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Repeated static contractions increase mitochondrial vulnerability toward oxidative stress in human skeletal muscle

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Sahlin, Kent, Jens Steen Nielsen, Martin Mogensen, and Michail Tonkonogi. Repeated static contractions increase mitochondrial vulnerability toward oxidative stress in human skeletal muscle. *J Appl Physiol* 101: 833–839, 2006. First published May 25, 2006; doi:10.1152/japplphysiol.01007.2005.—Repeated static contractions (RSC) induce large fluctuations in tissue oxygen tension and increase the generation of reactive oxygen species (ROS). This study investigated the effect of RSC on muscle contractility, mitochondrial respiratory function, and in vitro sarcoplasmic reticulum (SR) Ca^{2+} kinetics in human muscle. Ten male subjects performed five bouts of static knee extension with 10-min rest in between. Each bout of RSC (target torque 66% of maximal voluntary contraction torque) was maintained to fatigue. Muscle biopsies were taken preexercise and 0.3 and 24 h postexercise from vastus lateralis. Mitochondria were isolated and respiratory function measured after incubation with H_2O_2 (HPX) or control medium (Con). Mitochondrial function was not affected by RSC during Con. However, RSC exacerbated mitochondrial dysfunction during HPX, resulting in decreased respiratory control index, decreased mitochondrial efficiency (phosphorylated ADP-to-oxygen consumed ratio), and increased noncoupled respiration (HPX/Con post- vs. preexercise). SR Ca^{2+} uptake rate was lower 0.3 vs. 24 h postexercise, whereas SR Ca^{2+} release rate was unchanged. RSC resulted in long-lasting changes in muscle contractility, including reduced maximal torque, low-frequency fatigue, and faster torque relaxation. It is concluded that RSC increases mitochondrial vulnerability toward ROS, reduces SR Ca^{2+} uptake rate, and causes low-frequency fatigue. Although conclusive evidence is lacking, we suggest that these changes are related to increased formation of ROS during RSC.

calcium homeostasis; exercise; mitochondria; oxidative phosphorylation

REPEATED STATIC CONTRACTIONS (RSC) are characterized by a contraction phase (high-energy turnover and restricted blood flow) followed by a relaxation phase with low-energy turnover, hyperemia, and elevated P_{O_2}. RSC performed at low force but over an extended period of time have been associated with muscular disorders (15, 20). The etiology is unclear, but is considered to be multifactorial, involving both physiological and psychological mechanisms (14). RSC performed at higher intensities are frequent in certain sports (e.g., wrestling, sailing, speedway) and are associated with fatigue. The physiological and biochemical responses in muscle to RSC have been investigated only in a few studies.

RSC are associated with oxidative stress, and the generation of reactive oxygen species (ROS) appears to be related to the exerted force (31). Prior treatment of diaphragm muscle fiber bundles with antioxidants (*N*-acetylcysteine) attenuates fatigue development during RSC (21). Recent studies in humans also demonstrate that administration of *N*-acetylcysteine during exercise can delay fatigue in endurance-trained subjects (27) and suggest that ROS generation may have an important role in exercise-induced fatigue. Potential sources of ROS during RSC are the electron transport chain and the hypoxanthine-xanthine oxidase system (24, 35). Mitochondria are not only a source but also a target of ROS, and RSC may therefore deteriorate mitochondrial function. RSC have several physiological characteristics in common with ischemia-reperfusion, which is a condition known to induce excessive ROS load and tissue damage (17, 24). Studies in rat heart demonstrate that ischemia-reperfusion reduces mitochondrial ADP-stimulated respiration (state 3) (12, 23) and potentiates ROS-induced reduction in state 3 respiration (22). The oxygen cost of contraction increases successively during RSC (33, 40), and it was suggested (40) that the mechanism was related to reduced efficiency of oxidative phosphorylation (oxphos) [i.e., reduced phosphorylated ADP-to-oxygen consumed (P/O) ratio]. However, the effect of RSC on mitochondrial function and the vulnerability of mitochondria to ROS have not been investigated previously.

RSC result in a long-lasting depression of muscle force, which appears to be independent of muscle acidosis (7, 33, 39, 40). The mechanism of fatigue after RSC is unclear, but it has been hypothesized that RSC result in impaired excitation-contraction (E-C) coupling (40). A characteristic feature of impaired E-C coupling is low-frequency fatigue (LFF) (i.e., more pronounced decrease of force at low than at high frequencies of stimulation). LFF has been observed in humans after RSC (39), and the mechanism may be related to reduced sarcoplasmic reticulum (SR) Ca^{2+} release (8) or alternatively to a decreased Ca^{2+} sensitivity of the contractile proteins. Repeated isometric tetani in single mouse muscle fibers resulted in reduced Ca^{2+} sensitivity, which could be prevented by prior treatment with ROS scavenger (28). It is also known that exposure of single fibers to ROS alters Ca^{2+} handling at several sites (2). In humans, 30 min of RSC resulted in reduced SR Ca^{2+} uptake and intrinsic alterations in the Ca^{2+} pump (39). However, neither SR Ca^{2+} release nor contractility kinetics (rate of force development and relaxation) have been measured after RSC, and further studies are therefore required to investigate this issue.

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The purpose of this study was to test the hypothesis *1*) that RSC deteriorate mitochondrial function and increase the vulnerability toward oxidative stress, and *2*) that RSC alter muscle contractility and in vitro SR Ca^{2+} handling.

MATERIALS AND METHODS

Subjects. Ten healthy male subjects were investigated. Their age, weight, and height [mean (range)] were as follows: 26 (21–31) yr, 81 (65–98) kg, and 184 (179–190) cm. Total body fat, measured with impedance technique (Tanita body impedance TBF-300), was 15.5 ± 1.2% (mean ± SE). Subjects were mainly university students and were physically active on a recreational basis, including participation in various sports (in average two times per week) and cycling (average distance ~38 km/wk). Subjects agreed to participate in the experiment after having been informed of the purpose and potential risks involved. The project was approved by the local ethics committee at the Odense University Hospital (VF 20030129).

Experimental protocol. Before the main experiment (within a week), subjects were familiarized with the protocol of electrical stimulation. Subjects were also trained to perform maximal voluntary isometric contractions (MVC) until they were able to reach maximal torque repeatedly. Subjects were informed to abstain from physical activity 24 h before the experiment and to use the same transportation to the laboratory on both experimental days.

Subjects performed static contraction with the knee extensors of one leg at a target torque corresponding to 66% of MVC until fatigue (Fig. 1). Both the actual torque and the target torque were displayed on a computer screen in front of the subject. Fatigue was defined as the time point when subjects, despite verbal encouragement, were unable to maintain the target torque (>2 s at 10% below target torque). The contraction was repeated five times with 10-min rest in between. Changes in muscle contractility were investigated with a standardized test (see below) before and after the exercise period. The test was (except for immediately postexercise) preceded by warm up (10-min cycling at 80–120 W) and performed on the same leg in each subject (randomly chosen between the dominant and nondominant leg). Capillary blood (prewarmed fingertip) and muscle biopsy were taken after the contractility test. The two postexercise muscle biopsies were taken from the exercised leg, and the preexercise biopsy was taken from the other leg. The first postexercise biopsy was taken ~7 min after the test and thus 18 min after terminating RSC (Fig. 1). The rationale for this time delay was to maximize ROS generation, which is expected to reach a peak during the reperfusion period. Potential acute effects of RSC-induced metabolic perturbations are unlikely to affect the measured mitochondrial function and SR Ca^{2+} kinetics, since the organelles were studied in standardized solutions during the measurements. The preexercise biopsy was taken from a leg without prior contractility test, and it may therefore be argued that the observed changes of muscle mitochondrial function and/or SR Ca^{2+} kinetics are due to the contractility test rather than RSC. However, this seems rather unlikely, since the total duration of contraction during the test (including mainly submaximal electrical stimulation) was only ~8 s compared with >220-s contraction during RSC.

Muscle biopsy. After local anesthesia (2–3 ml 2% lidocaine), an incision was made through the skin and fascia (about one-third of the distance between patella and spina ilica anterosuperior) of vastus lateralis muscle. The muscle biopsy was taken with a modified Bergström needle with suction (3). The muscle specimen was dried on a filter paper placed on a glass plate (cooled on ice), and obvious connective tissue and fat were removed. The muscle specimen was divided into three lots, of which one (~10 mg) was immediately frozen in liquid nitrogen and later used for analysis of citrate synthase (CS) activity (see below). Another lot (35–50 mg) was used for determination of SR Ca^{2+} kinetics and was weighed and homogenized in 1:10 volumes (wt/vol) of ice-cold buffer (300 mM sucrose, 1 mM EDTA, 10 mM NaN_3, 40 mM Tris base, and 40 mM histidine at pH 7.8) at 0°C in a 1-ml glass homogenizer with a glass pestle (Kontes Glass Industry, Vineland, NJ). Before homogenization, the muscle sample was rinsed free of contaminating blood by washing in ice-cold buffer. This procedure is important to avoid artifacts due to the influence of blood on the assays of Ca^{2+} kinetics (30). The homogenate was divided in different portions and frozen in liquid nitrogen for later analyses of Ca^{2+} kinetics, protein, and myosin heavy chain (MHC) composition (see below). The last muscle portion (84–238 mg) was used for measurements of oxidative function of isolated mitochondria (see below).

Electrical stimulation and measurements of contractility. Muscle contractility was measured in a custom-made chair (knee angle 90°) with the trunk and hip firmly fixed with belts and the contracting leg fixed (2 cm proximal to the malleoli) in a metal cuff connected to a strain-gauge.

The procedure of electrical stimulation and measuring muscle contractility was similar to that previously described (30). Briefly, two surface electrodes (5 × 9 cm, Pals Axelgaard, Fallbrook, CA) were placed on vastus lateralis muscle. The muscle was stimulated (Digitimer DS7A, Digitimer, Welwyn Garden City, UK) with square-waved pulses of 100 μs with a cutoff voltage of 230 V. The stimulation protocol and data sampling (1 kHz) were controlled from a computer by predesigned macros in Spike 2 version 4.18 (Cambridge Electronic Design, Cambridge, UK) and controlled through a CED Micron 1401 II 16-bit A/D converter (Cambridge Electronic Design).

The muscle was stimulated with a current sufficient to elicit either maximal torque (twitch) or 25% of peak twitch torque (20 and 50 Hz). The following protocol was used: 20 Hz (1 s), 2-min pause, 20 Hz (1 s), 0.5-min pause, 50 Hz (1 s), 0.5-min pause, 50 Hz (1 s), 0.5-min pause, 1 Hz. Peak torque at 20 and 50 Hz was determined as the

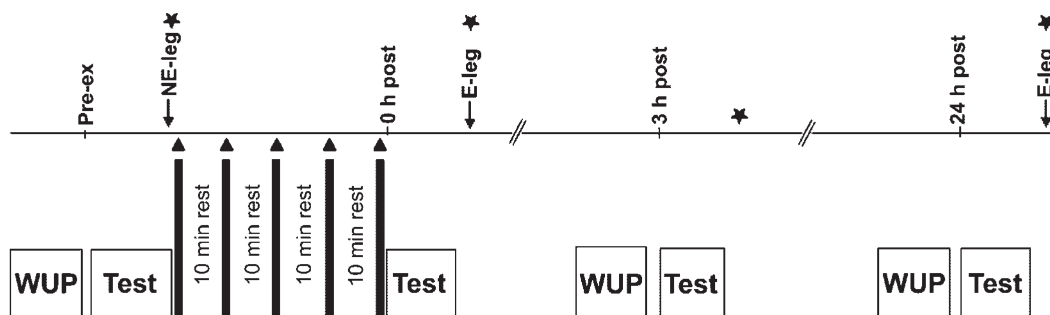

Fig. 1. Experimental protocol. Pre, preexercise; WUP, warm-up period (8 min cycling at 80 W and 2 min cycling at 120 W); test, period of muscle contractility test with both voluntary and electrical stimulated contractions (see MATERIALS AND METHODS for details). The arrows denote time of muscle biopsy (NE, nonexercise; E, exercise). The stars denote time of capillary blood. The black bars denote repetitive static contractions (RSC). Each bout of RSC was maintained to fatigue at 66% of maximal voluntary contraction torque (MVC).

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maximal torque produced during 0.4 s. One-half relaxation time ($RT_{½}$) was calculated as the time from the last stimuli to 50% of peak torque at 50 Hz, and one-half contraction time ($CT_{½}$) as the time from the first stimuli until 50% of peak torque. The degree of tetanic fusion during 20-Hz stimulation was calculated from the torque fluctuation (difference between peak and nadir torque) during the last four stimulations. After electrical stimulation, subjects performed three MVC separated by 45 s. Peak torque, i.e., 100% MVC, was defined as the maximal torque maintained during 1 s. The contractile measurements were performed on the same leg, and the coefficients of variation (CVs), calculated previously from duplicate measurements (30), were 6.5% ($RT_{½}$), 6.6% (MVC), 3.3% (20 Hz), and 1.2% (50 Hz).

SR Ca^{2+} uptake and release rates. The method used to determine the rates of Ca^{2+} uptake and release has been described in detail (30). Briefly, muscle homogenate (80 μl ~8 mg of muscle tissue) was mixed with 1 ml buffer [165 mM KCl, 22 mM HEPES, 7.5 mM oxalate, 11 mM NaN_3, 5.5 μM *N,N,N′,N′*-tetrakis(2-pyridylmethyl) ethylenediamine, 20 μM $CaCl_2$, 5 mM $MgCl_2$, and 1.5 μM indo 1 (pH 7.0 at 37°C)], and the reaction was initiated by adding ATP to a final concentration of 5 mM. Ca^{2+} concentration ($[Ca^{2+}]$) was determined fluorometrically (Ratiomaster RCM, Photon Technology International, Brunswick, NJ) at 37°C using the fluorescent Ca^{2+} indicator indo 1 (excitation wavelength 355 nm and emission wavelength 400 and 470 nm). The fluorescence was measured at 2 Hz over at least 250 s and converted to free $[Ca^{2+}]$, as previously described (30). After $[Ca^{2+}]$ had reached a plateau, Ca^{2+} release was initiated by adding 4-chloro-*m*-cresol (10 mM) (32), and the fluorescence followed during at least 30 s.

All raw data ($[Ca^{2+}]$ and absolute time) were imported into Matlab version 7.0.1 (The MathWorks, Natick, MA) and mathematically analyzed (Curve Fitting Toolbox version 1.1.1, The MathWorks). Initial free $[Ca^{2+}]$ was ≥800 nM in all assays of Ca^{2+} uptake. Data points between a free $[Ca^{2+}]$ of 800 nM and 20 s before initiating Ca^{2+} release were fitted to *Eq. 1*.

$$y = ae^{-bt} + c \qquad (1)$$

where y is the free $[Ca^{2+}]$, t is time, and a, b, and c are constants assigned from Matlab. The rate of Ca^{2+} uptake at a free $[Ca^{2+}]$ of 800 nM, and the time for the free $[Ca^{2+}]$ to reach 63% of the initial free $[Ca^{2+}]$ ($\tau = 1/b$) was calculated from *Eq. 1*. The data points during the first 30 s of release were mathematically fitted to *Eq. 2*.

$$y = a[1 - e^{-b(t-c)}] \qquad (2)$$

The initial rate of Ca^{2+} release was determined as the derivate in the first data point in the release process. The data points were well fitted to monoexponential functions, during both the uptake phase ($r^2 > 0.99$) and the release phase ($r^2 > 0.99$).

Protein content in the muscle homogenate was measured in triplicates with a standard kit (Pierce BCA protein reagent no. 23225). Assays of uptake and release rates of Ca^{2+} were performed in duplicates, and rates were expressed per milligram protein.

The CV as assessed in duplicate assays is larger for Ca^{2+} release rate (14%) than for Ca^{2+} uptake rate (5.5%), indicating a larger methodological variation and thus a lower statistical power of the former. The sampling rate (2 Hz) may limit the ability to accurately determine the rapid phase of Ca^{2+} release.

Fiber-type composition. MHC composition was analyzed in the same muscle homogenate as used for measurements of Ca^{2+} kinetics using gel electrophoresis, as previously described (4) and modified for humans (1). Briefly, muscle homogenate (80 μl) was mixed with 200 μl of sample buffer (10% glycerol, 5% 2-mercaptoethanol and 2.3% SDS, 62.5 mM Tris and 0.2% bromophenolblue at pH 6.8), boiled in water bath at 100°C for 3 min, and loaded (10–40 μl) on a SDS-PAGE gel [8% polyacrylamide (100:1 acrylamide-bisacrylamide), 30% glycerol, 67.5 mM Tris base, 0.4% SDS, and 0.1 M glycine]. Gels were run at 80 V for at least 42 h at 4°C, and MHC bands were made visible by staining with Coomassie. The gels were scanned (Linoscan 1400 scanner, Heidelberg, Germany), and MHC bands were quantified densitometrically (Phoretix 1D, nonlinear, Newcastle, UK).

Mitochondrial function. Isolation of mitochondria was performed as previously described (37). Briefly, muscle specimen was disintegrated with scissors, and mitochondria were isolated by proteinase treatment (Nagarse, 0.2 mg/ml, Sigma P-4789), followed by homogenization and subsequent differential centrifugation. The final mitochondrial pellet was resuspended (0.4 μl/mg initial muscle) in a medium consisting of (in mmol/l) 225 mannitol, 75 sucrose, 10 Tris, 0.1 EDTA, pH 7.40, and stored on ice until analysis.

Oxygen consumption in isolated mitochondria was measured polarographically using a Clark-type electrode (Hansatech DW1) in a water-jacketed glass chamber at a volume of 0.3 ml. The measurements were carried out at 25°C. Respiration in isolated mitochondria was analyzed in a reaction medium containing (in mmol/l) 225 mannitol, 75 sucrose, 10 Tris, 10 KCl, 10 K_2HPO_4, 0.1 EDTA, 5 pyruvate, 2 malate, 0.08 $MgCl_2$, pH 7.35. Mitochondrial suspension was added to the reaction medium, and state 3 respiration was initiated by the addition of ADP (final concentration 260 μM). When all of the added ADP had been phosphorylated to ATP, the respiratory rate returned to that before the addition of ADP (state 4). To elucidate potential loss of cytochrome *c*, state 3 was repeated after addition of cytochrome *c*. Respiratory control index (RCI) was calculated as the ratio of the respiratory rate in state 3 to that in state 4. The P/O ratio was quantified according to the method of Chance and Williams (9).

Mitochondria were incubated at 0°C for 30 min with freshly prepared H_2O_2 [final concentration 0.095 mM (HPX) or with suspension medium (Con)]. The order between HPX and Con was randomized between subjects but maintained constant in biopsies taken from the same subject.

Mitochondrial Ca^{2+} resistance. Mitochondrial Ca^{2+} resistance (MCaR) for opening of mitochondrial permeability transition pore was determined on isolated mitochondria, as previously described (16). An aliquot of mitochondrial suspension equivalent to 5×10^{-2} units CS, corresponding to 19.1 μg protein, was resuspended in 100 μl of buffer (pH 7.4) containing 150 mM KCl, 5 mM Tris, 5 mM K_2HPO_4, 5 mM malate, and 5 mM pyruvate. Equivalent pulses of $CaCl_2$ (2 μmol of Ca^{2+} per unit CS) were added to the mitochondria every 3 min, and mitochondrial swelling was monitored continuously as the change in absorbance at 540 nm until absorbance decreased rapidly. A rapid decrease in absorbance (limit >0.0175 absorbance units/min) indicates progressive swelling of mitochondria due to opening of mitochondrial permeability transition pore.

Plasma CK and thiobarbituric acid-reactive substances and muscle CS activity. Plasma creatine kinase activity was analysed with a standardized kit (Vitros, Ortho-clinical Diagnostic, Indigo Creek, NY). Total content of thiobarbituric acid reactive substances (TBARS) was analyzed in plasma using a fluorometrical method (6). Briefly, plasma (10 μl) was precipitated with phosphothungstic acid (10%), resuspended in a thiobarbituric acid reagent (0.67%), heated in water bath at 100°C for 60 min, and extracted with butanol. The fluorescence of the butanol phase was measured (excitation 515 nm, emission 545 nm), and TBARS was estimated with a standard curve constructed from tetraethoxypropane. All plasma samples from each subject were analyzed simultaneously and in duplicates (CV 10%).

CS activity was measured spectrophotometrically (25°C) in isolated mitochondria and in freeze-dried muscle and dissected free from nonmuscle constituents, using a technique described previously (37).

Mitochondrial lipid peroxidation (4-hydroxynonenal). Mitochondrial 4-hydroxynonenal (HNE) protein adducts were determined with Western blotting of samples taken pre- and 0.3 h postexercise. Equal amounts of mitochondria (as judged from CS activity) were loaded on SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane, and HNE protein

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adducts were detected with polyclonal antibodies (HNE12-S, Alpha Diagnosics, San Antonio, TX). After incubation with secondary antibodies conjugated with horseradish peroxidase, the membrane was incubated with detection reagent (ECL, Amersham Biosciences). An X-ray film was exposed to the membrane, and the optical density of the whole lane was quantified by using Molecular Analyst 1.5 (Bio-Rad).

Statistical methods. Values are presented as means ± SE. Statistical significance of differences was tested with a nonparametric analysis of variance (Friedman's test) followed by post hoc analysis with Wilcoxon's signed-rank test. Correlations between parameters were performed with Spearman rank correlation. Statistical significance was accepted at 5% level. All statistical analysis was conducted in Statview version 5.0 (SAS Institute, Cary, NC).

RESULTS

Muscle fiber-type distribution is reflected by the relative composition of MHC isozymes. MHC composition was not different between legs. When calculated from the average of the three biopsies from each subject, MHC composition was [mean ± SE (range)]: MHC I, 42.5 ± 2.2% (30.8–51.7%); MHC IIA, 56.8 ± 2.2% (47.9–68.0%) and MHC IIX, 0.9 ± 0.3% (0–1.9%).

The endurance during RSC decreased successively, being 52 ± 3, 47 ± 3, 42 ± 2, 40 ± 3, and 38 ± 3 s for the five sustained bouts of isometric contractions at 66% of MVC. Endurance was 26% lower during the last vs. the initial bout ($P < 0.05$). The sum of endurance during the five bouts (220 ± 13 s) was not correlated to the actual values or the change in values (post − pre) of mitochondrial function or Ca^{2+} kinetics. Maximal torque declined after RSC, and the depression was more pronounced during low-frequency stimulations (30% decline in twitch torque, $P < 0.05$) than during MVC (−14%; $P < 0.05$; Table 1). This was also reflected by a decline in 20- to 50-Hz force ratio (−15%, $P < 0.05$), which remained depressed 24 h postexercise (−11%; $P < 0.05$). The decline in 20- to 50-Hz torque ratio was related to the decline in twitch torque ($r = 0.83$, $P < 0.01$). $RT_{1/2}$, measured before RSC, was inversely related to the degree of torque fluctuation at 20 Hz ($r = -0.84$, $P < 0.01$; Fig. 2) and suggests that both parameters are related to torque relaxation. Following RSC, $RT_{1/2}$ decreased and torque fluctuation increased (Table 1). Both of these changes demonstrate that torque relaxation become faster after RSC.

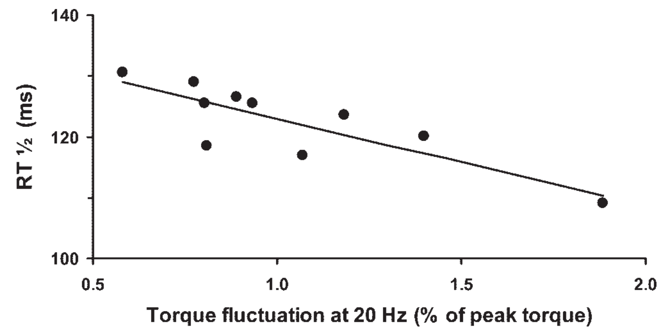

Fig. 2. Correlation between one-half relaxation time ($RT_{1/2}$) and fluctuation of torque at 20 Hz. $RT_{1/2}$ is the time between last stimuli (50 Hz) and decline of torque by 50%. Torque fluctuation denotes the relative degree of unfused torque, i.e., the average difference between the peak and nadir of torque (normalized to peak torque) during the last 4 stimuli. The figure shows values obtained preexercise ($r = -0.84$; $P < 0.01$, $n = 10$). $RT_{1/2}$ was also inversely correlated to torque fluctuation when measured at 3 h ($r = 0.72$; $P < 0.05$) and 24 h ($r = 0.82$; $P < 0.01$) postexercise but not at 0.3 h postexercise.

Ca^{2+} kinetics was measured in muscle homogenates. The average rate of SR Ca^{2+} uptake shortly after RSC was 11% lower than that after 24-h recovery ($P < 0.05$, Table 1) and that preexercise, albeit the latter did not reach statistical significance. SR Ca^{2+} release rate was not significantly changed ($P > 0.05$) by RSC.

RSC had no effect on mitochondrial function [state 3 and state 4 respiration, P/O ratio, resistance to Ca^{2+}-induced pore opening (MCaR) during Con; Table 2]. In vitro exposure of mitochondria to ROS resulted in lower state 3 respiration (−47%; $P < 0.001$), lower RCI (−43%; $P < 0.001$), and lower P/O ratio (−4%; $P < 0.01$). The ROS-induced impairment of mitochondrial function was exacerbated by RSC. When related to Con, there was a number of changes in mitochondrial function after RSC during HPX (Fig. 3), including reduced RCI ($P < 0.05$, pre- vs. 0.3 and 24 h postexercise), reduced P/O ratio ($P < 0.05$, pre- vs. 0.3 h postexercise), and increased noncoupled respiration (state 4) ($P < 0.05$, pre- vs. 24 h postexercise). Addition of cytochrome *c* could not reverse the ROS-induced deterioration of mitochondrial respiration (data not shown).

Plasma CK activity increased moderately after RSC and was 57% higher 24 h postexercise than that preexercise (333 ± 54

Table 1. *Effect of RSC on muscle contractility and in vitro Ca^{2+} kinetics*

	Pre	0 h Post	3 h Post	24 h Post
MVC, N·m	214±6	184±6*	197±7*†	215±8†‡
Twitch torque, N·m	38±1.5	26±2.5*	35±1*†	37±4†‡
20- to 50-Hz torque	0.75±0.02	0.64±0.03*	0.60±0.02*	0.67±0.02*†‡
Torque fluctuation at 20 Hz, %torque	1.03±0.12	1.27±0.12	1.37±0.13*†	1.17±0.12*‡
$CT_{1/2}$ 50 Hz, ms	102±4	109±4	100±3	101±4
$RT_{1/2}$ 50 Hz, ms	123±2	111±2*	114±2*†	120±2*†‡
SR Ca^{2+} release rate, μmol·mg protein^{-1}·min^{-1}	3.4±0.2	3.8±0.3		3.6±0.1
SR Ca^{2+} uptake rate, μmol·mg protein^{-1}·min^{-1}	3.8±0.1	3.4±0.2		3.8±0.1†

Values are means ± SE; $n = 10$ subjects. Pre, preexercise; Post, postexercise; MVC, maximal voluntary contraction torque; $CT_{1/2}$, one-half contraction time (time between first stimuli and 50% of peak torque at 50 Hz); $RT_{1/2}$, one-half relaxation time (time between last stimuli and 50% of peak torque at 50 Hz); torque fluctuation, relative degree of unfused torque, i.e., the average difference between the peak and nadir of torque (normalized to peak torque) during the last 4 stimuli; SR, sarcoplasmic reticulum. The first postexercise measurement of muscle contractility and Ca^{2+} kinetics (0 h Post) was performed 0.1–0.2 and 0.3 h postexercise, respectively. Twitch torque was induced by electrical stimulation of muscle with supramaximal current, whereas 20- and 50-Hz stimulation was performed with submaximal current corresponding to 25% of peak twitch torque. *$P < 0.05$ vs. preexercise; †$P < 0.05$ vs. 0 h postexercise; ‡$P < 0.05$ vs. 3 h postexercise.

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Table 2. *Effect of RSC on mitochondrial function during control*

	Pre	0.3 h Post	24 h Post
State 3, nmol O_2·unit CS^{-1}·min^{-1}	61.0±4.0	59.4±5.1	63.8±5.2
State 3 (+cytochrome *c*), nmol O_2·unit CS^{-1}·min^{-1}	53.7±3.5	54.3±4.7	58.8±4.9
State 4, nmol O_2·unit CS^{-1}·min^{-1}	6.1±0.7	5.6±0.3	5.2±0.4
RCI	10.5±0.8	10.5±0.5	12.7±1.2
P/O	2.65±0.06	2.65±0.06	2.64±0.05
MCaR, (no. of Ca^{2+} additions)	8.1±0.5	7.7±0.4	8.5±0.7
Muscle CS, mmol·min^{-1}·kg dry wt^{-1}	88.1±8.7	82.0±7.0	86.5±5.4

Values are means ± SE; n = 9 subjects [muscle citrate synthase (CS), n = 8]. State 3 and state 4, mitochondrial respiration with pyruvate-malate in the presence and absence of ADP, respectively; RCI, respiratory control index (state 3/state 4); P/O, ratio between phosphorylated ADP to oxygen consumed; MCaR (mitochondrial Ca^{2+} resistance) is the no. of Ca^{2+} additions (2 μmol of Ca^{2+}/unit CS) before mitochondrial swelling, which indicates opening of permeability transition pores. Postexercise values were not significantly different ($P > 0.05$) from preexercise values.

vs. 212 ± 70 U/l plasma; $P < 0.001$). There was no evidence of increased lipid peroxidation when measured as the concentration of TBARS in plasma [6.8 ± 0.7 (pre), 7.2 ± 0.3 (0 h), 6.9 ± 0.4 (3 h), and 7.5 ± 0.3 (24 h) μmol/l plasma, $P > 0.05$] or in urine (data not shown). The sensitivity of this method to detect local oxidative stress in muscle tissue may, however, be low (see DISCUSSION). Mitochondrial lipid peroxidation was measured as HNE protein adducts with Western blotting. There was no significant difference in HNE protein adducts in mitochondria isolated from muscle samples taken pre- and postexercise (93 ± 10% of preexercise value).

DISCUSSION

Mitochondrial function. In vitro ROS exposure of mitochondria from preexercise biopsies resulted in a pronounced decline in state 3 respiration, RCI, and P/O ratio (Fig. 3). Mitochondria isolated from muscle biopsies taken after RSC was more vulnerable to ROS exposure, as demonstrated by further depressions of RCI and oxphos, as well as increased noncoupled respiration (Fig. 3). These alterations remained when cytochrome *c* was added to the mitochondria, and thus the defects could not be attributed to a loss of cytochrome *c* due to damaged outer mitochondrial membrane. The augmented mitochondrial vulnerability to ROS after RSC is similar to that observed in rat cardiac tissue after ischemia-reperfusion (23), which was attributed to depletion of the mitochondrial antioxidant reserve (i.e., glutathione, GSH). Following ischemia-reperfusion of rat cardiac tissue, GSH decreased by 10 and 30% in the myocardium and mitochondria, respectively (23). Strenuous exercise in humans has previously been shown to reduce GSH in plasma (18) and in muscle (10, 27). Intravenous infusion with an antioxidant (*N*-acetylcysteine) prevented the exercise-induced fall in total glutathione and reduced glutathione in muscle of endurance-trained subjects (27). Furthermore, patients with circulatory shock exhibit both reduced GSH levels and mitochondrial dysfunction in skeletal muscle (11). Due to limited material, we were unable to measure mitochondrial glutathione status. However, because RSC is an ischemia-reperfusion type of exercise, it is possible that the increased mitochondrial vulnerability to ROS after RSC was caused by a reduction of the mitochondrial antioxidant reserve (e.g., GSH).

There are some studies that indicate that the vulnerability to oxidative stress is increased by endurance training. First, the ergogenic effect of intravenous infusion of antioxidants (*N*-acetylcysteine) was dependent on the training status of the subjects. Performance increased by 26% in endurance-trained subjects (27) but remained unchanged in recreationally active men (peak O_2 uptake < 48 ml O_2·min^{-1}·kg body wt^{-1}) (26). Furthermore, mitochondrial vulnerability to oxidative stress is increased in rats after rigorous swim training (23). The subjects included in the present study were active on a recreational basis, but were not classified as athletes. Although mitochondrial function was unaffected by RSC during Con, it can be speculated that mitochondria from endurance-trained athletes would be more susceptible to RSC than that from the present group of subjects.

Based on experiments in horses, it was concluded that maximal mitochondrial respiration was severely reduced after exhaustive exercise (19). However, later studies in humans, in whom more advanced techniques have been used, have not been able to confirm these data. Mitochondrial respiratory function, including oxphos, was maintained following fatiguing exercise of long duration (36), at high intensity (38), or of eccentric type (41). The results from the present study extend this list to RSC, which had no acute influence on mitochondrial function, including oxphos.

Muscle contractility and Ca^{2+} kinetics. Endurance at 66% MVC decreased successively during RSC. The relatively slow recovery of endurance (10-min rest between bouts) is consistent with previous findings (34) and is likely related to remain-

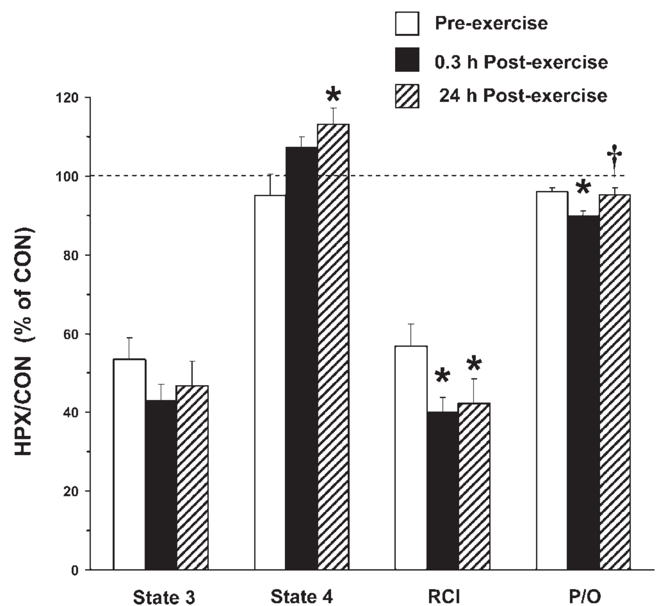

Fig. 3. Vulnerability of mitochondrial parameters to oxidative stress. Mitochondria were isolated from muscle biopsies taken preexercise and 0.3 and 24 h postexercise and incubated in 0.1 mM H_2O_2 (HPX) or mitochondrial suspension medium (Con) for 30 min. State 3 and state 4 denote mitochondrial respiration with pyruvate-malate in the presence and absence of ADP, respectively. RCI, respiratory control index (state 3/state 4); P/O, ratio between phosphorylated ADP to oxygen consumed. Values (means ± SE) are shown as percentage of corresponding values during Con. *$P < 0.05$ vs. preexercise. †$P < 0.05$ vs. 0.3 h postexercise.

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ing high muscle lactate. MVC decreased by 14% after RSC (∼10 min postexercise) and remained depressed 3 h postexercise. Previous studies have shown that muscle metabolic state is recovered 1 h after RSC, but that MVC remains reduced (39). The long-lasting depression of MVC observed in the present and previous studies (39) is, therefore, most likely caused by nonmetabolic factors, e.g., reduced SR Ca^{2+} release and/or reduced myofibrillar Ca^{2+} sensitivity. The observed long-lasting LFF (reduced 20- to 50-Hz torque ratio) after RSC is consistent with previous findings (39) and supports the hypothesis of disturbances in Ca^{2+} handling.

LFF is most likely a consequence of reduced cytosolic Ca^{2+} levels and/or reduced myofibrillar Ca^{2+} sensitivity. The rate of SR Ca^{2+} release, as measured in vitro, was not reduced after RSC (Table 1). However, this method only measures a late step in E-C coupling, and we cannot exclude that Ca^{2+} release in vivo is reduced due to disturbances in t-tubular function, defects in signaling between the t-tubular and the SR system, or reduced SR content of Ca^{2+}. Studies in isolated mouse muscle fibers showed that myofibrillar Ca^{2+} sensitivity decreased after RSC performed at body temperature (37°C) and that this could be prevented by ROS scavengers (28). Furthermore, exposure of muscle fibers to ROS (without RSC) reduced myofibrillar Ca^{2+} sensitivity (2). Thus it seems reasonable to suggest that the reduction in torque at low frequencies, which was observed after RSC, is caused by ROS-induced reduction in myofibrillar Ca^{2+} sensitivity and/or reduced SR Ca^{2+} release. Further studies are required to investigate this hypothesis.

The rate of SR Ca^{2+} uptake was lower in muscle biopsies taken shortly after RSC than those after 24-h recovery. The reduced rate of SR Ca^{2+} uptake is consistent with a previous study in humans, in whom 30 min of RSC resulted in a pronounced reduction in SR Ca^{2+} uptake rate and SR Ca^{2+} ATPase activity (39). Exposure of single fibers to ROS reduces the rate of Ca^{2+} removal (2), and the depression of SR Ca^{2+} uptake after RSC may, therefore, be an effect of increased ROS load. Conclusive evidence for a cause-effect relation is, however, lacking.

Torque relaxation is determined by the rate of Ca^{2+} removal from the cytosol and the rate of cross-bridge turnover. Everything else being the same, one would expect that a reduced maximal rate of Ca^{2+} uptake would result in a slower torque relaxation. However, previous studies have demonstrated that, despite a depressed Ca^{2+} pump function, relaxation of force was unchanged after prolonged exercise (5) or even more rapid after eccentric exercise (30). Similarly, in the present study, we observed a faster torque relaxation after RSC, despite a lower rate of Ca^{2+} uptake ($P < 0.05$; 0.3 h vs. 24 h postexercise). The anomaly between faster torque relaxation and slower maximal Ca^{2+} uptake may relate to changes in other parts of Ca^{2+} handling (i.e., reduced myofibrillar Ca^{2+} sensitivity or reduced in vivo Ca^{2+} release).

Unchanged mitochondrial resistance to Ca^{2+} overload. In a previous study, endurance exercise increased MCaR with almost 50% (16), and we suggested that this could serve as a protection of mitochondria to exercise-induced Ca^{2+} exposure. Preconditioning by exposure of muscle to brief periods of ischemia or contraction is known to protect cardiac tissue from subsequent periods of ischemia-reperfusion in (13), and skeletal muscle from, contraction-induced damage (25). There is evidence that the mechanism is related to activation of mitochondrial ATP-sensitive potassium channels, which attenuates mitochondrial Ca^{2+} overload (29). Being an ischemia-reperfusion type of exercise, RSC could be regarded as a form of preconditioning and thus expected to attenuate mitochondrial Ca^{2+} overload during subsequent periods of contractile activity. MCaR was, however, not significantly changed after RSC. However, MCaR is a measure of how much Ca^{2+} can be taken up by mitochondria before pore opening and does not include factors such as rate of Ca^{2+} accumulation or degree of Ca^{2+} overload. The unchanged MCaR does not exclude that RSC prevents mitochondria from Ca^{2+} overload.

Methodological considerations. Mitochondrial function can be studied with a number of techniques, including isolated mitochondria and permeabilized fibers. The reason for using isolated mitochondria in this study was that this model, in contrast to permeabilized fibers, can be used to assess intrinsic mitochondrial functional parameters, such as efficiency (P/O ratio), RCI, and PTP opening. An inherent limitation with this model is that only a fraction of the available mitochondria is harvested. The yield of harvested mitochondria was rather high in this study (∼28%), and there were no systematic differences in the yield between pre- and postexercise biopsies. However, we cannot exclude that the results of this study primarily reflect that of a subpopulation of mitochondria (e.g., subsarcolemmal mitochondria).

The rationale for the experimental protocol (i.e., RSC) was to maximize ROS generation. The model will induce large fluctuations of muscle oxygen tension with anoxia during the main part of the contraction and rapid, elevated P_{O_2} during the hyperemic recovery phase. This ischemic-reperfusion model is known to be associated with increased ROS generation. Furthermore, exercise to fatigue is known to induce adenine nucleotide catabolism (34), and the associated increase in substrate (hypoxanthine) and conversion of xanthine dehydrogenase into xanthine oxidase would provide a further source of ROS. Lipid peroxidation products (TBARS) were measured in plasma and urine but could not demonstrate an increased ROS load after RSC. However, it could be argued that the working muscle was too small (one-leg knee-extension) to influence markers of ROS (lipid peroxidation products) in plasma and urine. Furthermore, we measured the degree of lipid peroxidation in mitochondria by analyzing HNE protein adducts. Again, we were unsuccessful in demonstrating exercise-induced lipid peroxidation, i.e., markers of tissue damage.

We observed unchanged mitochondrial function after RSC but increased vulnerability to ROS exposure. It is possible that the antioxidative defense (e.g., glutathione) was sufficient to protect mitochondria against RSC-induced ROS load, but that this caused a reduction of the mitochondrial antioxidant reserve, rendering the mitochondria more vulnerable to subsequent ROS exposure. The absence of increased mitochondrial lipid peroxidation is consistent with this scenario.

In summary, RSC resulted in *1*) increased vulnerability of mitochondrial function to ROS exposure, *2*) reduced rate of SR Ca^{2+} uptake (0 h vs. 24 h postexercise), *3*) long-lasting muscle fatigue, especially at low frequencies, and *4*) a faster torque relaxation. Previous studies have demonstrated that most of these changes can be provoked by exposure of the muscle or mitochondria to ROS or are associated with ROS-generating conditions. Measurements of lipid peroxidation markers in

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plasma (TBARS) or mitochondria (HNE protein adducts) could not verify the hypothesis of an increased ROS load after RSC. Although conclusive evidence is lacking, we suggest that the observed changes are related to oxidative stress induced by RSC.

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