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Oxidative stress and the mitochondrial theory of aging in human skeletal muscle

Parise Gianni[a], Kaczor J. Jan[e], Mahoney J. Douglas[d], Phillips M. Stuart[a,d], Mark A. Tarnopolsky[b,c,d,f,*]

[a]*Department of Kinesiology, McMaster University, Hamilton, Ont., Canada*
[b]*Department of Pediatrics, McMaster University, Hamilton, Ont., Canada*
[c]*Department of Medicine, McMaster University, Hamilton, Ont., Canada*
[d]*Department of Medical Sciences, McMaster University, Hamilton, Ont., Canada*
[e]*Department of Biochemistry, Gdansk, School of Physical Education and Sport, J. Sniadecki University, Poland*
[f]*Department of Pediatrics and Medicine, McMaster University Medical Center, Room 4U4, 1200 Main Street West, Hamilton, Ont., Canada L8N 3Z5*

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Abstract

According to the mitochondrial theory of aging, an age-related increase in oxidative stress is responsible for cellular damage and ultimately cell death. Despite compelling evidence that supports the mitochondrial theory of aging in some tissues, data regarding aging skeletal muscle are inconsistent. We collected resting muscle biopsies from the vastus lateralis, and 24 h urine samples from, young ($N = 12$, $\sim$22 yr), and older ($N = 12$, $\sim$72 yr) men. Urinary 8-OHdG was significantly higher in older as compared to younger men (Old: 7714 $\pm$ 1402, Young: 5333 $\pm$ 1191 ng g^{-1} creatinine: $p = 0.005$), as were levels of protein carbonyls (Old: 0.72 $\pm$ 0.42, Young: 0.26 $\pm$ 0.14 nmol mg^{-1} protein: $p = 0.007$). MnSOD activity (Old: 7.1 $\pm$ 0.8, Young: 5.2 $\pm$ 1.8 U mg^{-1} protein: $p = 0.04$) and catalase activity (Old: 8.5 $\pm$ 2.0, Young: 6.2 $\pm$ 2.4 μmol min^{-1} mg^{-1} protein: $p = 0.02$) were significantly higher in old as compared to young men, respectively, with no differences observed for total or CuZnSOD. Full-length mtDNA appeared lower in old as compared to young men, and mtDNA deletions were present in 6/8 old and 0/6 young men ($p = 0.003$). The maximal activities of citrate synthase, and complex II + III, and IV were not different between young and old men, however, complex I + III activity was marginally higher in older as compared to younger men (Old: 2.5 $\pm$ 0.5, Young: 1.9 $\pm$ 0.5 μmol min^{-1} g^{-1} w.w: $p = 0.03$) respectively. In conclusion, healthy aging is associated with oxidative damage to proteins and DNA, a compensatory up-regulation of antioxidant enzymes, and aberrations of mtDNA, with no reduction in electron transport chain maximal enzyme activity.

Keywords: Oxidative damage; 8-OHdG; Protein carbonyls; Mitochondrial DNA damage; Antioxidant enzymes; Electron transport chain enzymes

1. Introduction

The mitochondrial theory of aging represents one of the leading theories on skeletal muscle aging (Harman, 1972). According to this theory, the aging process is mediated by a vicious cycle of events ultimately leading to cellular senescence. Central to this vicious cycle is an increase in oxidative stress, mediated by an increased production of reactive oxygen species (ROS), and/or a reduced antioxidant capacity. The electron transport chain (ETC) is thought to be the main producer of ROS in skeletal muscle, and it has been demonstrated that ROS, produced by the mitochondria, are maintained at a relatively high level inside the mitochondrial matrix (Lee and Wei, 1997). Given the proximity of mitochondrial DNA (mtDNA) and several functional mitochondrial proteins to the primary ROS generator, it is possible that these molecules are at a greater risk of incurring oxidative insults, potentially leading to mitochondrial dysfunction. Congruent with this hypothesis, markers of oxidative damage to DNA are much higher in the mitochondria as compared to the cytosol (Hamilton et al., 2001). Thus, theoretically, age-related oxidative stress is

* Corresponding author. Address: Department of Pediatrics and Medicine, McMaster University Medical Center, 1200 Main Street, Room 4U4, Hamilton, Ont., Canada L8N 3Z5. Tel.: +905-521-2100x75226; fax: +905-521-2656.
E-mail address: tarnopol@mcmaster.ca (M.A. Tarnopolsky).

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thought to lead to mitochondrial DNA (mtDNA) damage, which results in defective ETC proteins, reduced ETC activity, and enhanced production of ROS. Ultimately, these events may lead to cellular aging and cell death. Although this theory describes an elegant relationship between oxidants and aging, many of the studies designed to examine the mitochondrial theory have yielded equivocal or conflicting results.

The literature on human skeletal muscle strongly supports an age-associated increase in oxidative damage to proteins, lipid, and DNA (Mecocci et al., 1999; Pansarasa et al., 1999, 2000; Fano et al., 2001). Higher levels of lipid peroxidation, protein carbonyl content and 8-OHdG (oxidative DNA damage) have been demonstrated in human skeletal muscle of older individuals when compared to younger individuals (Mecocci et al., 1999; Pansarasa et al., 2000; Fano et al., 2001).

It has been suggested that age-related oxidative stress may be a function of a reduction in antioxidant capacity (Ji, 1993), but this has not been demonstrated in human skeletal muscle. In fact, all investigations of aging human skeletal muscle have reported significantly higher levels of at least one antioxidant enzyme (Pansarasa et al., 1999, 2000). Of the few reports in aging skeletal muscle, an increase (Pansarasa et al., 2000) or no change (Pansarasa et al., 1999) in catalase activity has been observed, along with increases in MnSOD, the mitochondrial isoform of superoxide dismutase (Pansarasa et al., 1999, 2000). Interestingly, CuZnSOD, the cytosolic isoform of superoxide dismutase, decreases with age (Pansarasa et al., 1999, 2000), suggesting that there may be a compartmentalization of the origin of oxidant stress in aging. Together, these results suggest that antioxidant capacity is not implicated as a mechanism in age-related oxidative stress, but rather is responsive to age-related oxidative stress.

In support of the mitochondrial theory of aging, a progressive relationship has been established between age and the accumulation of mtDNA deletions and mutations in human skeletal muscle (Melov et al., 1995; Kovalenko et al., 1997, 1998; Liu et al., 1998; Zhang et al., 1998; Cormio et al., 2000; Wanagat et al., 2001; Fayet et al., 2002; Yarovaya et al., 2002). Investigations in skeletal muscle homogenates have reported an accumulation of mtDNA point mutations (Munscher et al., 1993; Zhang et al., 1993; Murdock et al., 2000) as well as deletion products (Cortopassi and Arnheim, 1990; Linnane et al., 1990; Kovalenko et al., 1997) as a function of age, albeit to low levels of total mtDNA. However, isolated single muscle fibers presenting with ETC dysfunction accumulate mtDNA point mutations (Fayet et al., 2002; Lin et al., 2002) and deletion products (Cao et al., 2001; Wanagat et al., 2001) to significant proportion of total mtDNA. Together, this data suggests that age-associated mtDNA anomalies have physiological significance that may be undetectable in whole muscle homogenates. Conversely, it remains unclear whether or not anomalies in single fibers can account for the global effects observed in aging skeletal muscle.

Despite a strong relationship between aging and oxidative damage, and the recent correlation between ETC dysfunction and mtDNA deletions and mutations in single muscle fibers, the literature on the effect of aging on ETC function remains equivocal in humans. Many studies demonstrate a significant age-related reduction in ETC complex enzymes (Cardellach et al., 1989; Trounce et al., 1989; Boffoli et al., 1994; Hsieh et al., 1994), while others demonstrate no age-associated decrease in human skeletal muscle (Zucchini et al., 1995; Barrientos et al., 1996; Chretien et al., 1998; Rasmussen et al., 2003). It has been suggested that the reported age-related reductions in ETC function are not related to the aging process per se, but rather due to other confounding variables such as tobacco consumption and physical inactivity (Barrientos et al., 1996).

The purpose of the present study was to conduct a comprehensive examination of the mitochondrial theory of aging in an attempt to consolidate evidence of the individual components of the mitochondrial theory in human skeletal muscle. Particular attention was paid to oxidative damage, antioxidant enzyme activity, ETC function, and mtDNA.

2. Methods

2.1. Subjects

Healthy older men ($N = 12$, 72 ± 2 yr), and healthy young men ($N = 12$, 22 ± 3 yr) were recruited for the present investigation (Table 1). Subjects were recruited using a strict exclusion criteria including: coronary heart disease, congestive heart failure, uncontrolled hypertension, chronic obstructive pulmonary disease, diabetes mellitus, renal failure, major orthopedic disability, and smoking. Subjects were recreationally active, and had no known pathology or family history of mitochondrial disease. All subjects gave informed consent and the study was approved by the McMaster University and Hamilton Health Sciences Research Ethics Board.

Table 1
Subject characteristics

Dependent variable	Young ($n = 12$)	Old ($n = 12$)
Age (years)	22.8 ± 3.4	71.8 ± 6.2*
Height (cm)	175.4 ± 8.2	175.0 ± 5.0
Body mass (kg)	76.6 ± 13.1	88.1 ± 11.6*
Body fat (%)	25.9 ± 4.9	21.3 ± 3.8*
Fat-free mass (kg)	56.4 ± 8.1	68.3 ± 5.9*

Values are mean ± SD. * denotes a significant difference from young. Alpha was set at $p < 0.05$.

2.2. Tissue and urine collection

A muscle biopsy was taken from the *vastus lateralis* muscle of the dominant leg, 15–20 cm proximal to the knee joint using a modified Bergström needle (5 mm diameter) with suction modification. The biopsy specimen was dissected of fat and connective tissue and immediately frozen in liquid nitrogen. *Approximately 150 mg of muscle was collected from each subject*. All samples were stored at −80 °C for subsequent analysis.

Urine samples were collected at the time of the muscle biopsy. Urine was aliquoted into 2 ml conical polyethylene tubes and stored at −80 °C for subsequent analysis of 8-OHdG and creatinine.

All subjects were required to abstain from strenuous physical activity for 72 h prior to the testing session.

2.3. Markers of oxidative damage

Urinary 8-OHdG was determined using an enzyme-linked immunoassay. The assay was carried out in triplicate utilizing the manufacturer instructions (Genox Corp., Baltimore, MD, USA). Urinary creatinine levels were determined using a standard colorimetric UV spectrophotometric assay (Sigma Chemical Co., St Louis, MO, USA). Intra-assay coefficient of variation (CV) for 8-OHdG was 6.4 and 8.2% for creatinine, determined by comparing the variance between five replicates (*the mean% difference between replicates for the same sample*). 8-OHdG was expressed relative to creatinine to account for between subject differences in muscle mass.

Protein carbonyls were determined by measuring the reactivity of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) as previously described (Levine et al., 1990). Briefly, the tissue (~25 mg) was homogenized in ice cold Hepes buffer (pH 7.2–7.4), containing leupeptin (0.5 μg ml^{-1}), pepstatin (0.7 μg ml^{-1}), aprotinin (0.5 μg ml^{-1}), and phenylmethylsulfonyl fluoride (40 μg ml^{-1}). The soluble protein fraction was separated with centrifugation and then treated with streptamycin sulfate to remove nucleic acids. The protein sample was pipetted into two glass borosilicate tubes and precipitated with trichloroacetic acid (TCA). DNPH (10 mM in 2 M HCl) was added to one tube, and HCl (2 M) to the other. The tubes were incubated in a water bath at 37 °C for 1 h, with vortexing every 10 min. TCA (10% final concentration) was added to both tubes and pellets were recovered via centrifugation. Pellets were washed 3× with ethanol–ethyl acetate (1:1) to remove free DNPH. The precipitated protein was redissolved in 6 M guanidine solution, and the absorbance of both solutions was measured at 370 nm. All samples were analyzed in duplicate on a UV spectrophotometer (UV Visible Chemstation 8453, Hewlett Packard, Delaware City, DE).

2.4. Electron transport chain enzymes and citrate synthase

ETC Enzymes and Citrate Synthase. Tissue preparation and analysis were performed as previously described (Carter et al., 2001). Briefly, approximately 30 mg of wet muscle tissue was homogenized using a glass homogenizer. Assays were preformed on fresh homogenates *from previously snap-frozen tissue*.

Citrate synthase (CS) activity was determined by adding 10 μl of muscle homogenate to a solution of 1 ml 50 mM Tris–HCl buffer at pH 7.8 + 0.05% Triton X-100 plus 2 μl of acetyl CoA (30 mM in H_2O), 0.1 mM dithionitrobenzoic acid (DTNB) and reaction was started with addition of 10 μl of oxaloacetate in a cuvette warmed to 37 °C. Absorbance was recorded at 412 nm every 30 s for 3 min. CS activity was calculated and reported as μmol min^{-1} g $w.w^{-1}$. Three replicates for each sample were compared and intra-assay CV was 3.1%.

Complex I + III activity was measured by the reduction of cytochrome *c* in the presence of NADH, thus measuring the flux of electrons from Complex I through complex III to cytochrome *c*. This was achieved by adding 20 μl of muscle homogenate to 940 μl of potassium phosphate (KPi), 30 μl of oxidized cytochrome *c*, and 10 μl of NADH in a cuvette warmed to 37 °C. Absorbance was measured at 550 nm every 30 s for 3 min. In a separate cuvette, the same sample was analyzed under identical conditions, with the addition of rotenone (0.1 mM). The difference between the two cuvettes represents the rotenone sensitive complex I + III activity. Enzyme activity was calculated and reported in μmol min^{-1} g $w.w^{-1}$. Intra-assay CV was 6.6%.

Complex II + III activity was measured by the reduction of cytochrome *c* in the presence of succinate, thus measuring the flux of electrons from complex II through complex III to cytochrome *c*. Fifteen microliters of muscle homogenate was added to 930 μl of KPi buffer 30 μl of oxidized cytochrome *c*, and 25 μl of succinate (0.4 M) in a cuvette warmed to 37 °C. Absorbance at 550 nm was recorded every 30 s for 3 min. Enzyme activity was calculated and reported in μmol min^{-1} g $w.w^{-1}$. Intra-assay CV was 5%.

Complex IV was determined by measuring the oxidation of cytochrome *c*, by the flow of electrons through cytochrome oxidase. Stock cytochrome *c* (oxidized), was reduced by sodium ascorbate and KPi (10 mM). Fifteen microliters of muscle homogenate was added to 955 μl of KPi, and 30 μl of reduced cytochrome *c* to a cuvette warmed to 37 °C. Absorbance was recorded at 550 nm every 30 s for 3 min. Complex IV activity was calculated and reported in μmol min^{-1} g $w.w^{-1}$. Intra-assay CV was 4.5%.

All samples were analyzed in duplicate on a spectrophotometer as described above.

2.5. Western blots

Muscle samples (~30 mg) were homogenized and prepared for electrophoresis using methods previously

described (Tarnopolsky et al., 2001). The muscle homogenates from young and old men were probed using mouse monoclonal antibodies specific to human subunit II of cytochrome oxidase (Molecular Probes −A6404). This antibody recognizes a polypeptide with a molecular weight of ~25.6 kDa. Post-transfer blots were stained with coomassie blue to ensure equal protein loading between lanes. No difference in protein load were observed between lanes.

2.6. Antioxidant enzymes

Muscle catalase activity was determined by measuring the kinetic decomposition of H_2O_2. Muscle catalase activity was measured by combining 970 μl of K_2HPO_4 buffer (50 mM with 5.0 mM EDTA, and 0.01% Triton X-100, pH 7.2–7.4), with 30 μl of muscle homogenate. Ten microliters of H_2O_2 (1 M) was added to the cuvette and mixed by inversion to initiate the reaction. Absorbance was measured at 240 nm every 15 s for 2 min. Catalase activity was calculated and reported in μmol min^{-1} mg $protein^{-1}$. Intra-assay CV was 8.2%.

Muscle total SOD activity was determined by measuring the kinetic consumption of O_2^- by superoxide dismutase in a competitive reaction with cytochrome *c*. Muscle total SOD activity was measured by combining 955 μl of K_2HPO_4 buffer (50 mM with 0.1 mM EDTA, pH 7.8) containing partially acetylated cytochrome *c* (25 mg/100 ml) and xanthine (0.5 μM). Thirty microliters of muscle homogenate was added to the cuvette and mixed by inversion. Fifteen microliters of xanthine oxidase (0.2 U/ml) was added to initiate the reaction, and absorption at 550 nm was observed every 15 s for 2 min. One unit of SOD activity was defined as the amount of enzyme, which caused a 50% inhibition of the reduction of cytochrome *c*. Activity was calculated and expressed in U mg $protein^{-1}$.

In a separate cuvette, the same sample was analyzed under identical conditions with the addition of 10 μl of 0.2 M KCN (pH 8.5–9.5) for determination of Mn SOD activity. KCN is known to inhibit CuZn SOD activity as described by Higuchi and colleagues (1985). CuZn SOD was approximated by subtracting Mn SOD activity from total SOD activity, and was expressed in U mg $protein^{-1}$.

All antioxidant enzymes were measured using approximately 30 mg of wet muscle and all samples were analyzed in duplicate using a spectrophotometer as described above.

2.7. Mitochondrial DNA deletions

Mitochondrial DNA deletions were determined using long-range polymerase chain reaction (XL-PCR), as previously described (Kovalenko et al., 1997). Briefly, DNA was extracted from approximately 25 mg of wet muscle using a PUREGENE® DNA isolation kit, as per the instruction of the manufacturer (Gentra Systems, Minneapolis, MN). Two PCR primer sets were designed approximately 2000 bp apart, and all samples were run using both sets of primers. One set of primers were designed to begin amplification in the 16S rRNA region (3310L (30nt)—CCC ATG GCC AAC CTC CTA CTC CTC ATT GTA, 2690H (28nt)—TCT TGC TGT GTT ATG CCC GCC TCT TCA C) and a second pair was designed to begin amplification in the cytochrome *b* (cyt *b*) region (15148L (27nt)—GTG AGG CCA AAT ATC ATT CTG AGG GGC, 14841H (26nt)—TCT TGC TGT GTT ATG CCC GCC TCT TCA C). Methodological experiments determined that the optimal template concentrations for the 16s primers was 125 ng of total DNA, whereas the optimal template concentration for the cyt *b* primers was 100 ng of total DNA. Amplification was achieved using the Expand Long Template PCR System (1,681,842, Roche Biochemicals, Mannheim, Germany), utilizing a final concentration of 500 μM of each dNTP, 300 nM of each primer, 2.25 mM of $MgCl_2$ in 10 × Buffer 3, and 3.3 units of the Long Template Enzyme mixture. Mineral oil (30 μl) was used to prevent evaporation. Optimal conditions for the reactions consisted of 2 min hot-start at 92 °C, 10 cycles of 92 °C for 10 s to denature, 71 °C for 30 s for primer annealing, and 68 °C for 12 min and 30 s for elongation. An additional 16 cycles were performed at these same temperatures however for each subsequent cycle after the initial 10 cycles the elongation time was increased by 20 s. At the end of a total of 26 cycles there was a final 7 min elongation step at 68 °C. Ten microliters of the final product was run on an agarose gel containing ethidium bromide for 1 h at 100 V. Gels were visualized on a UV light gel documentation system, and analyzed using a commercially available software package. XL-PCR was used exclusively for *qualitative* analysis of mtDNA between young and old. Therefore, all results pertaining to mtDNA *content* were not subject to statistical analysis and are simply reported as observations.

2.8. Statistical analysis

Statistical analysis was performed using a commercially available software package (Statistica, V. 5.0, Tulsa, OK). All variables were statistically analyzed using an independent *t*-test. Alpha was established at $p < 0.05$, and all values were reported as mean ± standard deviation (SD). The effect of aging on the *prevalence* of mtDNA deletion products was analyzed using Fisher's Exact test.

3. Results

All subjects were not included in every variable measured due to insufficient muscle sample size from some subjects. Sample size is reported for each variable independently.

3.1. Subjects

Older men were significantly heavier ($p = 0.03$), had a greater fat-free mass ($p = 0.0006$), and had a lower % fat mass ($p = 0.017$) (Table 1). These results are evidence that we recruited healthy older individuals. Furthermore, these results suggest that any age-related changes we observed were due to a true effect of the aging process per se as opposed to other confounding variables such as inactivity.

3.2. Oxidative damage

Oxidative DNA damage in the form of urinary 8-OHdG was *on average* 44% higher in old ($N = 11$, 7714 ± 1402 ng g creatinine^{-1}) versus young men ($N = 11$, 5333 ± 1191 ng g creatinine^{-1}) ($p = 0.0003$) (Fig. 1A). In addition, oxidative damage in the form of protein carbonyls was *on average* 170% higher in old ($N = 9$, 0.72 ± 0.42 nmol mg protein^{-1}) versus young men ($N = 9$, 0.26 ± 0.14 nmol mg protein^{-1}) ($p = 0.007$) (Fig. 1B).

3.3. Antioxidant enzymes

Muscle catalase activity was *on average* 37% higher in the old ($N = 12$, 8.5 ± 2.0 μmol min^{-1} mg protein^{-1}) compared to young ($N = 12$, 6.2 ± 2.4 μmol min^{-1} mg protein^{-1})

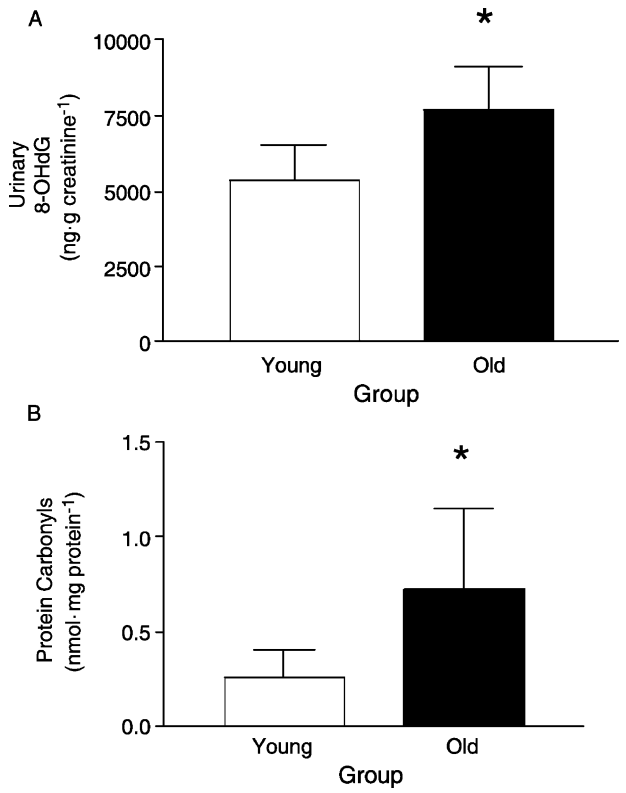

Fig. 1. (A) The effect of aging on levels of urinary 8-OHdG. (B) Demonstrates the effect of aging on levels of muscle protein carbonyls. * denotes a significant difference between young and old. Alpha was set at $p < 0.05$.

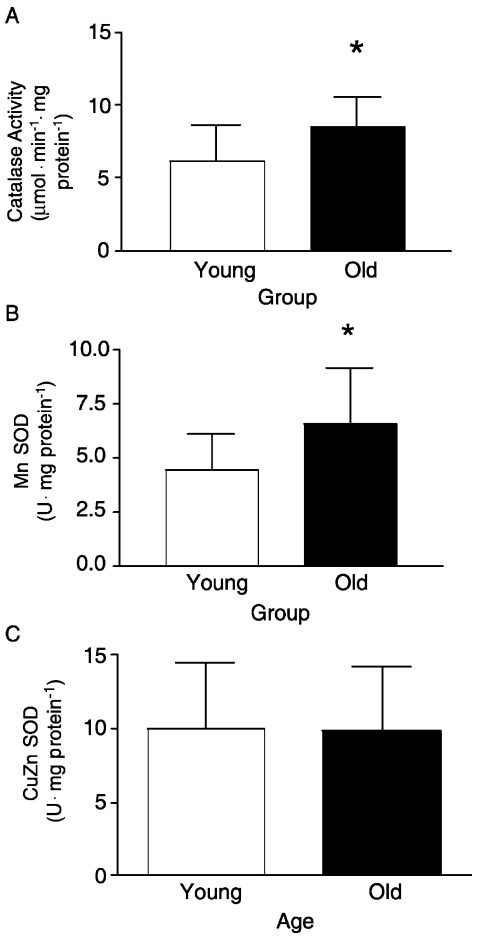

Fig. 2. (A) The effect of age on muscle catalase activity. (B) Demonstrates the effect of aging on muscle MnSOD activity. (C) Demonstrates the effect of aging on muscle CuZnSOD activity. * denotes a significant difference between young and old. Alpha was set at $p < 0.05$.

men ($p = 0.02$) (Fig. 2A). Mn SOD was also found to be *on average* 37% higher in old ($N = 6$, 7.1 ± 0.8 U mg protein^{-1}) versus young men ($N = 6$, 5.2 ± 1.8 U mg protein^{-1}) ($p = 0.04$) (Fig. 2B). Conversely, CuZn SOD was not different between groups (old: $N = 6$, 11.2 ± 2.8, young: $N = 6$, 10.8 ± 6.1 U mg protein^{-1}) (Fig. 2C).

3.4. Electron transport chain enzymes

Complex I + III activity was significantly higher in old ($N = 9$, 2.5 ± 0.5 μmol min^{-1} g^{-1} w.w) as compared to young ($N = 9$, 1.9 ± 0.5 μmol min^{-1} g^{-1} w.w) ($p = 0.03$) men, respectively. There were no other significant differences for any of the other oxidative enzymes (Table 2).

3.5. Mitochondrial DNA deletions

We observed significantly more mtDNA deletion products in the old versus the young ($p = 0.003$). Six of the eight older individuals examined presented with mtDNA deletions, whereas none of the eight young individuals had any visible deletion products. Older adults also appeared to

Table 2
ETC and CS activity in young and old men

Enzyme	Young ($n = 12$)	Old ($n = 12$)
Citrate synthase	11.5 ± 2.6	13.2 ± 4.5
Complex I + III	1.9 ± 0.5	2.5 ± 0.5*
Complex II + III	1.9 ± 0.6	1.8 ± 0.6
Complex IV	5.7 ± 2.7	6.7 ± 1.0

Values are mean ± SD, and are expressed as $\mu mol\ min^{-1}\ g^{-1}$ w.w.
* denotes a significant difference from young. Alpha was set at $p < 0.05$.

have less normal length mtDNA (37% *on average*) than young adults determined using optical density of DNA bands (Fig. 3). Results were similar using both primer pairs, however Fig. 3 depicts only results using the primers in the 16S rRNA region.

3.6. Western blots

Western blots for a mitochondrial DNA encoded subunit of COX revealed no age related differences in protein content (Fig. 4).

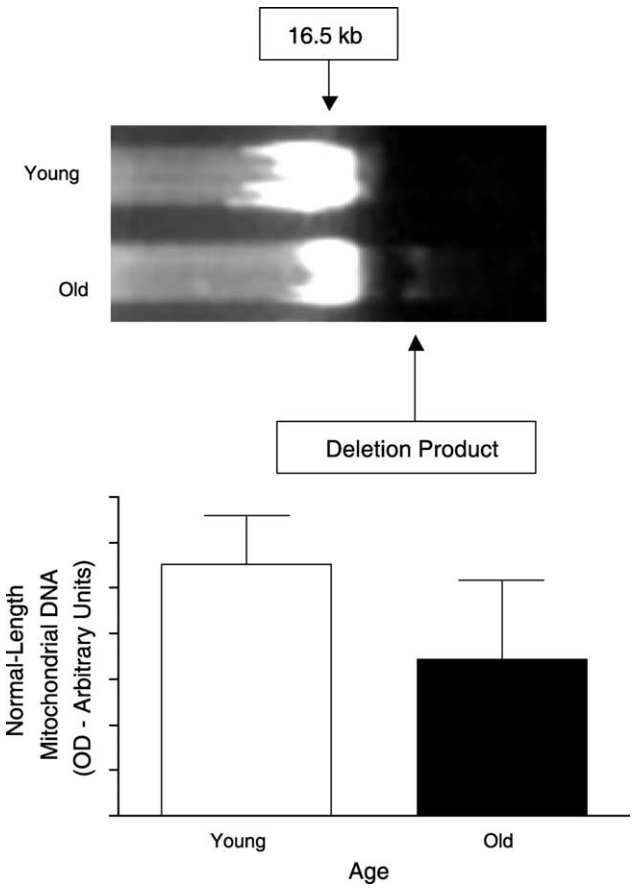

Fig. 3. The effect of age on mtDNA. Note an apparent reduction (37%) in normal length mtDNA. We also observed that 6/8 old subjects demonstrated an accumulation of deletion products, whereas none of the young subjects demonstrated any deletion products. This figure is representative of results using the 16S rRNA primer set.

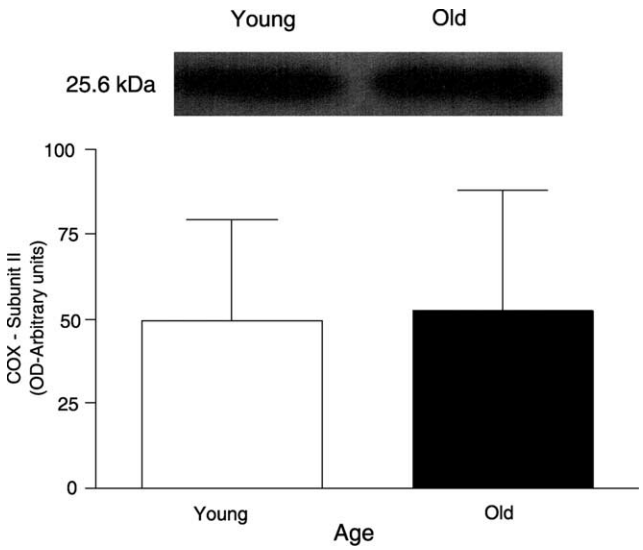

Fig. 4. Protein content of subunit II of COX was not different between young and old ($p > 0.05$).

4. Discussion

Although many investigations have examined components of the mitochondrial theory in isolation, the present study represents the most comprehensive examination of several aspects of the mitochondrial theory in human skeletal muscle simultaneously. Our data suggest that there is an age-related increase in oxidative damage to protein (carbonyls) and DNA (8-OH-2dG), associated with an increase in mitochondrial DNA deletions and an apparent reduction in full-length mtDNA. Furthermore, aging was associated with a significant increase in mitochondrial SOD, catalase but not cytosolic SOD. Interestingly, ETC maximal enzyme activity was not reduced by age suggesting that low level mitochondrial DNA deletions are not associated with ETC defects at the whole muscle level.

Oxidative damage to the cellular environment is a major tenet of the mitochondrial theory of aging. It is thought that accumulation of damaged products ultimately leads to cellular dysfunction, and eventual cell death. In the present study we demonstrated a *significantly* higher level of oxidative DNA damage, measured as urinary 8-OHdG, and an even more impressive higher level of total protein carbonyls in skeletal muscle, in older versus younger men. These results are in accordance with others who have demonstrated significant age-associated increases in oxidative damage to human muscle DNA, proteins and lipids (Mecocci et al., 1999; Pansarasa et al., 1999, 2000; Miro et al., 2000; Fano et al., 2001). The observed higher levels of urinary 8-OHdG is suggestive of an age-related increase in DNA damage, however we cannot distinguish whether the damage was incurred in mitochondrial or nuclear DNA. It is worth noting, however, that higher levels of 8-OHdG in old liver, heart and brain of rats and mice was

found to be 6–23 fold higher in mitochondrial as compared to nuclear DNA (Hamilton et al., 2001).

Miro and colleagues (2000) demonstrated that despite significant age-associated increases in lipid peroxidation of human heart, there was no measured correlation between oxidative damage and ETC enzyme activity. We also report normal ETC function despite age-associated increases in oxidative damage. Recent work suggested that protein in skeletal muscle is more sensitive to oxidative stress in old as compared to young rats (Radak et al., 2002) and our robust increase in protein carbonyls in the current study supports this finding. This suggests that oxidative damage may not be dependent upon, or result in ETC dysfunction, and that old proteins may simply be more susceptible to damage. However, it is important to note that the ETC enzyme activity in the current study represents the maximal in vitro activity and alterations in protein structure could alter other kinetic properties of the enzyme.

Oxidative stress can be the result of either an increase in the production of ROS or a reduction in the antioxidant capacity. Some studies have suggested that a reduction in antioxidant capacity contributes to age-related oxidative stress (Ji, 1993), however, more recent studies have demonstrated that antioxidant capacity adaptively increases with age in human skeletal muscle (Pansarasa et al., 1999, 2000). Consistent with these findings, we report a higher level of catalase activity in older men. Interestingly, Pansarasa and colleagues (1999, 2000) have reported an age-related increase in catalase activity, and in a separate investigation reported no change. Despite the variable results, neither of these studies suggested an age-related decrease in catalase activity, which might contribute to age-related oxidative stress. In the present study we also demonstrated an age-associated higher level of MnSOD activity in older versus younger men, whereas we observed no difference in CuZnSOD between age groups. MnSOD (SOD2) is localized to the inner membrane of the mitochondria, while CuZnSOD (SOD1) is found primarily in the cytosol. Compartmentalization of these isoforms suggests that they may be differentially affected, depending on the source of the oxidant stress. Pansarasa and colleagues (2000, 1999) demonstrated significantly higher levels of MnSOD in old men and women (66–91 yr), as compared to young men and women (17–40 years) in human skeletal muscle. Furthermore, they reported that total SOD activity was lower in the old than in the young, suggesting that CuZn SOD decreased with age since total SOD is comprised primarily of cytosolic CuZn SOD and mitochondrial Mn SOD. Together, these results suggest that aging is associated with a superoxide associated mitochondrial stress, with no apparent cytosolic stress. Given the short half-life of superoxide, and its inability to freely cross membranes (Lynch and Fridovich, 1978), any stress from the mitochondrial ETC will be realized only in the mitochondria. Conversely, H_2O_2 has a relatively long half-life and possesses the ability to freely cross membranes (Matsuo and Kaneko, 2000). Thus, we suggest that the cytosol may be associated with an age-related oxidative stress, potentially derived from the mitochondria, however not likely from superoxide. This concept is supported by our observation of an age-associated higher activity of catalase but not CuZnSOD. Together, results in the present study and those reported in the literature demonstrate a rather robust increase in MnSOD activity, no change or a decrease in CuZnSOD activity, and an increase in catalase activity. Therefore, we conclude that antioxidant enzymes respond to age-related oxidative stress by up-regulation, and reduced activity (Ji, 1993) does not appear to be the underlying mechanism of age-related oxidative stress.

It is suggested that an increased production of ROS leads to damage of mtDNA in the form of point mutations and deletions. As a consequence, these mutations and deletions are thought to lead to ETC dysfunction and enhance production of ROS. In the present study, using XL-PCR it appeared that older men had less full-length mtDNA, and that aging was associated with a significantly greater number of mtDNA deletion products. Although this method is largely qualitative, the results between the young and the old were striking and are in accordance with previously reported findings. It has previously been shown that aging is associated with a reduction in full-length mtDNA (Kovalenko et al., 1997). Moreover, evidence demonstrates an age-related accumulation of mtDNA deletion products (Melov et al., 1995; Kovalenko et al., 1997; 1998; Liu et al., 1998; Zhang et al., 1998; Cormio et al., 2000; Wanagat et al., 2001; Fayet et al., 2002; Yarovaya et al., 2002). The mechanism(s) responsible for such changes in the mitochondrial genome remain unclear. It is speculated that ROS are central to this process, however a causal link remains elusive. In loose support of this concept, we recently demonstrated that exercise associated with an increase in 8-OHdG, and total protein carbonyls, resulted in the acquisition of mtDNA deletion products in young individuals in the post exercise period, suggesting that oxidative stress may be the causal factor for the induction of anomalies in mtDNA (unpublished observations).

An unresolved issue, with respect to the role of mtDNA in aging, is the role of low-level mtDNA deletions in skeletal muscle. Traditionally, mtDNA deletions have been reported to accumulate to low levels in whole muscle homogenates (Linnane et al., 1990; Katayama et al., 1991; Cortopassi et al., 1992; Simonetti et al., 1992; Melov et al., 1995; Schwarze et al., 1995; Eimon et al., 1996; Kovalenko et al., 1997). Recent evidence, however, has demonstrated that mtDNA deletions and mutations accumulate to high levels in single fibers, and are associated with ETC dysfunction, as well as muscle fiber atrophy (Cao et al., 2001; Wanagat et al., 2001). Moreover, these deletions appear to preferentially amplify in a stochastic manner (Brierley et al., 1998). It is possible that low-level deletions in whole muscle homogenates simply reflect a relatively

small number of fibers with high levels of deletions products.

ETC function in human skeletal muscle with aging remains a controversial field of research. To date, results in human skeletal muscle remain equivocal, with studies demonstrating a significant age-related reduction in ETC complex enzymes (Trounce et al., 1989; Boffoli et al., 1994), and others demonstrating no age-associated decrease (Zucchini et al., 1995; Barrientos et al., 1996; Chretien et al., 1998; Rasmussen et al., 2003). Chretien and colleagues (1998), reported that there was no apparent relationship between aging and complex I, II, III, or IV activity in mitochondria isolated from human deltoid muscle. In addition, individual respiratory chain enzyme activities in human muscle were shown to be lower with age, however after including tobacco consumption and physical activity as confounding variables there was no apparent age-related decrease, suggesting that ETC function does not change as a result of 'normal' aging (Barrientos et al., 1996). A recent report by Rassmussen and colleagues (2003) demonstrated that there was no age-associated reduction in ETC enzyme activity, leading the authors to conclude that the mitochondrial theory of aging is not supported in human skeletal muscle. Indeed, we also report no decrease in ETC activity between young and old muscle using biochemical measurements of tissue homogenates. In support of this finding we observed no differences in protein expression for a mitochondrial-encoded sub-unit of cytochrome oxidase, despite a significant decrease in amplifiable full-length mtDNA. This may be due to an increase in mitochondrial mRNA stability with age (Barazzoni et al., 2000), and/or to the strict exclusion criteria we utilized in recruiting our subjects, accounting for the variables that Barrientos and colleagues (1996) identified as confounding for ETC function. Interestingly, we measured a significantly higher complex I + III activity in old muscle tissue as compared to young. The assays we used in these experiments followed NADH through the reduction of cytochrome *c* for complex I + III, and succinate through the reduction of cytochrome *c* for complex II + III. Given there was no difference between young and old in complex II + III activity, and complex III activity was constant in both of these assays, it is likely that the significantly higher activity of complex I + III can be attributed to complex I activity alone. This is the first time such an observation has been reported. It is possible that there are protein specific responses in the cell attempting to adapt to age-related changes to allow normal function. In the current study we used whole muscle homogenates in the analysis of ETC function, and thus may have missed subtle age-related changes in single fibers, as recently described (Brierley et al., 1998; Cao et al., 2001; Wanagat et al., 2001; Kopsidas et al., 2002). Alternatively, a healthy older population may demonstrate a true maintenance, or enhanced function in the ETC.

Based on the present findings we suggest that the mitochondrial theory of aging may be apparent in human skeletal muscle. Based on results in the current study, and those in the literature we propose a model of aging characterized by a degree of redundancy and adaptation throughout the aging process that allows the cell to function normally in the face of accumulating abnormalities. As others have reported (Mecocci et al., 1999; Pansarasa et al., 1999, 2000; Miro et al., 2000; Fano et al., 2001), we also report accumulating cellular abnormalities in the form of oxidatively modified DNA and proteins. In addition, as others have suggested (Linnane et al., 1990; Katayama et al., 1991; Cortopassi et al., 1992; Simonetti et al., 1992; Melov et al., 1995; Schwarze et al., 1995; Eimon et al., 1996; Kovalenko et al., 1997), we also report apparent decreases in full-length mtDNA, and an accumulation of deletions. A decrease in full-length mtDNA, with a significant number of visible deletion products, without a concomitant loss of ETC function suggests that there may be a level of redundancy to protect the cell against mitochondrial energy depletion. Congruent with this finding is data from experiments using cybrids that suggests that ETC function is maintained until approximately 90% of mtDNA is deleted (Attardi et al., 1995; Moraes and Schon, 1996). As cellular abnormalities accumulate, it is entirely possible that cellular adaptations occur to counteract these events. In the present investigation we report increases in antioxidant capacity, in particular MnSOD and catalase, in old as compared to young muscle. A somewhat unexpected finding was a specific up-regulation of complex I activity. Although this has never before been described in skeletal muscle, this apparent adaptation may represent an attempt to maintain ETC function. Perhaps in frail adulthood, the combination of accumulating abnormalities and a reduction in adaptive potential cross a threshold by which cellular function cannot be maintained in the 'normal' range. Furthermore, we hypothesize that crossing this threshold is associated with frailty and may be the cause of sharp

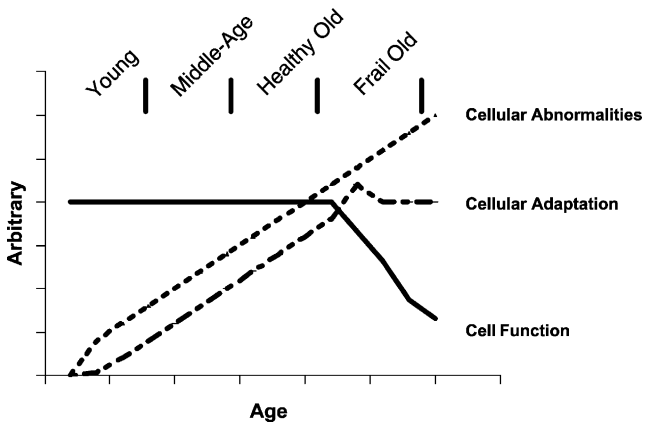

Fig. 5. A model of aging. Through redundancy and adaptation normal cell function is maintained in the presence of cellular abnormalities. Once a critical threshold is crossed (frail adulthood), with accumulating abnormalities, and reduced adaptability, there is a loss of cellular function. This theory is compatible with observations made in human skeletal muscle.

declines in muscle function (and ETC enzyme activity) with advanced age (Fig. 5).

In summary, our results lend support to the mitochondrial theory of aging in skeletal muscle. Increases in oxidative damage, mtDNA rearrangements, and antioxidant enzyme capacity all implicate oxidative stress as an important factor in the aging process of human skeletal muscle. Lack of a decrease in ETC function associated with age, measured in whole muscle homogenates, continues to cast doubt on the importance of low-level mtDNA deletions to whole muscle function. This phenomenon, however, may be indicative of cellular redundancy and adaptation and does not rule out the mitochondrial theory of aging as a potential mechanism underlying skeletal muscle aging.

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