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Electron paramagnetic spectroscopic evidence of exercise-induced free radical accumulation in human skeletal muscle

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Abstract

The present study determined if acute exercise increased free radical formation in human skeletal muscle. Vastus lateralis biopsies were obtained in a randomized balanced order from six males at rest and following single-leg knee extensor exercise performed for 2 min at 50% of maximal work rate (WR_{MAX}) and 3 min at 100% WR_{MAX}. EPR spectroscopy revealed an exercise-induced increase in mitochondrial ubisemiquinone ($UQ^{\cdot -}$) [0.167 ± 0.055 vs. rest: 0.106 ± 0.047 arbitrary units (AU)/g total protein (TP), $P < 0.05$] and α-phenyl-*tert*-butylnitrone-adducts (112 ± 41 vs. rest: 29 ± 9 AU/mg tissue mass, $P < 0.05$). Intramuscular lipid hydroperoxides also increased (0.320 ± 0.263 vs. rest: 0.148 ± 0.071 nmol/mg TP, $P < 0.05$) despite an uptake of α-tocopherol, α-carotene and β-carotene. There were no relationships between mitochondrial volume density and any biomarkers of oxidative stress. These findings provide the first direct evidence for intramuscular free radical accumulation and lipid peroxidation following acute exercise in humans.

Keywords: *EPR, spin-trapping, lipid peroxidation, antioxidants, skeletal muscle, mitochondria*

Introduction

Electron paramagnetic resonance (EPR) spectroscopy is the only direct technique that can detect and identify free radicals and has provided unique insight into the source, species and mechanisms of generation by contracting skeletal muscle. Davies et al. [1] were the first to establish an exercise-induced increase in the EPR signal intensity of a free radical signal in rodent hindlimb muscle. This signal was associated with lipid peroxidation, sarcolemmal membrane damage and decreased mitochondrial respiratory control. Located at $g = 2.004$, this spectrum was tentatively assigned to a mitochondrial ubisemiquinone ($UQ^{\cdot -}$) or flavin semiquinone radical which was later confirmed by Jackson et al. [2] in electrically stimulated rodent gastrocnemius muscle. Contemporary studies using alternative techniques in rodent and isolated skeletal muscle cells have since confirmed that contractile activity stimulates free radical and associated reactive oxygen and nitrogen species (ROS/RNS) generation [3].

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The early recognition that $UQ^{\cdot -}$ may be an important "upstream" initiator of exercise-induced oxidative stress has important biological consequences and implicates respiring mitochondria as a potential free radical source. A consequence of inadequate coupling of electron transfer at the NADH dehydrogenase and ubiquino/bc1 redox couple, $UQ^{\cdot -}$ is a reactive intermediate that can initiate superoxide ($O_2^{\cdot -}$) and subsequent hydrogen peroxide formation [4]. Early *in vitro* studies have estimated that mitochondrial electron "leak" accounts for 2% of the total oxygen consumed during State 4 (basal) respiration [5] though more recent evidence suggests that this is probably an overestimation. Hansford et al. [6] reported values of 0.4–0.8% for heart mitochondria respiring on physiological concentrations of succinate whereas values as low as 0.15% have been documented in rat skeletal muscle mitochondria utilizing palmitoyl carnitine [7].

Since oxygen flux through the active musculature can increase more than 100 times above resting values to support the energetic demands of exercise, mitochondrial electron "leak" has traditionally been considered the predominant mechanism responsible for the systemic increase in oxidative stress observed following exercise *in vivo*. However, this link remains controversial [8] since it is not entirely compatible with *in vitro* findings that have consistently identified a decrease in mitochondrial electron leak during the State 4 (basal respiration characterized by low O_2 consumption, high membrane potential, low ATP production) to State 3 (maximal respiration) transition [9,10].

In contrast, there are no studies to our knowledge that have documented free radical formation in human skeletal muscle following exercise. By combining invasive catheterization with an EPR spin-trapping approach, we have previously observed a regional outflow of free radicals across the exercising human quadriceps muscle [11,12]. However, since we did not perform muscle biopsies to assess complementary changes in intramuscular free radical formation, we could not establish whether contracting muscle was a potential contributory source of these second generation extravascular radicals.

Given the current lack of human data, the present study examined the effect of maximal exercise on free radical formation and lipid peroxidation in human skeletal muscle. We hypothesized (a) that exercise would overwhelm non-enzymic antioxidant defenses and increase the intramuscular concentration of $UQ^{\cdot -}$; (b) this would initiate lipid peroxidation resulting in an accumulation of lipid hydroperoxides (LOOH) and lipid-derived free radicals that could be observed by EPR spin-trapping, and (c) since mitochondrial volume density is directly related to maximal mitochondrial oxygen flux [13], that if respiring mitochondria were indeed a source of free radicals during exercise, we would observe a positive relationship between mitochondrial density and oxidative stress markers generated *in vivo*.

Materials and methods

Subjects

Six healthy males with a maximal oxygen uptake of 43 (mean) ± 5 (SD) ml/kg/min aged 26 (mean) ± 6 (SD) years old, volunteered to participate after written informed consent was obtained according to the ethical requirements of the University of California San Diego Human Subjects Protection Program. All procedures conformed to the code of Ethics of the World Medical Association (Declaration of Helsinki). A health history and physical examination was performed and it was specifically determined that subjects were not taking any analgesics, non-steroidal anti-inflammatories or antioxidant vitamin supplements. Subjects maintained their normal dietary patterns and refrained from any strenuous physical activities for at least 48 h prior to the study.

Exercise protocol and muscle biopsies

Each subject arrived at the laboratory following a 12 h overnight fast and performed two familiarization bouts on the single-leg dynamic knee extension (KE) apparatus prior to an incremental test to volitional exhaustion to determine WR_{MAX} with either the right or left leg (randomized balanced design) [14]. The KE model, first described by Andersen and Saltin [15], was selected as the exercise modality to ensure that muscular contractions were isolated to the quadriceps muscle (90° flexion to 170° extension). Anecdotal subject reports, force tracings, electromyography, intramuscular temperature measurements and T_2-weighted magnetic resonance imaging unequivocally confirm the isolation of mechanical work to this muscle group [16] which is an important methodological consideration.

The initial WR was set at 3–10 W and increased by 3–6 W/min (at a cadence of 60 rpm) according to the subject's predicted exercise capacity, resulting in a WR_{MAX} of 28 ± 8 W. On the final study day subjects returned to the laboratory following a 7 day recovery period and performed an acute bout of intense exercise that pilot studies had previously identified was sufficient to induce oxidative stress (DM Bailey et al. unpublished observations). This consisted of 2 min of KE exercise at 50% of their previously determined WR_{MAX} followed by 3 min at 100% WR_{MAX} with the original exercise-tested limb employed during the familiarization bouts.

Muscle biopsies were initially obtained from the vastus lateralis of the resting or exercised contra-lateral leg using a 5 mm diameter biopsy needle (Bergstrom)

attached to sterile tubing and syringe in a randomized balanced fashion. Thus, in three of the subjects (50%), a resting biopsy was obtained prior to the subsequent exercise biopsy, with an inter-sampling time difference of exactly 15 min. In the three remaining subjects, the resting biopsy was obtained 15 min following completion of the exercise challenge and immediate retrieval of the biopsy from the exercised contra-lateral leg.

All biopsies were obtained at an approximate depth of 3.5 cm, 15 cm proximal to the knee and slightly distal to the ventral midline of the muscle. Several minutes prior to exercise, lidocaine (0.5%) was used as the local anesthetic injected into four sites surrounding the biopsy site, but not infiltrating the site itself (ring block) and the scalpel incision was made. Facilitated by this pre-exercise preparation, post-exercise biopsy samples were obtained as rapidly as possible and immersed in liquid N_2 immediately, dissected with a pre-cooled glass rod and placed into three separate cryogenic glass vials. These samples were then stored at 77 K (Cryopak Series, Model CP100) for the analysis of free radicals via EPR spectroscopy using two different techniques (vials 1 and 2), for LOOH by spectrophotometry and lipid soluble antioxidants (LSA) by HPLC (vial 3).

Oxidative stress biomarkers

g = 2.004 signal. Frozen tissue samples (vial 1) were transferred using a forceps protected with polyvinyl chloride tubing to a quartz finger dewar (6 mm internal diameter) that contained liquid nitrogen. Mechanical manipulation of the tissue was unavoidable to ensure satisfactory placement in the dewar and thus tissue dissection was conducted under liquid nitrogen conditions using a ceramic mortar to avoid artefactual radical generation as previously identified [17]. This process was standardized to the best of our ability and rounded tissue pellets approximately 2 mm in diameter were quickly added to the dewar until they reached a vertical height of 4 cm, sufficient to completely fill the critical volume of the cavity. A pre-cooled glass rod was placed into the dewar securing the tissue in place thus preventing any movement. Samples were analyzed at 77 K on a Varian 104 X-band spectrometer equipped with an EPR-Ware data acquisition system. Spectrometer settings were maintained at: microwave power = 10 mW; frequency = 9.15 GHz; modulation frequency = 100 kHz; modulation amplitude = 4.0 G; gain = 8×10^3; magnetic field center = 3258 G; scan width = 400 G; scan time = 240 s; time constant = 0.128 s for 1 sweep. A control blank (dewar containing liquid nitrogen and the glass rod minus the tissue sample) did not generate any background signals.

Total protein (TP). For the purposes of normalizing the EPR signal relative to the TP concentration of tissue, pellets were blotted dry and weighed, then homogenized with 1 part butylated hydroxytoluene (200 μM dissolved in methanol) to 99 parts PBS (0.01 M) and adjusted to pH 7.4. This mixture was added at a concentration of 1 ml per 100 mg tissue and the supernatant analyzed for TP using a commercial spectrophotometric assay based on the Coomassie Blue reaction with proteins [Biorad, Munich Germany: 500-006, (Bradford assay)].

PBN-adducts. A separate portion of frozen tissue (vial 2) was transferred into a glass vial that contained 1 ml of a 190 mM/l solution of PBN dissolved in 0.9% NaCl and allowed to incubate at 295 K for 60 min. PBN was the trap of choice due to its stability and affinity for a variety of oxygen and carbon-centered radicals generated *in vivo*. The perfusate (800 μl) was added to an equal volume of toluene (HPLC-grade), vortexed for 30 s and centrifuged at 4000 rpm for 10 min. The adduct (200 μl) was pipetted into a 5 mm (o.d.) precision-bore quartz EPR sample tube (Wilmad Ltd, UK) and immediately vacuum degassed (West Technology Ltd, Bristol, UK) using a freeze (liquid N_2)/thaw procedure at a fixed vacuum of 10^{-3} Torr (Pirani 14 gauge detector, Edwards, APG-NW, West Sussex, UK) using a turbo molecular pump (Alcatel, ACT 200T, Annecy, France) for two cycles (total degassing time of 9 min). All procedures and chemicals were performed and stored in the dark to avoid photolytic degradation of the spin-trap. Samples were analyzed at 295 K using an EMX X-band EPR spectrometer fitted with an ER TM_{110} cavity (Bruker, Karlsruhe, Germany). The extraction efficiency for PBN was 85–90% as confirmed by UV spectroscopy. Spectrometer conditions were as follows: microwave power = 20 mW; frequency = 9.75 GHz; modulation frequency = 100 kHz; modulation amplitude = 0.5 G; gain = 1×10^5; magnetic field center = 3472 G; scan width = 40 G; scan time = 84 ms; time constant = 82 ms for 10 sweeps. EPR spectral parameters were obtained using commercially available software (Bruker Win EPR System, Version 2.11) and filtered identically. The average spectral peak-to-trough line height was considered a measure of the relative spin adduct concentration following conformation of peak-to-trough line-width conformity and double integration on selected samples. Samples were extracted from the glass vial, blotted dry and weighed. Since it is possible that the spin-trap may have influenced the TP assay, we chose to normalize the EPR signal intensity of the PBN-adducts relative to tissue mass.

Origin of PBN-adducts. To assess the possibility that PBN-adducts were generated from analogues

of LOOH, spin-trapping was performed at 37°C in a 4.5 ml aqueous reaction mixture containing a final concentration of 190 mM/l PBN, 8.3 μM $FeSO_4 \cdot 7H_2O$ and 0.13% cumene hydroperoxide (Cu-OOH).

Lipid hydroperoxides (LOOH) and lipid soluble antioxidants (LSA). TP from vial 3 was extracted as described and the remaining homogenate assayed for LOOH and LSA. LOOH was determined using the ferrous iron/xylenol orange (FOX) technique which incorporates the selective oxidation of ferrous to ferric ions by hydroperoxides [18]. This reaction yields a blue-purple coloured complex due to the selective binding of xylenol orange to ferric ions. Catalase was added to discriminate between authentic hydroperoxides reacting with ferrous ions, and H_2O_2 which may be present in the sample. Absorbance changes at 560 nm were monitored spectrophotometrically. The intra/inter-assay CV was <2 and 4%, respectively. For LSA, the remaining homogenate was assayed for α-tocopherol, α-carotene, β-carotene, retinol and lycopene using an HPLC method [19,20]. The intra/inter-assay CV's were both $<5\%$.

Tissue morphology and histology. An additional biopsy was obtained from the resting leg and separated into two smaller blocks (vials 4 and 5). The first tissue block was immediately frozen in liquid N_2 and 8 μm-thick transverse sections were cut at -24°C on a cryostat (Jung-Reichert Cryocut 1800) and kept at -20°C until histochemical processing. After 5 min fixation in a Guth and Samaha fixative at room temperature, sections were incubated at 37°C for 1 h in Pb-ATPase staining medium to stain for fiber types I and II [21]. The relative cross-sectional area and number of type I and type II fibers was estimated by point-counting using an eye-piece square grid test A100 on histochemical sections examined at a magnification of 250 × with a light microscope [21]. The second tissue block was immersion-fixed at 295 K in glutaraldehyde fixative (6.25% glutaraldehyde solution in 0.1 M sodium cacodylate buffer, total osmolarity = 1,100 mOsm, pH = 7.4) prior to ultra structural morphometric analysis (mitochondrial volume, myofibrular volume, and intramuscular fat) by low power electron microscopy according to established techniques [21]. Briefly, the glutaraldehyde-fixed samples were completely cut into thin longitudinal strips and processed for electron microscopy as described previously [21]. From each biopsy, four blocks were cut into 1 μm-thick transverse sections with an LKB Ultrotome III and stained with 0.1% aqueous Toluidine Blue solution. The angles of sectioning used to provide the transverse sections were determined as previously described [21]. Ultra thin sections (50–70 nm) were cut transversely to the muscle fiber axis from each block and were contrasted with uranyl acetate and bismuth subnitrate. Electron micrographs for morphometry were taken on 70 mm films with a Zeiss 10 electron microscope. All morphometric measurements were performed in a randomized and blinded fashion and the intra/inter-assay CV's were both $<5\%$. Quadriceps muscle mass was calculated according to established anthropometric methods [22].

Statistical analysis

Since all data were normally distributed (Shapiro–Wilk W test), the metabolic response to exercise was assessed using a paired samples *t*-test. The relationship between selected dependent variables was assessed using a Pearson Product Moment Correlation. Significance for all two tailed tests was established at an alpha level of $P \leq 0.05$ and data are expressed as a mean ± standard deviation (SD).

Results

Lipid soluble antioxidants (LSA)

Exercise increased ($P < 0.05$ vs. rest) the tissue concentration of α-tocopherol, α-carotene and β-carotene (Table I).

g = *2.004 signal*

All frozen tissue samples exhibited two distinct signals (lacking any hyperfine structure) as illustrated in Figure 1; an isotropic line at $g = 2.004$ consistent with mitochondrial $UQ^{\cdot-}$ and a high-field line located at $g = 1.960$ attributable to an iron-sulfur protein. A linear relationship was observed between the total protein concentration of the tissue and EPR signal intensities of the $g = 2.004$ and 1.960 lines (pooled resting and exercise data: $r = 0.56$, $P = 0.05$ and $r = 0.65$, $P < 0.05$ respectively). Since different sized

Table I. Effects of exercise on the intramuscular concentration of lipid soluble antioxidants.

Antioxidant	Rest (nmol/mg TP)	Exercise (nmol/mg TP)
α-tocopherol	2.88 ± 1.27	6.26 ± 5.02⋆
α-carotene	0.005 ± 0.005	0.012 ± 0.007⋆
β-carotene	0.004 ± 0.004	0.012 ± 0.010⋆
Retinol	0.034 ± 0.013	0.122 ± 0.193
Lycopene	0.023 ± 0.021	0.077 ± 0.056

Values are normalized for total tissue protein (TP) concentration and expressed as mean ± SD; ⋆ different ($P < 0.05$) compared to rest. Data are normally distributed.

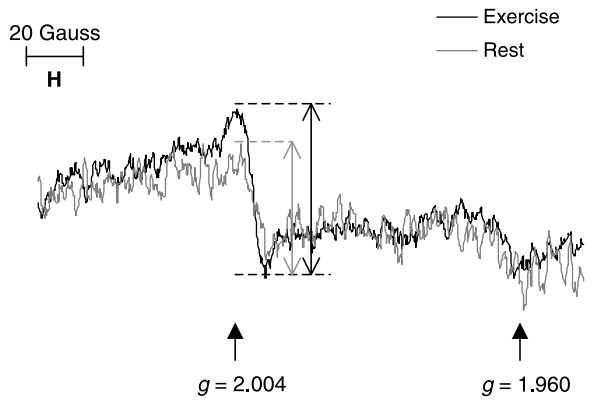

Figure 1. Typical EPR spectra of skeletal muscle at 77 K in one subject indicating the $g = 2.004$ and 1.960 signals consistent with a mitochondrial ubisemiquinone and iron-sulfur protein, respectively. No background signal was obtained when the dewar containing liquid nitrogen and the glass rod (minus the tissue sample) was scanned. Spectrometer settings: microwave power = 10 mW; frequency = 9.15 GHz; modulation frequency = 100 kHz; modulation amplitude = 4.0 G; gain = 8×10^3; magnetic field center = 3258 G; scan width = 400 G; scan time = 240 s; time constant = 0.128 s (1 sweep); H = magnetic field.

tissue samples were obtained from the rested and exercised limbs (dry mass: 187 ± 40 mg vs. 132 ± 43 mg respectively, $P < 0.05$), we chose to express all data (with the exception of PBN-adducts) relative to total TP concentration though the same qualitative outcome was apparent when biomarkers were expressed relative to tissue mass. Exercise increased the intramuscular concentration of $UQ^{\cdot -}$ from 0.106 ± 0.047 arbitrary units (AU)/g TP to 0.167 ± 0.055 AU/g TP ($P < 0.05$), as illustrated in Figure 2 whereas no changes were observed in the amplitude of the $g = 1.960$ signal. Based upon the isolation of the exercise to the quadriceps muscles, this equated to an exercise-induced increase of 0.050 ± 0.131 AU/kg active muscle mass ($P < 0.05$ vs. rest).

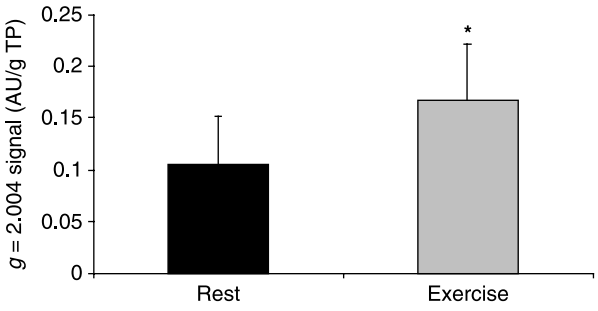

Figure 2. Effects of exercise on the EPR signal intensity of the $g = 2.004$ line detected in skeletal muscle at 77 K; values are normalized for total tissue protein (TP) concentration and expressed as mean ± SD; * different ($P < 0.05$) compared to rest. Data are normally distributed.

PBN-adducts

Analysis of the toluene extracts following incubation of tissue samples with PBN at room temperature revealed multiple adducts similar to those previously detected in human serum [11,12,23,24] (Figure 3). Computer simulation identified two species exhibiting hyperfine coupling constants of $a^N = 13.7$ G and $a_\beta^H = 1.9$ G (90% of total signal) and $a^N = 14.0$ G and $a_\beta^H = 4.0$ G (10% of total signal) consistent with the trapping of $LO^{\cdot}$ and $LC^{\cdot}$ radicals respectively [25]. Comparable spectral characteristics to the major signal were obtained following extraction of the aqueous reaction mixture containing Fe^{2+} and Cu-OOH ($a^N = 13.8$ G and $a_\beta^H = 2.0$ G) *in vitro*. As illustrated in Figure 4, exercise increased the concentration of PBN-adducts ($P < 0.05$ vs. rest) which was related to the exercise-induced increase in $UQ^{\cdot -}$ ($r = 0.76$, $P < 0.05$).

Lipid hydroperoxides (LOOH)

Figure 5 illustrates the exercise-induced increase in the tissue concentration of LOOH ($P < 0.05$ vs. rest) which was positively associated with the increase in PBN-adducts ($r = 0.78$, $P = 0.05$).

Muscle morphometry

The morphometric characteristics of the resting biopsy samples were as follows: % area of type I fibers: 37 ± 15%; % area type II fibers: 63 ± 15%;

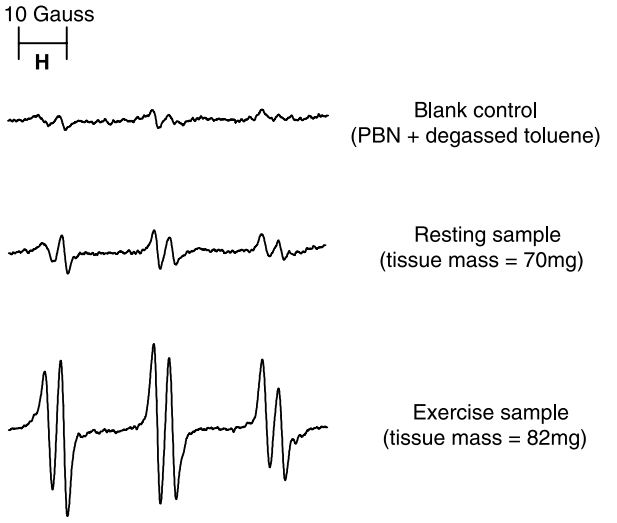

Figure 3. Typical EPR spectra of *ex vivo* trapped α-phenyl-*tert*-butylnitrone (PBN)-adducts extracted from thawed skeletal muscle in one subject. Nuclear hyperfine splittings ranged between: $a^N = 13.7$–14.0 G and $a_\beta^H = 1.9$–4.0 G. Spectrometer settings: microwave power = 20 mW; frequency = 9.75 GHz; modulation frequency = 100 kHz; modulation amplitude = 0.5 G; gain = 1×10^5; magnetic field center = 3472 G; scan width = 40 G; scan time = 84 ms; time constant = 82 ms (10 sweeps); H = magnetic field.

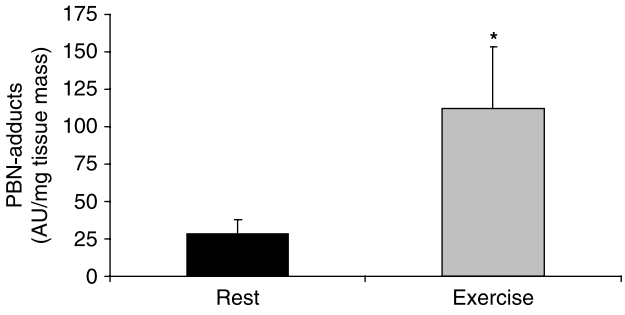

Figure 4. Effects of exercise on the EPR signal intensity of α-phenyl-*tert*-butylnitrone (PBN)-adducts (corrected for background signals) obtained from thawed skeletal muscle; values are normalized relative to tissue mass and expressed as mean ± SD; *different ($P < 0.05$) compared to rest. Data are normally distributed.

capillary:fiber ratio: 1.8 ± 0.8; mitochondrial volume density: 4.3 ± 0.7%; myofibrillar volume: 82 ± 1% and intramuscular fat: 0.5 ± 0.4%. No relationships were observed between mitochondrial volume density and the exercise-induced increase in the EPR signal intensities of the normalized $g = 2.004$ signal ($r = -0.09$, $P > 0.05$), PBN-adducts ($r = -0.16$, $P > 0.05$) or LOOH ($r = -0.35$, $P > 0.05$).

Potential order effect

In light of the randomized balanced design, the order in which the exercise biopsy was obtained (either after or before the resting biopsy was taken from the contralateral leg) did not have any extraneous effect on the exercise-induced increase in any of the biomarkers measured (data not shown).

Discussion

In agreement with our original hypotheses, these findings provide the first direct evidence of an exercise-induced increase in free radical formation and lipid peroxidation in human skeletal muscle.

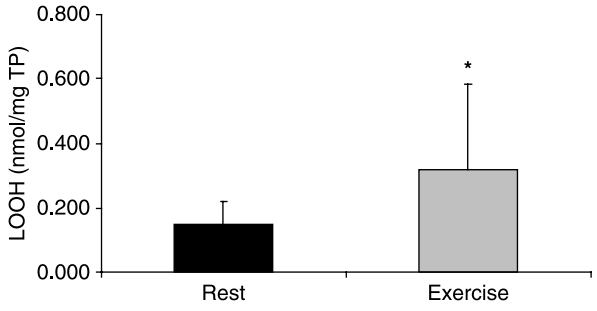

Figure 5. Changes in the intramuscular concentration of lipid hydroperoxides (LOOH) following exercise; values were normalized for total tissue protein (TP) concentration and expressed as mean ± SD; *different ($P < 0.05$) compared to rest. Data are normally distributed.

In spite of an uptake of LSA, EPR spectroscopy revealed an increase in the intramuscular concentration of mitochondrial $UQ^{\cdot -}$. This was associated with lipid peroxidation as indicated by an accumulation of LOOH and spin-trapped lipid-derived free radicals. Contrary to our original expectations, no relationships were observed between mitochondrial volume density and any biomarkers of exercise-induced oxidative stress.

Direct EPR spectroscopic evidence of exercise-induced free radical accumulation

The observation that exercise increased the intramuscular concentration of a distinct free radical signal located at $g = 2.004$ in humans confirms earlier findings in rodent muscle subject to repeated miometric or isometric contractions *in vivo* [1,2] and is the first to be reported following contractile activity in human skeletal muscle. This exercise effect persisted when the signal was normalized relative to tissue protein concentration or tissue mass, an important analytical consideration in light of the proportionality observed.

Identical signals have also been observed in the reperfused rodent heart [17] and contracting diaphragm [26] and are consistent with the stable carbon-centered intermediate $UQ^{\cdot -}$ formed during the one electron reduction of ubiquinone [27]. Since the mitochondrial electron transport chain (METC) contains an inner and outer pool of ubiquinone, $UQ^{\cdot -}$ is formed in the vicinity of the mitochondrial matrix and intermembrane space [28,29]. Autoxidation yields $O_2^{\cdot -}$, the stochiometric precursor of mitochondrial hydrogen peroxide (H_2O_2) which can diffuse rapidly into the cytoplasm and initiate lipid peroxidation [30].

Lipid peroxidation

The accumulation of LOOH and lipid derived radicals spin-trapped *in vitro* provides clear evidence for an exercise-induced increase in free radical-mediated lipid peroxidation in human skeletal muscle. The lipophilic adducts extracted from thawed tissue exhibited identical hyperfine splittings to the blood-borne species released across the contracting quadriceps muscle bed during identical exercise [11,12]. Thus, it is likely that these represent extra-mitochondrial species and are consistent with the trapping of $LO^{\cdot}$ and $LC^{\cdot}$.

However, it is important to emphasize that these adducts were probably formed *ex vivo* during the incubation phase with the spin-trap after the muscle biopsies had been obtained. In light of their instability [31], any intramuscular $LO^{\cdot}$ or $LC^{\cdot}$ formed during the exercise challenge would have reacted with other molecules *in vivo* prior to exposure to the spin-trap.

It is conceivable that transition metal ions released from metalloproteins during the exercise phase subsequent to increased sarcolemmal membrane permeability, which in addition to superoxide release from polymorphonuclear leucocytes and metabolic acidosis [12] may have catalyzed the reductive decomposition of existing LOOH to form these second-generation radicals.

An exercise-induced increase in mitochondrial $UQ^{\cdot -}$ may have further contributed to the metal-catalyzed reductive decomposition of LOOH to compound formation of these "downstream" EPR-detectable spin-trapped lipid-derived radicals ($UQ^{\cdot -} + M^{(n+1)+} \rightarrow UQ + M^{n+}; \rightarrow M^{n+} + LOOH \rightarrow M^{(n+1)+} + OH^- + LO^{\cdot} \xrightarrow{\beta\text{-scission}/-H^{\cdot}} LC^{\cdot}$). Though clearly downstream of the primary locus of generation, these lipid species are thermodynamically capable of initiating and propagating further oxidative tissue membrane damage [32]. The positive relationship observed between the exercise-induced increase in $UQ^{\cdot -}$, LOOH and PBN-adducts, though not dissociating cause from effect, may implicate skeletal muscle as the source and $UQ^{\cdot -}$ as the initiating or at least contributory species responsible for the extracellular formation of blood-borne free radicals previously detected across the contracting quadriceps muscle bed [11,12].

Though not a primary focus of the current study, our previous spin-trapping studies conducted *ex vivo* with human blood obtained from the femoral circulation using an identical exercise model attests to the significance of altered catalytic iron availability. *In vitro* addition of the iron promoter EDTA to whole blood in the presence of PBN or in untreated plasma resulted in a clear increase in the EPR signal intensity of PBN-adducts and the ascorbate free radical whereas a decrease was observed following separate addition of the iron chelator DTPA [12]. Finally, the fact that comparable spectral characteristics were observed following extraction of the aqueous reaction mixture containing Fe^{2+} and Cu-OOH *in vitro* further suggests that these secondary species may have evolved during the metal-catalyzed reductive decomposition of LOOH *ex vivo* subsequent to primary radical ($UQ^{\cdot -}$)-mediated damage to tissue membrane phospholipids initiated *in vivo* [12,33].

Mitochondria and oxidative stress

Borzone et al. [26] demonstrated a linear relationship between the EPR signal intensity of the $g = 2.004$ signal and rate of respiratory muscle oxygen uptake ($\dot{V}O_2$) during inspiratory resistive loading of the rodent diaphragm. These and earlier findings [1,2] suggest that increased METC activity may be responsible for the increased formation of mitochondrial $UQ^{\cdot -}$. Though not assessed in the present study, maximal KE exercise is associated with a considerable increase in metabolic rate which equates to a single-leg $\dot{V}O_2$ in excess of 1.4 l/min resulting in a high mass-specific O_2 flux equivalent to 60 ml/min/100 g active muscle [34].

However, this concept of "flux dependency" is not consistent with classical *in vitro* studies that have clearly identified a decrease in mitochondrial $O_2^{\cdot -}$ "leakage" during the transition from State 4 to State 3 respiration [9,10] subsequent to protective activation of mitochondrial uncoupling proteins [35]. Thus, it is possible that the mitochondrial contribution to exercise-induced oxidative stress *in vivo* has traditionally been overestimated.

In support of this concept, we have previously demonstrated an uncoupling between single-leg $\dot{V}O_2$ and free radical outflow from the contracting quadriceps muscle suggesting that exercise-induced free radical generation is more intimately regulated by a decrease in (extra)-mitochondrial PO_2 *per se* rather than increased electron flux as traditionally accepted [12,36]. Since mitochondrial volume density is directly related to maximal mitochondrial oxygen flux [13], the lack of any relationship observed between mitochondrial density and the exercise-induced increase in any of the biomarkers of oxidative stress provides additional, albeit indirect evidence against the flux concept.

It should be noted that there are marked variations in fiber types within the human vastus lateralis which may lead to variations in morphometric assessments within and between subjects. However, our group has found that with a focus upon consistent depth and location these assessments are highly reproducible, as assessed by multiple measures in the same subject both within and between legs (RS Richardson et al. unpublished observations).

Increased intramuscular lipid soluble antioxidants (LSA) in response to exercise

There is only one study in humans that has examined changes in the intramuscular concentration of α-tocopherol, arguably the most important chain-breaking antioxidant capable of scavenging peroxyl radicals [37], which was shown to decrease following eccentric exercise [38]. In contrast, we observed an exercise-induced increase in α-tocopherol and other selected LSA. This apparent discrepancy may be related to differences in the exercise models used and corresponding mechanics of muscle contraction. Unlike the typically pliometric contraction protocol employed in the earlier study [38], KE exercise involves miometric (concentric) contractions [14] which demand 20–25% less muscle energy consumption for a given work intensity [39] and impose comparatively less structural damage on skeletal muscle fibers [40].

The novel observation of an uptake or enrichment of LSA by skeletal muscle during exercise may represent a homeostatic antioxidant defense response to counter exercise-induced oxidative stress. Alternatively, it may simply reflect increased lipoprotein delivery and available pool for hydrolysis subsequent to a regional increase in quadriceps muscle blood flow (QMBF). In our previous studies, QMBF has been shown to increase substantially from 350 ml/min at rest to 9000ml/min which is equivalent to 385 ml/min/100 mg active tissue mass during maximal KE exercise [34]. Future studies will need to consider repetitive biopsy sampling and the simultaneous measurement of QMBF during incremental KE exercise to establish if LSA enrichment is indeed a perfusion-limited phenomenon. Changes in the intramuscular activities of antioxidant enzymes and glutathione, the major intracellular thiol in muscle [41], also warrant future consideration.

Experimental limitations

While the $UQ^{\cdot-}$ signal in muscle is known to be relatively stable [27], there was an unavoidable time delay between recovery of the biopsy sample following the exercise challenge and freezing (~60 s). Thus it is conceivable that the radicals detected probably underestimate the real-time concentration generated *in vivo* which is an acknowledged limitation of any *ex vivo* biopsy technique.[26,42] Finally, the small sample size and failure to include a resting control group are also recognized as potential limitations that may challenge the interpretive significance of our findings.

However, despite the small sample size, all data were normally distributed and statistical significance was consistently observed for the majority of parameters investigated. Thus, in light of the invasive procedures employed, it was considered unethical to "over-recruit" subjects since we had already satisfied the statistical power requirements. Finally, the randomized balanced design and incorporation of the contralateral leg were considered an acceptable compromize as our control comparators. We could not detect any evidence to suggest that the regional trauma associated with our biopsy technique contaminated the subsequent biopsy obtained contra-laterally hence we are confident that the changes observed were mostly attributable to exercise and not merely experimental artifact.[43–45] Future studies need to consider these limitations and confirm our observations.

In conclusion, these findings provide direct evidence for exercise-induced free radical formation and lipid peroxidation in human skeletal muscle though the interpretive limitations associated with the biopsy technique and small sample size are duly acknowledged. Application of an isolated exercise model has established contracting skeletal muscle as a major source of free radicals and mitochondrial $UQ^{\cdot-}$ as a contributory if not initiating species associated with lipid peroxidation. Further experiments are encouraged to determine if these radicals originate from respiring mitochondria.

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