories, Andover, MA, USA). Biopsies from m. vastus lateralis were collected at 10 and 60 min after the first bolus injection. Blood samples were collected regularly for 1 h after the first bolus injection. This procedure was performed before the resistance exercise session and repeated again exactly 1.5–2.5 h after the exercise session (Fig. 1).

Blood samples. Plasma from blood samples for the measurement of phenylalanine enrichment was analysed as previously described (Wolfe & Chinkes, 2005). Briefly, plasma was deproteinized with 500 μl 15% sulfosalicylic acid, and amino acids were purified using cation exchange chromatography. Purified amino acids were dried and thereafter derivatized with 80 μl (1:1, v/v) acetonitrile: N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) (Sigma-Aldrich) for 40 min at 70°C.

Muscle samples. Twenty-five to thirty milligrams of muscle was placed in 800 μl 10% perchloric acid (PCA), homogenized and centrifuged. The supernatant was collected for measurement of intracellular amino acid enrichment. The remaining pellet (bound protein) was washed three times with 2% PCA, two times with ethanol, and one time with diethyl ether, dried overnight at 50°C and hydrolysed overnight in 6 N HCl at 110°C. Amino acids from the bound and intracellular fractions were then purified by cation exchange chromatography and thereafter derivatized in the same way as for the blood samples.

Chromatography. Isotopic enrichments of the tert-butyldimethylsilyl (t-BDMS) derivatives of phenylalanine were determined by gas chromatography/mass spectrometry in electron impact ionization mode (Agilent 6890 GC, 5973 MS, Santa Clara, CA, USA).

Calculations. The muscle protein fractional synthetic rate (FSR) was directly calculated from the enrichment decay over time after the tracer bolus. FSR is calculated as the change in enrichment in tracer bound to muscle protein divided by the change in intracellular enrichment in muscle over the study period (Wolfe & Chinkes, 2005).

Statistics

A two-way ANOVA with repeated measures (time × group) was applied to test group differences before and after the 10 week training period and relative changes from before to after the exercise sessions (acute experiment). Holm–Sidak’s multiple comparisons test was used as a post hoc test to specify the significant group differences. Group differences in the relative changes (%) from before
to after the 10 week training period were tested with an unpaired Student’s t test. Relative changes within each group were assessed with a paired Student’s t test. Data sets not normally distributed (D’Agostino and Pearson omnibus normality test) were log-transformed before analysis (all data are illustrated in original form). The level of significance was set to 0.05. Values are given as mean and standard deviation (SD) in text and tables. The figures display maximum–minimum values, 25th and 75th percentiles, and the medians (boxplot), as some of the variables were not normally distributed. Outliers were defined by Tukey’s rule: values equal or greater than 1.5

Figure 2. Protein immunoblots
Panel of representative protein immunoblots for p38 MAPK (phosphorylated at Thr180/Tyr182 and total), p70S6K (phosphorylated at Thr389 and total), RPS6 (phosphorylated at Ser240/244 and total), eEF-2 (phosphorylated at Thr56 and total) and PRAS40 (phosphorylated at Thr246 and total). All these samples were run in duplicates. 4EBP1 (phosphorylated at Thr37/46 and total), ERK1/2 (phosphorylated at Thr202/Tyr204 and total) and AMPKα (phosphorylated at Thr172 and total) were run in singles with GAPDH as loading control. All samples from each participant were always quantified in the same blot.
times the interquartile range (IQR; 75th percentile minus the 25th percentile) plus the 75th percentile or smaller than 1.5 times IQR minus the 25th percentile. Outliers are identified individually in figures but not removed from the statistical calculations. Effect size was calculated as the differences between the group means divided by the combined SD. Graphpad Prism (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analyses.

Results

The participants in the vitamin C and E group and the placebo group completed the same number of strength training sessions: 37 ± 6 and 35 ± 6 sessions, respectively. The total load (repetitions × sets × kg × sessions) lifted during the 10 weeks training period was 200,000 ± 90,600 kg and 190,000 ± 92,900 kg for the vitamin C and E group and the placebo group, respectively (P = 0.7). The adherence to the supplements was adequate: vitamin C and E group at 93 ± 9% and placebo group at 96 ± 6%. Furthermore, the total energy and macronutrient consumption and the habitual intake of vitamin C and E were similar in the two groups (Table 3).

Vitamin C and E levels in plasma were not measured in this study. However, the supplements (same production batch) were tested on another group of participants of similar age range (Paulsen et al. 2014b), and a robust increase in both vitamins C and E during 11 weeks of supplementation was observed, 52 ± 55% and 31 ± 21%, respectively (both P < 0.001). No changes were observed in the placebo group.

Muscle strength

During the 10 week training period, both the vitamin C and E group and the placebo group increased 1RM in leg exercises (knee-extensions and leg curl) and upper body exercises (biceps curl and triceps press; P < 0.01; Fig. 3). The placebo group tended to increase strength more than the vitamin C and E group in the upper body exercises (P = 0.057; Effect size = 0.74); however, the group difference was only significant for biceps curl (17.1 ± 17.0% vs. 7.6 ± 5.0%; P = 0.04; Effect size = 0.86). A similar trend was observed in the isometric maximal voluntary contraction (MVC) for the knee-extensors (Fig. 4; P = 0.1; Effect size = 0.46). Moreover, the specific strength was decreased in the vitamin C and E group (P = 0.03), and the changes tended to be different from the placebo group (Effect size = 0.78; Fig. 4).

Muscle hypertrophy

The cross-sectional areas of the upper arm and the thigh muscles increased similarly in both groups during the 10 weeks of strength training (vitamin C and E: 8.5 ± 6.7%...
(upper arm) and 3.8 ± 4.0% (thigh); placebo: 7.6 ± 7.0% (upper arm) and 3.8 ± 4.7% (thigh); \( P < 0.01 \) for all; Fig. 5). Both appendicular lean mass (arms and legs; vitamin C and E: 2.4 ± 3.0%; placebo: 3.4 ± 2.9%; both \( P < 0.01 \); Fig. 5) and total lean mass increased equally in the two groups (Table 4). Fat mass and bone mineral content were unchanged in both groups (Table 4).

The mean cross-sectional area of individual muscle fibres did not significantly change in the vitamin C and E group (pre: 6336 ± 2393 \( \mu \text{m}^2 \) vs. post: 6805 ± 2046 \( \mu \text{m}^2 \); 12 ± 25%; \( P = 0.28 \); Table 5) or the placebo group (pre: 5651 ± 1847 \( \mu \text{m}^2 \) vs. post: 6086 ± 2021 \( \mu \text{m}^2 \); 9 ± 12%; \( P = 0.06 \); Table 5).

**Acute experiment**

In the acute experiment, muscular fatigue was manifested as reduced MVC force after the exercise session in both groups (vitamin C and E: −20 ± 8%, placebo: −16 ± 7%; both \( P < 0.01 \)), but the force-generating capacity had

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**Figure 4. Changes in maximal isometric contraction force**

Upper panel: changes in maximal voluntary contraction force (MVC) during isometric conditions (90 deg in the knee-joint) after vs. before 10 weeks of strength training. Lower panel: changes in specific strength (MVC force divided by quadriceps cross-sectional area) after vs. before 10 weeks of strength training. Boxplots show maximum–minimum values, 25–75th percentiles, and medians. •, outliers (Tukey’s rule); *within-group changes. The \( P \) value for a between-group comparison (statistical tendency) is given in the figure. C + E vit, vitamin C and E group.

**Figure 5. Changes in muscle cross-sectional area and appendicular lean mass**

Upper and middle panels: changes in cross-sectional area (CSA) of upper arm (m. brachialis, m. biceps brachii, m. triceps brachii) and thigh (m. quadriceps and hamstring muscles) after vs. before 10 weeks of strength training. Lower panel: appendicular muscle mass (arms and legs) after vs. before 10 weeks of strength training. Boxplots show maximum–minimum values, 25–75th percentiles, and medians. •, outliers (Tukey’s rule); *within-group changes. C + E vit, vitamin C and E group.
recovered in both groups within 24 h (Fig. 6). There were no differences between groups.

Muscle protein fractional synthetic rate (FSR). The FSR rate was increased after the exercise session with no differences between groups ($P = 0.42$; Fig. 7).

Ubiquitination. The total level of ubiquitinated proteins was increased in the placebo group 100 and 150 min after exercise, and these changes were significantly different from the vitamin C and E group ($P = 0.023$ and $P = 0.005$, respectively; Fig. 7).

Protein signalling. Phosphorylation levels of the upper band of p38 MAPK were increased in the placebo group 100 and 150 min after the exercise session ($P < 0.01$), and the increases were significantly larger than in the vitamin C and E group 10 min after exercise ($P = 0.01$), but there were no significant group differences ($P = 0.09$; Fig. 8). ERK1/2 phosphorylation increased only in the placebo group (Fig. 8). When the 100 and 150 min time points were combined for ERK1/2, the exercise-induced phosphorylation was higher in the placebo group than in the vitamin C and E group ($P = 0.023$).

Discussion
The present study tested the hypothesis that vitamin C and E supplementation would blunt the muscular adaptation to strength training in young, recreationally trained individuals. There were three primary findings. (1) Vitamin C and E supplementation did not affect the gain in muscle mass during 10 weeks of strength training, while increases in muscle strength were partly suppressed in the vitamin C and E group. (2) In response to a standardized strength training session, signalling pathways associated with muscle hypertrophy (MAPKs

### Table 4. Body composition before and after the 10-week intervention period

<table>
<thead>
<tr>
<th></th>
<th>Vitamin C and E</th>
<th>Placebo</th>
<th>$P$ values for group difference (% change)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre Post % change</td>
<td>Pre Post % change</td>
<td></td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>76.6 $\pm$ 11.9 78.2 $\pm$ 12.3 $^{<em>}$ 2.2 $\pm$ 1.6 $^{</em>}$</td>
<td>72.8 $\pm$ 14.2 73.9 $\pm$ 15.0 $^{*}$ 1.3 $\pm$ 2.7</td>
<td>0.31</td>
</tr>
<tr>
<td>Fat free mass (kg)</td>
<td>59.7 $\pm$ 10.1 60.7 $\pm$ 10.3 $^{<em>}$ 1.8 $\pm$ 1.6 $^{</em>}$</td>
<td>59.0 $\pm$ 13.2 60.6 $\pm$ 14.3 $^{<em>}$ 1.7 $\pm$ 1.6 $^{</em>}$</td>
<td>0.89</td>
</tr>
<tr>
<td>Bone mineral content (kg)</td>
<td>3.1 $\pm$ 0.6 3.1 $\pm$ 0.5 0.7 $\pm$ 1.5</td>
<td>3.0 $\pm$ 0.6 3.0 $\pm$ 0.6 0.3 $\pm$ 1.5</td>
<td>0.45</td>
</tr>
<tr>
<td>Lean mass (kg)</td>
<td>56.6 $\pm$ 9.6 57.6 $\pm$ 9.8 $^{<em>}$ 1.8 $\pm$ 1.8 $^{</em>}$</td>
<td>56.0 $\pm$ 12.6 57.5 $\pm$ 13.8 $^{<em>}$ 1.9 $\pm$ 1.8 $^{</em>}$</td>
<td>0.93</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>16.9 $\pm$ 4.7 17.5 $\pm$ 5.3 3.6 $\pm$ 11.9</td>
<td>13.8 $\pm$ 3.3 13.9 $\pm$ 3.9 $^{*}$ $\pm$ 0.3 $\pm$ 12.0</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Values are means and standard deviations. Within-group changes: $^{*}P \leq 0.01$.

### Table 5. Fibre cross-sectional area of type 1 and type 2 fibres and fibre-type distribution

<table>
<thead>
<tr>
<th></th>
<th>Vitamin C and E</th>
<th>Placebo</th>
<th>$P$ values for group difference (% change)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre Post % change</td>
<td>Pre Post % change</td>
<td></td>
</tr>
<tr>
<td>Fibre type 1 (%)</td>
<td>46 $\pm$ 15 44 $\pm$ 14 $^{*}$ 1.2 $\pm$ 8.1</td>
<td>50 $\pm$ 12 47 $\pm$ 13 $^{<em>}$ $^{</em>}$ 2.1 $\pm$ 7.2</td>
<td>0.77</td>
</tr>
<tr>
<td>CSA fibre type 1 ($\mu$m$^2$)</td>
<td>5687 $\pm$ 1339 5933 $\pm$ 907 $^{*}$ 7.3 $\pm$ 17.9</td>
<td>5239 $\pm$ 1223 5252 $\pm$ 1399 $^{*}$ 3.7 $\pm$ 14.7</td>
<td>0.63</td>
</tr>
<tr>
<td>CSA fibre type 2 ($\mu$m$^2$)</td>
<td>6987 $\pm$ 3158 7333 $\pm$ 2734 $^{*}$ 11.1 $\pm$ 29.0</td>
<td>6171 $\pm$ 2468 6694 $\pm$ 2818 $^{*}$ 9.6 $\pm$ 16.2</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Values are means and standard deviations. CSA, cross-sectional area.
Strength training and vitamin C and E supplementation

Antioxidants interfere with cell signalling after exercise

We investigated several steps in signalling pathways involved in the regulation of protein synthesis, and we observed that vitamin C and E supplementation blunted the activation of the MAP kinases p38 and ERK1/2, as well as p70S6K. Interestingly, our observations are closely in line with those of Makanae et al. (2013). Indeed, Makanae et al. described blunted activation of ERK1/2 and p70S6K in overloaded m. plantaris of rats supplemented with high doses of vitamin C. Similarly, Wretman et al. (2001) reported that ERK1/2 activation could be inhibited by the antioxidant N-acetyl-cysteine in isolated rodent m. extensor digitorum longus muscles after concentric contractions. Thus, contraction-induced activation of ERK1/2 and p38 MAPK appears to be redox sensitive and therefore inhibited by antioxidant supplements. Lending support to these rodent studies, Michailidis et al. (2013) recently showed that N-acetyl-cysteine blunted the activation of both p38 MAPK and p70S6K after eccentric exercise in humans. The supplement also seemed to delay the recovery of muscle function. The later observation was, however, not supported in the present study as we found no effect of supplementation on the recovery of force-generating capacity after a standardized strength training session (see discussion).
below). In a study with endurance training (Paulsen et al. 2014b), using the same supplementation as in the present study, we observed certain effects on the mRNA levels of MAPK1 (ERK2). Thus, evidence accumulates and points towards MAPK kinases being activated by oxidative stress and therefore sensitive to antioxidant supplementation.

4EBP1 phosphorylation decreased in the placebo group in contrast to the C and E vitamin group, but the changes were not different between groups. A decrease in 4EBP1 activation after resistance exercise, which in theory may slow the protein synthesis rate, has been reported in recent studies (Koopman et al. 2007; Deldicque et al. 2008; Mascher et al. 2008); however, the decrease may be avoided

![Figure 8. Changes in the phosphorylation status of p38 MAPK upper and lower bands, and ERK1/2 100 and 150 min after a single session of strength training, compared to before (pre 1) exercise levels](image-url)

Pre 1, biopsy obtained 80 min before exercise. Pre 2, biopsy obtained 30 min before exercise. •, outliers (Tukey’s rule); #, difference between groups; * within-group changes from pre 1. C + E vit, vitamin C and E group.
by whey protein consumption (Hulmi et al. 2009), which was not provided in the present study. Phosphorylation of AMPKα, RPS6, RPAS40, and eEF-2 was not changed after exercise in either group.

The divergent effects of vitamin C and E supplementation on the phosphorylations of the mTORC1 downstream targets p70S6K and 4EBP1 deserves comment. The fact that p70S6K phosphorylation at Thr^{389} was blunted by the supplementation while no

Figure 9. Changes in the phosphorylation status of AMPKα, PRAS40, 4EBP1, p70S6K, RPS6 and eEF-2 100 and 150 min after a single session of strength training, compared to before (pre 1) exercise levels
Pre 1, biopsy obtained 80 min before exercise. Pre 2, biopsy obtained 30 min before exercise. Boxplots show maximum–minimum values, 25–75th percentiles, and medians. * outliers (Tukey’s rule); # difference between groups; * within-group changes from pre 1. C + E vit, vitamin C and E group.
effect was seen in the phosphorylation of 4EBP1 suggests that the supplement did not directly affect mTORC1 activity. It is plausible that the supplementation attenuated the increase in p70S6K phosphorylation by diminishing the phosphorylation of the auto-inhibitory domain of p70S6K via blunted MAPK signalling (ERK1/2, p38, JNK) (Martin et al. 2014). Furthermore, p70S6K and 4EBP1 may compete as substrates for mTORC1 (Dennis et al. 2013), implying that the higher p70S6K phosphorylation in the placebo group would explain the reduction in 4EBP1 phosphorylation in this group only. Thus, assuming that mTORC1 activity is more important than p70S6K activity in regulating the protein synthesis machinery could explain why we found no differences between groups in muscle protein fractional synthetic rate.

Interestingly, p66shc is potentially an important player in this process as it can regulate reactive oxygen species activity, activate both MAPKs and p70S6k, and it can be modulated by ascorbic acid (vitamin C) (Natalicchio et al. 2009; Ramieri et al. 2010; Kirmani et al. 2013). RPS6, downstream of p70S6K and ERK1/2, did not display increased phosphorylation after the resistance exercise session, which is in contrast to other reports (Dreyer et al. 2006; Glover et al. 2008). Similarly to us, Lundberg et al. (2012) observed p70S6K (Thr389) phosphorylation without a corresponding RPS6 phosphorylation 15 min and 3 h after resistance exercise in recreationally trained individuals. The lack of RPS6 could be explained by the influence of other regulators, such as S6K2 (Pende et al. 2004). Furthermore, RPS6 appears to be activated by amino acids (Meyuhas, 2008), and the combination of resistance exercise and protein intake induces a much stronger RPS6 activation than exercise and placebo (Hulmi et al. 2009). No protein supplement was ingested between the exercise session and the biopsies in the present study. Finally, we cannot rule out that our sampling points made us miss certain mechanistically important cell signalling processes.

We did not succeed in measuring protein breakdown rate. Any potential effects of antioxidant supplementation on the protein breakdown rate, and thus the net balance between synthesis and breakdown, are therefore not known. We did, however, observe more ubiquitination in the placebo group than the supplemented group during the first hours after exercise. Accumulation of ubiquitinated proteins indicates that the vitamin C and E supplement may have hindered the exercise-induced increase in the ubiquitin–proteasome proteolytic pathway, possibly by reducing the oxidation of proteins. The blunted ubiquitination may be associated with the reduced p38 MAPK phosphorylation in the vitamin C and E group because p38 MAPK regulates ubiquitin ligase expression (Glass, 2005; Kim et al. 2009).

The blunted ubiquitination by the vitamin C and E supplement is seemingly in contrast with the findings of Makanae et al. (2013), where the atrogin levels (ubiquitin ligase) were lower in the placebo group than in the antioxidant group. This discrepancy is difficult to disentangle and could be related to several factors, e.g. different assay, species and muscle loading model. On the other hand, an alternative explanation for our findings is that the accumulation of ubiquitinated proteins observed after exercise in the placebo group was caused by reduced proteasome activity. In this scenario, the rate of ubiquitination might have been similar, but the supplement may, by reducing the cellular oxidative stress, have affected the proteasome activity positively (Shang & Taylor, 2011) and, thereby, decreased the amount of ubiquitinated proteins for degradation.

**Recovery of muscle function**

Intensive exercise induces fatigue, which may be followed by prolonged muscle weakness (Paulsen et al. 2012). To accelerate recovery from exercise, several approaches and treatments have been tested (Bloomer, 2007), and supplements containing vitamin C and/or E have been investigated in previous studies. The results are mixed as authors have reported positive effects (Jakeman & Maxwell, 1993; Thompson et al. 2001), no effects (Beaton et al. 2002; Thompson et al. 2003; Avery et al. 2003; Mastaloudis et al. 2006; Teixeira et al. 2009), and adverse effects (Close et al. 2006). The purported effect of antioxidant supplementations is to reduce the allegedly negative event caused by reactive oxygen and nitrogen species (RONS) generated during exercise and in the recovery period (e.g. due to inflammation). As most studies, we found no effect of vitamin C and E supplements on the recovery of force-generating capacity after the strength training session. This finding indicates that RONS are not major contributors in the prolonged recovery process after normal resistance exercise or that the supplement was ineffective in this matter. Unfortunately, we have no measurements of the oxidative stress levels in the exercised muscles.

**Changes in muscle strength**

In accordance with previous strength training studies without supplement interventions (Deschenes & Kraemer, 2002; Wernbom et al. 2007), muscle growth and improved 1RM muscle strength were evident in both the vitamin C and E supplement group and the placebo group. The augmented upper body strength tended, however, to be larger in the placebo group (Effect size = 0.7). In fact, for biceps curl 1RM, the increase was statistically larger in the placebo group than in the vitamin C and E
group. Similarly, MVC for the knee-extensor increased only in the placebo group, but the difference between groups was statistically uncertain (Effects size = 0.5). The indicated negative effects of supplementation on strength improvement were peculiarly not reflected in the measurements of muscle growth, which were similar for both groups. Purportedly, the hampered development of strength was due to changes in muscle quality and reduced specific strength. Without any redox-measurements, we can only suggest that the supplementation unfavourably altered the redox conditions for force generation (Niess & Simon, 2007). Alternatively, the supplementation may have reduced the quality in force-generating structures as an attenuated ubiquitination in response to exercise potentially may have slowed the protein turnover.

Human studies

The present study is the first to investigate the effects of high dosages of vitamin C and E on younger adults undergoing traditional, heavy-load, strength training. Previous human studies are therefore not directly comparable. Of some relevance, Theodorou et al. (2011) observed no effects of vitamin C and E supplementation (1000 mg day\(^{-1}\) and 400 IU day\(^{-1}\), respectively) on either strength increases or markers of redox status in blood and muscle after 4 weeks of eccentric exercise training (note that the supplementation preceded the training period by 5 weeks). Contrary to our observations, Bobeuf et al. (2010, 2011) reported that vitamin C (1000 mg day\(^{-1}\)) and E (400 IU day\(^{-1}\)) supplementation supposedly facilitated muscle growth in elderly participants during resistance exercise training (6 months). More positive responses to antioxidant supplementation may, however, be expected in elderly individuals due to age-related alterations in the redox conditions of skeletal muscles (Gomez-Cabrera et al. 2013). Indeed, Ryan et al. (2010) demonstrated improved concentric work capacity of old rats, but not young rats, supplemented with vitamin C and E. However, such observations await support from human studies. Also, the dose effect and the timing of the dosing should be further investigated.

Perspectives

The present study clearly demonstrates that antioxidant supplements, such as vitamins C and E, can alter exercise-induced cellular signalling (MAPK and p70S6K) and ubiquitination. However, although adverse effects on strength development were found, consistent long-term effects on muscle hypertrophy were not detected. The present study may have been underpowered and too short lasting to elucidate all the physiological effects of the supplementation. Moreover, individual responses to strength training (Hubal et al. 2005) combined with the lack of standardized protein intake post-exercise (Cermak et al. 2012) may have diluted some of the supplementation effects in the present study. Future studies should provide a standardized protein source (e.g. milk or protein bar) after each exercise session in order to maximize hypertrophy, increase the length of the intervention period (e.g. 21 weeks), and recruit a higher number of participants. In order to reduce individual responses, future studies should also strive to recruit individuals with a similar training background, e.g. team sport athletes.

Conclusion

The aim of this study was to investigate the effects of vitamin C and E supplementation on adaptations to strength training. Our results are equivocal because we observed an inhibition of the acute protein signalling after a standardized strength exercise session, but there were no significant group differences in muscle protein fractional synthetic rate or muscle growth over 10 weeks of training. Still, the gains in muscle strength were partly blunted in the vitamin C and E group. It therefore appears that vitamin C and E supplementation can interfere with the cellular signalling after exercise, such as MAPKs, and ubiquitination, but redundancy in the pathways may overpower most of these effects, and adaptions over time are less affected. Nevertheless, we suggest that young, healthy individuals who exercise for improved strength and muscle growth should avoid consuming high dosages of vitamins C and E close to the exercise sessions because, if anything, the effects tend to be undesirable.

References


Strength training and vitamin C and E supplementation


Additional information

Competing interests

None declared.

Author contributions

H.W. and I.G. All authors approved the final version for publication.

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