Age, Vol. 21, 109-117,1998

OXIDATIVE STRESS AND MITOCHONDRIAL FUNCTION IN SKELETAL MUSCLE: EFFECTS OF AGING AND EXERCISE TRAINING

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ABSTRACT

The rate of oxidative phosphorylation was investigated *in isolated mitochondria from hindlimb* muscles of young (4.5 mo) and old (26.5 mo) male Fischer 344 rats with or without endurance training. Further, the susceptibility of the muscle mitochondria to exogenous reactive oxygen species was examined. State 3 and 4 respiration, as well as the respiratory control index (RCI), were significantly lower in muscle mitochondria from aged vs. young rats ($P<0.05$), using either the site 1 substrates malate-pyruvate (M-P) and 2-oxoglutarate (2-OG), or the site 2 substrate succinate. In both young and old rats, training increased state 4 respiration with M-P, but had no effect on state 3 respiration, resulting in a reduction of RCI. Training also increased state 4 respiration with 2-OG and decreased RCI in young rats. When muscle mitochondria were exposed to superoxide radicals ($O_2\cdot^-$) and hydrogen peroxide (H_2O_2) generated by xanthine oxidase and hypoxanthine, or H_2O_2 alone *in vitro,* state 3 respiration and RCI in both age groups were severely hampered, but those from the old rats were inhibited to a less extent than the young rats. In contrast, state 4 respiration was impaired by $O_2\cdot^-$ and/or H_2O_2 to a greater extent in the old rats. Muscle mitochondria from trained young rats showed a greater resistance to the $O_2\cdot^-$ and/or H_2O_2-induced state 3 and RCI inhibition than those from untrained young rats. Muscle from aged rats had significantly higher total activities of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), and glutathione reductase than that from young rats, however, training increased SOD and GPX activities in young but not old rats. The results of this study suggest that mitochondrial capacity for oxidative phosphorylation is compromised in aging skeletal muscle. Further, the increased *mitochondrial resistance to reactive oxygen species demonstrated in aged and young trained muscles may be attributed to enhanced antioxidant enzyme activities.*

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KEY WORDS: Aging, antioxidant enzyme, mitochondria, oxidative phosphorylation, reactive oxygen species, skeletal muscle, training

INTRODUCTION

Aging has been hypothesized to be caused by the deleterious and accumulative effects of reactive oxygen species (ROS) occurring throughout the life span (1). Mitochondria are a major site of ROS generation as well as a primary target for ROS in the cell (2,3). It is noticed that mitochondria from aged organisms have a lower respiratory capacity and demonstrate some defects of bioenegetic function (4). Many studies have shown that ROS production is increased in the mitochondria with aging due to the biochemical alterations occurring at the electron transport chain (ETC) and that mitochondrial deterioration may contribute to aging itself (5-7). However, recent findings suggest that biological mechanisms of aging are complex and should not be simplistically explained by an increase in mitochondrial ROS production (8-11). Furthermore, the effects of aging on mitochondria appear species- and tissue-specific (12,13). In the skeletal muscle, one of the less studied organs, the literature is inconsistent. Some authors have reported a decrease of state 3 respiration of muscle mitochondria with age, but this effect dependents upon substrates used in the experiments and the reduction is generally small compared to changes in other organs and tissues (4, 14,15).

Exercise training increases muscle mitochondrial protein involved in oxidative phosphorylation, resulting in a reduced rate of O_2 consumption by the individual respiratory chain at a given work load (16). Assuming that the rate of ROS production is proportional to the rate of mitochondrial O_2 utilization at state 4 or state 3 respiration, the leakage of ROS from each individual respiratory chain is expected to be reduced after training. Furthermore, training enhances both enzymatic and non-enzymatic antioxidant defenses in skeletal muscle, which provide an important protection against ROS-induced oxidative damage in the mitochondria both at young and old age (17-19). However, there is some controversy as to whether training can improve age-related deterioration of muscle mitochondrial function (20,21). Moreover, little attention has been given to the potential roles of ROS and antioxidant defense system in the manifestation of these changes.

Thus, the purpose of the present study was three-fold: (a) to investigate the age-related alterations of mitochondrial respiratory function in skeletal muscle; (b) to examine whether exercise training can attenuate an age-related deterioration of muscle mitochondrial function; and (c) to determine the susceptibility of muscle mitochondria to an imposed *in vitro* oxidative insult. Finally, muscle antioxidant enzyme activities were measured to evaluate their potential role in protecting against ROS-induced damage.

METHODS AND PROCEDURES

Animals

Male Fischer 344 rats (24 mo) were obtained from the National Institute on Aging rat colony (Indianapolis, IN). Male Fischer 344 rats (2 mo) were purchased from the Harlan Sprague-Dawley Co. (Indianapolis, IN). After arrival, rats were individually housed in a temperature controlled (22°C) room at the animal facilities of the University of Illinois College of Medicine, and maintained with a Purina rat chow and tap water *ad libitum*. The 12-12 hr dark-light cycle was reversed (7:00-19:00 dark; 19:00-7:00 light). Body weight of the rats were monitored carefully throughout the experimental period. At the time of kill, the mean ages for the young and old rats were 4.5 and 26.5 mo, representing young adult and aged group, respectively.

Exercise training

Following a 1 wk acclimatization period, each age group was randomly divided into an exercise training and a sedentary control group. Training consisted of a progressive running protocol on a Quinton small animal treadmill and lasted for 10 wk. Rats at both ages began running at mild intensity at 10 m/min, 0% grade for 10 min/day, 5 days/wk. For the young rats, duration and intensity were gradually increased during the first 4 wk, reaching 27 m/min, 15% grade for 60 min/day, 5 days/wk. This training intensity corresponded to ~80% pre-training maximal oxygen consumption (VO_2 max) of rats at this age (22), and it was maintained for the remaining 6 wk. For the old rats, treadmill speed and grade were increased slowly and more cautiously for the first 8 wk. The final workload was 15 m/min with 5% grade and this intensity, corresponding to ~75% pre-training VO_2 max at this age of rats (23), was sustained for the last 2 wk. All training sessions were scheduled during the dark cycle when the rats were active and fed. Three old rats could not finish the 10-wk training period and were killed between 7 and 9 wk of training. Three old sedentary rats were selected to be killed to match the ages of the trained rats. Since the three less-trained old rats did not show different muscle mitochondrial respiration patterns compare to the mean of the group, data from these animals were included in the current report. The young and old sedentary rats were run on the treadmill at low speed (<10 m/min), 2-5 times/wk, in order to simulate the stress associated with the handling and treadmill.

Mitochondrial preparation and respiratory measurements

All rats were killed 48 hours after their last exercise training bout to minimize any acute effects of exercise. To avoid day to day variation, one trained rat and one sedentary rat were killed on the same day. Rats were killed by decapitation. The mixed vastus lateralis (VL) muscles of one of the hindlimbs were quickly removed and placed in an ice-cold suspension medium containing 0.25 M sucrose, 1.0 mM EDTA, 5 mM HEPES, 0.2% fatty acid-free albumin, and 13 units of collagenase (pH= 7.4), for mitochondrial preparation as previously described (24). The deep portions of the VL muscle from the other hindlimb was then removed and quickly frozen in liquid N_2 for antioxidant enzyme activity measurement. The volume of the medium was adjusted so that the weight-to-volume ratio was 1:10. The muscle was immediately minced with scissors and then homogenized with a Potter-Elvehjem glass homogenizer at 0-4°C. The homogenate was filtered through four layers of medical gauze and spun at 700 g for 10 min in a Beckman J2-21M/E centrifuge. The supernatant was saved on ice. The pellet was resuspended in the original suspension medium and centrifuged again at 700 g for 10 min. The pellet from the second spin was discarded. Both supernatants were combined and centrifuged at 12,000 g for 10 min. The resulting mitochondrial pellets were washed and resuspended in 15 ml of a wash solution (0.25 M sucrose, 1 mM EGTA, pH 7.4) and centrifuged again at 12,000 g for 10 min. The final mitochondrial pellets obtained were suspended in 0.25 M sucrose and 2 mM EDTA (pH = 7.4) for respiratory measurements performed immediately after the mitochondria were prepared. Protein concentration was immediately assessed by the Bradford assay.

Mitochondrial respiratory function was measured polarographically with a Biological Oxygen Monitor System (model YSI 5300, Yellow Spring Instruments, Columbus, OH) connected to a Micro Chamber (volume 0.67 ml, Instech, Inc., Plymouth Meeting, PA) at 30°C, according to (25) with slight modifications (24). The mitochondrial respiration medium consisted of 130 mM KCl, 5 mM $MgCl_2$, 20 mM NaH_2PO_4, 20 mM Tris HCl, and 30 mM glucose (pH = 7.4). After the addition of the air-equilibrated medium and the establishment of a stable base line for 2 min, ~0.4 mg of mitochondrial protein was added into the respiration chamber, followed by the addition of one of the substrates: 2.0 mM malate /2.0 mM pyruvate (M-P), 4.0 mM 2-oxoglutarate (2-OG), or 4.0 mM succinate (with 2.4 µmol rotenone). The state 3 and 4 respirations and the respiratory control index (RCI) were defined according to Chance and Williams (26). State 3 respiration was initiated with the addition of 750 nmol ADP. The amount of ADP was selected based on a preliminary dose-response analysis using malate-pyruvate as substrate, wherein a maximal state 3 respiration and RCI were observed at ~750 nmol ADP in muscle mitochondria from young rats. For old rats state 3 respiration showed a decline at ADP concentration greater than 500 nmol. We chose to use 750 nmol ADP

that was expected to reveal a greater age difference in muscle mitochondria. All measurements were made in duplicates or triplicates immediately after mitochondria were prepared, and measurements were completed within 3 h after the excision of the muscles, during which the mitochondria were kept on ice. There was no significant alteration of the mitochondrial RCI between the first and last measurements within each rat.

To study the susceptibility of muscle mitochondria to ROS *in vitro*, the above experiments were repeated in the presence of superoxide radicals ($O_2 \cdot^-$) plus hydrogen peroxide (H_2O_2), generated by xanthine oxidase (XO, EC 1.1.3.22) with hypoxanthine (Hx) as substrate, or H_2O_2 alone. The concentrations of ROS used were determined by dose-response analyses performed with mitochondria isolated from the same muscle group of young rats. XO (0.13 units/ml) and various concentrations of Hx were mixed in an Eppendorf micro test tube under aerobic conditions at room temperature, lightly vortexed, and transferred into the mitochondrial respiratory chamber immediately before the addition of mitochondria. The inhibitory effect of $O_2 \cdot^-$ and H_2O_2 on state 3 respiration was greater with 2-OG than with M-P and succinate (Fig. 1A). A concentration of 22 μM Hx was chosen because higher Hx concentrations showed little additional inhibitory effect with all three substrates used. H_2O_2 at various concentration was pre-diluted from a 30% stock (Sigma) and added into the chamber immediately before the experiment. H_2O_2 inhibited state 3 respiration with both M-P and 2-OG in a dose-dependent manner, whereas it showed a moderate stimulatory effect with succinate (Fig. 1B). The dose chosen for H_2O_2 was 78 μM which caused approximately 60% inhibition of state 3 respiration with M-P or 2-OG. All ROS inhibition experiments were carried out in duplicates or triplicates for each rat, and all measurements were completed within 3 h after the excision of the muscles.

Antioxidant Enzyme Activities

Activities of total SOD (EC 1.15.1.1), GPX (EC 1.11.1.9), GR (EC 1.6.4.2.), and CAT (EC 1.11.1.6) were determined in the muscle homogenate derived from the deep portions of the VL muscle as previously described (27). Muscle homogenate was freeze-thawed three times to release total enzyme activity. Briefly, SOD activity was assayed according to (28) at 30°C. The amount of enzyme that causes 50% inhibition of epinephrine autooxidation is defined as 1 unit. GPX was assayed at 37°C according to (29) with H2O2 used as the substrate. GR activity was measured at 30°C according to (30). CAT was assayed at 20°C by the method of (31), with the following modifications: 1 volume of muscle homogenate (750-g supernatant, 1:10 wt/vol) was added to 0.01 volume of 95% ethanol to decompose complex II. After a 30 min incubation period at 0°C, 0.01% digitonin and 1% Triton X-100 were added to the muscle. The supernatant was diluted with phosphate buffer (0.1 M, pH 7.4) before assay.

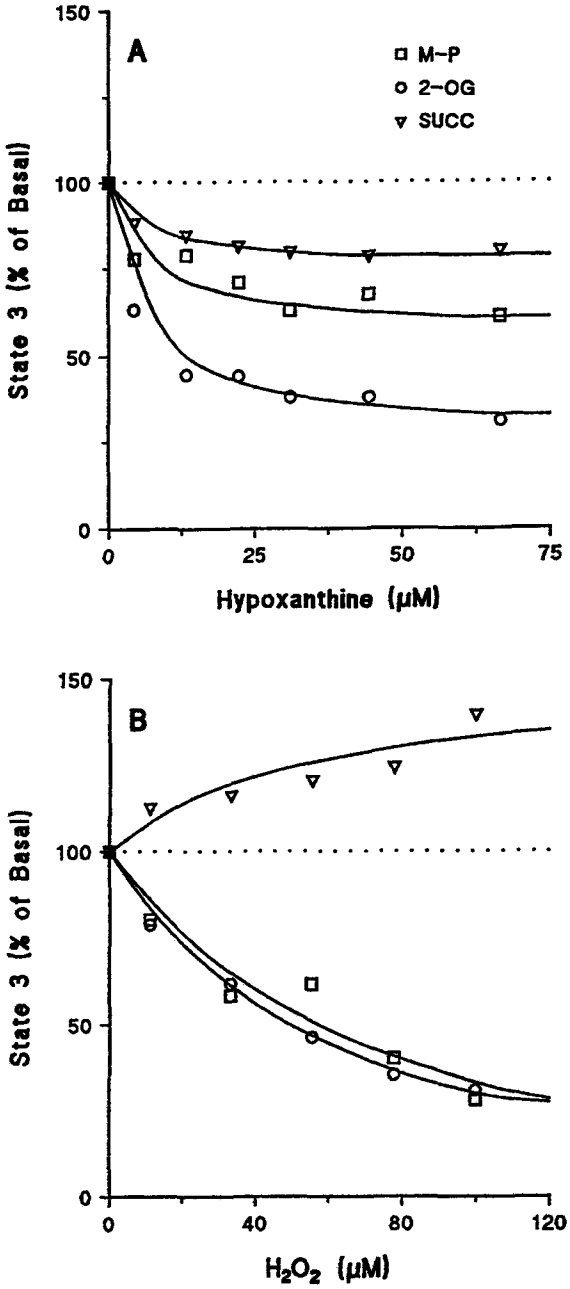

Figure 1: Effect of various concentrations of hypoxanthine in the presence of 0.13 unit/ml xanthine oxidase (A) and H_2O_2 (B) on mitochondrial state 3 respiration, represented as a percentage of basal respiration. Each data point represents the mean of duplicate measures from 2-3 young rats using malate (2mM)-pyruvate (2mM), 2-oxoglutarate (4mM), or succinate (4mM) as substrates.

Statistics

Data were analyzed with a three-way analysis of variance method. When a significant F value was found for a main treatment effect (age vs. training vs. ROS), Fisher's Least Significant Difference (LSD) multiple comparison was performed to test the significant levels of differences between means. $P < 0.05$ was considered statistically significant.

RESULTS

Old rats had significantly lower body weight (P<0.05) than the young rats at the time of killing (Table 1). Exercise training had no effect on the body weight of the rats. Heart weights were also significantly lighter in the old vs. young rats (P<0.05), and showed no effect with training regardless of age. The heart-to-body weight ratio was not altered by either aging or training. Protein concentration in the VL muscle was not different among the various treatment groups (Table 1).

Table 1. Body Weight, Heart Weight, Heart/Body Weight Ratio, and Protein Concentration

	Young Control (n=6)	*Trained (n=4)*	*Old Control (n=9)*	*Trained (n=11)*
Body Wt. (g)	506±18	512±13	387±16*	393±9*
Heart Wt. (g)	1.8±0.09	1.7±0.09	1.4±0.04*	1.4±0.04*
Heart/Body Wt. (g/kg)	3.6±0.1	3.3±0.2	3.6±0.2	3.6±0.1
Protein (mg/g wet wt)	134±5	121±6	129±5	119±3

Values are means ± SEM with the numbers of rats per treatment group indicated in parenthesis. * p<0.05, young vs. old. Protein concentration was measured in the homogenate of vastus lateralis muscle.

State 4 respiration of muscle mitochondria was not affected by age with M-P, but showed a 32% reduction with 2-OG (P<0.05) and a 60% reduction with succinate (P<0.05) (Table 2). State 3 respiration was dramatically decreased with both site 1 and site 2 substrates in the old rats compared to the young rats. The reductions in the control rats were 76, 80, and 76% using M-P, 2-OG, and succinate, respectively (P<0.05). RCI with all three substrates also showed significant reductions with old age, resulting mainly from much larger decreases in state 3 than state 4 respiration rates (Table 2).

Table 2. Mitochondrial Respiration Rates and Respiratory Control Index in Rats

	Young Control (n=6)	*Trained (n=4)*	*Old Control (n=9)*	*Trained (n=11)*
Malate/Pyruvate				
State 4	17.3 ± 1.3	28.1 ± 3.9+	14.9 ± 1.9	22.7 ± 3.1+
State 3	131 ± 14	125 ± 18	30.9 ± 6.2*	27.6 ± 3.6*
RCI	7.7 ± 0.8	4.5 ± 0.5+	2.0 ± 0.2*	1.3 ± 0.1*+
2-Oxoglutarate				
State 4	16.2 ± 1.5	23.1 ± 5.3+	11.0 ± 1.9*	13.8 ± 2.1*
State 3	100 ± 9.8	100 ± 17	19.7 ± 3.3*	17.3 ± 2.3*
RCI	6.4 ± 0.6	4.6 ± 0.6+	1.8 ± 0.1*	1.3 ± 0.1*
Succinate				
State 4	38.5 ± 1.6	34.3 ± 6.0	15.1 ± 2.4*	18.2 ± 2.3*
State 3	68.6 ± 4.5	46.5 ± 7.6	16.8 ± 2.8*	21.1 ± 2.4*
RCI	1.8 ± 0.07	1.4 ± 0.05+	1.1 ± 0.05*	1.2 ± 0.03*

Values are means ± SEM with the number of rats per treatment group indicated in parenthesis. Units for state 4 and 3 respiration: ng AO·min^{-1}·mg^{-1} protein. * P<0.05, young vs. old. + P<0.05, trained vs. control.

Training significantly increased state 4 respiration in both young and old rats when M-P was used as substrate (P<0.05, Table 2). State 4 respiration with 2-OG was also increased with training (P<0.05, main effect), but post-hoc test revealed significant training effect only in the young rats (P<0.05). There was no difference in state 4 respiration with succinate between the trained and control rats. Training did not alter state 3 respiration with either M-P, 2-OG, or succinate regardless of animal's age. Mitochondrial RCI with M-P was decreased with training in both young and old rats (P<0.05), mainly as a result of the increased state 4 respiration. Training also decreased RCI with 2-OG (P<0.05, main effect). With succinate as substrate, a significant age and training interaction was detected, with a lower RCI shown in the trained young rats vs. untrained young rats (P<0.05).

After being treated with $O_2^{\cdot-}$ and/or H_2O_2, mitochondrial respiratory properties were markedly affected, shown as a percent of untreated basal values (Fig. 2 & 3). State 4 respiration with M-P was significantly elevated by 70% (P<0.05) with XO+Hx treatment in young sedentary rats, whereas it was decreased by 27% (P<0.05) in old sedentary rats (Fig. 2a). Training abolished the stimulation of state 4 respiration with XO+Hx in the young rats (P<0.05). State 4 respiration with 2-OG and succinate showed a modest inhibition with XO+Hx in all groups and there was no significant difference between the various groups. With H_2O_2 treatment (Fig. 2b), state 4 respiration with M-P was not affected in young rats, but was decreased by ~40% (P<0.05) in old rats. Trained young rats had a lower state 4 respiration compared to control rats (P<0.05). State 4 respiration with 2-OG showed a significant reduction with H_2O_2 in both young and old rats, but old rats had lower rates than young rats (P<0.05). H_2O_2 had no significant effect on state 4 respiration with succinate regardless of age or training status.

XO+Hx treatment significantly decreased state 3 respiration rates with all substrates (P<0.01, Fig. 3a). The extent of inhibition on state 3 respiration with M-P was similar between old and young rats, however, old rats demonstrated less inhibitory effect than young rats when 2-OG and succinate were used as substrates (P<0.05). Furthermore, trained young rats showed significantly smaller $O_2^{\cdot-}$ and H_2O_2-induced inhibition of state 3 respiration than control young rats regardless of the substrate used (P<0.05). This training effect, however, was not observed in the old rats. Mitochondria pre-exposed to H_2O_2 showed a dramatic reduction of state 3 respiration both with M-P and 2-OG, but not with succinate (Fig. 3b). Old rats were less susceptible to H_2O_2-induced state 3 inhibition with M-P compared to young rats (P<0.05). Trained young rats maintained a higher level of state 3 respiration with 2-OG than their sedentary counterparts (P<0.05).

RCI with both M-P and 2-OG showed a significant reduction with XO+Hx treatment in the young rats (P<0.05), whereas the changes in RCI were not significant in the old rats (Fig. 4a). Interestingly, RCI with succinate was not significantly affected by XO+Hx. Training enhanced mitochondrial resistance to $O_2^{\cdot-}$ and H_2O_2 as RCI was maintained at higher levels for all three substrates in trained young rats, and for M-P and 2-OG in trained old rats, vs. their untrained counterparts (P<0.05). These findings with respect to RCI were essentially held true in mitochondria respiring on M-P

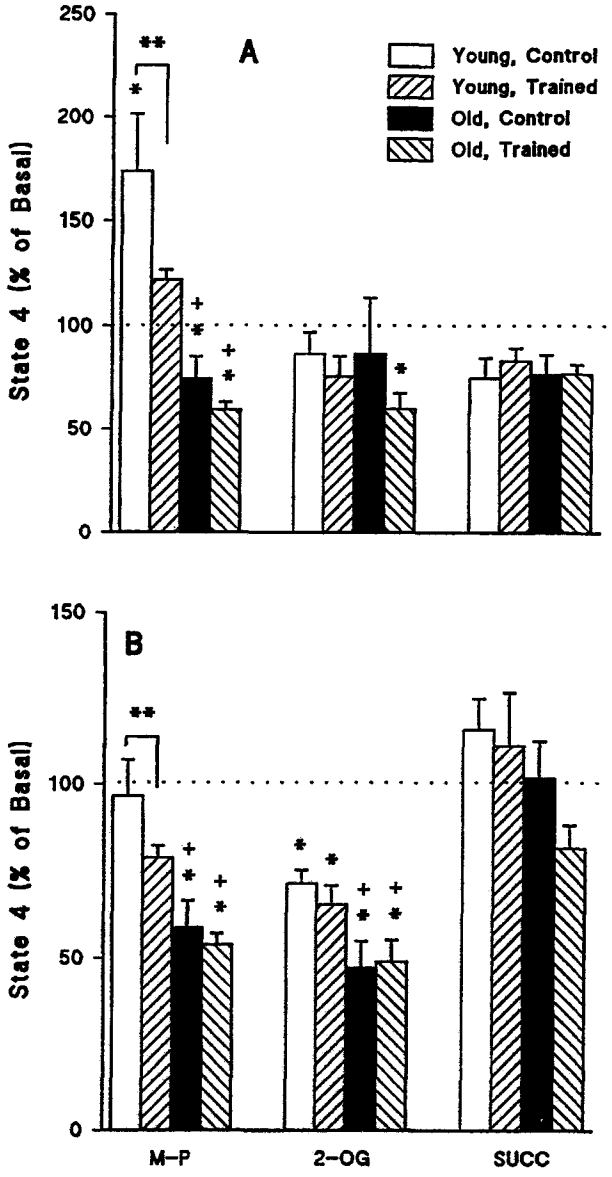

Figure 2: State 4 respiration in muscle mitochondria pre-exposed to HX+XO (A) and H_2O_2 (B). Data are represented as a percentage of basal state 4 respiration, with malate-pyruvate (M-P), 2-oxoglutarate (2-OG), and succinate (SUCC) as substrates. Values are mean ± SEM of duplicate or triplicate measurements for numbers of animals specified in Table 1. * p<0.05, treated vs. basal respiration. + p<0.05, young vs. old rats. ** p<0.05, trained vs.control.

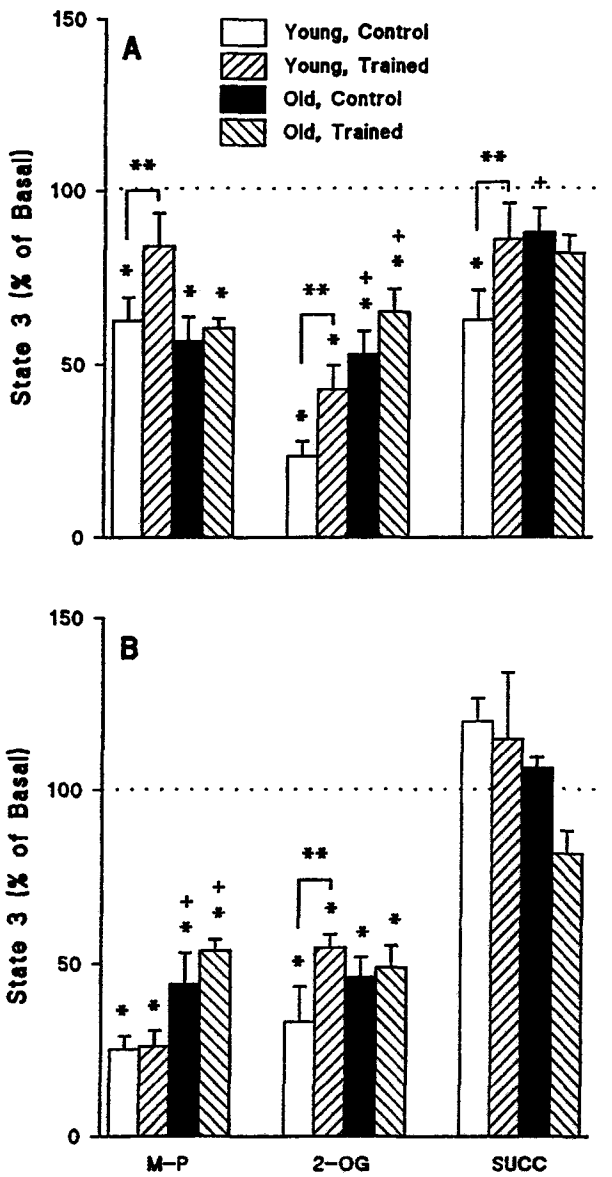

Figure 3: State 3 respiration in muscle mitochondria pre-exposed to HX+XO (A) and H_2O_2 (B). Data are represented as a percentage of basal state 3 respiration, with malate-pyruvate (M-P), 2-oxoglutarate (2-OG), and succinate (SUCC) as substrates. Values are mean ± SEM of duplicate or triplicate measurements for numbers of animals specified in Table 1. * p<0.05, treated vs. basal respiration. + p<0.05, young vs. old rats. ** p<0.05, trained vs.control.

and 2-OG and pre-treated with H_2O_2 (Fig. 4b). No significant H_2O_2 effect on RCI was observed in mitochondria respiring on succinate regardless of age and training status.

Activities of SOD, GPX, GR, and CAT were significantly higher in the old rats compared to young rats (Fig. 5). Training resulted in a 28% increase in total SOD activity (P<0.05) in the young rats, whereas old rats showed no change. Training also increased GPX activity by 50% (P<0.05) in the young rats. Again, this training effect was not seen in the old rats. GR and CAT activity did not alter with training regardless of age.

DISCUSSION

Aging is known to cause significant deterioration of the bioenergetic capacity of skeletal muscle, which is largely controlled by the mitochondrial oxidative phosphorylation (4, 21). Previous studies have reported a wide-range of age-associated alterations of mitochondrial respiration from no change to 20-60% reduction, depending on the substrate used, the muscle from which mitochondria are isolated, and the species (4). In the present study, we found that state 4 respiration rate was decreased only slightly with site 1 substrates and by 60% with site 2 substrate, while state 3 respiration was

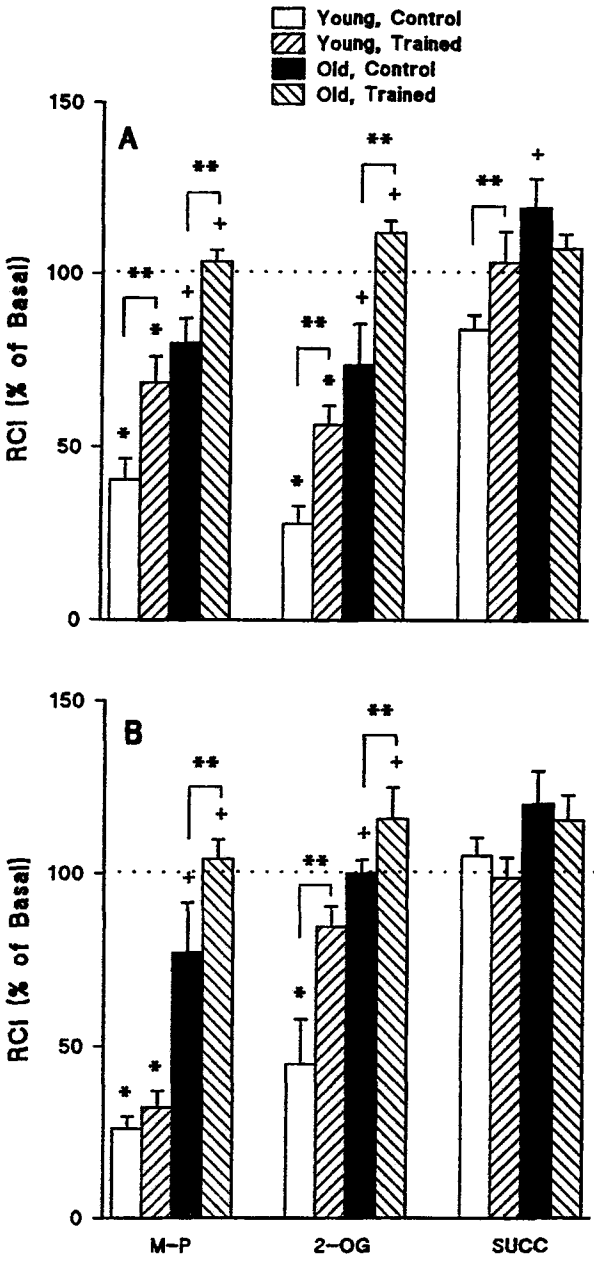

Figure 4: Respiratory control index (RCI) in muscle mitochondria pre-exposed to HX+XO (A) and H_2O_2 (B). Data are represented as a percentage of basal RCI with malate-pyruvate (M-P), 2-oxoglutarate (2-OG), and succinate (SUCC) as substrates. Values are mean ± SEM of duplicate or triplicate measurements for numbers of animals specified in Table 1. * p<0.05, treated vs. basal respiration. + p<0.05, young vs. old rats. ** p<0.05, trained vs.control.

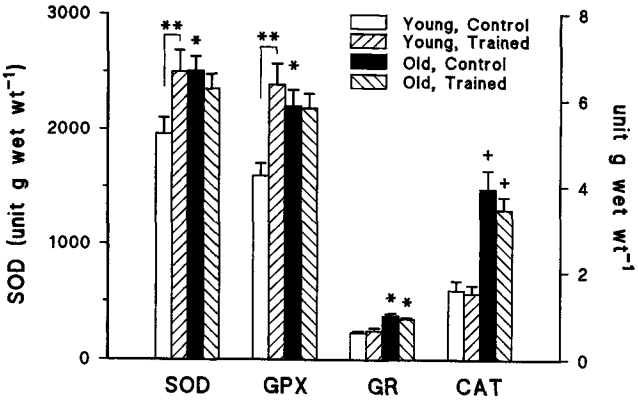

Figure 5: Activity of antioxidant enzymes in vastus lateralis muscle. Values are means SEM with the number of rats per treatment group indicated in Table 1. SOD, superoxide dismutase (units/g wet wt); GPX, glutathione peroxidase; GR, glutathione reductase (mmol/min/g wet wt); CAT, catalase (K ˙ 10^{-3}/g wet wt). * p<0.05; + p<0.01, old vs. young rats. ** p<0.05, trained vs. control rats.

75-80% lower with either site 1 or site 2 substrate, in the senescent muscle compare to young ones. Further, we reported that aging dramatically reduced RCI by ~60-70% despite concomitant decreases in state 4 respiration. These large reductions of mitochondrial respiratory properties, especially with succinate as substrate, indicate that both ETC and ATP synthase complex were deteriorated in senescent skeletal muscle. It is well known that mitochondrial oxygen consumption in the presence of succinate and ADP (state 3) is a measure of both ETC and ATP synthase function, whereas state 4 respiration indicates the integrity of ETC *per se* (26,32). The large reduction of state 3 respiration rates in the old rats might reflect decreased activities of mitochondrial oxidative enzymes and ETC proteins in skeletal muscle (4, 21). It could also be in part due to the high concentration of ADP (750 nmol) used in the current study. Darnold et al. (33) emphasized the importance of ADP concentration during state 3 respiration in revealing age differences of mitochondrial function. A significant reduction of state 3 respiration in liver mitochondria from 27 vs. 6.5 mo old rats was reported when 650 nmol ADP was used, whereas with low doses of ADP no age difference was found. A decrease in mitochondrial adenine nucleotide translocase activity was suggested to explain this large age effect (34,35). Thus, aged mitochondria appear to lack the ability to increase oxidative phosphorylation in the presence of a high concentration of ADP resulting from rapid ATP hydrolysis to meet the energy demand. This could be an important reason for observed age differences in skeletal muscle performance during heavy physical exertion.

Muscle mitochondria from aged rats showed lower rates of state 4 respiration than those from young rats after being exposed to equal doses of $O_2^{\cdot-}$ and/or H_2O_2. Because state 4 respiration is not limited by substrate levels, this finding agrees with several previous authors who have shown that ETC and high energy phosphate translocase are the most vulnerable mitochondrial components to ROS at old age (3,7,34). These enzymes are located on the lipid-rich mitochondrial membrane which is vulnerable to peroxidative damage such as fluidity loss and ion channel blockage occurring at old age (7,36). Membrane protection by α-tocopherol and ubiquinol may also be compromised with old age (37). In contrast to state 4, state 3 respiration with 2-OG and succinate was found to be less susceptible to XO+Hx

and state 3 respiration with M-P was less susceptible to H_2O_2 in the aged mitochondria compared to the young ones. Similarly, RCI with the various substrates in the old rats also demonstrated more resistance to $O_2^{\cdot-}$ and/or H_2O_2 (Fig. 4, except RCI with succinate treated with H_2O_2). The reason for the decreased susceptibility of state 3 respiration to ROS in aged muscle mitochondria is largely unknown. One possible explanation is that metabolic enzymes providing reducing equivalents, such as pyruvate and malate dehydrogenases, 2-oxoglutarate dehydrogenase and succinate dehydrogenase, are better protected from $O_2^{\cdot-}$ and/or H_2O_2 at old age, because they are located in the mitochondrial matrix. This cell compartment enjoys abundant protection from antioxidant enzymes and reduced glutathione (GSH), both of which have shown age-related adaptations (19,27,38,39). An alternative and complementary explanation would be that aging itself already severely impaired mitochondrial function, so that further exposure to ROS has less of an effect when expressed as a percentage of the basal levels. More specifically, aging could cause a decrease of critical thiol groups at the active site of enzymes, resulting in a decreased sensitivity of these active sites to oxidants in a specific pattern. Despite the uncertainty of the mechanisms involved, our data clearly indicate that although mitochondria from senescent muscle are functionally deteriorated, they are more resistant to further oxidative damage by ROS due to age-related adaptations yet to be fully understood.

The current study reveals that training is a double-edged sword regarding mitochondrial function. Training decreased mitochondrial RCI with site 1 substrates M-P and 2-OG caused primarily by an increase in state 4 respiration, while state 3 respiration was intact (Table 2). State 4 respiration with M-P also showed a greater susceptibility to H_2O_2 in the trained young rats compared to controls (Fig. 2b). A plausible reason would be that intensive training had caused some muscle mitochondrial membrane damage, resulting in a proton leakage, since state 4 respiration is known to be increased in damaged mitochondria (7, 40). Consistent with this explanation were the findings that an acute bout of exhaustive exercise increased state 4 respiration in the mitochondria of liver, skeletal muscle (41) and heart (24), accompanied by loss of endoplasmic and sarcoplasmic reticulum latencies and increased lipid peroxidation. Thus, strenuous physical training may damage muscle mitochondria. On the other hand, training attenuated ROS-induced inhibition of state 3 respiration in the young rats, and of RCI at both ages. Since training *per se* does not compromise state 3 respiration rate, we contend that the delivery of reducing power to ETC, controlled by the various metabolic enzymes, are better protected in the trained state. This may be accomplished in part by training induction of SOD and GPX activities in the young rats (Fig. 5). A major training adaptation of antioxidant enzymes occurs in the muscle mitochondria (27,42), and GSH content in the muscle used for mitochondrial preparation has also shown an increase after endurance training (43). Thus, a moderate damage of mitochondria may stimulate training adaptation which allows the muscle to withstand a higher level of oxidative stress.

Mitochondria respiring on M-P and 2-OG showed more prominent inhibition of state 3 respiration and RCI with XO+Hx treatment than those respiring on succinate. Furthermore, succinate-supported mitochondrial respiration was almost completely unaffected by H_2O_2. Since both M-P and 2-OG produce NADH (some $FADH_2$ are also produced), whereas $FADH_2$ is the primary source of reducing power with succinate, our data suggest that ROS have a more detrimental effect on complex 1 than complex 2. These substrate- and ROS-specific mitochondrial responses appear perplexing, however, the various components in the mitochondrial ETC have demonstrated differential susceptibility to ROS. Zhang et al. (44) showed that $O_2^{\cdot-}$ inactivated NADH dehydrogenase and NADH oxidase more efficiently (i.e., requires less dose to reach a greater inhibition) than succinate dyhydrogenase and succinate oxidase. Further, H_2O_2 was found to be a poor inhibitor of most enzyme systems tested. Recently, Herrero and Barja (45) showed that H_2O_2 production was higher using M-P than using succinate as respiratory substrate in rat and pigeon heart mitochondria. They conclude that complex 1 is the primary site for mitochondrial free radical generation. Therefore, it is not surprising that training elicited little effect on muscle mitochondrial respiratory properties with succinate in both young and aged rats.

In summary, the present study demonstrates that both aging and physical training can elicit profound effects on mitochondrial oxidative phosphorylation at the basal state and under imposed oxidative stress. The specific responses are dependent upon respiratory substrates and ROS used.

ACKNOWLEDGEMENTS

The present research was supported in part by a grant from the American Federation for Aging Research, and a grant-in-aid by the American Heart Association. Aged rats were generously supplied through an NIA/NIH pilot study research support grant. C. Leeuwenburgh was a recipient of the Student Stipend Award of the AHA (Illinois Affiliate).

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