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Advanced aging causes diaphragm functional abnormalities, global proteome remodeling, and loss of mitochondrial cysteine redox flexibility in mice

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ABSTRACT

Aim: Inspiratory muscle (diaphragm) function declines with age, contributing to exercise intolerance and impaired airway clearance. Studies of diaphragm dysfunction in rodents have focused on moderate aging (~24 months); thus, the impact of advanced age on the diaphragm and potential mechanisms of dysfunction are less clear. Therefore, we aimed to define the effects of advanced age on the mechanics, morphology, and global and redox proteome of the diaphragm.

Methods: We studied diaphragm from young (6 months) and very old male mice (30 months). Diaphragm function was evaluated using isolated muscle bundles. Proteome analyses followed LC-MS/MS processing of diaphragm muscle.

Results: Advanced aging decreased diaphragm peak power by ~35% and maximal isometric specific force by ~15%, and prolonged time to peak twitch tension by ~30% ($P < 0.05$). These changes in contractile properties were accompanied, and might be caused by, decreases in abundance of calsequestrin, sarcoplasmic reticulum Ca^{2+}-ATPase, sarcalumenin, and parvalbumin that were revealed by our label-free proteomics data. Advanced aging also increased passive stiffness ($P < 0.05$), which might be a consequence of an upregulation of cytoskeletal and extracellular matrix proteins identified by proteomics. Analyses of cysteine redox state indicated that the main diaphragm abnormalities with advanced aging are in metabolic enzymes and mitochondrial proteins.

Conclusion: Our novel findings are that the most pronounced impact of advanced aging on the diaphragm is loss of peak power and disrupted cysteine redox homeostasis in metabolic enzymes and mitochondrial proteins.

1. Introduction

Age-related loss of muscle mass and function are significant predictors of morbidity and mortality (Kalyani et al., 2014). Weakening of limb muscles is well documented and extensively investigated in aging, and this weakness is accentuated in advanced aging (Lindle et al., 1997; Metter et al., 1997). However, loss of inspiratory muscle size and function with age is often overlooked. Multiple reports show decreases in maximal inspiratory muscle pressure (MIP) with aging, which becomes more pronounced with advanced age (Britto et al., 2009; Polkey et al., 1997; Tolep et al., 1995). This age-related loss in MIP does not impair resting ventilatory function. However, the decline in MIP will compromise expulsive behaviors like coughing and sneezing that require maximal force. As MIP decreases, mobility disability, morbidity, and mortality increase significantly (Buchman et al., 2009; Buchman et al., 2008).

The diaphragm is the main inspiratory muscle and a key contributor to measures of MIP. Animal studies show that the aged diaphragm exhibits reduced force-generating capacity, atrophy, and increased stiffness (Powers et al., 1996; Kim et al., 2012; Greising et al., 2013; Greising et al., 2015; Elliott et al., 2016; Cacciani et al., 2014; Imagita et al., 2009). These studies have utilized animal models of moderate aging, which roughly approximates 70 years of age for humans. However, the impact of advanced aging on diaphragm morphology and function is less clear. Determination of these variables is imperative given the increasing proportion of the population living past 70 years of age. Moreover, the proteomic changes associated with diaphragm abnormalities in aging have not been fully characterized.

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Muscle weakness results from atrophy and/or dysfunction of the contractile machinery. In both human and murine skeletal muscle, fast twitch glycolytic fibers are more susceptible to age-related atrophy relative to the more oxidative fibers (Holloszy et al., 1991; Nilwik et al., 2013; Lexell, 1995). Additionally, healthy aging is associated with a fiber type shift from fast to slow in humans and rodents (Larsson et al., 1993; Schiaffino and Reggiani, 2011; Mitchell et al., 2012).

Abnormalities in a number of cellular pathways, such as autophagy, apoptosis, and protein post-translational modifications in general, have been consistently associated with age-related loss of skeletal muscle mass and function (Marzetti et al., 2009; Thompson, 2009). Importantly, mitochondrial dysfunction and loss of redox homeostasis have been considered drivers of this pathology (Marzetti et al., 2009; Chabi et al., 2008; Jackson, 2013). Endogenous generation of reactive oxygen species (ROS) during muscle contractions is requited for correct cellular signaling and plays a key role in skeletal muscle repair and adaptation (Horn et al., 2017). ROS are thought to regulate cellular signaling through reversible modifications of regulatory Cysteine (Cys) residues (or protein thiols) in redox sensitive proteins, subsequently affecting enzymatic activity, gene expression, metabolism, and cellular function (Powers and Jackson, 2008). In skeletal muscle physiology, several processes have been reported as sensitive to the intracellular redox environment including excitation-contraction coupling, but excessive levels of ROS can result in irreversible oxidation and affect muscle contractile properties (Ferreira and Reid, 2008; Debold, 2015). Reversible and irreversible modifications of Cys residues have been identified in ryanodine receptor channels, sarcomeric proteins involved in calcium binding, force generation, and shortening velocity. (Dutka et al., 2017; Gross and Lehman, 2013; Sun et al., 2013).

In the present study, we aimed to define the physiological effects of advanced age on diaphragm passive and contractile properties, fiber type distribution, and fiber cross sectional area. Based on previous studies in moderate aging, we hypothesized that diaphragm from very old mice would demonstrate isometric and isotonic contractile dysfunction, increased stiffness, decreased percentage of type IIb fibers, and fiber atrophy. In order to gain insights into the molecular mechanisms responsible for these phenotypic changes we performed a global label free proteomic analysis that included the relative quantification of the oxidation state of regulatory Cys residues to determine if advanced aging had significant effects on the muscle proteome and in particular on the intracellular redox environment.

2. Methods

2.1. Animals

We obtained young (6 months, $n = 11$, 30 ± 2.5 g) and very old male C57BL/6 mice (30 months, $n = 6$, 34.8 ± 2.9 g) from the National Institute on Aging. We determined, using G*Power software ($\alpha \leq 0.05$ and $\beta \geq 0.80$) (Faul et al., 2007), that this number of animals would be sufficient to detect a 20% difference in maximal diaphragm force based on our pilot data and previously published results on diaphragm (Greising et al., 2013; Greising et al., 2015). For proteomics analyses, we arbitrarily matched the number of young (6) and old animals (6). Mice were housed at the University of Florida under 12 h:12 h light-dark cycle and had access to standard chow and water ad libitum. All procedures conformed to the guiding principles for use and care of laboratory animals of the American Physiological Society and were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC 201408469).

2.2. Diaphragm preparation

On the day of the experiment, mice were deeply anaesthetized via inhalation of isoflurane (5% induction; 2–3% maintenance), and we performed a laparotomy and thoracotomy. None of the mice studied had gross organ pathology based on visual inspection during laparotomy and thoracotomy. The diaphragm was quickly excised and placed immediately in ice-cooled Krebs Ringer solution (in mM: 137 NaCl, 5 KCl, 1 $MgSO_4$, 1 NaH_2PO_4, 24 $NaHCO_3$, and 2 $CaCl_2$) followed by removal of the heart. Bundles of the diaphragm muscle were then rapidly dissected and further prepared for each specific analysis as described below.

2.3. Passive and contractile mechanical properties

A costal diaphragm bundle maintaining a segment of the rib and central tendon was dissected for attachment to a muscle mechanics apparatus (Aurora Scientific, 300C L-R model). The bundle was kept in Krebs Ringer solution gassed with a mixture of 95% O_2 and 5% CO_2 throughout the procedure. The rib was tied to a metal pin located on a glass rod, and the central tendon was attached to the force transducer with 4.0 silk suture. To determine the static passive length-tension relationship and find optimal length for isometric contraction, the bundle was slowly stretched until passive force was ~30 mN. The position of the lever arm was recorded and the muscle stimulated repeatedly (120 Hz, 600 mA, 0.25 ms pulse) at 1 min intervals, with the bundle being progressively shortened by 0.3 mm and force allowed to reach a steady state before each stimulation. Active and passive (static) forces were recorded, and the muscle was placed at the length that elicited the highest active force (optimal length, l_0). The preparation was then warmed to 37 °C, allowing 10 min for thermo-equilibration, before measurements of isometric and isotonic contractile properties.

We measured isometric force during twitch (1 Hz) and maximal tetanic stimulations (300 Hz, current and pulse as above). The maximal tetanic force (P_o) produced by each muscle was used as reference for isotonic contractions in the force-velocity protocol. Isotonic release steps followed 5 min after the maximal tetanic stimulation to determine the force-velocity relationship, where maximal tetanic contractions lasted 300 ms with an isometric (250 ms) and isotonic phase (50 ms). During the isotonic phase, the load was clamped at a force corresponding to 4–75% P_o and the muscle was allowed to shorten. The interval between each stimulation was two minutes. We measured shortening velocity 15 to 25 ms after the initial change in length, within the linear portion of the tracing (see example under *Results*). We normalized shortening velocity per l_0 and force per cross-sectional area (CSA, kN/m^2). To estimate the bundle CSA, diaphragm bundle weight (g) was divided by bundle length (cm) multiplied by muscle specific density (1.056 g/cm^3) (Close, 1972). The force-velocity curve was plotted and fitted using the Hill equation (Hill, 1938). We used parameters of the Hill equation to determine maximal shortening velocity (V_{max}, velocity extrapolated to zero force) and calculated power output (in W/kg) as Force (N/kg) × Velocity (m/s). Peak power was defined as the highest power obtained from the calculated Power-Force relationship.

2.4. Fiber typing and cross sectional area

The diaphragm bundle allotted for histology was embedded in Tissue-Tek OCT freezing medium, frozen in liquid-nitrogen-cooled isopentane, and stored at −80 °C. We sliced the diaphragm bundles into 10 μm cross sections at approximately −20 °C using a cryostat (Leica, CM 3050S model). Sections were incubated in 1:200 wheat germ agglutinin (WGA) Texas Red (Molecular Probes) for 1 h at room temperature, washed in PBS (3 × 5 min), permeabilized with 0.5% Triton X-100 solution (5 min), washed in PBS (5 min), and incubated in primary antibodies in a humid chamber (90 min). We used primary antibodies for myosin heavy chain (MyHC) type I (A4.840, 1:15; Developmental Studies Hybridoma Bank) and MyHC type IIa (SC-71, 1:50; Developmental Studies Hybridoma Bank). After the primary antibody incubation, sections were washed in PBS (3 × 5 min) and exposed to fluorescently conjugated secondary antibodies (60 min)