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Original Contribution

Age-related alterations in oxidatively damaged proteins of mouse skeletal muscle mitochondrial electron transport chain complexes

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ABSTRACT

Age-associated mitochondrial dysfunction is a major source of reactive oxygen species (ROS) and oxidative modification to proteins. Mitochondrial electron transport chain (ETC) complexes I and III are the sites of ROS production and we hypothesize that proteins of the ETC complexes are primary targets of ROS-mediated modification which impairs their structure and function. The pectoralis, primarily an aerobic red muscle, and quadriceps, primarily an anaerobic white muscle, have different rates of respiration and oxygen-carrying capacity, and hence, different rates of ROS production. This raises the question of whether these muscles exhibit different levels of oxidative protein modification. Our studies reveal that the pectoralis shows a dramatic age-related decline in almost all complex activities that correlates with increased oxidative modification. Similar complex proteins were modified in the quadriceps, at a significantly lower level with less change in enzyme and ETC coupling function. We postulate that mitochondrial ROS causes damage to specific ETC subunits which increases with age and leads to further mitochondrial dysfunction. We conclude that physiological characteristics of the pectoralis vs quadriceps may play a role in age-associated rate of mitochondrial dysfunction and in the decline in tissue function.

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Introduction

Sarcopenia, or muscle weakness, is a major physiological sign of aging in humans [1–4]. The mitochondrial theory of aging proposes that increasing oxidative stress resulting from progressive mitochondrial dysfunction is a mechanism of mammalian aging and may be an important physiological characteristic of muscle aging [5,6]. It is well established that although ROS are generated by multiple compartments, e.g., the plasma membrane NADPH oxidases [7], peroxisomal lipid metabolism, and cytosolic enzymes such as cyclogenases, the majority (~90%) of ROS is generated by mitochondrial dysfunction [8]. The generation of mitochondrial ROS, in vivo, is linked to the processes of oxidative phosphorylation carried out by the mitochondrial electron transport chain complexes. Thus, leakage of electrons, i.e., mitochondrial ROS, is produced in vivo, from ETC complexes during normal respiration, particularly from Complex I (CI) and Complex III (CIII) [9–11]. Increased ROS production and oxidative stress during the aging process are, therefore, attributed to mitochondrial dysfunction, and the age-associated decline in tissue function is proposed to be a consequence of dysfunctional mitochondria [12–14].

Mitochondrially generated ROS are difficult to measure directly both in vitro and in vivo. Although the production of ROS in CI and CIII has been estimated by electron paramagnetic resonance [15–17], the short half-life of these radicals makes their accurate measurement difficult. Since the products of ROS reaction with proteins are stable and easily measured we have proposed that the amount of modified protein may be indicative of the level of oxidative modification in tissues [13]. Furthermore, since oxidatively modified proteins have been shown to impair the functions of these proteins [18–21] we have initiated these studies to characterize the role of these modifications on the development of mitochondrial dysfunction in aged pectoralis (aerobic) and quadriceps (anaerobic) skeletal muscle.

The covalent oxidative protein modifications caused by reactions with free radicals are more stable and easily detectable, and thus have been used as molecular markers of oxidative stress [17,22–24]. The relative abundance of modified proteins has been used in our laboratory to indicate the level of oxidatively modified proteins that accumulate in aged tissues [22–24]. Protein modifications caused by

Abbreviations: ACAD1, acetyl-CoA dehydrogenase 1; ACADV1, acetyl-CoA dehydrogenase V1; ATP5A1, complex V α chain; ATP5B, complex V β chain; BN-PAGE, blue-native polyacrylamide gel electrophoresis; CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CV, complex V; CoQ, coenzyme Q; COX1, cytochrome *c* oxidase subunit 1; COX2, cytochrome *c* oxidase subunit 2; CS, citrate synthase; DNP, 2,4-dinitrophenylhydrazone; DNPH, 2,4-dinitrophenylhydrazine; ETC, electron transport chain; FH1, fumarate hydratase 1; HNE, 4-hydroxynonenal; ISP, Rieske iron-sulfur protein; MALDI-TOF-TOF, matrix-assisted laser desorption/ionization–time of flight-time of flight; MDA, malondialdehyde; MDH2, malate dehydrogenase 2; MPP, mitochondrial processing peptidase; NDUFS1, NADH dehydrogenase Fe–S subunit 1; NDUFS2, NADH dehydrogenase Fe–S subunit 2; ROS, reactive oxygen species; SDHA, succinate dehydrogenase subunit 1; UQCRC1, Core 1 subunit; UQCRC2, Core 2 subunit; UQCRFS1, Rieske iron–sulfur protein.

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ROS include the formation of lipid peroxidation adducts (4-hydroxynonenal or HNE and malondialdehyde or MDA) on lysine, histidine, and cysteine, nitration of tyrosine and cysteine, and carbonylation of lysine, arginine, proline, and threonine [18,19,25,26]. Oxidatively modified proteins have been detected by immunoblotting using antibodies specific for these modifications and subsequently identified by mass spectrometry [17,22–24]. In addition, such oxidative modifications to proteins can result in reduction of function associated with aging and age-associated diseases [18,19,21–27]. These oxidative modifications are, therefore, molecular markers that provide insight into the cumulative effects of oxidative stress on the molecular mechanisms of aging and development of age-associated diseases.

In this study we analyzed mitochondria isolated from young, middle-aged, and old mouse pectoralis and quadriceps skeletal muscles because of their aerobic vs anaerobic physiological characteristics, to determine whether aging affects the activities of their ETC complexes CI–CV. We chose, specifically, the pectoralis which is an adductor, internal rotator, and flexor of the shoulder; its fibers, which are slow twitch (type I), consist of high myoglobin levels which improves the delivery of oxygen, and high mitochondrial content, are primarily red muscle and highly aerobic. Secondly, we chose the quadriceps which consists of fast-twitch type I fibers whose physiological characteristics include fewer mitochondria and less myoglobin. The large store of glycogen and high levels of glycolytic enzymes enable these fibers to respire anaerobically. We tested, therefore, whether specific ETC CI–CV proteins of the highly aerobic pectoralis and primarily anaerobic quadriceps muscles are differentially susceptible to oxidative modification due to their location within the mitochondria, whether the levels of these modifications increase, and whether their functions are altered with aging. To achieve this we identified the oxidatively modified subunits of ETC CI–CV and correlated the levels of protein modification with changes in enzyme activities. Blue-native polyacrylamide gel electrophoresis (BN-PAGE) was used to resolve intact ETC complexes [28] followed by second-dimension denaturing SDS-PAGE to resolve individual complex subunits. Protein abundance of each complex was measured using antibodies to specific complex subunits to quantify age-related changes [22,23]. Using immunobloting with antibodies recognizing specific types of oxidative damage, we detected proteins that were carbonylated, and modified by HNE, MDA, and tyrosine nitration. Proteins shown to be differentially modified were identified by MALDI-TOF-TOF mass spectrometry. These studies identify whether there is a differential susceptibility of highly aerobic skeletal muscle ETC complexes compared to the anaerobic skeletal muscle. Finally, we determined whether there is a direct correlation between increased protein modification on enzyme function associated with the ETC complexes that might suggest a progressive increase in endogenous oxidative stress during aging. Our study systematically identifies the effects of oxidative protein modification on ETC activity, on possible specificity of targeted oxidative modification and whether these modifications play a role in causing symptoms of the aging, of the pectoralis and quadriceps.

Materials and methods

Animals

Young (3–5 months), middle–aged (12–14 months), and old (20–22 months) male C57BL/6 mice, purchased from the National Institute on Aging (Bethesda, MD), were maintained in a pathogen-free facility at 27°C, 45–55% humidity, on a 14/10 light/dark cycle and fed ad libitum on an NIH low-fat diet (Purina) 4% fat by weight.

Mitochondrial isolation

Mice were sacrificed by cervical dislocation and their skeletal muscles were harvested immediately, rinsed in ice-cold PBS, and prepared for subcellular fractionations. Mitochondria were prepared from the pooled muscles of 9 young, 10 middle-aged, and 8 old C57BL/6 male mice. Mitochondrial isolation was carried out at 4°C as described with minor modifications [22,24,29].

Enzyme activities

Enzyme activities were measured at room temperature using a Beckman Coulter DU 530 spectrophotometer (Beckman Coulter, CA). Citrate synthase activity was measured as described [22,30]. Rotenone-sensitive Complex I (CI) activity, malonate-sensitive Complex II (CII) activity, antimycin A-sensitive Complex III (CIII) activity, KCN-sensitive Complex IV (CIV) activity, and oligomycin-sensitive Complex V (CV) activities were assayed as described [22,27,31]. CI–III and CII–III coupled assays were performed as described [25,32–34].

Coenzyme Q levels

Total mitochondrial coenzyme Q was quantified using an HPLC method described previously [22,33,34].

Polyacrylamide gel electrophoresis

BN-PAGE and SDS-PAGE were carried out by established methods [26] with minor modifications as described previously [17,22,28].

Immunobloting

Immunoblot analysis was performed as described [22,24].

MALDI-TOF-TOF: Sample preparation

Individual ROS-modified protein bands were excised from second-dimension SDS-PAGE run simultaneously with the gels that were immunoblotted and analyzed by the Proteomics Core Facility at UTMB. Gel samples were cut into ~1-mm-size pieces and placed into separate 0.5-ml polypropylene tubes. Ammonium bicarbonate buffer (50mM; 100μl) was added to each tube and the samples were then incubated at 37°C for 30min. After incubation, the buffer was removed, 100μl of water was added to each tube, and the samples were incubated again at 37°C for 30min. After incubation, the water was removed and 100μl of acetonitrile was added to each tube to dehydrate the gel pieces. The samples were vortexed, and after 5min the acetonitrile was removed. Acetonitrile (100μl) was again added to each of the sample tubes and vortexed, and acetonitrile removed after 5min. The samples were then placed in a speedvac for 45min to remove excess solvent.

Lyophilized trypsin (20μg; Promega Corp.) was added to 2ml of 25mM ammonium bicarbonate, pH 8.0. The trypsin solution was then vortexed and added to each sample tube in an amount (~10μl) to just cover the dried gel. The samples were then incubated at 37°C for 6h.

After digestion, 1μl of the sample solution was spotted directly onto a MALDI target plate and allowed to dry. Alpha-cyano-4-hydroxycinnamic acid (1μl; Aldrich Chemical Co.) matrix solution (50/50 acetonitrile/water at 5mg/ml) was then applied on the sample spot and allowed to dry. The dried MALDI spot was blown with compressed air (Decon Laboratories, Inc.) before inserting into the mass spectrometer.

Mass spectrometry

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF-MS) was used for protein identification. Data were acquired with an Applied Biosystems 4800 MALDI TOF/TOF Proteomics analyzer. Applied Biosystems software package included 4000 Series Explorer (v. 3.6 RC1) with Oracle Database Schema

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