

# Effects of vitamin E and $\alpha$ -lipoic acid on skeletal muscle contractile properties

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<sup>1</sup>Center for Exercise Science, University of Florida, Gainesville, Florida 32611; <sup>2</sup>Department of Molecular and Cell Biology, University of California, Berkeley, California 94720; and <sup>3</sup>Department of Physiology, University of Kuopio, Kuopio, Finland 70211

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**Coombes, Jeff S., Scott K. Powers, Benjamin Rowell, Karyn L. Hamilton, Stephen L. Dodd, R. Andrew Shanely, Chandan K. Sen, and Lester Packer.** Effects of vitamin E and  $\alpha$ -lipoic acid on skeletal muscle contractile properties. *J Appl Physiol* 90: 1424–1430, 2001.—Initial experiments were conducted using an in situ rat tibialis anterior (TA) muscle preparation to assess the influence of dietary antioxidants on muscle contractile properties. Adult Sprague-Dawley rats were divided into two dietary groups: 1) control diet (Con) and 2) supplemented with vitamin E (VE) and  $\alpha$ -lipoic acid ( $\alpha$ -LA) (Antiox). Antiox rats were fed the Con rats' diet (AIN-93M) with an additional 10,000 IU VE/kg diet and 1.65 g/kg  $\alpha$ -LA. After an 8-wk feeding period, no differences existed ( $P > 0.05$ ) between the two dietary groups in maximum specific tension before or after a fatigue protocol or in force production during the fatigue protocol. However, in unfatigued muscle, maximal twitch tension and tetanic force production at stimulation frequencies  $\leq 40$  Hz were less ( $P < 0.05$ ) in Antiox animals compared with Con. To investigate which antioxidant was responsible for the depressed force production, a second experiment was conducted using an in vitro rat diaphragm preparation. Varying concentrations of VE and dihydrolipoic acid, the reduced form of  $\alpha$ -LA, were added either individually or in combination to baths containing diaphragm muscle strips. The results from these experiments indicate that high levels of VE depress skeletal muscle force production at low stimulation frequencies.

antioxidants; contraction; redox; oxidation; reactive oxygen species

IT WAS RECOGNIZED OVER A DECADE AGO that the redox status of skeletal muscle fibers is altered by heavy exercise (8). Indeed, exercising muscle produces reactive oxygen species (ROS) such as superoxide anions and hydroxyl radicals (10). Furthermore, it is now clear that intense muscular contractile activity can result in oxidative stress as indicated by altered muscle glutathione levels and an increase in both protein oxidation and lipid peroxidation (2, 8, 15, 26, 32). When proteins and lipids become oxidized by ROS, muscle force production is diminished (22). Reducing agents such as antioxidants can protect cells against ROS-induced

oxidative stress (21). Theoretically, it follows that increasing the intracellular levels of antioxidants within a muscle cell should provide greater protection against these oxidizing agents and reduce fatigue. However, Reid et al. (22) have demonstrated that low levels of ROS are required for optimum muscle contractile function. Specifically, they demonstrated that addition of the antioxidant enzymes (i.e., catalase and superoxide dismutase) resulted in diminished in vitro muscle contractile performance in unfatigued muscle. Further, the addition of strong synthetic antioxidants such as dithiothreitol (3), *N*-acetylcysteine (NAC) (13), and DMSO (23) to an organ bath containing skeletal muscle also results in depressed skeletal muscle force production. Combined, these studies indicate that administration of synthetic antioxidants may negatively impact skeletal muscle contractile properties in unfatigued muscle. At present, it is unknown whether similar effects occur when antioxidants are consumed in the diet in large quantities; this forms the basis for the current experiments.

Vitamin E (VE) is an important dietary antioxidant in biological systems because of its association with the cell membrane and its ability to act directly on ROS and prevent lipid peroxidation (6). After quenching a radical, VE is converted to a radical (chromanoxyl form) and loses its antioxidant capacity. Recent research has focused on the ability of VE to be reduced from this chromanoxyl form back to its native state via other antioxidants. This regeneration of VE can be achieved by dihydrolipoic acid (DHLA), the reduced form of  $\alpha$ -lipoic acid ( $\alpha$ -LA) (12). In addition to its ability to recycle VE,  $\alpha$ -LA is also a potent antioxidant because of its capacity to quench ROS in both the aqueous and lipid phases of the cell (19). Indeed, the combination of VE and  $\alpha$ -LA has been shown to act synergistically to protect cells against oxidative damage such as ischemia-reperfusion injury, neurodegeneration, and cataract formation (19). Hence, we selected the combination of the antioxidants VE and  $\alpha$ -LA to determine whether these dietary antioxidants impact skeletal muscle contractile function.

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Therefore, the primary purpose of these experiments was to determine the effects of dietary supplementation with VE and  $\alpha$ -LA on skeletal muscle contractile function in situ. On the basis of previous studies, we hypothesized that consumption of large quantities of dietary antioxidants would negatively impact muscle force production in unfatigued skeletal muscle. Our results supported this hypothesis and revealed that supplementation with  $\alpha$ -LA and VE resulted in a right shift in the muscle force-frequency curve. These findings prompted a second series of experiments designed to elucidate whether this right shift in the force-frequency curve was due to VE or  $\alpha$ -LA alone or to the combination of the two antioxidants. To address this question, we used the *in vitro* rat diaphragm preparation and investigated the effects of these antioxidants both collectively and individually.

## METHODS

### *In Situ Experiment (Experiment 1)*

Female Sprague-Dawley rats (4 mo old,  $n = 28$ ) were divided into 2 dietary groups: 1) control (Con) and 2) supplemented with VE and  $\alpha$ -LA (Antiox). Con animals were fed an AIN-93M purified diet containing 75 IU/kg diet VE (DL- $\alpha$ -tocopheryl acetate). The Antiox animals were fed the AIN-93M purified diet with an additional 10,000 IU/kg diet DL- $\alpha$ -tocopheryl acetate (Hoffmann-LaRoche, Nutley, NJ) and 1.65 g/kg  $\alpha$ -LA (Asta Medica, Frankfurt, Germany). Animals were fed their respective diets for 8 wk before the measurement of muscle contractile properties. Diets were professionally prepared by Harlan Teklad (Madison, WI). To ensure that all animals consumed equal amounts of their respective diets, animals were pair fed. This was achieved by first pairing animals (14 pairs) on the basis of body weight, allocating one to each group, and housing them individually. In seven of the pairs, the animal in the Con group was given the weight of food that the corresponding pair from the Antiox group consumed the previous day. In the other seven pairs this was reversed so that the animal from the Antiox group received the weight of food that the corresponding pair from the Con group consumed the previous day. Weight gain was equal between groups (data not presented).

On completion of the feeding period, animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg) and ventilated (tidal volume = 0.7 ml/100 g body mass; breathing frequency = 80 breaths/min and positive end-expiratory pressure = 1.0 cmH<sub>2</sub>O) with room air using a Columbus small animal ventilator (CIV-101, Columbus, OH) to maintain a constant arterial partial pressure of O<sub>2</sub> (Pa<sub>O<sub>2</sub></sub>) of ~90 mmHg. Pa<sub>O<sub>2</sub></sub> was determined via blood gas analysis (IL 1610, Instrumentation Laboratories, Lexington, MA) with 75- $\mu$ l samples taken from the arterial catheter. Pa<sub>O<sub>2</sub></sub> was maintained by increasing the respiratory frequency when necessary.

Rectal temperature was monitored, and body temperature was maintained at 37°C with a heating blanket. After a surgical plane of anesthesia was reached, the sciatic nerve was located and cut, with the distal stump placed in a bipolar electrode. The tendon of the tibialis anterior (TA) muscle was cut close to the bone and attached to an isotonic force transducer (Piezo Systems, Cambridge, MA, model 400). The TA was chosen for the experiment because of its mixed-fiber-type composition. The transducer output was amplified and differentiated by operational amplifiers and underwent analog-

to-digital conversion for analysis with a computer-based data acquisition system (GW Instruments-Series II, Somerville, MA).

Muscle temperature and moisture were maintained with a heating lamp and saline-soaked gauze, respectively. The origin of the TA muscle was anchored with a bone nail placed in the femur perpendicular to the limb. Previous experiments in our laboratory indicate that circulation to the muscle was not affected by the procedure (unpublished results). Calibration of the force transducer was done by applying known weights on the lever. The TA was stimulated to contract via stimulation of the sciatic nerve with a Grass Instruments S48 stimulator (Quincy, MA). Twitch measurements were used to determine optimal muscle length ( $L_o$ ) initially and throughout the experiment. Maximal specific tension ( $P_o$ ), twitch tension ( $P_t$ ), and a force-frequency curve were then determined at  $L_o$  before and after a 60-min fatigue protocol.

$P_o$  was measured with a series of isometric tetanic contractions using a supramaximal stimulus train (250 ms, 60 V, and 150 Hz). Each tetanic contraction was separated by a 2-min recovery period to prevent fatigue between contractions. The absolute maximal force was then divided by the wet weight of the TA to obtain  $P_o$  (N/kg).  $P_t$  was measured with a 2-ms twitch contraction at 60 V. The force-frequency curve was determined by stimulating the muscle to contract every 2 min at 10, 20, 30, 40, 60, 80, 120, 160, 200, and 250 Hz (60-V, 250-ms trains). The fatigue protocol consisted of 60-V, 15-Hz trains every 2 s. The duty cycle [ratio of the period of muscle contraction (250 ms) to the duration of a cycle of contraction and rest (2,250 ms)] was 1:9.

Immediately after the contractile measurements, the animal was euthanized with a lethal injection of pentobarbital sodium (100 mg/kg). The TA muscle was removed, trimmed of fat and connective tissue, weighed, frozen in liquid nitrogen, and stored at -80°C for future biochemical analyses. The contralateral TA was also removed for comparative biochemical measurements.

*Measurement of VE levels in muscle.* The concentration of VE in the TA was determined with high-performance liquid chromatography (HPLC) using the protocol of Cort et al. (7). Samples from both the contracted and the contralateral limb were homogenized in acetone using a mechanical homogenizer (Ultra-Turrax T25, IKA Works, Cincinnati, OH). Samples were extracted twice with petroleum ether and then reconstituted in isoctane and analyzed for VE content. The isoctane extract (20  $\mu$ l) was injected onto a 250  $\times$  4 mm, 10- $\mu$ m LiChrosorb SI column (Baird and Tatlock, Dagenham, UK).

*Measurement of  $\alpha$ -LA levels in muscle.* Tissue levels of  $\alpha$ -LA were measured according to Panigrahi et al. (20), with slight modification. Frozen samples (0.5–0.6 g) were ground to powder in liquid nitrogen with a mortar and pestle then homogenized on ice in 2 ml of 20% metaphosphoric acid using a Teflon homogenizer. The homogenate was ultrasonicated intermittently for 100 s (10-s periods with 10 s rest). After hexane evaporation under nitrogen, the extract was solubilized in a solvent containing 50% 0.2 M monochloroacetic acid, 30% acetonitrile, and 20% methanol. Samples were filtered (0.22  $\mu$ m) and frozen at -80°C for HPLC analysis. Samples (0.2 ml) were separated on a Alltima C18 column (250  $\times$  4.6 mm, 5  $\mu$ m; Alltech Associates, Deerfield, IL) using a mobile phase consisting of 50% 50 mM sodium biphosphate in water, 30% acetonitrile, and 20% methanol and a flow rate of 1 ml/min.  $\alpha$ -LA was detected at a retention time of 9.5 min using a Coulechem II multielectrode electrochemical detector (ESA model 5100A, ESA, Chelmsford, MA). The electrodes were set at the following potentials: *electrode 1*, +0.45 V;

electrode 2, +0.85 V; and guard cell: +0.90 V. Racemate mixture of lipoate (50% DHLA, 50%  $\alpha$ -LA), used as a standard, was provided by ASTA Medica (Frankfurt, Germany). With the use of this method, a linear ( $r^2 = 0.996$ ) standard curve in the range 0.2–0.75 nM of lipoate was obtained.

**Lipid peroxidation measurements.** To determine the amount of oxidative damage in the TA, levels of lipid peroxidation were measured by two methods. First, malondialdehyde levels were measured spectrophotometrically using the thiobarbituric acid-reactive substance (TBARS) method described by Mihara and Uchiyama (16). 1,1,3,3-Tetraethoxypropane was used as the standard for this assay. Secondly, lipid hydroperoxides were quantified using the ferrous oxidation/xylenol orange technique described by Hermes-Lima et al. (11). Cumene hydroperoxide was used as the standard for this assay, and hydroperoxide values are expressed in cumene hydroperoxide equivalents (CHE). In our hands, the coefficients of variation for the TBARS and the lipid hydroperoxide assays are ~3 and 4%, respectively.

### *In Vitro Protocol (Experiment 2)*

The TA muscle was not used in our in vitro experiments because the diameter of this muscle is too great to permit gases to diffuse to and from deep fibers in a nonperfused in vitro preparation. Hence, to avoid this problem, we selected the costal diaphragm for study because the diameter of this muscle in the rat is not diffusion limited in an in vitro preparation. Similar to the TA, the costal diaphragm is a mixed-fiber muscle. Importantly, the in vitro rat diaphragm preparation is a well-established model to study skeletal muscle function in vitro (9, 10, 13, 21–23).

Four-month-old female Sprague-Dawley rats ( $n = 32$ ) were anesthetized with an intraperitoneal injection of pentobarbital sodium (90 mg/kg). When the animal reached a surgical plane of anesthesia, the diaphragm was quickly excised with the ribs and central tendon intact and placed in Krebs-Ringer solution (containing in mM: 137 NaCl, 5 KCl, 2 CaCl<sub>2</sub>, 1 MgSO<sub>4</sub>, 1 NaH<sub>2</sub>PO<sub>4</sub>, and 24 NaHCO<sub>3</sub>) equilibrated with 95% O<sub>2</sub>-5% CO<sub>2</sub> (pH 7.4). Two small strips of muscle (~3.0 × 20–25 mm) from the ventral costal diaphragm were carefully cut with a portion of rib and central tendon retained on each strip to enable the attachment of a clamp without compromising constituent fibers.

Figure 1 illustrates the protocol of *experiment 2*. Isometric contractile properties of each strip were measured simultaneously using a dual-bath setup. Each muscle strip was suspended vertically between two lightweight Plexiglas clamps in a 100-ml temperature-controlled bath (Harvard Scientific, Holliston, MA). Each strip was connected to an isometric force transducer (Grass FT-03D) and mounted on a micrometer for adjustment of muscle length. The baths contained Krebs-Ringer solution with 25  $\mu$ M D-tubocurarine chloride and equilibrated with 95% O<sub>2</sub>-5% CO<sub>2</sub>. Field stimulation was created along the entire length of each muscle strip with platinum plate electrodes (4 × 37 mm) using a modified Grass Instruments S48 stimulator (Quincy, MA). The stimulator was modified to produce sufficient amperage for field stimulation in both baths simultaneously. Output from each transducer was amplified and differentiated by operational amplifiers and underwent analog-to-digital con-

version for analysis using a computer-based data acquisition system (GW Instruments-Series II).

At the beginning of each protocol, the strips were maintained at 25°C to minimize temperature-dependent deterioration. Each strip was adjusted to its  $L_o$  by systematically altering the length of the muscle using a micrometer while evoking twitch contractions (140 V, 2-ms duration). Once  $L_o$  was established,  $P_o$  was measured using tetanic contractions (100 Hz, 330 ms, 140 V). Maximal force generation during tetanic contractions was normalized to cross-sectional area (CSA) and expressed as newtons (N/cm<sup>2</sup>). CSA was estimated using the formula CSA (cm<sup>2</sup>) = muscle mass (g)/[muscle length (cm) × muscle density (g/cm<sup>3</sup>)], assuming muscle density = 1.056 g/cm<sup>3</sup> (30). Response of the diaphragm muscle strip to increasing stimulus frequency was assessed at  $L_o$ , by the application of 20-, 30-, 40-, 60-, 80-, 120-, 160-, and 200-Hz pulses applied in 330-ms trains at 140 V. A 2-min recovery period was used between contractions. At the completion of the force-frequency protocol, the temperature of the muscle bath was increased to 37°C, and either an antioxidant treatment or the vehicle (alcohol) was added to the muscle baths. The following antioxidant treatments were investigated: 1) combination of VE (400  $\mu$ M) and DHLA (100  $\mu$ M), 2) VE (400  $\mu$ M), 3) DHLA (100  $\mu$ M), 4) VE (200  $\mu$ M), or 5) VE (100  $\mu$ M). Each experimental treatment was prepared by adding the compound(s) to 500  $\mu$ l of alcohol and vortexing in an Eppendorf tube until dissolved. Thirty minutes after the increase in bath temperature and the addition of the aforementioned compound(s) to the baths,  $P_o$ ,  $P_t$ , and the force-frequency relationship were assessed. Note that alcohol did not affect contractile properties of the muscle strip in the control bath.

Fatigue of the diaphragm strips was then determined by monitoring the decrease in force development over time. The muscle strips were stimulated simultaneously with tetanic contractions (40 Hz, 250 ms) every 2 s (duty cycle = 1:9). This pattern of stimulation was chosen to elicit a 40–50% reduction in force in 30 min. Fatigue was determined at 37°C because Diaz et al. (9) have shown that antioxidants are more likely to have an effect on diaphragm fatigue resistance in vitro at this temperature.

### *Data Analysis*

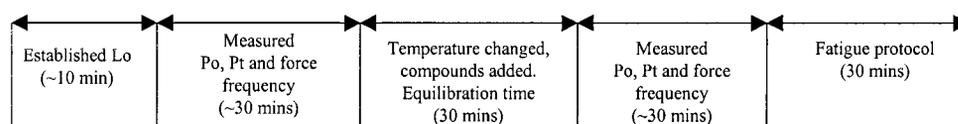
Comparisons between groups for each dependent variable were made using either a one-way or two-way (repeated-measures) ANOVA where appropriate. For multiple comparisons, a Bonferroni correction was used. Differences were evaluated via post hoc analysis using a Scheffé's test. Significance was established a priori at  $P < 0.05$ .

## RESULTS

### *Experiment 1*

Supplementation with VE and  $\alpha$ -LA resulted in a significant increase ( $P < 0.05$ ) in the VE and lipoate content of the tibialis anterior (TA) (Fig. 2). In addition, the contractile protocol was associated with a significant loss ( $P < 0.05$ ) of VE from the stimulated muscle only in supplemented animals.

Fig. 1. Schematic illustration of the protocol used in *experiment 2*.  $L_o$ , optimal muscle length;  $P_o$ , maximal specific tension;  $P_t$ , maximal twitch tension.



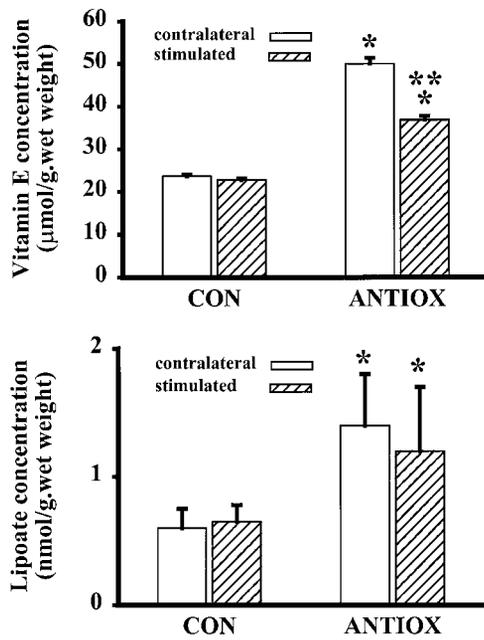


Fig. 2. Vitamin E (top) and  $\alpha$ -lipoic acid (bottom) concentrations from the tibialis anterior of rats fed either a control diet (Con;  $n = 14$ ) or a diet supplemented with vitamin E and  $\alpha$ -lipoic acid (Antiox;  $n = 14$ ). Values are means  $\pm$  SE. \*Significantly different from control diet animal ( $P < 0.05$ ); \*\*significantly different from contralateral muscle using 1-way ANOVA ( $P < 0.05$ ).

A comparison of the contractile properties of the TA from Con and Antiox animals before the fatigue protocol is presented in Table 1. Supplementation with VE and  $\alpha$ -LA had no significant effect ( $P > 0.05$ ) on  $P_o$  and the tetanic-twitch ratio ( $P_o/P_t$ ). There was, however, a significant decrease ( $P < 0.05$ ) in  $P_t$  in supplemented animals.

Figure 3 illustrates the force-frequency response before and after the fatigue protocol. Before the fatigue protocol, muscle force production was significantly depressed ( $P < 0.05$ ) at stimulation frequencies  $\leq 40$  Hz in Antiox animals. However, after the fatigue protocol, no group differences existed ( $P > 0.05$ ) in force production at any stimulation frequency.

No differences existed ( $P > 0.05$ ) in TA force production between Con and Antiox animals throughout the 60-min fatigue protocol (Fig. 4). Furthermore, no differences existed ( $P > 0.05$ ) between groups in muscle contractile properties after the fatigue protocol (data not shown).

Table 1. Contractile measurements from the tibialis anterior of rats fed either a control diet or a diet supplemented with vitamin E and  $\alpha$ -lipoic acid

	<i>n</i>	$P_o$ , N/kg	$P_t$ , N/kg	$P_o/P_t$
Con	11	$1.98 \pm 0.06$	$0.72 \pm 0.03$	$2.8 \pm 0.1$
Antiox	12	$1.83 \pm 0.06$	$0.58 \pm 0.04^*$	$3.2 \pm 0.2$

Values are means  $\pm$  SE; *n*, no. of rats per group.  $P_o$ , maximal tetanic tension;  $P_t$ , maximal twitch tension;  $P_o/P_t$ , tetanic-twitch ratio; Con, rats fed control diet; Antiox, rats fed a diet supplemented with vitamin E and  $\alpha$ -lipoic acid. \*Significantly different from control using a 1-way ANOVA ( $P < 0.05$ ).

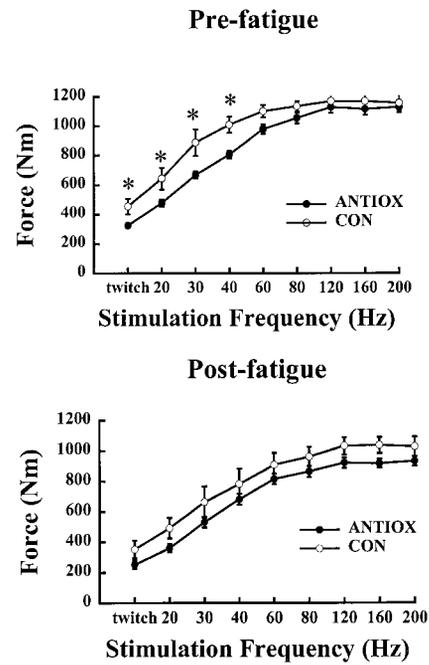


Fig. 3. Force-frequency curves for tibialis anterior of Con ( $n = 11$ ) or Antiox rats ( $n = 12$ ) measured before (top) and after (bottom) a fatigue protocol ( $P < 0.05$ ). Values are means  $\pm$  SE. \*Significantly different from Antiox using multiple *t*-tests with Bonferroni correction ( $P < 0.001$ ).

Two markers of lipid peroxidation were used to determine the effects of the contractile protocol on oxidative damage of the TA in *experiment 1*. Figure 5 contains the values for TBARS and CHE in both experimental groups. Similar results were obtained by using the two markers of lipid peroxidation. In the Con diet group, the contractile protocol resulted in a significant increase ( $P < 0.05$ ) in both TBARS and CHE levels in the stimulated TA compared with the contralateral muscle. An important finding was that the stimulated TA from the Antiox animals had significantly lower ( $P < 0.05$ ) TBARS and CHE levels compared with the contracted TA from Con animals.

Experiment 2

Table 2 shows the initial contractile measurements from the rat diaphragm in vitro experiment. Addition

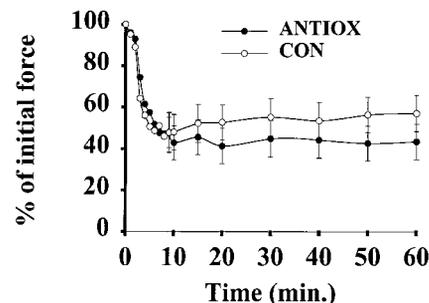


Fig. 4. Average force production of the tibialis anterior undergoing a 60-min fatigue protocol from Con ( $n = 11$ ) or Antiox rats ( $n = 12$ ). Values are means  $\pm$  SE. No differences between group means at any time point using 2-way repeated-measures ANOVA with Bonferroni correction ( $P > 0.001$ ).

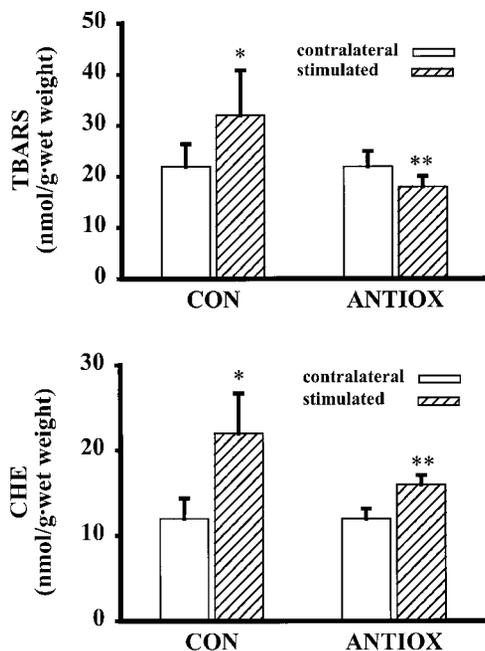


Fig. 5. Thiobarbituric acid-reactive substance (TBARS; *top*) and cumene hydroperoxide equivalent (CHE; *bottom*) concentrations in the tibialis anterior of Con ( $n = 11$ ) or Antiox rats ( $n = 12$ ). Values are means  $\pm$  SE. \*Significantly different from contralateral muscle (Con); \*\*significantly different from Con animal (stimulated) using 1-way ANOVA ( $P < 0.05$ ).

of VE and DHLA to the preparation did not impact ( $P > 0.05$ )  $P_o$  or  $P_o/P_t$ .

Similar to the in situ experiment, there was a significant decrease ( $P < 0.05$ ) in  $P_t$  and maximal force production at frequencies  $< 40$  Hz when either the combination of VE (400  $\mu$ M) and DHLA (100  $\mu$ M) or VE (400  $\mu$ M) was added to the bath (Fig. 6). Compared with control, no differences existed in the force-frequency relationship when either DHLA (100  $\mu$ M), VE (200  $\mu$ M) or VE (100  $\mu$ M) was added to the bath (data not shown).

Finally, there were no differences in (in vitro) muscle fatigue between control and any of the antioxidant treatments. Figure 7 shows data comparing control with the combination of VE (400  $\mu$ M) and DHLA (100  $\mu$ M). The data for the other treatments are not shown.

Table 2. *Contractile measurements from experiment 2*

	$n$	$P_o$ , N/cm <sup>2</sup>	$P_t$ , N/cm <sup>2</sup>	$P_o/P_t$
Control	5	27.2 $\pm$ 0.8	6.8 $\pm$ 0.4	4.0 $\pm$ 0.6
Vit E (100 $\mu$ M)	5	27.1 $\pm$ 1.2	6.7 $\pm$ 0.4	4.0 $\pm$ 0.3
Vit E (200 $\mu$ M)	5	27.4 $\pm$ 0.9	5.7 $\pm$ 0.5	4.8 $\pm$ 0.6
Vit E (400 $\mu$ M)	6	26.9 $\pm$ 1.3	5.1 $\pm$ 0.6*	5.3 $\pm$ 0.7*
DHLA (100 $\mu$ M)	5	27.2 $\pm$ 1.2	6.5 $\pm$ 0.8	4.2 $\pm$ 0.7
Vit E (400 $\mu$ M)/DHLA (100 $\mu$ M)	6	26.5 $\pm$ 1.1	4.9 $\pm$ 0.7*	5.4 $\pm$ 0.7*

Values are means  $\pm$  SE;  $n$ , no. of rats per group. Vit E, vitamin E; DHLA, dihydrolipoic acid. \*Significantly different from control using a 1-way ANOVA ( $P < 0.05$ ).

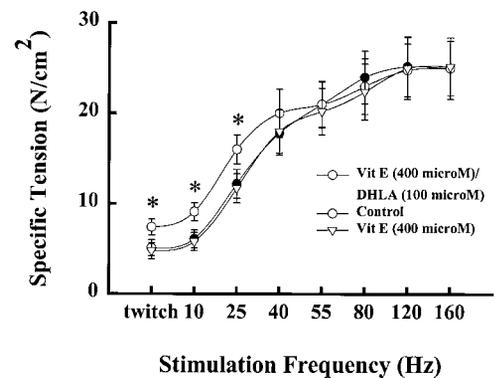


Fig. 6. Comparison of the force-frequency curves for diaphragm strips incubated in either 1) the dissolving medium (control,  $n = 5$ ); 2) vitamin E (Vit E; 400  $\mu$ M,  $n = 6$ ); or 3) Vit E (400  $\mu$ M) and dihydrolipoic acid (DHLA; 100  $\mu$ M,  $n = 6$ ). Values are means  $\pm$  SE. Strips incubated in either 400  $\mu$ M or the combination of 400  $\mu$ M Vit E and 100  $\mu$ M DHLA produced less force at frequencies  $< 40$  Hz using 1-way ANOVA with Bonferroni correction ( $P < 0.001$ ).

## DISCUSSION

### Summary of Principal Findings

To our knowledge, this is the first study to examine the effects of dietary supplementation of dietary antioxidants on skeletal muscle contractile function in situ. Our results indicate that 8 wk of supplementation with VE and  $\alpha$ -LA significantly increased the levels of these antioxidants within the TA muscle. Interestingly, although dietary supplementation with antioxidants reduced the magnitude of contraction-induced lipid peroxidation in the TA muscle, antioxidant supplementation did not improve the fatigue resistance of this muscle during an in situ fatigue protocol. Furthermore, in nonfatigued muscle, the TA from antioxidant-supplemented animals generated less tetanic force at stimulation frequencies  $\leq 40$  Hz. On the basis of this result, we performed a second series of experiments using an in vitro rat diaphragm preparation to determine which antioxidant was responsible for this loss of contractile force. When muscle strips were incubated in either 400  $\mu$ M of VE alone or a combination of VE (400  $\mu$ M) and DHLA (100  $\mu$ M), a reduction in submaximal tetanic

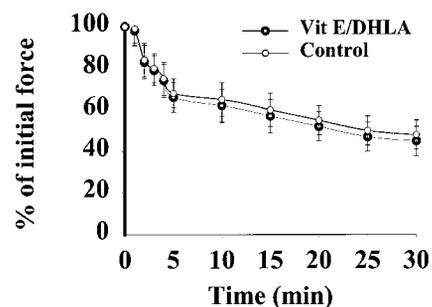


Fig. 7. Average force production of diaphragm strips during a fatigue protocol. Strips were incubated in either 400  $\mu$ M Vit E and 100  $\mu$ M DHLA ( $n = 6$ ) or the dissolving medium ( $n = 5$ ) during a fatigue protocol. Values are means  $\pm$  SE. No differences were found between group means at any time point using 2-way repeated-measures ANOVA with Bonferroni correction ( $P > 0.001$ ).

force generation was observed (i.e., right shift in the force-frequency relationship). However, incubation with DHLA alone (up to 100  $\mu\text{M}$ ) or low levels of VE alone ( $<200 \mu\text{M}$ ) did not alter the normal force-frequency relationship. Collectively, these results indicate that dietary supplementation with high doses of VE can result in a depression in muscle contractile function at low stimulation frequencies.

#### *Dietary Antioxidants and Muscle Force Production*

Again, our results indicate that dietary consumption of high doses of antioxidants impairs muscle force production in situ. This finding supports previous in vitro studies indicating that exogenous antioxidants have a negative effect on skeletal muscle contractile function in unfatigued muscle (9, 13, 22, 23, 25). For example, Reid et al. (22) demonstrated that isolated diaphragm fiber bundles incubated with the antioxidant enzymes catalase and superoxide dismutase display depressed submaximal tetanic contractile force generation. Similarly, investigators have demonstrated that incubation of skeletal muscle with NAC resulted in a depression of submaximal tetanic force production (9, 13). Collectively, the current in situ data and previously published in vivo results indicate that high levels of antioxidants can negatively effect both twitch and submaximal tetanic contractions in unfatigued muscle. Collectively, these observations support the notion that a redox balance must exist for optimal skeletal muscle contractile function.

How does redox balance influence muscle force production? The literature indicates that several redox-sensitive skeletal muscle proteins play active roles in the contractile process. For example, the ryanodine-sensitive calcium release channel of the sarcoplasmic reticulum (SR) has multiple sulfhydryl groups that are sensitive to redox modulation by ROS or reactive nitrogen species (1, 24). We postulate that in the present study the antioxidant supplementation led to an increased antioxidant concentration in the SR membranes, leading to a reduction of regulatory sulfhydryls on the calcium release channel. This would result in a redox depression of calcium transients during twitch and low-frequency tetanic stimulation, leading to lower muscle force production. Nonetheless, based on the present data alone, this postulate is conjecture because it is not possible to determine the exact redox mechanism(s) responsible for the impaired muscle force production at low stimulation frequencies. This is an interesting area for future research.

Combined, these data indicate that optimal contractile function depends on the redox state of some or all of the cellular elements that participate in muscular contraction. The present study did not determine the VE content of organelles such as the SR. Nonetheless, given the lipid composition of the SR, it seems likely that both the in vivo supplementation and the in vitro incubation resulted in additional VE availability at the SR membrane; this could have modified redox balance and impacted contractile function. Additional studies

are required to determine the molecular mechanisms responsible for the VE interference with muscle force production during submaximal tetanic stimulation.

The observation that dietary supplementation of dietary antioxidants in rats is capable of interfering with skeletal muscle contractile function at low stimulation frequencies may have implications to humans consuming megadoses of antioxidants. Indeed, the dietary levels of VE provided to animals in the current experiments results in blood levels of VE in the rat that are comparable to blood VE levels in humans taking VE doses of 1,000 IU/day ( $91 \pm 6 \mu\text{M}$ ) (29). Nonetheless, whether or not dietary supplementation with high doses of VE would impair human muscle performance is unknown; this is an interesting area for further research.

#### *Dietary Antioxidants and Muscle Fatigue*

A number of studies have reported that exogenous antioxidants protect against skeletal muscle fatigue (4, 9, 13, 14, 18, 23, 28, 31). Most of these studies have applied synthetic or enzymatic antioxidants and employed in vitro muscle preparations (4, 9, 13, 23, 28, 31). For example, NAC has been shown to reduce fatigue in both rabbit (28) and rat diaphragms (9, 13). Further, Barclay and Hansel (4) used DMSO to demonstrate that skeletal muscle fatigue can be attenuated with antioxidant administration in both an in vitro mouse soleus model and an in situ canine gastrocnemius-plantaris preparation. These authors concluded that oxidative stress contributes to muscular fatigue.

Although dietary antioxidant supplementation attenuated exercise-induced lipid peroxidation in the TA muscle in our experiments, our data indicate that neither the in situ nor in vitro models of muscular fatigue were affected by antioxidant administration. This observation agrees with a number of studies investigating the ergogenic potential of dietary supplementation with antioxidants. In this regard, three studies have concluded that dietary antioxidants do not improve muscular performance (5, 17, 27). However, a recent study has reported a performance benefit of antioxidants. Specifically, Lands et al. (14) used a cysteine donor to augment endogenous glutathione synthesis and reported an improvement in human exercise tolerance during short-duration leg exercise (i.e., isokinetic cycling). Additional research to corroborate these findings at varying exercise intensities and durations is warranted.

#### *Summary and Conclusions*

The purpose of these experiments was to determine the effects of the dietary antioxidants VE and  $\alpha\text{-LA}$  on skeletal muscle contractile function. Our experiments demonstrate that dietary supplementation with VE and  $\alpha\text{-LA}$  results in a right shift in the muscle force-frequency relationship. Subsequent experiments using the rat in vitro diaphragm model revealed that the depressed contractility of skeletal muscle at low stimulation frequencies was due to the effects of VE. Re-

sults from both experiments indicated that these antioxidant supplements had no effect on skeletal muscle fatigue. We conclude from these observations that the dietary antioxidant VE, in high doses, has a negative effect on skeletal muscle function.

## REFERENCES

1. **Aghdasi B, Zhang JZ, Wu Y, Reid MB, and Hamilton SL.** Multiple classes of sulfhydryls modulate the skeletal muscle  $\text{Ca}^{2+}$  release channel. *J Biol Chem* 272: 3739–3748, 1997.
2. **Alessio HM, Goldfarb AH, and Cao G.** Exercise-induced oxidative stress before and after vitamin C supplementation. *Int J Sport Nutr* 7: 1–9, 1997.
3. **Andrade FH, Reid MB, Allen DG, and Westerblad H.** Effect of hydrogen peroxide and dithiothreitol on contractile function of single skeletal muscle fibres from the mouse. *J Physiol (Lond)* 509 (Pt 2): 565–575, 1998.
4. **Barclay JK and Hansel M.** Free radicals may contribute to oxidative skeletal muscle fatigue. *Can J Physiol Pharmacol* 69: 279–284, 1991.
5. **Brady PS, Brady LJ, and Ullrey DE.** Selenium, vitamin E and the response to swimming stress in the rat. *J Nutr* 109: 1103–1109, 1979.
6. **Burton GW and Traber MG.** Vitamin E: antioxidant activity, biokinetics, and bioavailability. *Annu Rev Nutr* 10: 357–382, 1990.
7. **Cort W, Vicente TS, Waysek EH, and Williams BD.** Vitamin E content of feedstuffs determined by high-performance liquid chromatographic fluorescence. *J Agric Food Chem* 31: 1330–1333, 1983.
8. **Davies KJ, Quintanilha AT, Brooks GA, and Packer L.** Free radicals and tissue damage produced by exercise. *Biochem Biophys Res Commun* 107: 1198–1205, 1982.
9. **Diaz PT, Brownstein E, and Clanton TL.** Effects of *N*-acetylcysteine on in vitro diaphragm function are temperature dependent. *J Appl Physiol* 77: 2434–2439, 1994.
10. **Diaz PT, She ZW, Davis WB, and Clanton TL.** Hydroxylation of salicylate by the in vitro diaphragm: evidence for hydroxyl radical production during fatigue. *J Appl Physiol* 75: 540–545, 1993.
11. **Hermes-Lima M, Willmore WG, and Storey KB.** Quantification of lipid peroxidation in tissue extracts based on Fe(III) xylenol orange complex formation. *Free Radic Biol Med* 19: 271–280, 1995.
12. **Kagan VE, Serbinova EA, Forte T, Scita G, and Packer L.** Recycling of vitamin E in human low density lipoproteins. *J Lipid Res* 33: 385–397, 1992.
13. **Khawli FA and Reid MB.** *N*-acetylcysteine depresses contractile function and inhibits fatigue of diaphragm in vitro. *J Appl Physiol* 77: 317–324, 1994.
14. **Lands LC, Grey VL, and Smountas AA.** Effect of supplementation with a cysteine donor on muscular performance. *J Appl Physiol* 87: 1381–1385, 1999.
15. **Meydani M, Evans W, Handelman G, Fielding RA, Meydani SN, Fiatarone MA, Blumberg JB, and Cannon JG.** Antioxidant response to exercise-induced oxidative stress and protection by vitamin E. *Ann NY Acad Sci* 669: 363–364, 1992.
16. **Mihara M and Uchiyama M.** Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal Biochem* 86: 271–278, 1978.
17. **Nielsen AN, Mizuno M, Ratkevicius A, Mohr T, Rohde M, Mortensen SA, and Quistorff B.** No effect of antioxidant supplementation in triathletes on maximal oxygen uptake,  $^{31}\text{P}$ -NMRS detected muscle energy metabolism and muscle fatigue. *Int J Sports Med* 20: 154–158, 1999.
18. **Novelli GP, Bracciotti G, and Falsini S.** Spin-trappers and vitamin E prolong endurance to muscle fatigue in mice. *Free Radic Biol Med* 8: 9–13, 1990.
19. **Packer L, Witt EH, and Tritschler HJ.**  $\alpha$ -Lipoic acid as a biological antioxidant. *Free Radic Biol Med* 19: 227–250, 1995.
20. **Panigrahi M, Sadguna Y, Shivakumar BR, Kolluri SV, Roy S, Packer L, and Ravindranath V.**  $\alpha$ -Lipoic acid protects against reperfusion injury following cerebral ischemia in rats. *Brain Res* 717: 184–188, 1996.
21. **Reid MB, Haack KE, Franchek KM, Valberg PA, Kobzik L, and West MS.** Reactive oxygen in skeletal muscle. I. Intracellular oxidant kinetics and fatigue in vitro. *J Appl Physiol* 73: 1797–1804, 1992.
22. **Reid MB, Khawli FA, and Moody MR.** Reactive oxygen in skeletal muscle. III. Contractility of unfatigued muscle. *J Appl Physiol* 75: 1081–1087, 1993.
23. **Reid MB and Moody MR.** Dimethyl sulfoxide depresses skeletal muscle contractility. *J Appl Physiol* 76: 2186–2190, 1994.
24. **Salama G, Abramson JJ, and Pike GK.** Sulfhydryl reagents trigger  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum of skinned rabbit psoas fibres. *J Physiol (Lond)* 454: 389–420, 1992.
25. **Sams WM Jr, Carroll NV, and Crantz PL.** Effect of dimethylsulfoxide on isolated-innervated skeletal, smooth, and cardiac muscle. *Proc Soc Exp Biol Med* 122: 103–107, 1966.
26. **Sen CK, Atalay M, Agren J, Laaksonen DE, Roy S, and Hänninen O.** Fish oil and vitamin E supplementation in oxidative stress at rest and after physical exercise. *J Appl Physiol* 83: 189–195, 1997.
27. **Sharman IM, Down MG, and Sen RN.** The effects of vitamin E and training on physiological function and athletic performance in adolescent swimmers. *Br J Nutr* 26: 265–276, 1971.
28. **Shindoh C, DiMarco A, Thomas A, Manubay P, and Supinski G.** Effect of *N*-acetylcysteine on diaphragm fatigue. *J Appl Physiol* 68: 2107–2113, 1990.
29. **Simons LA, von Konigsmark M, Simons J, Stocker R, and Celermajer DS.** Vitamin E ingestion does not improve arterial endothelial dysfunction in older adults. *Atherosclerosis* 143: 193–199, 1999.
30. **Taylor JA and Kandarian SC.** Advantage of normalizing force production to myofibrillar protein in skeletal muscle cross-sectional area. *J Appl Physiol* 76: 974–978, 1994.
31. **Travaline JM, Sudarshan S, Roy BG, Cordova F, Leyenson V, and Criner GJ.** Effect of *N*-acetylcysteine on human diaphragm strength and fatigability. *Am J Respir Crit Care Med* 156: 1567–1571, 1997.
32. **Witt EH, Reznick AZ, Viguie CA, Starke-Reed P, and Packer L.** Exercise, oxidative damage and effects of antioxidant manipulation. *J Nutr* 122: 766–773, 1992.