Skeletal Muscle Aging in F344BN F1-Hybrid Rats: I. Mitochondrial Dysfunction Contributes to the Age-Associated Reduction in \( VO_{2\text{max}} \)

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Although mitochondrial DNA damage accumulates in aging skeletal muscles, how this relates to the decline in muscle mass-specific skeletal muscle aerobic function is unknown. We used a pump-perfused rat hind-limb model to examine maximal aerobic performance (\( VO_{2\text{max}} \)) in young adult (YA; 8–9-month-old), late middle aged (LMA; 28–30-month-old) and senescent (SEN; 36-month-old) Fischer 344 × Brown Norway F1-hybrid rats at matched rates of convective \( O_2 \) delivery (\( QO_2 \)). Despite similar muscle \( QO_2 \) during a 4-minute contraction bout, muscle mass-specific \( VO_{2\text{max}} \) was reduced in LMA (15%) and SEN (52%) versus YA. In plantaris muscle homogenates, nested polymerase chain reaction revealed an increased frequency of mitochondrial DNA deletions in the older animals. A greater reduction in the flux through electron transport chain complexes I–III than citrate synthase activity in the older animals suggests mitochondrial dysfunction consequent to mitochondrial DNA damage with aging. These results support the hypothesis that a reduced oxidative capacity, due in part to age-related mitochondrial dysfunction, contributes to the decline in aerobic performance in aging skeletal muscles.

AGING is associated with a general decline in physiological function that leads to increased morbidity and mortality. Among the most well-known changes in the exercise response is a reduction in maximal oxygen consumption (\( VO_{2\text{max}} \)) with increasing age (1–3), a change that is intimately tied to impaired mobility with aging (4). Whereas an age-related reduction in convective \( O_2 \) delivery (blood flow \( \times \) arterial oxygen content) has been a primary explanation for the reduction in \( VO_{2\text{max}} \) with aging (5,6), the role contributed by an intrinsic reduction in skeletal muscle aerobic function has only recently been established (7). Specifically, in these latter experiments it was shown that late middle aged rat skeletal muscles exhibit a lower mass-specific \( VO_{2\text{max}} \) than young adult muscles even when perfused at a similar rate of convective \( O_2 \) delivery (arterial \( O_2 \) content \( \times \) blood flow), revealing an impairment at one or more points in the movement of \( O_2 \) from blood to cytochrome oxidase in the muscle mitochondria (7).

Aging is associated with significant alterations in skeletal muscle, such as reduced muscle mass (8,9) and reduction of the activity of some mitochondrial enzymes (10–12), although not all studies are in agreement with this latter point (e.g., 13). Whereas these alterations in skeletal muscle are influenced by reduced levels of physical activity with aging, a portion of these changes are believed to be a consequence of the biological process(es) of aging (14–18). In this respect, the oxidative stress theory of aging states that some of the physiological decrements typical of increasing age can be ascribed to the life long accumulation of intracellular damage induced by the generation of free radicals (19). In particular, it is significant that the mitochondrial genome is more prone to oxidative damage because of its location (attached to the inner mitochondrial membrane), a lack of histone proteins, and less efficient repair mechanisms than nuclear DNA, rendering it some 16-fold more susceptible to oxidative damage than nuclear DNA (20). Thus, it has been argued that oxidative damage over time leads to mitochondrial DNA mutation deletions that result in dysfunctional mitochondria, and that skeletal muscle is one tissue that is particularly susceptible to this phenomenon (15,16). Since skeletal muscle \( VO_{2\text{max}} \) is a function of an interaction between \( O_2 \) supply and mitochondrial oxidative capacity (21), it stands to reason that mitochondrial dysfunction, due to oxidative damage, would also be a contributor to the reduction in \( VO_{2\text{max}} \) with increasing age.

To address this issue, we examined changes in skeletal muscle function and biochemistry in young adult (8- to 9-month-old), late middle-aged (28- to 30-month-old), and senescent (36-month-old) Fischer 344 × Brown Norway F1-hybrid (F344BN) rats. We hypothesized that under similar conditions of skeletal muscle convective \( O_2 \) delivery, mass-specific \( VO_{2\text{max}} \) of the skeletal muscles in senescent animals would be even lower than seen previously in skeletal muscles from late middle aged animals compared to young adult animals (7). Furthermore, we hypothesized that the decline in muscle mass-specific \( VO_{2\text{max}} \) with increasing age would be associated with genetic (e.g., mitochondrial DNA deletions) and biochemical (greater decrease in activity of a biochemical
Table 1. Summary of Animals Common to the Current Study and Two of Our Previous Studies

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Note: CSA = citrate synthase activity; Flux I–III = the flux through electron transport chain complexes I–III.

“X” denotes an animal included in the specified study.

pathway containing mitochondrial DNA-encoded peptides versus a nuclear encoded mitochondrial enzyme) evidence of mitochondrial dysfunction consequent to accumulation of oxidative damage.

METHODS

Animals

All experiments were conducted after obtaining the approval of the University of Calgary Animal Care Committee. Three groups of specific pathogen-free male F344BN rats were obtained from the National Institute on Aging to represent young adult (8- to 9-month-old; n = 13), late middle-aged (28- to 30-month-old; n = 18), and senescent (36-month-old; n = 9) animals, based on previously published survival curves for this strain of rat (22), in a relative comparison to survival curves for humans (23). Rats were housed two per cage (with filter bonnets) in the Faculty of Medicine vivarium at the University of Calgary for a minimum of 1 week prior to experiments (12:12 hour light/dark cycle, 22°C), and were provided water and Purina rat chow ad libitum. As described previously (7), necropsies were performed postexperiment to detect any abnormalities or lesions within each animal. The internal assessment involved an examination of the internal organs and tissues looking for specific identifiable lesions (24,25). To prevent contamination of the data by the presence of disease, animals demonstrating tissue abnormalities or lesions were excluded from the data set, as recommended by NIA guidelines (24). Note that one 28-month-old animal and three of the 36-month-old animals were excluded on this basis (large tumors in each case). As such, the final data set comprises n = 13 (8- to 9–month-old), 17 (28- to 30-month-old), and 6 (36-month-old) animals.

Physical Activity

The amount of voluntary physical activity over a 72-hour period was quantified in four of the 8-month-old, four of the 28- to 30-month-old [both published previously in (26)], and all six of the 36-month-old animals. As described previously (26), this involved placing individual rats in a standard cage (21 × 20 × 42 cm) that was resting on two aluminum lever arms instrumented with strain gauges on their fulcrum. Measurements from each rat were collected online using a data acquisition system (Dataq DI-700; Dataq Instruments, Inc., Akron, OH) connected to a laptop computer running Windaq Pro+ software (Dataq Instruments). Data analysis of physical activity recordings was performed off-line with Matlab (ver. 6.5; The MathWorks, Inc., Natick, MA) and involved integrating the time during which the voltage signal was elevated above the baseline voltage associated with small movements, such as shifts in body weight and grooming behavior (baseline determined for each animal individually). As such, these measurements are taken as an indicator of locomotor activity. As noted previously (26), the first 2 hours of recording were discarded for each animal to exclude the exploratory behavior associated with transfer to a new environment (27).

Surgical Procedures

After anesthetizing the animal with Pentobarbital Sodium (i.p., 75 mg/kg), the right iliac artery and vein were ligated and the right gastrocnemius–plantaris–soleus muscle group was removed, trimmed free of fat and connective tissue, and weighed. The right plantaris muscle from 10 of the 8- to 9-month-old, 11 of the 28- to 30-month-old, and all 6 of the 36-month-old animals was frozen in liquid nitrogen and stored at −70°C for subsequent morphological (data not shown) and/or biochemical (below) analyses. Following this, 12 of the 8–9-month-old, 10 of the 28–30-month-old, and all 6 of the 36-month-old animals were prepared for surgical perfusion of the left hind limb (21,28). Note that the metabolic and contractile data during hind-limb perfusion for eight of the 8- to 9-month-old animals and seven of the 28- to 30-month-old animals were part of a recently published study (7) (Table 1).

Preparation for hind-limb perfusion and muscle contractions began with isolating the left sciatic nerve in the avas-
cular space between the biceps femoris muscles. The inferior gluteal nerve was then severed to prevent stimulation of the upper hind-limb muscles, and the sciatic nerve was cut proximally in preparation for electrical stimulation via a platinum hook electrode. The Achilles tendon was cut, with a portion of the calcaneous intact, and secured by 1.0 silk thread to a force transducer (FT-10; Grass Instruments, Quincy, MA). The animal was then placed on a heating pad and moved to a stereological base plate where a metal clamp attached around the proximal femur and another clamp attached to the ankle was secured to immobilize the distal hind limb during force measurements.

Catheters were inserted into the iliac artery (22 ga) and vein (20 ga) and advanced into the respective femoral artery and vein to initiate perfusion to the hind limb. Ligatures were placed around the iliac artery inside the abdominal wall (immediately proximal to the inguinal ligament) and around the femoral vein to secure the arterial and venous catheters in place. The hind limb was wrapped in warm saline soaked gauze, saran wrap (encompassing a thermistor probe connected to a heat lamp), and aluminum foil to maintain a muscle temperature of 37°C. The perfusion medium consisted of bovine erythrocytes reconstituted in a Krebs–Henseleit buffer (pH 7.4) containing 4.5% bovine serum albumin, bovine erythrocytes (hematocrit 42%), 5 mM glucose, 100 mU/mL insulin, and 0.15 mM pyruvate. Hematocrit (~42%) was verified by direct observation in centrifuged capillary tubes and yielded an average hemoglobin concentration of 14.3 ± 0.2 g/dL. Prior to entering the hind limb, the perfusion medium was gassed with 95% O₂ and 5% CO₂ using an oxygenator (4 L flask containing 7 m of silastic tubing), yielding an average arterial O₂ content of 20.7 ± 0.2% by volume. Flow was controlled using a peristaltic pump (Gilson Minipuls 3; Gilson, Inc., Middleton, WI), with flow verified after each experiment by timed blood collection through the arterial catheter. A pressure transducer (PT-300; Grass Instruments) was placed at the height of the hind limb for determination of total perfusion pressure during perfusion conditions.

**Experimental Protocol**

Perfusion rate was incrementally increased to elicit a flow-induced vasodilatory response until the desired rate of perfusion was achieved (~30 min). Note that the desired flow rate was selected to permit matching of mass-specific blood flow to the contracting muscles between age groups, as done previously (7). Briefly, this involved measuring the mass of the gastrocnemius–plantaris–soleus muscle group in the contralateral (right) hind limb and using this value to select the rate of perfusion on the pump that would yield a similar rate of mass-specific muscle blood flow for each animal. Tetanic stimulation, elicited by square wave pulses (200 ms trains at 100 pulses/s, each 0.2 ms in duration), was used to elicit muscle contractions at a frequency of 60 tetani/minute for 4 minutes, since this frequency yields the highest VO₂ for this preparation (29). Muscle length and voltage (~7 volts) were adjusted to yield maximum force development. Blood samples were drawn anaerobically every 30 s during contractions from the arterial blood and venous effluent and were analyzed for PO₂, PCO₂, O₂ saturation (SO₂), [hemoglobin], and [lactate] [note: lactate was only measured in a subset of animals; see our companion paper (30)] by a blood gas analyzer (Stat Profile M3; Nova Biomedical; Waltham, MA). Blood oxygen content was calculated using the formula: \[ O_2 \times SO_2 \times 1.39 + 0.003 \times PO_2 \]. VO₂ across the hind limb was determined from the product of blood flow and the arteriovenous O₂ content difference, with VO₂max taken as the sample having the lowest venous percent O₂ saturation (arterial O₂ content and blood flow were held constant).

**Normalization Procedures**

As demonstrated by Gorski and colleagues (28), total perfused muscle mass in the hind limb in this preparation includes all of the hind-limb muscles (hamstrings, quadriceps, gastrocnemius, plantaris, soleus, tibialis anterior, and remaining tibial muscles) except the gluteal muscles (c.f. 29). The total perfused muscle mass was measured in all of the 36-month-old animals, and in three each from the 8- to 9-month-old and 28- to 30-month-old animals. Using this data, the relationship between total perfused muscle mass and the total contracting muscle mass [the latter was measured in all animals and includes the gastrocnemius–plantaris–soleus muscle group, tibialis anterior, and remaining tibial muscles (28)] was determined for each age group, and total perfused muscle mass was estimated from these relationships in the remaining animals. Total noncontracting perfused muscle mass was calculated as the difference between the total perfused muscle mass and the total contracting muscle mass and comprises 72 ± 1%, 71 ± 1%, and 80 ± 2% of the total perfused muscle mass in the 8- to 9-month-old, 28- to 30-month-old, and 36-month-old animals, respectively. As done previously (7,29), since most of the VO₂ measured at rest is contributed by these noncontracting tissues, VO₂ during contractions was normalized to the mass of the contracting muscles after subtracting the VO₂ contributed by the noncontracting tissues (calculated based upon values observed at rest).

**Blood Flow Distribution**

As described previously (21), following the contraction bout ~280,000 colored microspheres (15 μm diameter, Dye Tak; Triton Technology, Nottinghamshire, U.K.) were drawn from a known stock concentration and injected slowly (while reducing the pump output to maintain perfusion pressure) into a side arm port situated proximal to the arterial catheter. Saline (2 mL) was slowly infused immediately after the microsphere infusion to ensure that all microspheres reached the hind limb. The gastrocnemius, plantaris, and soleus muscles were excised and heated in a water bath (60°C) in centrifuge tubes containing 4 M KOH until each muscle was digested (≥60 min). The content of each centrifuge tube, along with a reference sample (stock solution), was individually filtered through 8 μm pore membranes (Whatman Nucleopore, Clifton, NJ) to trap the microspheres. The membranes were placed in microcentrifuge tubes containing 1 ml of N, N-dimethyl-formamide to release the color of the spheres. Absorbance of the resulting solution was analyzed using a spectrophotometer (Ultrospec 2100 Pro; Biochrom, Berlin, Germany) after 10 minutes at a wavelength of 448 nm to determine the number.
of microspheres in each sample (calculated based on the regression equation provided by the lot manufacturer). The blood flow to the gastrocnemius–plantaris–soleus muscle group was determined as the product of the total hind-limb blood flow and the proportion of microspheres found in the gastrocnemius–plantaris–soleus muscle group. Similarly, muscle convective $O_2$ delivery was calculated as the product of the blood flow to the gastrocnemius–plantaris–soleus muscle group and the arterial $O_2$ content. As done previously (7), $O_2$ extraction across the contracting muscles was estimated as the quotient of the mass-specific $VO_2_{max}$ and blood flow to the gastrocnemius–plantaris–soleus muscle group.

**Biochemistry**

The flux through mitochondrial electron transport chain complexes I–III [which contains 8 of the 13 polypeptides encoded by the mitochondrial genome (31)] and citrate synthase activity (entirely nuclear encoded) was determined using spectrophotometric methods. Muscles were thawed on ice and then spun at 60000 g at 4°C for 1 hour. The supernatant was removed and the resulting muscle homogenates were stored at −70°C until assayed.

As done previously (7,21), to assess the flux through complexes I–III, homogenates were thawed on ice and the rate of reduction of cytochrome-c at 38°C was followed spectrophotometrically. Each homogenate was thawed on ice and placed in a glass homogenizer at 4°C in phosphate buffer (pH 8.0) containing 0.05 M Tris–HCl and 0.67 M sucrose, and the resulting muscle homogenates were stored at −70°C until assayed.

Brieﬂy, each homogenate was thawed on ice and then spun at 600 g for 3 minutes. These samples were then diluted by adding homogenizing medium to reach a 1:22 dilution. Each sample was vortexed for 20 seconds, sonicated in an ice bath for 3 times. The final dilution was then performed by adding homogenizing medium to reach a 1:440. Citrate synthase activity was measured according to the method of Srere (32), with the exception that the homogenizing medium used in this assay was the same as that used in preparing samples for measuring the flux through complexes I–III described above (such that both enzyme pathways could be determined from the same homogenate sample).

Briefly, each homogenate was thawed on ice and then spun at 600 g for 3 minutes. These samples were then diluted by adding homogenizing medium to reach a 1:22 dilution. Each sample was vortexed for 20 seconds, sonicated in an ice bath for 3 times. The final dilution was then performed by adding homogenizing medium to reach a 1:440. Citrate synthase activity was determined by measuring the rate of production of the mercaptide ion spectrophotometrically at 412 nm (Biochrom Ultrospec 2100 Pro) after the addition of 20 µl of homogenate to 1.0 M phosphate buffer (pH 8.0), 0.1 M NaN3, 1% aqueous cytochrome-c, 0.01 M NADH, and bringing the total volume to 1 ml with double distilled H2O. Each sample was measured in triplicate and the average activity over the linear portion of the absorbance versus time relationship was used to represent the flux through complexes I–III (21). Citrate synthase activity was measured according to the method of Srere (32), with the exception that the homogenizing medium used in this assay was the same as that used in preparing samples for measuring the flux through complexes I–III described above (such that both enzyme pathways could be determined from the same homogenate sample).

**Mitochondrial DNA Analysis**

Mitochondrial DNA deletions were assessed in homogenates of plantaris muscle used in biochemical analyses (above) from four of the 8- to 9-month-old, four of the 28- to 30-month-old, and five of the 36-month-old animals. Note that the primers used spanned the region of the mitochondrial genome containing 10 of the 13 mitochondrial DNA-encoded components of the electron transport chain: ND3, ND4L, ND4, ND5, and ND6 in complex I; cytochrome b in complex III; COX II and COX III in complex IV; and ATP6 and ATP8 of complex V.

Total DNA was recovered by adding 1 M Tris–HCl (pH 8.0), 0.5 M EDTA, and 20% SDS to each homogenate to give a final concentration of 10 mM, 0.1 M, and 0.5%, respectively. Pancreatic RNAase (400 µg/ml) was then added to each homogenate and incubated at 37°C for 1 hour. Proteinase K (100 µg/ml) was then added to each homogenate and incubated at 50°C for 3 more hours. DNA was subsequently extracted with phenol, and quantified using spectrophotometry, according to the method described by Sambrook and Russell (33). Following DNA quantification, a stock DNA solution of 50 ng/µl was prepared and used for polymerase chain reaction (PCR). Mitochondrial DNA was amplified from the DNA stock using the PCR Takara Ex Taq hot start version kit (Takara Bio Inc., Japan) and the following forward and reverse primers: 5'-TCCCCCTCAGTTTAAACCAGA-3' (forward); 5'-GGCGGAATGTATAGCTCGTTG-3' (reverse), with 30 thermocycles, consisting of 30 seconds at 94°C, 25 seconds at 60°C, and 90 seconds at 72°C. The primary PCR product was then further amplified using nested PCR, to determine deletions within the mitochondrial DNA itself. This was achieved using the Takara hot start version PCR kit (as described above) and the following forward and reverse primers: 5'-CCGGCCGCTTAAACCAAGCTACAGT-3' (forward); 5'-TGCGCGGCTGAGCTG-3' (reverse), with 30 thermocycles, consisting of 30 seconds at 94°C, 25 seconds at 66°C, and 90 seconds at 72°C. Intact and deleted mitochondrial DNA was then photographed under UV light following gel electrophoresis (1% agarose gel with ethidium bromide, 160 volts for 1.5 hours). Total amplifiable mitochondrial DNA was estimated from the optical density of the largest bands in each lane using image analysis software (Sigmascan Pro 5.0; SPSS, Inc., Chicago, IL) calibrated for optical density units.

**Statistical Analysis**

Values are reported as means ± standard error (SE). Differences between 8-, 28- to 30-, and 36-month-old animals were assessed by Student’s t test (ratio of the flux through complex I–III and citrate synthase activity; combined older groups), one-way analysis of variance (ANOVA) (i.e., animal characteristics, physical activity, perfusion conditions, contractile and metabolic responses, total amount
The relationship between distal muscle; Gastroc–plantaris–soleus muscle group. N month-old group and 5 animals in the 36-month-old group.

Despite a greater body mass, the mass of the gastrocnemius–plantaris–soleus muscle group was progressively reduced (8–9 mo old) in the older animals (see above), muscle mass-specific blood flow to the gastrocnemius–plantaris–soleus muscle group was not different between the 8- to 9-month-old, 28- to 30-month-old, and 36-month-old animals. Similarly, blood flow distribution between the gastrocnemius, plantaris, and soleus muscles was not different between groups (Figure 1). Finally, there was no difference in the mass-specific convective O\textsubscript{2} delivery.

The mass of the contracting muscles (gastrocnemius muscle, plantaris muscle, soleus muscle, tibialis anterior muscle, and remaining deep tibial muscles) was significantly reduced with increasing age. The net perfusion pressure (difference between total perfusion pressure and the pressure required to overcome the resistance of the perfusion system) was lower in the 36-month-old animals versus the 28- to 30- and 8- to 9-month-old animals (Table 3). Blood flow to the whole hind limb was less in the 36-month-old and 28- to 30-month-old versus the 8- to 9-month-old animals. However, because the muscle mass was lower in the older animals (see above), muscle mass-specific blood flow to the gastrocnemius–plantaris–soleus muscle group was not different between the 8- to 9-month-old, 28- to 30-month-old, and 36-month-old animals. Similarly, blood flow distribution between the gastrocnemius, plantaris, and soleus muscles was not different between groups (Figure 1). Finally, there was no difference in the mass-specific convective O\textsubscript{2} delivery.

![Figure 1. Blood flow distribution in the gastrocnemius–plantaris–soleus muscle group. SOL = soleus muscle, PLA = plantaris muscle, GAS = gastrocnemius muscle, Combined = gastrocnemius–plantaris–soleus muscle group as a whole. Note that n = 11 for the 8- to 9-month-old group, n = 10 for the 28- to 30-month-old group, and n = 5 for the 36-month-old group. Values are means ± SE.](image-url)
between age groups (Figure 2), showing that muscle convective O₂ delivery was well matched between groups.

**Metabolic Responses**

When expressed in absolute terms, the hind-limb resting VO₂ in the 36-month-old animals was lower compared with the 8- to 9-month-old and 28- to 30-month-old animals. However, when expressed relative to the total estimated perfused muscle mass, the resting VO₂ was not different between groups (Table 4; \( p = .085 \)), which is similar to our previous observations in young adult and late middle-aged rats (7). Detailed treatment of the force measurements made in a subset of these animals during the contraction bout can be found in our companion paper (30). The force at VO₂max was progressively lower in the 28- to 30-month-old and 36-month-old animals compared with the 8- to 9-month-old animals. Whether expressed in absolute terms (Table 4) or normalized to the mass of the contracting hind-limb muscles, VO₂max in the 28- to 30- and 36-month-old animals was significantly less than the 8- to 9-month-old animals, despite very similar rates of convective O₂ delivery (Figure 2). In this regard, the difference in VO₂max between 8- to 9-month-old and 28- to 30-month-old animals (15%) was slightly less than that observed previously in a smaller number of animals (22%) (7). The estimated gastrocnemius–plantaris–soleus muscle group oxygen extraction at VO₂max was significantly lower in the 36-month-old compared with the 28- to 30-month-old and 8- to 9-month-old animals.

**Mitochondrial DNA Analyses**

Whereas there were no mitochondrial DNA deletions seen in the 8- to 9-month-old animals, there was a progressive increase in the frequency of mitochondrial DNA deletions in 28- to 30-month-old and 36-month-old animals (Figure 3). Similarly, the optical density of the 7591 bp bands on the PCR gel were 40% lower in the 36-month-old animals compared with young adult animals (Figure 4), suggesting a reduced amount of intact total amplifiable mitochondrial DNA in the senescent animals.

**Muscle Oxidative Capacity**

There was a progressive reduction (8- to 9-month old > 28- to 30-month old > 36-month-old rats) in the flux through electron transport chain complexes I–III with increasing age. In contrast, citrate synthase activity was not different between 8- to 9-month-old and 28- to 30-month-old animals, but was significantly reduced in 36-month-old versus 8- to 9-month-old animals (Figure 5). In comparing the relative changes between these two enzyme pathways with aging, the 36-month-old animals were pooled with the 28- to 30-month-old animals because the number of animals in the 36-month-old group was not large enough to yield sufficient statistical power and there were no significant differences in the ratio of the two enzyme pathways between these two groups. In this comparison, the flux through complexes I–III was reduced to a greater extent than citrate synthase activity in the older animals, such that the ratio of the two enzyme pathways between these two groups was significantly less in the 36-month-old group than that observed previously in a smaller number of animals (15%) was slightly less than that observed previously in a smaller number of animals (22%) (7). The estimated gastrocnemius–plantaris–soleus muscle group oxygen extraction at VO₂max was significantly lower in the 36-month-old compared with the 28- to 30-month-old and 8- to 9-month-old animals.

**DISCUSSION**

The objective of this study was to test the hypothesis that the decline in skeletal muscle mass-specific VO₂max with aging is due, in part, to mitochondrial dysfunction consequent to accumulation of damage to the mitochondrial genome. To this end, we examined contractile and metabolic responses in the distal hind-limb muscles of young adult, late middle aged, and senescent rats using an in situ pump-perfused hind-limb preparation to permit matching of skeletal muscle convective oxygen delivery between groups. Our results showed that the decline in muscle mass-specific VO₂max previously reported between young adulthood and late middle age (7) accelerates between late middle age and senescence in rat distal hind-limb skeletal muscles perfused at similar rates of convective oxygen delivery. Whereas the decline in voluntary physical activity previously reported between young adulthood and late...
middle age (26) also accelerated between late middle age and senescence, the decline in muscle oxidative capacity in the older animals (28- to 30- and 36-month-old animals combined) was characterized by a relatively greater decline in flux through electron transport chain complexes I–III than citrate synthase activity in homogenates prepared from the plantaris muscle. As such, these latter results suggest that the decline in oxidative capacity was not entirely explained by the decline in physical activity, but was also affected by age-associated mitochondrial dysfunction. Consistent with this interpretation, there was an increased frequency of mitochondrial DNA deletions and a reduced amount of intact total amplifiable mitochondrial DNA in homogenates prepared from the plantaris muscle with aging. As such, our results support the hypothesis that a portion of the decline in skeletal muscle VO2max with aging is due to mitochondrial dysfunction consequent to age-associated mitochondrial DNA damage.

Critique of the Model

The animal model used in our studies is the F344BN rat, which was developed by the National Institute on Aging for aging research. This rat strain lives considerably longer and exhibits fewer tissue pathologies at any given absolute age than either of the parental strains (Fischer 344 or Brown Norway) (34). The ages of the animals in the current study were chosen to represent young adult (8- to 9-month-old), late middle-aged (28- to 30-month-old), and senescent (36-month-old) animals, based on published survival characteristics for this strain of rat (22). Although maturational differences between humans and rats prevent an exact comparison, if we use survival characteristics as the basis for our comparison, the human equivalents are roughly 20 years of age (young adult), 60 years of age (late middle aged), and 80 years of age (senescent) (23).

Previous studies of the F344BN rat have shown that it exhibits a progressive loss of muscle with aging (35,36), like humans (37–39). In this regard, there was a 17% decrease in the mass of the distal hind-limb muscles (i.e., total contracting muscle mass; Table 2) between young adult and late middle age, and a decrease of 53% between young adult and senescence in our study. Based upon Lexell’s data examining changes in cross-sectional area in whole vastus lateralis muscle from human cadavers aged 15–83 years of age.

Figure 3. Ethidium bromide–stained agarose gel of intact (arrow at 7591 bp) and fragmented mitochondrial DNA extracted from homogenates of plantaris muscles of 8- to 9-month-old, 28- to 30-month-old, and 36-month-old F344BN rats. Far right lane is a marker denoting the size of mitochondrial DNA deletion products. Note the presence of deleted regions of mitochondrial DNA in both the 28- to 30-month-old and 36-month-old animals but not in the 8- to 9-month-old animals.

Figure 4. Optical density of the 7591 bp bands (representing intact mitochondrial DNA) on the agarose gel (see Figure 3) in 8- to 9-month-old (n = 4), 28- to 30-month-old (n = 4), and 36-month-old (n = 5) F344BN rats. Results show a reduced amount of intact total amplifiable mitochondrial DNA in the 36-month-old animals. Values are means ± SE. *p < .05 versus other groups.
Figure 5. The flux through mitochondrial electron transport complexes I–III and citrate synthase activity in homogenates prepared from the plantaris muscle. Units refer to the rate of appearance of reduced cytochrome-c (Complex I–III), or the appearance of the mercaptide ion (citrate synthase). Note that $n = 11$ for the 8- to 9-month-old group, $n = 11$ for the 28- to 30-month-old group, and $n = 6$ for the 36-month-old group. Values are means ± SE. *$p < .05$ versus other groups; †$p < .05$ versus 8- to 9-month-old group.

(38), this degree of muscle mass loss is very similar to that observed in humans between young adulthood and late middle age (~22%), and between young adulthood and senescence (~50%). With the caveat that this comparison is confined to examples of locomotor skeletal muscles, not only is the degree of muscle atrophy similar with aging in the F344BN rat and humans, but also an accelerated loss of muscle after late middle age is apparent in both species. Since a decline in physical activity is known to affect skeletal muscle mass and function, it is also noteworthy that, like humans, there is a decline in voluntary physical activity with aging in F344BN rats. Lastly, we have reported an accumulation of mitochondrial DNA deletions in homogenates prepared from aged muscles compared to that obtained from young adult muscles in rats. This is consistent with prior studies showing that skeletal muscle from both rats and humans accumulate mitochondrial DNA deletions with aging (18,40,41). Thus, on the basis of these comparisons, the F344BN rat is a useful model for providing insight into the effects of aging in human skeletal muscle.

Based upon the higher mass-specific blood flows observed in the distal hind-limb muscles of young adult rats during treadmill running (42), it is likely that the highest $V_O^2_2$ attained for these muscles is greater in vivo than seen with the in situ pump-perfused model used in our study. In this respect, the high degree of fatigue seen with our electrical stimulation protocol [see Figure 1 in companion paper (30)], and the resulting intracellular perturbation, might be considered to compromise the aerobic metabolic response. However, in previous experiments in our laboratory where we used a gradual increase in contraction frequency, despite a more gradual fatigue of the stimulated muscles, this did not affect the maximal $V_O^2_2$ attained (29). We have since observed that this is also true in 8- to 10-month-old and 35-month-old F344BN rats (R.T. Hepple, unpublished observations). As such, the $V_O^2_2_{max}$ values observed in this study are the maximum achievable for these perfusion conditions (29), and, therefore, can be used to gain insight into the factors that contribute to the decline in skeletal muscle aerobic function with aging. Further to this point, whereas it is not technically feasible to control convective $O_2$ delivery in human studies, the pump-perfused rat hind-limb preparation is well suited to this purpose.

Reasons for Reduced $V_O^2_2_{max}$ With Aging

Although a decline in whole body $V_O^2_2_{max}$ with increasing age is well established (1,3,43), the causes of this decline remain to be clarified. The physiological determinants of $V_O^2_2_{max}$ during whole body exercise in young adult humans or animal models involve the coordinated actions of multiple systems, beginning at the lung and ending with cytochrome oxidase in the mitochondria of contracting skeletal muscles. Although a comprehensive review of the evidence is beyond the scope of this manuscript, alterations in $O_2$ delivery (44–47) and mitochondrial oxidative capacity (48,49) affect $V_O^2_2_{max}$ in a near-proportional manner. Most recently, it was shown that when reduced oxygen delivery was combined with reduced mitochondrial oxidative capacity the reduction in $V_O^2_2_{max}$ was greater than either intervention performed independently, revealing an interaction between oxygen delivery and mitochondrial oxidative capacity in determining $V_O^2_2_{max}$ (21). The implication of this last point is that even though maximal mitochondrial oxidative capacity may appear to be in relative excess of that required in vivo at $V_O^2_2_{max}$, it apparently still exerts an influence on $V_O^2_2_{max}$ (21).

Based on the preceding evidence, it is likely that a reduced capacity at several points in the chain of events linking oxygen transport and oxygen utilization contributes to reduce $V_O^2_2_{max}$ during whole body exercise with aging. In this regard, whereas a reduced cardiac output (5,6,50) and mal-distributed blood flow (51–53) are thought to impair $V_O^2_2_{max}$ with aging, few studies have addressed the contribution that alterations within the contracting muscles play in this response. Consistent with prior evidence from whole body exercise responses in humans suggesting that skeletal muscle changes (e.g., a reduced oxidative capacity) contribute to the decline in $V_O^2_2_{max}$ with aging (54,55), a reduced ability to use oxygen was recently observed in skeletal muscles of late middle aged rats (7), and is further supported by our current findings. Specifically, by using a pump-perfused rat hind-limb preparation to achieve similar rates of convective oxygen delivery to the contracting muscles across age groups (where blood flow and its distribution was not different between groups), the lower $V_O^2_2_{max}$ observed in the skeletal muscles of the late middle aged [current results and (7)] and senescent animals (current results) can be attributed to differences in the movement of oxygen from the blood to the tissues. As such, the factors that could be involved in the lower $V_O^2_2_{max}$ seen in the aged muscles include the structural (i.e., capillary number) and functional (i.e., erythrocyte hemodynamics) capillary surface area, myoglobin concentration, and mitochondrial oxidative capacity. It is, therefore, pertinent that the anatomical capillarization is not compromised in distal hind-limb skeletal muscles of late middle aged (56) or senescent rats (57).
Similarly, the erythrocyte hemodynamics exhibit minor changes in resting spinotrapezius muscles of older rats (58), although the hemodynamics during muscular contractions remain to be examined. In addition, a prior study reported no change in myoglobin concentration between the ages of 9 months and 25 months in the gastrocnemius muscle of Sprague-Dawley rats (59). This leaves changes in mitochondrial oxidative capacity as a likely contributor to the decline in \( VO_{2\text{max}} \) seen in the aged muscles.

**Flux Through Complexes I–III and \( \dot{VO}_{2\text{max}} \)**

The current results show that the flux through electron transport chain complexes I–III was reduced by 43% and 60% in 28- to 30-month-old and 36-month-old rats, respectively. We chose to examine the flux through electron transport chain complexes I–III as a marker of mitochondrial oxidative capacity for two reasons. Firstly, we (21), and Terjung’s group (48,49), have previously shown that acute reduction in the flux capacity of this pathway (using the complex III inhibitor, myxothiazol) results in a proportional reduction in \( VO_{2\text{max}} \), showing that this pathway plays an important part in determining maximal aerobic function in skeletal muscles. Secondly, this enzyme pathway reflects the biochemical consequences of age-associated alterations in the mitochondrial genome because it contains several mitochondrial DNA-encoded peptides (31). As seen in Figure 6, where we have plotted our previous results using myxothiazol to acutely reduce the flux capacity through complexes I–III together with the current results, it is apparent that whereas the senescent animals fall on the line depicting the relationship between \( VO_{2\text{max}} \) and the flux capacity through complexes I–III, the late middle-aged animals have a considerably higher \( VO_{2\text{max}} \) than would be predicted by this relationship. [In our previous study (21), \( VO_{2\text{max}} \) values were normalized to the mass of the gastrocnemius–plantaris–soleus muscle group, rather than the mass of the entire contracting distal hind-limb muscles (as done in the current study). Thus, we have estimated the mass of the distal hind-limb muscles for our previous results based upon the relationship between the mass of the gastrocnemius–plantaris–soleus muscle group and the entire distal hind-limb muscles in 49 animals studied in our laboratory (total distal mass = 302 + \{1.660 × gastrocnemius–plantaris–soleus muscle group mass\}; \( r^2 = .84, p < .001 \), and used these values to normalize our prior \( VO_{2\text{max}} \) data (21) in the same manner used in the current study.] Thus, on the basis of the aforementioned points, the decline in flux through this biochemical pathway must be contributing to the decline in muscle mass-specific \( VO_{2\text{max}} \) with aging observed in our study.

The dissociation of \( \dot{VO}_{2\text{max}} \) and the flux capacity through complexes I–III in the late middle aged animals may indicate some compensation at this age. Alternatively, because the relationship between \( VO_{2\text{max}} \) and the flux through electron transport chain complexes I–III was derived from experiments utilizing the complex III inhibitor, myxothiazol (21), it is possible that complex III activity in the late middle aged group is reduced in proportion to \( VO_{2\text{max}} \) but that a relatively greater decline in complex I is obscuring this relationship. Indeed, previous studies have shown that complex I often exhibits the greatest degree of dysfunction with aging (60–62), lending support to this explanation. The fact that the senescent animals fall on the line predicted by the relationship between \( VO_{2\text{max}} \) and the flux through complexes I–III suggests that the decline in complex I and complex III activity in senescence is more proportional. Further studies using assessment of the activity of individual components of the electron transport chain are required to address these possibilities.

**Effects of Aging and Physical Inactivity on Mitochondrial Oxidative Capacity**

The accumulation of mitochondrial DNA damage (e.g., due to the accumulated effects of oxidative stress with aging) has been linked to a decreased mitochondrial electron transport chain function in tissues exhibiting a high aerobic metabolic activity [e.g., skeletal muscle, cardiac muscle, neurons (16,31,63)]. Whereas a decline in skeletal muscle oxidative capacity with aging has been well documented (10–12); with noted exceptions (13,64), distinguishing between a decline which is due to reduced physical activity versus that which is due to aging processes (such as mitochondrial DNA damage) requires consideration of mitochondrial biochemistry.

Although physical inactivity leads to a reduced oxidative capacity, the activity of individual mitochondrial enzymes relative to one another in a given volume of mitochondria remains constant across wide differences in mitochondrial content when comparing physically active versus sedentary animals (65). In contrast, aging has been shown to cause a relatively greater loss in the activity of mitochondrial enzymes that contain mitochondrial DNA-encoded polypeptides (i.e., electron transport chain complexes I, III, IV, and V) than mitochondrial enzymes that are entirely nuclear encoded (e.g., complex II) (62,66). Furthermore, this dissociation in the activities of mitochondrial enzymes is thought to be the result of aging-associated damage to the
mitochondrial genome [secondary to oxidative stress (18,19)], which causes impaired function of the complexes containing polypeptides encoded by the affected regions (16,18,67). Thus, although the observed reduction in voluntary physical activity likely is contributing to a reduction in oxidative capacity with aging in our study, the relatively greater reduction in the flux through electron transport chain complexes I–III than citrate synthase activity suggests that a portion of the reduction in oxidative capacity with increasing age is due to mitochondrial dysfunction.

Müller-Hocker and colleagues were among the first to show that, in extraocular muscles obtained from elderly subjects, fibers exhibiting very low activities of cytochrome oxidase also had very high levels of mitochondrial DNA mutations (68). More recently, mitochondrial DNA deletions have been found in cytochrome oxidase–deficient fibers from aged human limb skeletal muscles (16) and aged rat limb skeletal muscles (67). Indeed, the better maintained proportionality of mitochondrial enzymes with aging seen in long-term calorically restricted animals (62) is consistent with the lower incidence of mitochondrial DNA damage seen with caloric restriction (66,69). Furthermore, a recent study showed that generation of mice with defective mitochondrial DNA polymerase results in a markedly shortened life span, in conjunction with an earlier age-associated accumulation of mitochondrial DNA damage and cytochrome oxidase deficient cells in heart (70). Supporting the idea that mitochondrial DNA mutations are linked to mitochondrial dysfunction, we observed an age-associated increase in the frequency of deletions in mitochondrial DNA extracted from homogenates of the plantaris muscle in conjunction with biochemical evidence of mitochondrial dysfunction. Studies from patients with mitochondrial myopathies suggest that the ratio of mutant to normal mitochondrial DNA (mitochondrial heteroplasmy) within individual cells needs to be quite high (≥60% mutation loads) to affect electron transport chain function (71–73). Given that our measurements were made on whole muscle (not individual cells), it is likely that despite a relatively low deletion load detected in the late middle-aged group, some individual cells may have reached levels necessary to impair electron transport chain function.

The marked reduction in the amount of intact total amplifiable mitochondrial DNA in the senescent animals could limit mitochondrial gene expression and thus, compromise mitochondrial biogenesis in the aged muscles (74). In this latter regard, a reduced amount of mitochondrial DNA in skeletal muscle with aging has been observed in humans (75,76) and rats (74), a change thought to result from exhaustion of mitochondrial DNA repair mechanisms consequent to age-associated acceleration in mitochondrial DNA damage (75). This explanation is supported by the current results showing that a reduced amount of intact amplifiable mitochondrial DNA coincides with a dramatic increase in mitochondrial DNA deletions in the senescent muscles.

We hypothesized that the decrease in oxidative capacity resulting from production of dysfunctional electron transport chain complexes with aging contributes to a reduction in the muscle oxidative capacity, and thus, impairs the muscle’s aerobic function. In support of this hypothesis, we observed that muscle mass-specific VO2max was progressively reduced with increasing age even when provided with similar levels of convective oxygen delivery; a difference that is due in large part to the lower mitochondrial oxidative capacity. Since our results suggest that a portion of the decline in oxidative capacity can be attributed to mitochondrial dysfunction, at least a portion of the decline in VO2max must also be due to mitochondrial dysfunction. Our results are similar in this regard to a recent study of creatine-stimulated respiration in single permeabilized skeletal muscle fibers from aged humans which also concluded that mitochondrial dysfunction contributed to reduce skeletal muscle aerobic function with aging (77). Future studies using strategies for limiting mitochondrial dysfunction with aging (e.g., through dietary manipulations such as caloric restriction) should prove useful in further testing for a link between oxidative stress, mitochondrial dysfunction, and impaired aerobic function of aging skeletal muscles.

Summary

Our current results expand upon our previous study showing reduced ability of late middle aged skeletal muscles to use O2 even when provided similar levels of convective O2 delivery as young adult muscles (7), and show that this decline is accelerated between late middle age and senescence. These results reveal an impairment at one or more points in the flux of O2 from blood to cytochrome oxidase in myocyte mitochondria in aged muscles. In this regard, the relatively greater decline in the flux through electron transport chain complexes I–III than citrate synthase activity with aging is indicative of mitochondrial dysfunction that is the result of age-related mitochondrial DNA damage. Consistent with this notion, not only was there a progressive increase in mitochondrial DNA deletions between late middle age and senescence, there was also a dramatic decrease in the intact total amount of amplifiable mitochondrial DNA in senescent muscles. Therefore, our results support the hypothesis that a portion of the decline in skeletal muscle mass-specific VO2max is due to age-associated mitochondrial dysfunction.

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