AGING AND ACUTE EXERCISE ENHANCE FREE RADICAL GENERATION IN RAT SKELETAL MUSCLE

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Bejma, J., and L. L. Ji. Aging and acute exercise enhance free radical generation in rat skeletal muscle. J. Appl. Physiol. 87(1): 465–470, 1999.—Reactive oxygen species (ROS) are implicated in the mechanism of biological aging and exercise-induced oxidative damage. The present study examined the effect of an acute bout of exercise on intracellular ROS production, lipid and protein peroxidation, and GSH status in the skeletal muscle of young adult (8 mo, n = 24) and old (24 mo, n = 24) female Fischer 344 rats. Young rats ran on a treadmill at 25 m/min and 5% grade until exhaustion (55.4 ± 2.7 min), whereas old rats ran at 15 m/min and 5% grade until exhaustion (58.0 ± 2.7 min). Rate of dichlorofluorescin (DCFH) oxidation, an indication of ROS and other intracellular oxidants production in the homogenate of deep vastus lateralis, was 77% (P < 0.01) higher in rested old vs. young rats. Exercise increased DCFH oxidation by 38% (P < 0.09) and 50% (P < 0.01) in the young and old rats, respectively. DCFH oxidation in isolated deep vastus lateralis mitochondria with site 1 substrates was elevated by 57% (P < 0.01) in old vs. young rats but was unaltered with exercise. Significantly higher DCFH oxidation rate was also found in aged-muscle mitochondria (P < 0.01), but not in homogenates, when ADP, NADPH, and Fe3+ were included in the assay medium without substrates. Lipid peroxidation in muscle measured by malondialdehyde content showed no age effect, but was increased by 20% (P < 0.05) with exercise in both young and old rats. Muscle protein carbonyl formation was unaffected by either age or exercise. Mitochondrial GSH/GSSG ratio was significantly higher in aged vs. young rats (P < 0.05), whereas exercise increased GSSG content and decreased GSH/GSSG in both age groups (P < 0.05). These data provided direct evidence that oxidant production in skeletal muscle is increased in old age and during prolonged exercise, with both mitochondrial respiratory chain and NADPH oxidase as potential sources. The alterations of muscle lipid peroxidation and mitochondrial GSH status were consistent with these conclusions.

aging; exercise; free radical; muscle; oxidative damage

AGING HAS BEEN HYPOTHESIZED to be caused by the deleterious and cumulative effects of reactive oxygen species (ROS) occurring throughout the life span (9). In skeletal muscle, increased ROS production has been hypothesized to cause various pathogenic conditions associated with old age (23). Mitochondria, as the major site of ROS generation as well as a primary target of ROS in the cell, are particularly important in age-related deterioration of muscle function (5, 30). Numerous studies have shown that aged muscles are under constant and increasing insult by ROS, as indicated by enhanced lipid peroxidation, protein oxidation, and alteration of GSH status and antioxidant enzyme activities (8, 12, 19, 32). However, most of the evidence so far has been indirect, and there is sparse research directly measuring muscle ROS production in aging skeletal muscle.

Strenuous physical exercise increases ROS production and causes various forms of oxidative damage in skeletal muscle. However, because of the lack of reliable and convenient methods to measure ROS, only scarce data exist regarding ROS generation in skeletal muscle during exercise. Using electron spin resonance (ESR) spectroscopy, Jackson et al. (11) reported that free radical signals in intact muscle fibers were increased during stimulated contraction. Although ESR provides great sensitivity, quantitation of ROS production is relatively poor. With greater precision, Reid et al. (29) showed that the oxidation rate of dichlorofluorescin (DCFH), a synthetic probe for intracellular oxidants, was dramatically increased in contracting diaphragm muscle compared with resting muscle, wherein superoxide radicals were suggested to be the primary oxidant causing DCFH oxidation. Using in situ feline skeletal muscle as a model, O’Neill et al. (26) reported increased hydroxyl radical production in contracting triceps muscle in proportion to maximal tension development. Reid (28) recently proposed that, in addition to ROS, nitric oxide (NO) may play an important role in skeletal muscle and that muscles involving repetitive contraction may produce sufficient amount of NO to influence oxidant-antioxidant balance. However, most of these studies were not carried out in vivo, and therefore the species, sources, and quantity of oxidants produced in skeletal muscle during whole body exercise are still poorly understood. Furthermore, no data are currently available regarding exercise-induced oxidant generation in senescent muscle. Thus the present study was undertaken to test the following hypotheses: 1) oxidant production rate is higher in aged than in young skeletal muscle; 2) an acute bout of prolonged exercise at similar intensity increases muscle oxidant produc-
tion to a greater extent in aged than in young muscle; 3) mitochondrial electron transport chain and NADPH oxidase are two major sources of muscle oxidant generation; and 4) enhanced oxidant production with age and exercise can cause increased muscle oxidative damage such as lipid peroxidation, protein oxidation, and alteration of GSH status.

METHODS AND PROCEDURES

Animal care. Female Fischer 344 rats at 8 mo of age (young adult; Y; n = 24) and 25 mo (old; O; n = 24) were obtained from the National Institute of Health rat colony. On arrival, animals were caged individually in a temperature-controlled room (22°C) with a 12:12-h reverse light-dark cycle (0700–1900 dark; 1900–0700 light). Rats were fed a Purina chow diet and tap water ad libitum. Animal-use protocol was approved by the University of Wisconsin-Madison Research Animal and Resource Center Review Committee before the study began. After a 1-wk acclimation period, both Y and O rats were randomly assigned to one of the following two subgroups: acutely exercised (E; n = 12) or rested controls (R; n = 12).

Exercise protocol. All rats (both E and R) were accustomed to treadmill running. Exercise was performed on a Quinton rodent treadmill, beginning at 5 m/min at 0% grade for 10 min/day, 3 days/wk. Over a 3-wk period, the intensity was gradually increased to 25 m/min and 5% grade for the Y rats and 15 m/min and 5% grade for the O rats while a 10-min duration was maintained. On the day of experiment, YE and OE rats performed an acute bout of treadmill running to exhaustion at the above mentioned speeds and grades, respectively. These intensities represent ~75% of maximal oxygen uptake for both the young and old rats, according to Brooks and White (2) and Lawler et al. (18). Exhaustion was defined as the inability of a rat to right itself when being laid on its side. No rat ceased exercise because of foot or any other type of injury.

Tissue preparation. Immediately after the exhaustive exercise bout, rats were decapitated and exanguination followed. The liver, heart, and deep vastus lateralis (DVL) muscles from both hind legs were quickly removed. DVL from one leg was immediately placed in liquid nitrogen and stored at ~80°C. The other DVL was placed in a mitochondrial isolation buffer containing 0.25 M sucrose, 1.0 mM EDTA, 5.0 mM HEPES, 0.2% fatty acid-free albumin, and 13 units/ml of collagenase (ph 7.4) at a weight-to-volume ratio of 1:10. Tissues were minced thoroughly and homogenized with a motor-driven Potter-Elveljem glass homogenizer at 0–4°C at low speed. DVL homogenate was filtered through four layers of medical gauze to remove connective tissue debris. A portion of the homogenate was used to determine oxidant production (see below). The remaining homogenate was used to isolate mitochondria according to the methods described in Ji et al. (14). The final pellets containing mitochondria were suspended in 0.25 M sucrose (ph 7.4). Preparation of muscle mitochondria took ~50 min after the animals’ death.

Determination of oxidant production. Generation of muscle ROS and other oxidants was evaluated in both the homogenate and isolated mitochondria under either basal or induced condition by using DCFH as a probe, according to LeBel and Bondy (16) as modified by Kim et al. (15). The DCFH assay is sensitive to both ROS and NO (including its derivatives) in the defined assay conditions because of the existence of NO synthase in muscle homogenate and mitochondria (26). Once in the homogenate or when defusing across mitochondrial membrane, DCFH-diacetate is cleaved by an esterase, and the resultant DCFH can be oxidized to the highly fluorescent dichlorofluorescein (DCF). In the basal state, the assay buffer contained (in mM) 130 KCl, 5 MgCl₂, 20 NaH₂PO₄, 20 Tris-HCl, and 30 glucose (ph 7.4), plus 2 malate and 2 pyruvate as site 1 substrates. The compositions of this solution were identical to those of a mitochondrial respiratory medium at state 4 (4). In the induced state, 0.1 mM FeCl₃, 1.7 mM ADP, and 0.1 mM NADPH were included in the basal buffer but without the substrates. Both basal and induced assay media contained 5.0 µM of DCFH-diacetate dissolved in 1.25 mM methanol. For muscle homogenate, 500 µl of DVL homogenate (~5 mg protein) were included in both assay media and incubated at 37°C for 15 min. To verify that DCFH concentration was saturating allowing for maximal rate of oxidation for at least 60 min, 1 µmol of H₂O₂ was added into the mixture at the end of assay (final concentration 330 µM). The DCFH oxidation rate was found to increase several fold immediately, indicating that sufficient DCFH was still available. For mitochondrial assay, isolated mitochondria were incubated in the respective buffers at 37°C for 15 min to allow DCFH-diacetate to cross mitochondrial membrane. The solution was then centrifuged at 12,000 g for 8 min, and the supernatant containing excess DCFH-diacetate not crossing the mitochondrial membrane was discarded. The mitochondrial pellets were resuspended, and 50 µl of the suspension (~2 mg protein) were used for assay. DCF formation was followed at the excitation wavelength of 488 nm and emission wavelength of 525 nm for 30 min by using a Hitachi F-2000 fluorescence spectrometer. The rate of DCFH conversion to DCF was linear for at least 60 min, corrected with the autooxidation rate of DCFH without protein. All assays were carried out in duplicates. DCF production was proportional to the amount of protein added in a wide range. The units were picomoles DCF formed per minute per milligram protein.

Lipid peroxidation and protein oxidation. Peroxidative damage to cellular lipid constituents was determined by measuring malondialdehyde (MDA) in butanol extracts, according to Uchiyama and Mihara (33), with modifications as follows: 10 mM butylated hydroxytoluene and 200 mM ferrous sulfate were included in the assay mixture. Sealed tubes were incubated for 15 min at 99°C. MDA content was calculated based on a standard curve using 1,1,3,3-tetraethoxypropane as a standard.

Muscle protein oxidation was evaluated by measuring carbonyl formation using 2,4-dinitrophenylhydrazine (DNPH) as a reagent, according to Levine et al. (20), with some modifications. Briefly, 200–300 mg of frozen DVL tissues were homogenized at 0–4°C in 5 mM potassium phosphate buffer (ph 7.4; wt/vol 1:10), including 0.1% Triton X and protease inhibitors leupeptin (1.0 µg/ml), pepstatin (1.4 µg/ml), and aprotinin (1.0 µg/ml). The homogenate was centrifuged at 500 g for 3 min, and 900 µl of supernatant were transferred to a microcentrifuge tube including 100 µl of 10% streptomycin sulfate (in 50 mM HEPES). The samples were vortexed vigorously and incubated at room temperature for 15 min. The samples were centrifuged at 6,000 g for 10 min at 4°C, and the supernatant was used. After reacting with 10 µM DNPH-2 N HCl in the dark, protein was precipitated with 20% trichloric acid, followed by centrifugation at 14,000 g for 10 min. The pellets were washed three times to remove excess DNPH, suspended in 6 M guanidine HCl (in 20 mM KH₂PO₄, pH 2.3), vortexed, and allowed to dissolve overnight. The absorbance of the samples was measured at 366 nm. Carbonyl content was calculated by using a molar absorption coefficient of 22,000 M⁻¹·cm⁻¹.
RESULTS

Body weight and exercise duration. Body weight of the O rats was significantly higher than that of Y rats (Table 1). There was no difference in body weight between E and R rats in either Y or O group. Time of running to exhaustion was not significantly different for Y (55.4 ± 2.7 min) and O (58.0 ± 2.7 min) rats.

Oxidant production in muscle homogenate. Oxidant production, as revealed by DCFH oxidation rate in DVL homogenate, was increased with age and after acute exercise under basal condition (Fig. 1, top). OR and OE rats had 77 and 92%, respectively (P < 0.01), higher DCFH oxidation rate than did YR and YE rats. DCFH oxidation was 38% (P < 0.087) greater in YE than in YR group and 50% (P < 0.01) greater in OE vs. OR group. In the induced condition, there was no aging or exercise effect on DCFH oxidation in muscle homogenate (Fig. 1, bottom).

Oxidant production in muscle mitochondria. Muscle mitochondria from O rats demonstrated a higher rate of oxidant production than those from Y rats under both basal and induced conditions (Fig. 2). OR animals showed a 57% (P < 0.01) higher DCFH oxidation rate than did YR rats in the basal condition (Fig. 2, top). In the induced condition, the age difference between OR and YR rats was ~100% (P < 0.05; Fig. 2, bottom). There was also a significant age-exercise interaction revealed by a 46% (P < 0.05) higher DCFH oxidation in YE vs. YR rats but a 23% (P > 0.05) decrease in OE vs. OR group.

Lipid peroxidation and protein oxidation. An acute bout of exercise resulted in a significant increase in muscle lipid peroxidation (Table 1). Exercise-induced MDA content was 18% higher in YE vs. YR animals and 26% higher in OE vs. OR rats (P < 0.05). MDA content in DVL showed no significant difference between Y and O rats. There was no significant change in protein carbonyl content among various treatment groups. Also, muscle protein content was unaffected by either age or exercise (data not shown).

Mitochondrial GSH status. Muscle mitochondria from O rats tended to have higher GSH (P < 0.12) and total glutathione contents (P < 0.13) than those from Y rats (Table 1). GSSG content was not affected by age; however, GSH/GSSG was significantly elevated in OR vs. YR rats (P < 0.05). Exercise did not affect mitochondrial GSH content, but it significantly increased GSSG levels by 32 and 41% (P < 0.05) in YE and OE rats, respectively, compared with YR and OR groups. Further-

Table 1. Body weight, endurance time, and muscle malondialdehyde, protein carbonyl, and mitochondrial glutathione contents in rats

<table>
<thead>
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<th></th>
<th>Rested (n=12)</th>
<th>Exercised (n=12)</th>
<th>Rested (n=12)</th>
<th>Exercised (n=12)</th>
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<tr>
<td>Body wt, g</td>
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<td>203 ± 4</td>
<td>277 ± 8*</td>
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<td>MDA, nmol/g wet wt</td>
<td>101 ± 10</td>
<td>120 ± 10*</td>
<td>106 ± 10</td>
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<td>Carboxyl, nmol/mg protein</td>
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<td>GSH, nmol/mg protein</td>
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<td>GSSG, nmol/mg protein</td>
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<td>GSH/GSSG</td>
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Values are means ± SE with number of rats indicated in parenthesis. Young, 8-mo-old rats; Old, 25-mo-old rats. MDA, malondialdehyde; GSH, reduced glutathione; GSSG, glutathione disulfide; TGSH, total glutathione; NA, not applied. *P < 0.05, Exercised vs. Rested; †P < 0.05; ‡P < 0.01, old vs. young.
more, GSH/GSSG was decreased with exercise in both Y and O rats ($P$, 0.05).

DISCUSSION

The present study has generated some interesting and significant data. First, the study supported a long-standing hypothesis that generation of oxygen free radicals and other reactive oxidants may be increased in aged skeletal muscle. Second, an acute bout of exercise at similar relative workload and duration enhanced muscle oxidant production in both young and old skeletal muscle, accompanied by increased oxidative stress.

The DCFH assay in the present study has been adopted to measure ROS production in a wide range of tissues, including nerve cells, isolated liver mitochondria and microsomes, and isolated diaphragm muscle bundles (15, 17, 29). Although DCFH is sensitive to ROS, it does not appear to be ROS specific (17). Recent work suggests that DCFH may be readily oxidized by NO in skeletal muscle because of the presence of NO synthase (28). Thus the assay may be a nondiscriminative measurement of most, if not all, intracellular oxidants, including ROS, reactive nitrogen species, and other low-molecular-weight oxidants such as ascorbyl radicals. Another important consideration is that DCFH oxidation rate was measured in the presence of intact intracellular antioxidant systems in the muscle homogenate and mitochondria, which would compete with DCFH for oxidants. Hence, the differences in the rate of DCFH oxidation observed among various experimental groups may be viewed as combined effects of increased oxidant production minus antioxidant defense capacity. Despite these limitations, our work was among the first to directly measure oxidant production in muscle homogenate and mitochondria, associated with in vivo exercise in young and old animals.

Numerous studies have shown that senescent skeletal muscle is subjected to enhanced oxidative stress indicated by lipid peroxidation, protein oxidation, and DNA damage (5, 8, 32). A disturbance of cellular redox status and upregulation of antioxidant enzymes and GSH content found in aged muscle have also been suggested to reflect an increased ROS production (12, 19, 21, 27). However, few studies have directly measured ROS levels in aged muscle. Our data provided definitive evidence that oxidant production in the DVL, an important hindlimb muscle involved in locomotion, was dramatically increased (~80%) as the animals grew older. Furthermore, mitochondrial electron transport chain (ETC) at state 4 appeared to be a major source of oxidants, which showed a prominent increase (57%) at old age. A significant portion of the oxidants measured in muscle homogenate was probably attributed to mitochondrial ROS production, although NO may also be generated by type III NO synthase in this organelle (28). These findings were consistent with the hypothetical role of mitochondria in cellular free radical generation and with previous data showing the highest rate of H$_2$O$_2$ production in state 4 (3, 24, 31). Moreover, the large age differences in DCFH oxidation rate shown with site 1 substrate pyruvate-malate suggest that Complex 1 (NADH reductase) is a major source of ROS in aged mitochondria (10, 25). It is noteworthy that antioxidant defense capacity has been shown to increase with age in rat skeletal muscle, especially in the mitochondria (13, 19, 21). Thus the actual differences in muscle oxidant production rate between young and old rats might be even higher than those reported in the present study.

Mitochondrial ETC did not seem to be the sole source of oxidant production in aged muscle, because age difference in DCFH oxidation rate was much larger in the muscle homogenate (77%) than in the isolated mitochondria (57%) in the basal condition. Among the possible cellular pathways mentioned above, we only examined the potential role of NADPH oxidase and found that oxidant production through this pathway was doubled in aged muscle mitochondria. In the presence of ADP and Fe$^{3+}$, NADPH oxidase catalyzes a one-electron transfer from NADPH to oxygen to produce superoxide radical. This enzyme, once thought to be located only on plasma membrane, has now been found to be present in many cellular components, including the mitochondria. Using a similar DCFH assay, Kim et al. (15) demonstrated that liver mitochondrial oxidant production was increased two- to three-
fold under the induced condition and that aged rats showed a higher oxidant level than did young rats. Glinn et al. (7) showed in beef mitochondria that NADPH in the presence of ADP and Fe3+ stimulated lipid peroxidation to a greater extent than NADH and that this increase was despite the presence of rotenone, a Complex I inhibitor. Our preliminary work also revealed that adding site 1 substrates (pyruvate-malate) to the induced assay medium depressed DCFH oxidation rate with or without rotenone, possibly because of an oxygen shunt through the ETC (1). Thus ROS production in the induced state via NADPH oxidase is independent of mitochondrial ETC. Although the assay included much higher concentrations of NADPH, ADP, and Fe3+ than those present in the muscle in vivo, an estimate of the maximal potential of ROS production through this pathway (an indirect measure for maximal velocity of NADPH oxidase) is important in assessing age-related changes in muscle mitochondria.

Muscle MDA and protein carbonyl contents in the aged rats were not significantly elevated as a result of the increased oxidant production. The reason for this dissociation of oxidants and oxidative damage could be related to animals' age and muscle fiber type under investigation. Large increases in muscle lipid peroxidation were reported in rats at 24 vs. 4 mo of age but not vs. 12 mo of age in one of our previous studies (19). Dramatic increases in lipid peroxidation were also found in rats of much older age (31 mo) (12). The age difference in the present study (8 vs. 24 mo) might not be sufficiently large to reveal age-enhanced lipid peroxidation. Also, we have previously reported much greater age differences in MDA levels in soleus (type 1) compared with DVL (type 2a) muscle (19). Nevertheless, muscle mitochondrial GSH content and GSH/GSSG were increased in aged muscle, reflecting an increased oxidative stress and subsequent adaptation. Because mitochondria lack the enzymes in the γ-glutamyl cycle and GSH is transported into the mitochondria via a membrane-borne energy-dependent system (22), our results suggest that mitochondrial GSH transport in skeletal muscle may be enhanced at old age.

Rate of DCFH oxidation in DVL muscle homogenate was significantly increased as a result of an acute bout of exhaustive running in both young and old rats. Our data demonstrated unequivocally that strenuous exercise enhances free radical generation in working muscle (6, 11, 26, 29). Although only <5 min elapsed from animal's death to exposure of muscle homogenate to DCFH, it is still uncertain whether the observed rate of oxidant production approaches the in vivo rate during exercise. However, based on data from several recent studies, the in vivo rate of exercise-induced oxidants is expected to be even higher. For example, O'Neill et al. (26) showed that hydroxyl radical production decreased rapidly, within the first 1–2 min, after muscle contraction ceased. Therefore, the present method likely underestimated the real-time oxidant production during exercise. Because DCFH oxidation was measured in the presence of state 4 substrates, mitochondria were expected to be a major source of oxidants. However, data obtained in isolated muscle mitochondria did not show an exercise effect in either the young or old rats. There are two possible explanations for this discrepancy. First, the damaging effect of exercise might be short lived and diminished during the mitochondrial isolation procedure, which took about an hour after the rats were killed. This explanation was argued against by the findings that both young and old exercised rats demonstrated significantly higher GSSG levels and lower GSH/GSSG ratio in the mitochondria. Alternatively, there might be additional intracellular sources that made a significant contribution to oxidant production in the muscle cells. Exercise has been shown to activate xanthine oxidase, polymorphonutrophil infiltration, and NO synthase in the skeletal muscle, all of which may generate various ROS or reactive nitrogen species (23, 29). According to Fig. 2, bottom, NADPH oxidase was activated after exercise in the mitochondria from young rats, which might be one of the potential sources.

Exercise increased oxidant production to a greater extent in aged muscle than in young muscle. Because no exercise effect was observed in aged-muscle homogenate or mitochondria in the induced state, NADPH oxidase did not seem to be a main contributor. Previous work suggests that both aging and strenuous exercise per se are associated with a higher rate of electron leakage from mitochondrial ETC (6, 24, 25). In the present study, although young and old rats ran at the same relative workload and for the same duration, the absolute exercise intensity was much lower for the aged rats. Yet, aged rats showed a greater rate of oxidant production and similar exercise-induced lipid peroxidation and GSH oxidation in DVL muscle, compared with young rats. These results indicate that, despite the adaptation of antioxidant enzyme activities and GSH content (13, 19, 21, 27), senescent muscles are more susceptible to exercise-induced oxidative stress. Meydani and Evans (23) made a similar conclusion based on their finding that aged human subjects required higher doses of vitamin E supplementation to reach a similar level of protection against muscle lipid peroxidation due to exercise.

In summary, both aging and exhaustive exercise resulted in increased oxidant production in DVL muscle. Mitochondrial ETC and NADPH oxidase appeared to contribute to the age-related increase in ROS production, but the exercise-induced muscle oxidants were probably from multiple sources. These data have extended the relevance of the free radical theory of aging to senescent skeletal muscles that are especially vulnerable to oxidative damage caused by strenuous exercise.

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