

## Forum Original Research Communication

# Mitochondrial H<sub>2</sub>O<sub>2</sub> Production Is Reduced with Acute and Chronic Eccentric Exercise in Rat Skeletal Muscle

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### ABSTRACT

Oxidative stress with acute/chronic exercise has been so far examined using exercise involving a combination of concentric and eccentric contractions, but skeletal muscles are likely to be injured to a greater extent by pliometric contractions. In the present study, the effects of acute and chronic bouts of downhill running exercise on mitochondrial hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) generation (fluorimetric detection of a dimer with homovanillic acid in presence of horseradish peroxidase) and oxygen consumption in conjunction with antioxidant enzymes activity were examined. The results show that acute eccentric exercise was accompanied by a significantly reduced mitochondrial H<sub>2</sub>O<sub>2</sub> production that is likely due to a decrease in complex I of the electron transport chain (ETC). On the other hand, eccentric training leads to positive adaptations, reflected by a higher citrate synthase activity and decreased mitochondrial H<sub>2</sub>O<sub>2</sub> production. The decrease in mitochondrial H<sub>2</sub>O<sub>2</sub> cannot be attributed to alterations in antioxidant capacities but rather to changes in mitochondrial membrane composition characterized by an increased polyunsaturated to saturated fatty acids ratio, and decreased contents in arachidonic acid and plasmalogens. These results suggest that changes in mitochondrial membrane properties with eccentric training can affect H<sub>2</sub>O<sub>2</sub> production by muscle mitochondria. It is hypothesized that these changes resulted in a mild uncoupling sufficient to reduce electron back flow through complex I of the ETC, the major generator of reactive oxygen species by skeletal muscle mitochondria. *Antioxid. Redox Signal.* 8, 548–558.

### INTRODUCTION

MANY REPORTS SHOW that exercise causes oxidative stress (see Refs. 19 and 20 for reviews). This was shown through direct detection of free radical generation by exercising muscles (9) and confirmed by release of superoxide anion and nitric oxide into the extracellular medium from contracting skeletal muscle cells (33) and by an increase in oxidative biomarkers such as protein carbonyls and thiobarbi-

turic acid reactive substances (TBARS, 37). Exercise can activate a number of intracellular sources for reactive oxygen species (ROS), including phagocytes, xanthine oxidase, catecholamines, and peroxisomal oxidative enzymes. But the mitochondrial electron transport chain (ETC) is generally considered as the major source of ROS during exercise (19). Indeed, during the course of normal oxidative phosphorylation, a small fraction of oxygen consumed is converted into superoxide free radicals (O<sub>2</sub><sup>•-</sup>), that are quickly converted

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into hydrogen peroxide ( $H_2O_2$ ) (5). Because exercise increases whole body and tissue rates of oxygen consumption, it has been hypothesized that the increase in mitochondrial oxidative stress induced by exercise was responsible for tissue damages (19, 20).

So far, most of the studies examining oxidative stress with acute/chronic exercise were performed using exercise involving a combination of concentric and eccentric contractions, but activated skeletal muscles are more likely to be injured by lengthening than by shortening contractions that could explain the greater muscle damages reported with eccentric exercise (10, 36, 38). Interestingly, intraperitoneal injection of polyethylene glycol-superoxide dismutase before exercise attenuates muscle fiber injury observed 10 min after eccentric exercise (51). These results suggest that free radicals contribute to the initial eccentric contraction-induced injury of skeletal muscle fibers. On the other hand, skeletal muscle injury resulting from downhill running can be prevented by only 5 days of intermittent downhill running at low speed (38), suggesting that an adaptation took place in response to the initial injury and subsequent recovery from eccentric exercise, which then acted to protect the active muscles (8, 11, 31, 44). Even though many studies have examined muscle oxidative stress caused by exercise, many questions still remain unanswered, such as (a) Does eccentric exercise cause an increase in mitochondrial ROS generation? (b) Does attenuation effect with eccentric training relate with a reduction in mitochondrial ROS generation and/or adaptation of antioxidant enzymes?

To answer those questions we determined  $H_2O_2$  production and oxygen consumption in isolated mitochondria from quadriceps muscles of rats submitted to acute or chronic eccentric exercise (downhill treadmill running). Determination of antioxidant enzymes activities (superoxide dismutase [SOD], catalase [CAT], and glutathione peroxidase [GPx]) was also performed. As there is evidence showing that the rate of ROS production can be affected by changes in fatty acid composition of mitochondrial membrane (18), and because eccentric contractions were reported to affect the fatty acid composition of the muscle membrane phospholipids (17), the second aim of the present study was dedicated to the analysis of fatty acid composition of mitochondrial lipids in order to determine a possible link between fatty acid composition and ROS production with eccentric exercise.

## METHODS AND MATERIALS

### *Animal care*

The present investigation was performed according to the ethical principles laid down by the French Agricultural Department and the European Convention for the protection of Vertebrate Animals used for Experimental and Scientific purposes (Council of Europe N° 123, Strasbourg, 1985). Six week-old, pathogen free male Wistar rats were obtained from Harlan (Gannat, France). The animals were caged and maintained at room temperature 22°–25°C on a 12:12 h light:dark cycle with food (AO3, UAR Villemoisson, France) and water provided *ad libitum*.

### *Chronic and acute eccentric exercise*

Eccentric exercise was performed on a rodent treadmill with a  $-7^\circ$  inclination. The treadmill was equipped with an electric shock grid on the rear to provide exercise motivation to the animals.

**Chronic eccentric exercise protocol.** After familiarization to a daily low intensity exercise on a motorized rodent treadmill (5 m/min for 5 min), animals showing willingness to run were assigned to a chronic exercise group (CE,  $n = 8$ ) and the others remained sedentary during the training protocol ( $n = 16$ ). This protocol consisted of an 8-week intermittent training program with five sessions per week. The first week begun with four bouts (5 min at 20 m/min) with 2 min rest between bouts. The second week consisted of six bouts (2.5 min at 30 m/min) and 1 min of rest. The same number and duration of bouts were maintained during the third and fourth weeks but at higher speeds (32.5 m/min and 35 m/min, respectively). Exercise intensity and duration were then kept constant during the last 4 weeks of training.

**Acute eccentric exercise protocol.** Two weeks before acute exercise, half of the sedentary rats (SED) were accustomed to run at 5 m/min for 5 min during three sessions per week. Exercise was initiated at 8 m/min, followed by a gradual speed increase every 5 min up to 30 m/min. Thereafter, the rats ran for 4 min at 30 m/min and 1 min at 40 m/min until exhaustion. Rats were considered exhausted when the animal touched the rear of the treadmill more than three times within a minute. In a separate group, sedentary rats were acutely exercised and serum creatine kinase (CK) was evaluated with a reagent kit (Sigma, Inc., L'Isle d'Abeau Chesnes, France) from blood collected immediately at the end or after 3 and 6 h of recovery from the exercise bout.

### *Tissue preparation*

Sedentary rats were sacrificed either at rest (SED,  $n = 8$ ) or immediately after acute exercise (AE,  $n = 8$ ). Trained animals were killed 48 h after their last running bout, the time at which maximum muscle damage has been previously reported to occur with downhill running (38). Rats were sacrificed by decapitation between 08:00 and 10:00 a.m. and the deep portion of quadriceps muscle from both legs were quickly removed and used for mitochondrial extraction and muscle fatty acid composition. Gastrocnemius muscles were frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until antioxidant and oxidative enzymes assays.

### *Isolation of muscle mitochondria*

Quadriceps muscles were quickly removed and placed in ice-cold isolation medium (100 mM sucrose, 50 mM KCl, 5 mM EGTA, and 50 mM Tris/HCl, pH 7.4). Muscles were freed of extracellular fat and connective tissue, minced with scissors, and incubated with 1 mg/g muscle wet weight of protease (Subtilisin A, Sigma, France) for 1 min and then homogenized with a Teflon glass homogenizer. The mixture was diluted 1:2 (v:v) with isolation medium without protease and

centrifuged at 800 *g* for 10 min. The resulting supernatant was filtered through cheesecloth and centrifuged at 8000 *g* for 10 min to obtain the mitochondrial pellet. The pellet was washed twice with isolation medium and then centrifuged at 8000 *g* for 10 min. Finally, the pellet was re-suspended in ice-cold storage medium (250 mM sucrose, 20 mM Tris/HCl, 1 mM EGTA, pH 7.4). After protein quantification by the Biuret method with BSA as standard, mitochondria were diluted to 20 mg/ml with storage medium. All centrifugation were performed at 4°C.

### Mitochondrial $O_2$ consumption

Oxygen consumption was measured with a Clark oxygen electrode (Gilson oxygraph 5/6H, Gilson France, Bron, France) in a 1.5 ml glass cell, thermostatically controlled at 30°C, with constant stirring. The respiratory medium, saturated with room air, contained 200 mM sucrose, 10 mM potassium phosphate, and 20 mM Tris/HCl, pH 7.4, with 0.1% fatty-acid-free BSA. The control state of respiration (state 4) was initiated by the addition of pyruvate/malate (5 mM/2.5 mM) or succinate (5 mM) with rotenone (5  $\mu$ M). Addition of ADP (100  $\mu$ M) initiated the active state of respiration (state 3). The efficiency of the mitochondrial oxidative phosphorylation was assessed by the following parameters: (a) the ADP-to- $O$  ratio, which measures the efficiency of the phosphorylating system; (b) the state 3/state 4 ratio which measures the degree of control imposed on oxidation by phosphorylation (respiratory control ratio, RCR).

### Mitochondrial $H_2O_2$ production

The rate of mitochondrial  $H_2O_2$  release was measured at 30°C following the linear increase in fluorescence ( $\lambda_{ex}$  312 nm and  $\lambda_{em}$  420 nm) due to oxidation of homovanillic acid (HVA) by  $H_2O_2$  in the presence of horseradish peroxidase (HRP) on a SFM-25 fluorometer (Kontron Instruments, Dardilly, France), as described previously (4, 39). Reaction conditions were 0.2 mg of mitochondrial protein per ml, HRP (6 U/ml), HVA (0.1 mM) and pyruvate/malate (5 mM/2.5 mM) or succinate without rotenone (5  $\mu$ M), as substrates, in the same incubation buffer used for oxygen consumption measurements. Known concentrations of  $H_2O_2$  were used to establish a standard concentration curve. Addition of catalase showed a dose-dependent drop of fluorescence. There was no increase of fluorescence in absence of substrates or mitochondria. Measurements of oxygen consumption and  $H_2O_2$  release were performed at the same temperature with the same concentration of substrates, inhibitors, and ADP.

### Mitochondrial fatty acid composition

Determination of fatty acid composition was performed according to the method of Morrison and Smith (29). Total lipids were extracted from mitochondrial pellets and partitioned in chloroform/methanol (2:1, v/v). The lower organic phase was evaporated to dryness under reduced pressure, and the lipids were taken up in 250  $\mu$ l of toluene: methanol (2:3 v/v) and treated by 250  $\mu$ l of  $BF_3$  (boron fluoride) for 90 min at 100°C. The reaction was stopped by adding 750  $\mu$ l of  $K_2CO_3$  (10% in water) and the resulting fatty acid methyl es-

ters were extracted by 1 ml of iso-octane. The organic phase was carefully taken after centrifugation at 1500 *g* for 10 min and evaporated under  $N_2$ . Fatty acid methyl esters were then solubilized in 50  $\mu$ l of iso-octane and 1  $\mu$ l aliquot was analyzed by gas chromatography. Separation was performed on a Hewlett-Packard 6890 gas chromatograph equipped with a capillary column (60 m  $\times$  0.25 mm  $\times$  0.25  $\mu$ m) coated with a stationary phase SP2380 (Supelco). Helium was used as carrier gas. The split/splitless injector and the flame ionization detector were set at 230°C and 250°C, respectively. Two hundred and eighty ml/min of air and 30 ml/min of hydrogen were used for the detector. Analysis started at 57°C for 2 min, then the oven temperature was raised at 20°C/min to 150°C and then at 4°C/min until 250°C, and finally held at 250°C for 4 min. Identification of fatty acid methyl esters was done by comparison with standards. Results were expressed as percent of the total fatty acids content.

### Citrate synthase activity

Citrate synthase (CS, EC.4.1.37) activity was determined as described by Srere (42) in a medium containing DTNB (0.1 mM) and acetyl-CoA (0.3 mM) by monitoring the decrease in absorbance at 412 nm for 3 min after the addition of oxaloacetic acid (0.5 mM).

### Lipid peroxidation; antioxidant enzymes activities

Estimation of malondialdehyde, a lipid peroxidation marker, was evaluated by the thiobarbituric reactive acid substances (TBARS) method according to Ohkawa *et al.* (32). For enzymatic determinations, a portion of frozen gastrocnemius muscle was homogenized with a potter Elvehjem at 4°C, in buffer containing  $KH_2PO_4$  (100 mM), DTT (1 mM), and EDTA (2 mM), pH 7.4. After centrifugation (3000 *g* for 5 min), the supernatant was used for enzymatic assays. Superoxide dismutase (SOD, EC.1.15.1.1) activity was assayed by monitoring the rate of acetylated cytochrome *c* reduction by superoxide radicals generated by the xanthine-xanthine oxidase system (15). The assay for total activity of glutathione peroxidase (GPx, EC. 1.11.1.9) coupled the reduction of cumene hydroperoxide to the oxidation of NADPH by glutathione reductase, and this coupled reaction was monitored at 340 nm (45). The activity of catalase (CAT, EC. 1.11.1.6) was determined by the method of Aebi (1). This technique used the first-order rate constant of the decomposition of  $H_2O_2$  by tissue CAT at 20°C. One unit of catalase activity was calculated by using  $k = (2.3/dt)(\log A_1/A_2)$ , where  $k$  is CAT activity,  $dt$  is change in time,  $A_1$  is initial absorbance, and  $A_2$  is final absorbance. All enzymes activities are expressed in U/100 mg of protein.

### Western blot analysis

For UCP3 analysis, samples of gastrocnemius containing 40  $\mu$ g protein were mixed with equal volumes of 2 $\times$  sodium dodecyl sulphate (SDS) loading buffer, incubated at 90°C for 5 min and subjected to one-dimensional SDS-PAGE on 12% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes and immunological detection was performed by using antibodies against UCP3 [Chemicon

AB3046 (1:1,000)]. The binding of antibodies was detected with a horseradish peroxidase-coupled anti-rabbit [Bio-Rad (1:3,000)] secondary antibody, and an enhanced chemiluminescence (ECL) detection kit (Amersham).

### Statistical analysis

All values reported are means  $\pm$  SEM. Groups were calculated using analysis of variance (Statview 4.02, Abacus Concepts, Inc, Berkeley, CA). Fisher's protected least significant difference for multiple comparisons was used post hoc when significant *F* ratios were obtained and significance was accepted at the *p* < 0.05 level.

## RESULTS

### Body mass

Trained animals ( $366 \pm 7$  g) displayed a lower body weight than sedentary rats ( $397 \pm 7$  g, *p* = 0.002). On the other hand, acute exercise ( $61 \pm 2$  min) was accompanied by a slight decrease in body weight from  $390 \pm 11$  to  $384 \pm 11$  g. Citrate synthase (CS) activity, a marker of oxidative capacity, was significantly higher in CE ( $1252 \pm 33$  U/mg protein) than in SED ( $1147 \pm 23$  U/mg protein). There was no significant change of CS activity after an acute bout of exercise ( $1195 \pm 79$  U/mg protein) as we have already seen with acute exhaustive exercise (40).

### Oxidative stress status

Serum creatine kinase was not different between SED and CE groups (Fig. 1A). Immediately after acute exercise, serum CK remained unchanged, but increased progressively thereafter (3 and 6 h after the end of exercise). Muscle lipid peroxidation as appreciated from TBARS was not affected by acute eccentric exercise but increased significantly after training (Fig. 1B).

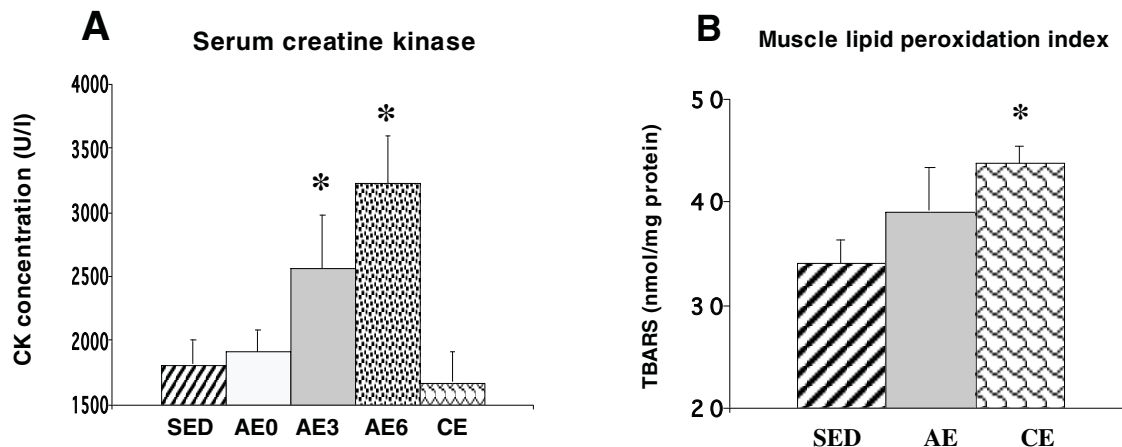
### Mitochondrial release of $H_2O_2$

The rate of  $H_2O_2$  generation by skeletal muscle was clearly dependent upon the substrates used for energizing mitochondria. In nonphosphorylating conditions (state 4) with pyruvate/malate as substrates that provide NADH to complex I of ETC, the rate of  $H_2O_2$  release was somewhat 8–9 times lower than with succinate providing  $FADH_2$  to complex II of the ETC.  $H_2O_2$  production was significantly increased by addition of rotenone to mitochondria energized with pyruvate/malate but decreased drastically with succinate (Table 1).

State 4 mitochondrial  $H_2O_2$  production decreased significantly after acute exercise (AE) as compared to rested rats (SED) with pyruvate/malate but not with succinate as substrates. On the other hand, eccentric trained rats (CE) displayed a lower  $H_2O_2$  production ( $-25\%$ ) with both substrates (Table 1). While the rate of  $H_2O_2$  production was similar in phosphorylating and nonphosphorylating conditions when mitochondria were energized with pyruvate/malate, it was drastically decreased following ADP addition with succinate. The increase in  $H_2O_2$  production after rotenone addition to pyruvate/malate supplemented mitochondria unequivocally demonstrates the capacity of complex I of the ETC for ROS generation (4) which was similar in rested and exercised rats (Table 1). On the other hand, the severe reduction of ROS generation by rotenone in succinate-supplemented mitochondria shows that the physiologically relevant ROS production supported by succinate occurs in complex I through reversed electron transfer (4, 43).

### Mitochondrial oxygen consumption

State 4 oxygen consumption in CE was higher than in SED (*p* < 0.05) and AE (*p* = 0.06) groups when mitochondria were energized with pyruvate/malate, but not with succinate (Table 2). By contrast, oxygen consumption in state 3 was not modified by acute or chronic exercise, irrespective of the substrates provided to mitochondria (pyruvate/malate or succi-



**FIG. 1.** Effects of acute (AE) or chronic (CE) eccentric exercise on serum creatine kinase and muscle thiobarbituric acid reactive substances (TBARS) (A and B). Values are means  $\pm$  SEM. AE0, AE3, AE6: values obtained immediately after acute exercise (0) or after 3 or 6 h of recovery from acute exercise. \*Significantly different from SED (sedentary rats).

TABLE 1. EFFECT OF ACUTE OR CHRONIC ECCENTRIC EXERCISE ON  $H_2O_2$  RELEASE FROM SKELETAL MUSCLE MITOCHONDRIA INCUBATED WITH PYRUVATE/MALATE AND SUCCINATE

A. Pyruvate/Malate	State of respiration	SED	AE	CE
		(pmol $H_2O_2$ /min/mg prot)		
	4	83.1 ± 5.5	63.1 ± 6.4*	64.0 ± 6.5*
	3	62.0 ± 5.7	44.2 ± 8.4	53.0 ± 5.0
	4 + Rot	333 ± 30	269 ± 27	274 ± 29
B. Succinate	State of respiration	SED	AE	CE
		(pmol $H_2O_2$ /min/mg prot)		
	4	698.3 ± 53.0	575.6 ± 41.1	529.0 ± 46.4*
	3	3.0 ± 10.0	n.d.	7.7 ± 6.6
	4 + Rot	35.1 ± 8.6	7.8 ± 15.5*	39.9 ± 12.7

Values are means ( $n = 6-8$ /group) ± SEM. AE, acutely exercised rats; CE, chronically exercised rats. n.d., not detectable; SED, sedentary rats. *State 4*: mitochondrial  $H_2O_2$  rate in absence of ADP; *State 3*, mitochondrial  $H_2O_2$  rate in presence of ADP; *4 + Rot*: mitochondrial  $H_2O_2$  rate after addition of rotenone.

\*Significantly different from SED.

nate). The RCR (state 3 to state 4 ratio), indicating the good quality of our mitochondrial preparations, was significantly reduced in CE compared to SED and AE rats with pyruvate/malate. In mitochondria incubated with succinate, the ADP/O ratio was significantly decreased by acute exercise, but not with chronic exercise.

### Mitochondrial fatty acid composition

Mitochondrial fatty acid composition was slightly modified by acute eccentric exercise as evidenced by a small increase in

monounsaturated fatty acids and in the MUFA/ PUFA ratio (Table 3). In contrast, chronic eccentric exercise was accompanied by a significant increase in the PUFA/SAT ratio due to a reduced content in saturated fatty acids and a slight nonsignificant increase in polyunsaturated fatty acids. In addition, oleic [18:1 (n-9)] and vaccenic acids [18:1 (n-7)] increased with chronic exercise leading to a significantly higher 18:1 to 16:0 ratio. Whereas long chain unsaturated fatty acids (n-3, n-6) were not affected by training, both arachidonic acid (20:4 n-6) and plasmalogens were significantly lower in trained as compared to sedentary or acutely exercised animals.

TABLE 2. EFFECT OF ACUTE OR CHRONIC ECCENTRIC EXERCISE ON RESPIRATORY PARAMETERS FROM SKELETAL MUSCLE MITOCHONDRIA INCUBATED WITH PYRUVATE/MALATE AND SUCCINATE

A. Pyruvate/Malate	Respiratory parameters	SED	AE	CE
		(ng atoms O/min/mg of protein)		
	State 4	19.91 ± 0.69	19.85 ± 0.46	22.71 ± 1.0*
	State 3	162.17 ± 3.6	173.32 ± 9.3	168.76 ± 6.8
	RCR	8.18 ± 0.14	8.71 ± 0.31	7.47 ± 0.18*
	ADP/O	2.29 ± 0.09	2.18 ± 0.08	2.34 ± 0.12
B. Succinate	Respiratory parameters	SED	AE	CE
		(ng atoms O/min/ng of protein)		
	State 4	77.0 ± 3.31	83.1 ± 6.40	79.4 ± 4.27
	State 3	341.7 ± 12.3	352.6 ± 19.4	333.9 ± 11.5
	RCR	4.46 ± 0.12	4.34 ± 0.29	4.24 ± 0.12
	ADP/O	1.34 ± 0.03	1.24 ± 0.03*	1.36 ± 0.04†

Values are means ( $n = 6-8$ /group) ± SEM. AE, acutely exercised rats; CE, chronically exercised rats; SED, sedentary rats. *State 4*, oxygen consumption in absence of ADP. *State 3*, ADP-stimulated respiration. *RCR* was calculated as the ratio between state 3 and 4. *ADP/O* ratio was calculated as the amount of ADP consumed divided by the corresponding oxygen consumed.

\*Significantly different from SED; †significantly different from AE.



TABLE 3. EFFECTS OF ACUTE OR CHRONIC ECCENTRIC EXERCISE ON FATTY ACID COMPOSITION OF SKELETAL MUSCLE MITOCHONDRIA

Fatty acids	Percentage (%)		
	SED	AE	CE
16:0	19.23 ± 0.23	19.37 ± 0.32	18.83 ± 0.26
18:0	16.80 ± 0.20	16.51 ± 0.14	16.67 ± 0.22
Saturated	36.24 ± 0.21	36.12 ± 0.30	35.67 ± 0.14*
18:1	5.80 ± 0.10	6.20 ± 0.22	6.14 ± 0.07*
MUFA	6.67 ± 0.12	7.16 ± 0.32	6.99 ± 0.12
Σn-3	9.52 ± 0.15	9.41 ± 0.21	9.78 ± 0.21
Σn-6	42.96 ± 0.25	42.68 ± 0.40	43.37 ± 0.43
Σn-6/Σn-3	4.52 ± 0.08	4.55 ± 0.12	4.43 ± 0.10
20:4 n-6	15.18 ± 0.21	14.86 ± 0.32	14.39 ± 0.29*
PUFA	52.83 ± 0.26	52.30 ± 0.36	53.49 ± 0.29†
PUFA/Sat.	1.46 ± 0.015	1.45 ± 0.02	1.50 ± 0.01†
MUFA/PUFA	0.13 ± 0.00	0.13 ± 0.00	0.14 ± 0.00
Plasmalogens	4.27 ± 0.05	4.42 ± 0.11	4.02 ± 0.08*,†
18:1/16:0	0.156 ± 0.04	0.168 ± 0.07	0.172 ± 0.05*

Values are means ( $n = 6-8/\text{group}$ ) ± SEM. AE, acutely exercised rats; CE, chronically exercised rats; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SED, sedentary rats.

\*Significantly different from SED; †significantly different from AE.

### Antioxidant enzymes activities

Mn-SOD activity was lower in AE than in CE rats ( $p < 0.05$ ) but not different from SED. On the other hand the activities of CAT, GPx, and Cu,Zn-SOD in skeletal muscle were not affected by acute or chronic eccentric exercise (Fig. 2).

### UCP3 content

The relative amount of UCP3 in skeletal muscle was not affected by acute or chronic eccentric exercise (Fig. 3).

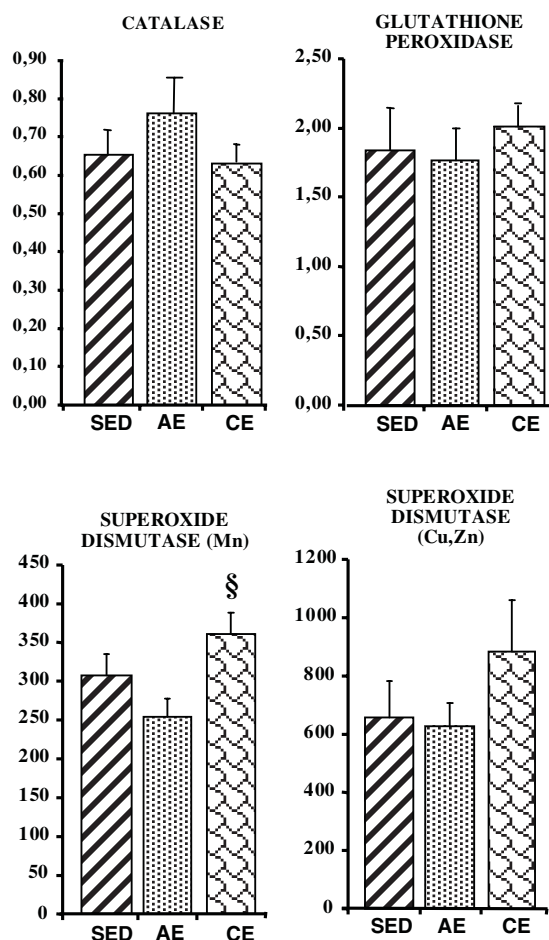
## DISCUSSION

It is generally accepted that strenuous or unaccustomed physical exercise can induce oxidative stress in contracting skeletal muscles, the intensity of which depends on the nature of exercise, the type of muscle investigated, and level of endogenous antioxidants (9, 19, 20, 34, 35, 37, 48, 49). The effects of exercise on oxidative stress have been extensively examined during the last decade, but so far most of the exercise protocols involved a combination of concentric and eccentric contractions (see Ref. 19 for a recent review). By contrast, mitochondrial function and antioxidative defense with eccentric exercise has been only scarcely investigated (50) even though greater muscle damage occurs with eccentric than concentric exercise (36).

### Effect of acute eccentric exercise

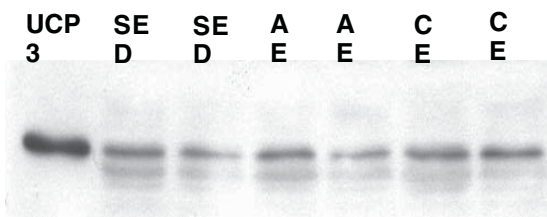
Recently Walsh *et al.* (50) examined muscle mitochondrial oxidative phosphorylation after eccentric exercise and concluded that such exercise, although causing muscle damage (31, 34, 50) did not impair oxidative function in muscle. To our knowledge, however, the impact of acute eccentric exer-

cise on mitochondrial ROS generation has never been evaluated. Two principal sites of ROS generation have been identified in mitochondria, complex I and complex III of the ETC, the relative importance of each complex varying with experimental conditions and between tissues and species (4, 5, 43). In the present study, we found that acutely exercised rats displayed a significantly reduced rate of  $\text{H}_2\text{O}_2$  production when mitochondria were incubated with pyruvate/malate (Table 1), whereas the reduction did not reach the level of significance with succinate. The addition of rotenone to mitochondria respiring on pyruvate/malate (Table 1) induced a significant increase in ROS production that indicates that complex I in skeletal muscle can generate superoxide when it is fully reduced and inhibited by rotenone, but that the rate is very low when complex I is not inhibited by rotenone. In agreement with previous studies (4, 5, 22, 43) we found that the majority of ROS produced by mitochondria are due to the reversed electron transport through complex I as evidenced by the 85–90% reduction by rotenone addition to mitochondria energized with succinate. Interestingly, the magnitude of the rotenone-induced decrease in ROS production was greater in mitochondria from acutely exercised animals than sedentary animals. These data suggest that acute eccentric exercise reduces not only back electron flow through complex I of the electron transport chain but also mitochondrial  $\text{H}_2\text{O}_2$  generation at the level of complex III of the ETC. Recently we have shown decreasing activities of mitochondrial complexes I and IV after a bout of exhaustive exercise, no matter the time of sampling, suggesting that mitochondria are the main target of exercise induced ROS attack (28). Thus, in this case, we can speculate that the reduction in mitochondrial  $\text{H}_2\text{O}_2$  production after a bout of exhausting eccentric exercise could be due to a decrease in complex I activity (the main ROS generator within ETC). Alternatively, we can hypothesized that the reduced rate of mitochondrial  $\text{H}_2\text{O}_2$  production was due to a de-



**FIG. 2.** Effects of acute or chronic eccentric exercise on catalase, glutathione peroxidase, and mitochondrial (Mn) and cytosolic (Cu, Zn) superoxide dismutase activities. Antioxidant activities are expressed as U/100 mg protein. Values are means  $\pm$  SEM ( $n = 8$ /group). AE, acutely exercised rats; CE, chronically exercised rats; SED, sedentary rats. <sup>§</sup>Significantly different from AE.

crease in Mn-SOD localized in the mitochondrial matrix in close proximity to the primary endogenous source of superoxide. However, the decrease in Mn-SOD activity after acute exercise did not reach the level of significance (Fig. 2).



**FIG. 3.** Effects of acute (AE) or chronic eccentric (CE) exercise on skeletal muscle UCP3 content. As a positive control, we used 293 cells transfected with an adenoviral vector driving the expression of the human UCP3 cDNA.

On the other hand, neither the cytosolic superoxide dismutase isoform (Cu,Zn-SOD), nor GPx nor CAT were affected by acute exercise, in agreement with some studies (1). ROS production is greatly dependent on the pH gradient ( $\Delta$ pH) across the mitochondrial inner membrane (22) and any change in the proton electrochemical gradient by addition of an uncoupling agent (27, 41) or by modifying UCP gene expression (7, 12) was found to affect ROS generation. In this respect, acute intermittent eccentric exercise interspersed with short (2–4 min) resting periods was recently reported to be accompanied by an increased UCP3 mRNA 48 h after downhill running exercise (6). While mRNA increase is frequently referred to as increase in gene expression, physiological consequences do not occur until there is an increase in the concentration of the protein encoded by the gene. In the present study, UCP3 mRNA was not determined but we found that UCP3 protein level was not changed by acute eccentric exercise (Fig. 3). Two recent studies (14, 21) provided evidence that acute exercise did not result in UCP3 protein accumulation immediately after exercise cessation but UCP3 increased significantly 18–20 h after the exercise bout (21).

In the present study, we provided evidence that mitochondria are unlikely to be a significant source of ROS during exercise, but several other systems (myeloperoxidase, xanthine oxidase, NADPH oxidase, intracellular  $\text{Ca}^{2+}$ ) or infiltration by phagocytes could potentially increase oxidative stress within skeletal muscles. These extramitochondrial ROS generators were not involved in the early response to eccentric exercise since neither TBARS accumulation (Fig. 1B) nor phagocytes infiltration (51) were observed after acute eccentric contraction and basal intracellular calcium was not different 24h after an acute downhill treadmill running bout (23). The results of the present study indicate that muscles subjected to pliometric contractions during downhill running can buffer changes in intracellular  $\text{Ca}^{2+}$  (23) perhaps until a point where the damage repair mechanisms are activated. Indeed, it takes several hours before to see intracellular accumulation of  $\text{Ca}^{2+}$  (23) and heat shock proteins (19, 46) and increase in serum creatine kinase (Fig. 1A).

Among tissue macromolecules, polyunsaturated fatty acids (PUFA) are the most sensitive to free radical damage, and Perez *et al.* (34) showed that a single bout of eccentric exercise led to increased content formation of carbonyls (an index of protein oxidation) and mitochondrial membrane fluidity after eccentric exercise, suggesting that lipid composition of mitochondria could be affected by such exercise. Indeed, Helge *et al.* (17) have recently shown that muscle membrane phospholipids fatty acid composition was affected 48 h after eccentric contractions. In the present study, we did not find changes in mitochondrial lipid composition immediately after acute exercise (Table 3) in agreement with Meydani *et al.* (26).

### Effects of chronic eccentric exercise

In the present study, we found that  $\text{H}_2\text{O}_2$  generation was reduced by chronic eccentric exercise when mitochondria were incubated with either pyruvate/malate or succinate (Table 1). The effects of training on  $\text{H}_2\text{O}_2$  production by skeletal muscle mitochondria have been only scarcely exam-

ined and it was shown that mitochondrial  $\text{H}_2\text{O}_2$  generation was reduced by 10 weeks of forced swimming (49) but not by 5 months of voluntary wheel running (39). The discrepant results on  $\text{H}_2\text{O}_2$  production reported in chronically exercised rats could be explained by the significant increase in mitochondrial protein content with forced exercise (49) that did not occur with voluntary wheel running (39). This assumption is somewhat supported by the increase in citrate synthase activity following chronic eccentric exercise. Nevertheless, mitochondrial  $\text{H}_2\text{O}_2$  production remained significantly lower in chronically exercised animals when expressed relative to  $\text{O}_2$  consumption ( $6.6 \pm 0.5$  versus  $9.2 \pm 0.5$  pmoles  $\text{H}_2\text{O}_2/\text{ng}$  atom O consumed with succinate as substrate, for CE and SED, respectively). The rate of superoxide production by complex I through reverse electron transport is very sensitive to protonmotive force (22, 27). Thus, it can be hypothesized that increased expression of UCP3 with eccentric training (6) was responsible for the lower  $\text{H}_2\text{O}_2$  production measured in trained animals (Table 1). However, UCP3 protein level was not changed by chronic eccentric exercise (Fig. 3). It has to be kept in mind that there is a link between UCP3 levels and increase in mitochondrial markers (e.g., citrate synthase; 21) and we can speculate that the modest mitochondrial changes with eccentric training reported in present study were not sufficient to enhance UCP3 (Fig. 1). It remains possible that eccentric training might cause intrinsic changes in mitochondrial function (mild uncoupling) and prevent excess formation of ROS in absence of significant alterations in UCP expression (47). This assumption is partly supported by the slight increase in nonphosphorylating mitochondrial  $\text{O}_2$  consumption (state 4) with pyruvate/malate but not with succinate as substrate (Table 2). Our inability to see an increase in  $\text{O}_2$  consumption with succinate might be due to the necessity of incubating skeletal muscle mitochondria with rotenone when energized with succinate.

The decreased rate of  $\text{H}_2\text{O}_2$  generation by skeletal mitochondria with eccentric training could be due to an interaction between superoxide with nitric oxide derived from mitochondrial/endothelial cell nitric oxide synthase (16), since reactive nitrogen species could remove superoxide anion and therefore reduce  $\text{H}_2\text{O}_2$  production. Indeed, exercise training has been reported to increase eNOS in fast-twitch, oxidative-glycolytic skeletal muscle (25).

By using either pyruvate/malate or succinate, both without and with rotenone, we were able to discriminate the site of mitochondrial  $\text{H}_2\text{O}_2$  production affected by eccentric exercise. In the present study, we found that the rate of  $\text{H}_2\text{O}_2$  production by muscle mitochondria respiring on pyruvate/malate was much lower than the rate measured with succinate (Table 1), in agreement with previous data (4, 43). As evidenced by a drastic decrease in mitochondrial  $\text{H}_2\text{O}_2$  production with succinate and rotenone, we found that the major contributor to mitochondrial generation is the reverse electron transport through complex I of the ETC (22, 27, 43). However, the small  $\text{H}_2\text{O}_2$  production through forward electron transport within complexes I and III of the ETC was also reduced by chronic exercise ( $64.0 \pm 6.5$  versus  $83.1 \pm 5.5$  pmoles  $\text{H}_2\text{O}_2/\text{min}/\text{mg}$  protein with pyruvate/malate as substrates for CE and SED, respectively). The reduced  $\text{H}_2\text{O}_2$  production after CE could be due to a lower activity of complex I of the

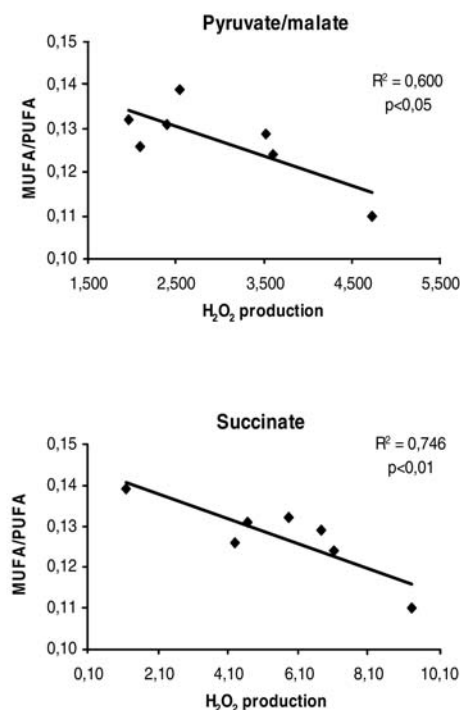
ETC. In the present study, we did not evaluate the activity of complex I of the ETC; but in a previous study, we found that activity of complex I was reduced at the end of exhaustive exercise and remained depressed during a 6 h recovery period (28). Therefore, it is possible that activity of complex I of the ETC in our trained animals was not fully recovered 48 h after their last bout of exercise.

Interestingly, mitochondrial fatty acid composition was affected by chronic eccentric exercise as evidenced by increased PUFA/SAT and 18:1/16:0 ratios but a decreased content of arachidonic acid (20:4 n-6) and of total plasmalogen fatty acids in trained rats. These results are consistent with those reported by Andersson and colleagues (2), showing an increase in 18:1 (n-9) to C16:0 ratio after training that could be attributed to an increase in the  $\Delta 9$ -desaturase activity (17) resulting in a reduced level of SAT fatty acids (Table 3). We can speculate that regular eccentric exercise influences the fatty composition of mitochondrial membrane that hypothetically may contribute to changes in membrane fluidity/permeability (3, 18, 34) and influences ROS production.

However, the basal rate of pyruvate/malate  $\text{H}_2\text{O}_2$  production by heart mitochondria was not modified by increasing the fatty acid double bond content of mitochondrial membranes (18). Direct relationship between changes in fatty acid profile of skeletal muscle mitochondria and decrease in ROS generation in chronically exercised rats cannot be determined from the present results. However, we found a negative correlation between MUFA/PUFA ratio and  $\text{H}_2\text{O}_2$  production by muscle mitochondria in CE rats ( $r^2 = -0.60$ ,  $p < 0.05$  with pyruvate/malate and  $r^2 = -0.74$ ,  $p < 0.01$  with succinate) (Fig. 4) in agreement with Mataix and colleagues (24) who suggested that MUFA/PUFA ratio is a predictive factor of the magnitude of the hydroperoxide production. Even though mitochondrial  $\text{H}_2\text{O}_2$  production was reduced in CE rats, chronic eccentric exercise enhanced oxidative stress as appreciated by TBARS levels (Fig. 1) and an increased membrane susceptibility to ROS attacks during eccentric exercise could be somewhat supported by the lower level of plasmalogens and arachidonic acid (ARA) in mitochondrial membrane from trained rats (Table 3). Reduced contents of these fatty acids (ARA, plasmalogens) are likely correlated because several phospholipids which contain vinyl-ether linkage in their sn-1 position (a characteristic of plasmalogens) possess also ARA in their sn-2 position (Table 3). Previous studies have shown that plasmalogens are efficient antioxidants by preventing propagation of lipid peroxidation (13, 30). It is tempting to speculate that both plasmalogens and ARA contributed to protection of mitochondria against oxidative attacks during adaptive process induced by eccentric training.

The influence of training on various antioxidant enzymes activity has been extensively examined (see, for example, Refs. 19 and 20) and high-intensity exercise training was found to be generally superior to low-intensity for upregulation of antioxidant enzyme activities (35). In the present study, we found that none of the antioxidant enzymes (SOD, GPx, CAT) examined in the present study were affected by CE exercise (Fig. 2). It is likely that our intermittent exercise protocol (15–20 min/day, 5 days/weeks) did not permit substantial upregulation of antioxidant enzymes even though mi-





**FIG. 4. Correlation between  $H_2O_2$  production and MUFA/PUFA ratio in chronically exercised rats.**  $H_2O_2$  production is expressed in pmol  $H_2O_2$ /natom O consumed in the presence of pyruvate/malate and succinate as substrates (State 4).

tochondrial SOD activity was significantly higher after chronic than acute eccentric exercise (Fig. 2).

In conclusion, the present study provides evidence that, contrary to our expectation, mitochondrial ROS production decreases immediately after acute eccentric exercise. This decrease could be linked to changes in complex I activity of the electron transport chain and/or to reduced mitochondrial SOD activity. On the other hand, eccentric training resulted in a lower mitochondrial  $H_2O_2$  production that appeared to be due either to a sustained decreased in complex I activity or to changes in mitochondrial membrane properties that can induce a mild uncoupling barely detectable by oxygraphic measurements.

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## ABBREVIATIONS

ARA, arachidonic acid; CAT, catalase; CK, creatine kinase; CS, citrate synthase; ETC, electron transport chain;

GPx, glutathione peroxidase; HRP, horseradish peroxidase; HVA, homovanillic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; RCR, respiratory control ratio; ROS, reactive oxygen species; SAT, saturated fatty acids; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TBARS, thiobarbituric reactive acid substances; UCP3, uncoupling protein 3.

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