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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 (6months) 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.

Keywords

Skeletal muscle; Stiffness; Weakness; Protein oxidation; Proteomics

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Conflict of interest

The authors have no conflict of interest to declare.

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.

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 required 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 ($\alpha = 0.05$ and $\beta = 0.80$) (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 MgSO₄, 1 NaH₂PO₄, 24 NaHCO₃, and 2 CaCl₂) 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% O₂ and 5% CO₂ 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_0) 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_0 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) $\times$ 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 $10 \mu\text{m}$ 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 ($3 \times 5 \text{ min}$), 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 ($3 \times 5 \text{ min}$) and exposed to fluorescently conjugated secondary antibodies (60 min) (Goat $\times$ Mouse IgM Alexa 350 and Goat $\times$ Mouse IgG Alexa 488, Invitrogen). Sections were then washed in PBS ($3 \times 5 \text{ min}$), allowed to dry, and imaged.

We acquired and merged images using an inverted fluorescence microscope (Axio Observer) and Zen Pro software (Carl Zeiss Microscopy). To quantify fiber type distribution and fiber cross-sectional area, we used semi-automatic muscle analysis using segmentation of histology (SMASH) code, run in MATLAB software (Smith and Barton, 2014).

2.5. Global and thiol redox proteomics

The diaphragm bundles allotted to proteomics analysis were snap-frozen in liquid nitrogen and stored on dry ice or -80°C until further processing. The procedures for protein extraction and cysteine labeling, LC-MS/MS, and label-free and redox MS Quantification were described in detail previously (McDonagh et al., 2014). Briefly, samples were lysed in a thiol blocking buffer containing a light isotopic form of the alkylating reagent N-ethylmaleimide, d(0) NEM. Excess d(0) NEM was removed by desalting and reversibly oxidized Cys residues were reduced with tris(2-carboxyethyl)phosphine (TCEP). The newly TCEP-reduced Cys residues were alkylated with the heavy isotopic form of NEM, d (5) NEM. Finally, all samples were digested with trypsin, and the peptides were analyzed by LC-MS/MS using QExactive (Thermo) mass spectrometer as previously described (McDonagh et al., 2014). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaino et al., 2016) partner repository with the dataset identifier PXD005014. Supporting information files 1–3, contain a summary of the data analysis, a list of all identified and quantified proteins and a list of significantly changed proteins respectively.

2.6. Statistical analysis

Data are shown as mean $\pm$ SE. The data passed tests for normality and homogeneity of variance, and all comparisons between groups were conducted using Student's *t*-tests, except ANOVA was used for fiber distribution (Prism 6, GraphPad Software Inc., La Jolla, CA). For contractile and fiber type analyses, we declared statistical significance when $P < 0.05$. We performed global quantification of proteins using PEAKS software. We considered protein abundance to be significantly changed when there was a fold change ≥ 1.5 , significance of $-10 \log P \geq 20$ (which is equivalent to $P \leq 0.01$), each protein with at least 3 unique peptides, and a false discovery rate of 1%. PEAKS software also includes a post-translational modification (PTM) algorithm, where PTMs are assessed under the same criteria as for protein abundance. Supporting information files 4–12 contain the list of labelled Cys residues as detailed by McDonagh et al. (2014). We conducted targeted analysis of differentially labelled Cys residues using Skyline (Schilling et al., 2012) to relatively quantify the ratio of the Cys containing peptides labelled with both “light” d(0) NEM for reduced Cys residues and “heavy” d (5) NEM for reversibly oxidized Cys residues.

3. Results

3.1. Passive and contractile properties

Our analyses of diaphragm passive mechanical properties revealed that advanced age increased diaphragm bundle stiffness compared to young controls (Fig. 1). We also found that advanced aging depressed maximal specific force by approximately 15% ($P < 0.05$) and prolonged time to peak tension during twitch by approximately 30% ($P < 0.05$) (Fig. 2). However, there were no differences in twitch specific force and V relaxation time between old and young diaphragm bundles (Fig. 2, B). In addition, aging decreased peak power by 35% ($P < 0.05$) and a/P_o by 45% ($P < 0.05$), with no change in maximal shortening velocity (Fig. 3).

3.2. Fiber type distribution and cross sectional area

Immunohistochemistry analysis of diaphragm bundles showed a change in diaphragm fiber type distribution with advanced age. To our surprise, type IIa fibers within aged diaphragm demonstrated a ~ 15% higher cross-sectional area (CSA) compared to those from young diaphragm (Fig. 4). The CSAs of type I and type IIb/x fibers was not different between young and very old mice (Fig. 4). Relative to young mice, old mice showed ~ 8% decrease in type IIb/x fibers and ~ 8% increase in type IIa fibers ($P < 0.05$) (Fig. 4, C).

The relative contribution of each fiber type to the total diaphragm muscle fiber CSA was estimated as previously described (Elliott et al., 2016). Briefly, the average CSA of each fiber type was multiplied by that fiber type's average relative contribution, e.g., CSA of type I $\times$ relative proportion of type I. These 3 products were summed, and then each of the 3 products was divided by the grand total. In old mice, there was an increase (~ 15%, $P < 0.05$) in the relative contribution of type IIa fibers to the total diaphragm CSA with an associated decrease in the relative contribution of type IIb/x fibers (Fig. 4, D).

3.3. Label-free proteomic analysis of diaphragm muscle from young and very old mice

Shotgun proteomics detected over 1200 proteins in all samples. Of these, 63 were significantly altered in young versus aged diaphragm samples: 18 were downregulated with age and 45 were upregulated with age using the criteria of at least 3 unique peptides/protein and $P < 0.01$ (Fig. 5, A and Supp File 3). Proteins that were significantly altered in expression between groups were analyzed by the database for annotation, visualization, and integrated discovery (DAVID) (Huang et al., 2009). DAVID analysis allowed for functional annotation clustering of proteins that were upregulated or downregulated with age. Several proteins of interest within these functional groups are highlighted in a volcano plot (Fig. 5, B). Notably, proteins that were upregulated in old mice involved the extracellular matrix and space (enrichment score 4.27, $P = 2.5 \times 10^{-6}$) and cytoskeleton (enrichment score 1.08, $P = 2.3 \times 10^{-2}$). Proteins involved in carbohydrate metabolism, especially glycolysis, were downregulated with age (enrichment score 4.18, $P = 5.1 \times 10^{-6}$). Interestingly, functional clusters related to calcium regulation were enriched in both upregulated and downregulated proteins.

3.4. Redox analysis of Cys-containing proteins from diaphragm of young and aged mice

The redox proteome of the diaphragm from young and old mice was quantified using differential labeling of reduced and reversibly oxidized cysteine residues. Proteome Discovery software was used to generate lists of peptides with d(0), d(5), or both NEM modifications. In the young mouse diaphragm, 340 Cys-containing proteins were identified. Of these Cys-containing proteins, 6 had irreversibly oxidized Cys-residues ($-SO_2H$ or $-SO_3H$) only, 255 had reduced Cys-residues (identified with d(0) NEM) only, 19 had reversibly oxidized Cys-residues identified with d(5) NEM only, and 60 had both reduced and reversibly oxidized Cys-residues on identical amino acid sequences (identified with both d(0) and d(5) NEM i.e., redox proteins) (Fig. 6, A). In the aged mouse diaphragm, 347 Cys-containing proteins were identified, and 278 of these were similarly identified in the young diaphragm. Of the 347 Cys-containing proteins identified in the aged diaphragm, 7 had oxidized Cys-residues only, 254 had reduced Cys-residues only, 30 had reversibly oxidized

Cys-residues only, and 56 had both reduced and reversibly oxidized Cys-residues on identical amino acid sequences (i.e., proteins with redox peptides) (Fig. 6, A). There were 48 redox proteins identified by Proteome Discoverer in common between young and old samples. Interestingly, of the 12 unique redox proteins to young samples, 7 were related to mitochondria according to functional annotation clustering (enrichment score 3.06, $P = 8.7 \times 10^{-5}$) (Table 1). Subsequent targeted analysis with Skyline using the MS1 and retention times of these individual peptides allowed the relative quantification of the redox state of their specific Cys residues labelled with both the heavy and light NEM (Table 2).

The greatest quantifiable difference between differential labeling results for young and very old samples was in proteins containing reversibly oxidized Cys-residues, (i.e., 19 in young and 30 in old). There was considerable overlap in young and old for proteins with reversibly oxidized Cys-residues (e.g., Calreticulin, Cathepsin D, NADH dehydrogenase Fe-S protein 5 and Cytochrome C oxidase subunit 6b), but there were also proteins that emerged with reversibly oxidized residues uniquely in young (7 proteins) and old (18 proteins) (Fig. 6, B; Supp Files 7 and 11).

Targeted analysis of peptides identified from fragmentation of parent ions with d(0) and d(5) NEM allowed the calculation of average ratio intensity d(0)/d(5) NEM for specific redox sensitive Cys residues (Table 2). The reduced/reversibly oxidized Cys quantification indicates a preferentially reduced state (ratios > 1.0) or a preferentially oxidized state (ratio < 1.0) of a specific redox cysteine residue. Fig. 6C shows cysteine residues (and proteins) with a fold change $\geq 10\%$ in redox state for old relative to young animals.

4. Discussion

The main impact of advanced aging on diaphragm contractile function was a loss of peak power as opposed to the loss of maximal diaphragm specific force previously reported in moderate murine aging (24 months) (Greising et al., 2013; Greising et al., 2015). The diaphragm muscle's power is likely a main determinant of an individual's potential to complete expulsive behaviors (e.g., coughing and sneezing). Additional physiological observations in advanced aging include increased stiffness and diminished maximal tetanic force that are consistent with previous reports in moderate aging. The functional changes in the diaphragm were reflected by decreases in proteins involved in calcium regulation and carbohydrate metabolism and increases in cytoskeletal and ECM-related proteins evident from the global label free proteomic analysis. Furthermore, the redox proteomic analysis of advanced aged diaphragm showed an increase in the number of proteins containing reversibly oxidized Cys residues. Our proteomics findings are important to inform future studies testing cause-and-effect relationship between changes in diaphragm protein abundance or redox state and functional abnormalities in advanced aging.

4.1. Stiffness

The increase in passive stiffness with advanced age described in this study is consistent with previous findings in moderate aging (Gosselin et al., 1994). An increase in stiffness can impair the expiratory phase of expulsive behaviors and compromise airway clearance. Static passive stiffness measured in intact muscles or muscle bundles is determined by elastic

components of the cytoskeleton, sarcomere, and connective tissue. (Gillies and Lieber, 2011; Sieck et al., 2013) We observed a ~1.6 to 13-fold increase in cytoskeletal and ECM proteins. Thus, our data suggest that a denser cytoskeletal and ECM network contributes to increased diaphragm stiffness with aging.

Beyond its structural role and potential contribution to stiffness, the ECM is also a major determinant of cell signaling, proliferation, differentiation, and repair (Hynes, 2009). Our proteomics results highlighted increases in several components of the ECM. These include bi-glycan, which has both structural roles in binding collagen and signaling relevance with possible pro-inflammatory effects; (Neill et al., 2015) fetuin-A, which inhibits protease action on the ECM; (Hedrich et al., 2010) and inter- α -trypsin inhibitor (Serpina3k), which binds hyaluronic acid and stabilizes the ECM (Bost et al., 1998).

4.2. Contractile function

The main impact of advanced aging on diaphragm contractile function in our study was compromised isotonic properties. The a/P_o ratio and peak power were decreased by ~ 35% and 45%, respectively (Fig. 3). The decrease in a/P_o reflects an accentuated curvature of the force-velocity relationship, which reveals that shortening velocity is impaired to a greater extent at submaximal forces than at the extremes (“unloaded” and isometric portion) of the force-velocity relationship. Impairments in peak power may result from slowed shortening velocities, lower specific force, or both. Previous studies in old hamsters and mice show either preserved isotonic contractile properties or decreased peak power (Lynch et al., 1997; Zhang and Kelsen, 1990). The decreased peak power in old hamsters was mainly attributed to loss of specific force (Zhang and Kelsen, 1990). Loss in peak power that is largely dependent on decreased specific force may be specific to early/ moderate aging (Tallis et al., 2014). We observed a 15% reduction in maximal specific force with advanced age compared to an approximately 30% decrease previously reported in moderately aged mouse diaphragm (Greising et al., 2013; Greising et al., 2015). These findings suggest that mice surviving to a very old age have attenuated loss of diaphragm maximal specific force. In advanced aging, however, lower specific force along with slowing of shortening velocities in the sub-maximal range contribute to decreased peak power. Therefore, unlike moderate aging, abnormalities in cross-bridge kinetics occur in advanced aging and influence diaphragm power. Such loss in power will contribute to impair activities that require rapid diaphragm contractions, such as breathing during exercise and reflex expulsive behaviors responsible for airway clearance (e.g., coughing).

Our label free proteomic results suggest that there were significant changes in proteins regulating calcium handling and metabolism with advanced age that could affect contractile function. We found that calsequestrin 1 (CASQ1), sarco-endoplasmic reticulum calcium ATPase 1 (SERCA1), sarcalumenin, and parvalbumin decreased in advanced aging. Calsequestrin is involved in calcium storage in the sarcoplasmic reticulum and release by the ryanodine receptor channel. Knockdown of CASQ1 in skeletal muscle slows the kinetics of sarcoplasmic reticulum calcium release (Zhao et al., 2010). Thus, the decrease in diaphragm CASQ1 likely contributed to the slowed time to peak tension observed in our study. The SERCA pump promotes relaxation by actively sequestering calcium from the cytosol,

whereas sarcalumenin is a calcium-binding protein localized predominantly in the longitudinal sarcoplasmic reticulum, and parvalbumin buffers cytosolic calcium. The downregulation of CASQ1, SERCA1, sarcalumenin, and parvalbumin in the very old diaphragm would be expected to slow relaxation kinetics (Gehlert et al., 2015), yet diaphragm twitch 1/2 relaxation time was unchanged with advanced aging. It is worth noting that effects of moderate aging on twitch characteristics are controversial (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; Kelley and Ferreira, 2017).

Notably, our label-free proteomics analyses did not detect changes in relative abundance of contractile apparatus proteins between young and very old diaphragm. Sample preparation for quantification of such proteins for use in label-free proteomics analysis is the same as that used for traditional Western blot (except for the extraction of myosin heavy chain, which requires the use of a high-salt extraction buffer) (e.g., Roberts et al., 2013). The shotgun proteomic approach applied in this study is complicated by the high abundance of sarcomeric proteins in skeletal muscle that could mask changes in low abundant regulatory proteins, considering that low and high abundant proteins may differ up to seven orders of magnitude (Murgia et al., 2017). Nevertheless considering the stringent criteria applied, we consider the protein expression levels detected as valid, and conclude that selective loss of sarcomeric proteins is not the primary mechanism responsible for diaphragm weakness in advanced age. From a metabolic standpoint, proteins involved with carbohydrate metabolism, especially glycolysis, decreased in very old mouse diaphragm. A decline in glycolytic enzymes will accelerate fatigue during high-intensity shortening contractions.

4.3. Fiber type and CSA

We found a fiber type shift from type IIb/x to type IIa fibers in the advanced aged diaphragm. Moderate aging also causes a fast-to-slow fiber type shift in rodents (Greising et al., 2013; Elliott et al., 2016; Imagita et al., 2009). In our hands, the aged type IIa fibers also had larger CSA compared to young type IIa fibers. There are reports that the aged rodent diaphragm experiences atrophy (Kim et al., 2012; Greising et al., 2013; Elliott et al., 2016), however others have demonstrated no changes, (Gosselin et al., 1992; Kavazis et al., 2012) or increased fiber CSA with age in agreement with our results (Cacciani et al., 2014). A possible explanation for increased CSA of type IIa fibers is abnormal denervation/reinnervation events with age that lead to denervation of type IIb/x fibers and then reinnervation of those fibers by a type IIa motor neuron. In such a scenario, the reinnervated type IIb/x fibers would shift toward the type IIa myosin heavy chain phenotype (which could explain the fast to fast-oxidative fiber type shift in our study) while still maintaining the larger CSA of their previous type IIb/x type. Thus, an increase in CSA with age may reflect incomplete remodeling of previously type IIb/x fibers as they acquire type IIa metabolic and protein expression features, and not hypertrophy per se. Consistent with this notion, aging causes neuromuscular junction instability and increases denervation/reinnervation events (Hepple, 2014; McDonagh et al., 2016).

4.4. Label-free and redox proteomics – beyond connections to mechanical properties and fiber size

In general, the significant changes in cytoskeletal and ECM proteins together with the changes observed in calcium handling proteins would support the physiological effects on stiffness and contractile function observed in advanced age as discussed above. However, the global proteomic results also highlighted alternative mechanisms that could play a significant role in the physiological changes observed. We detected almost a 30-fold increase in chitinase-3-like 3 protein (Chi3l3, Ym1) in diaphragm with advanced aging. The protein is a typical marker of alternatively activated M2a macrophages that are induced by IL-4, IFN- γ , and IL-13 (Maresz et al., 2008; Wang et al., 2015) and stimulate collagen synthesis by fibroblasts. (Song et al., 2000) Aged limb muscles of mice show increases in M2a macrophages, a factor that is dictated by the age of bone marrow derived cells and contributes to skeletal muscle fibrosis (Wang et al., 2015). The substantial increase in diaphragm Ym1 suggests infiltration of M2a macrophages that is characteristic of ‘wound healing’ and muscle regeneration.

Our redox proteomics approach detected an increase in the number of proteins identified with only reversibly oxidized cysteine residues in advanced age. Moreover, the quantity of proteins identified independently with both light and heavy isoforms of NEM, which we have defined as “redox peptides”, indicates redox flexibility (McDonagh et al., 2014). Proteins involved with mitochondria function demonstrated redox flexibility only in young diaphragm samples (Table 1). Several of the mitochondria redox sensitive proteins detected are involved in metabolism including malate dehydrogenase (Cys285), isocitrate dehydrogenase (Cys235/23), and proteins involved in electron transport chain. The functional relevance of these mitochondrial findings is limited in that we did not test for associated compromise in muscle fatigue properties. Future contractile function experiments should determine whether abnormal redox dynamics of these mitochondrial proteins exacerbates fatigue.

Advanced aging also altered the redox state of various other Cys residues (Fig. 6C and Table 2). Among the most notable changes was higher oxidation of cysteine residues in N-Myc downstream regulator gene 2 (NDRG2), GAPDH, and fructose-bisphosphate aldolase. NDRG2 regulates cell-growth (Takahashi et al., 2005) and differentiation (Hu et al., 2006), and is susceptible to oxidative modification in cardiac muscle (Yao et al., 2015). The oxidized form of GAPDH may facilitate apoptosis (Nicholls et al., 2012), and oxidation of cysteine residues within aldolase may inactivate the enzyme (Offermann et al., 1984). Other relevant changes in redox state of Cys residues occurred in the catalytic sites of Prdx5 (Cys96) and Prdx6 (Cys47), which are involved in oxidant scavenging and redox shuttling. Maintenance of an optimal redox state of these enzymes is likely imperative for proper cell function.

4.5. Methodological considerations

One of the limitations of a data dependent shotgun proteomic analysis is that only the most abundant ions are selected for MS/MS fragmentation and hence identification of the peptide. If a peptide was detected in one set of samples only, it does not necessarily signify that it is

not present in the sample set being compared. Nevertheless, this technique does provide an indication of the global redox changes occurring. Moreover, interpretation of our label free proteomics results must take into consideration any genetic reprogramming accompanying the diaphragm fiber type shift with advanced age. Based on our criteria of at least a 1.5 fold difference in abundance, we consider alternative mechanisms than fiber type shift contribute to the changes in protein expression. Furthermore, contrasting our global label-free proteomics results with those reported for limb muscle suggests that the diaphragm is uniquely affected by aging. For instance, gastrocnemius from 24 month old mice show increased expression of CASQ1 and SERCA1, which is diametrically opposite to our results (McDonagh et al., 2014).

Finally, it is worth noting that we studied 30 month old mice (~ 25% survival rate), which roughly models 80 years of age in humans (Life span as a biomarker. The Jackson Laboratory; Turturro et al., 1999). Previous studies of isometric and isotonic properties have utilized mice at ~ 24 months of age (70–75% survival rate), which roughly models 70 years of age in humans (Greising et al., 2013; Greising et al., 2015; Lynch et al., 1997; Life span as a biomarker. The Jackson Laboratory; Turturro et al., 1999). Perhaps inherent physiological differences, including better diaphragm (or skeletal muscle) isometric force, at ~ 24 months of age may have allowed a subset of mice to live for 30 months. Alternatively, if physiological differences outside of the respiratory musculature permitted these very old mice to survive when others did not, they may have experienced compensatory adaptation in their diaphragm muscle to account for the increased work of breathing in advanced age. These selection hypotheses are alternatives to our conclusion of attenuated contractile dysfunction in advanced age relative to moderate aging.

5. Conclusion

The main impact of advanced aging on diaphragm function was a loss of peak power and increased stiffness. Our proteomics analysis identified lower abundance of calcium regulatory proteins (SERCA, calsequestrin, and sarcalumenin) and increased abundance of ECM and cytoskeletal proteins as potential mechanisms underlying impaired contractile properties and elevated stiffness in the diaphragm with advanced aging. Redox proteomics revealed oxidation of glycolytic proteins and loss of redox flexibility in mitochondrial proteins, which may impair metabolism and accelerate fatigue. Altogether, our study identified functional and molecular changes that are likely to impair rapid, forceful, and repetitive diaphragm contractions required for airway clearance behaviors and to sustain ventilation during exercise.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Very old diaphragm is stiffer than young controls. (A) Relationship between passive tension (N/cm²) and length (normalized to bundle's optimal length) in young (white circles) and very old (black circles) diaphragm. (B) Using data shown in panel A, Young's elastic modulus of diaphragm bundles calculated as change in tension normalized to change in strain (~6% from optimal length). $N=8$ (young) and 6 (old). * $P < 0.05$.

Fig. 2.

Diaphragm isometric twitch and tetanic contractile properties. Advanced aging causes loss of maximal specific force (A) and prolongs time to peak tension (C), with no effects on twitch specific force (B) and 1/2 relaxation time (D). Young (white bars) and very old (black bars) diaphragm. $N=8$ (young) and 6 (old). $*P < 0.05$.

Fig. 3.

Diaphragm isotonic contractile properties. A) Example of tracings of force and length during an isotonic release experiment. Dashed lines illustrate region selected for measurement of force and shortening velocity. B) Representative profiles of diaphragm force-velocity and force-power relationship for young (white circles) and very old mice (black circles). C) Maximal shortening velocity (V_{max}) extrapolated from force-velocity relationship. D) Peak power. E) Curvature of the force-velocity relationship (a/P_0). This value determined as constant a divided by maximal specific force (P_0), determined by using the Hill equation to fit the force-velocity relationship. $N=6$ (young) and 8 (old). * $P < 0.05$.

Fig. 4. Diaphragm fiber type and morphology. A) Representative transverse cross-sections of young and very old diaphragm. Blue = type I fibers, green = type IIa fibers, and black = type IIb/x fibers. B) fiber cross-sectional area (CSA), C) fiber type distribution, and D) relative contribution of fiber types to total diaphragm muscle fiber CSA (D). Data are from young (white bars) and very old mice (black bars). $N=11$ (young) and 5 (old). $*P < 0.05$.

Fig. 5. Relative protein abundance in diaphragm from young and very old mice. A) Heat map of significantly up- and down-regulated proteins in diaphragm from young and very old mice detected by PEAKS label-free software ($P < 0.01$, min fold change > 1.5 , quality 0.8, and at least three unique peptides). B) Volcano plot showing distribution of proteins with significant change in abundance. Selected proteins of interest are highlighted per functional groups. CHO = carbohydrate, ECM = extracellular matrix, PRVL = parvalbumin, CSQ1 = calsequestrin 1, SERCA1 = sarco-endoplasmic reticulum calcium ATPase 1, SRL =

sarcalumenin, S100A6 and S100A11 = S100 calcium binding proteins, GAPDH = glyceraldehyde 3-phosphate dehydrogenase, ALDOA = aldolase A, PFK = phosphofructokinase, UGP = UDP-glucose pyrophosphorylase, BGN = biglycan, ITIH2 = inter- α -trypsin inhibitor heavy chain 2.

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Fig. 6. Notable changes in the redox proteome in advanced age. (A) Number of proteins containing oxidized Cys only (purple), reversibly oxidized Cys only (green), redox Cys peptides (red), and reduced Cys only (blue). (B) Proteins containing reversibly oxidized Cys residues in young (yellow), aged (blue), and both (green) diaphragm samples. The number of reversibly oxidized proteins increased from 19 to 30 with age. (C) For thiol redox proteomics, a ratio > 1.0 reflects increased cysteine oxidation and ratio < 1.0 reflects decreased oxidation with aging. Cysteine redox data are from residues with difference 10% between young and old.

Table 1

List of redox proteins involved in mitochondrial function detected only in young diaphragm.

Accession	Protein
P70404	Isocitrate dehydrogenase [NAD] subunit gamma 1, mitochondrial (Idh3g)
Q9JHI5	Isovaleryl-CoA dehydrogenase, mitochondrial (Ivd)
P99029	Peroxiredoxin-5, mitochondrial (Prdx5)
P99028	Cytochrome b-c1 complex subunit 6, mitochondrial (Uqcrh)
Q9D051	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial (Pdhb)
Q01768	Nucleoside diphosphate kinase B (Nme2)
P48962	ADP/ATP translocase 1 (Slc25a4)

Note. Label-free MS/MS generated raw data which were run through PEAKS software for identification using Mascot score 20.

Table 2

List of all redox proteins detected, the redox state of selected redox Cys residues labelled with both d(0) NEM and d(5) NEM was calculated in Skyline using the ratio of the average ion intensity of parent ions. The m/z values and retention times of selected peptides were applied in the targeted approach using Skyline open software. Proteins in bold indicate that there were significant differences in abundance between young and very old samples.

Accession	Protein	Redox cys	Adult red/ox	old red/ox	Adult vs. old	(-)10logP
P63101	14-3-3 protein zeta/delta (Ywhaz)	94	12.94	13.11	1.00:1.41	19.28
P61982	14-3-3 protein gamma (Ywhag)	189	6.62	4.97		
P60597	2-oxoglutarate dehydrogenase, mitochondrial (Ogdh)	112	20.82	20.27	1.00:1.20	3.97
		395	25.75	22.71	1.00:0.92	4.1
		565	12.64	13.41		
		904	6.74	4.44		
Q99KI0	Aconitate hydratase, mitochondrial (Aco2)	126	6.36	7.22	1.00:1.16	5.29
		385	2.03	2.27		
		592	1.99	2.02		
P68134	Actin, alpha skeletal muscle (Acta1)	219	8.82	8.22	1.00:1.01	0.36
		259	3.67	3.67		
		287	8.14	8.26		
P48962	ADP/ATP translocase 1 (Slc25a4)	160	32.43	37.85	1.00:0.90	5.37
P47738	Aldehyde dehydrogenase, mitochondrial (Aldh2)	388	17.60	16.17	1.00:1.17	5.22
P45376	Aldose reductase (Akr1b1)	200	10.86	14.10	1.00:0.86	2.96
		289/94	4.18	5.35		
Q61838	Alpha-2-macroglobulin (A2m)	340	5.39	6.05	1.00:1.57	25.88
P05201	Aspartate aminotransferase, cytoplasmic (Got1)	391	11.45	12.53	1.00:1.12	8.04
P05202	Aspartate aminotransferase, mitochondrial (Got2)	106	43.21	40.70	1.00:1.10	4.48
		187	10.14	8.45		
		295	8.99	11.51		
Q9DCX2	ATP synthase subunit d mitochondrial (Atp5h)	101	3.72	3.87	1.00:0.97	2.82
P56382	ATP synthase subunit epsilon, mitochondrial (Atp5e)	19	13.95	14.19	1.00:1.18	5.67
Q9CZU6	Citrate synthase, mitochondrial (Cs)	359	8.92	8.10	1.00:0.72	27.95
P18760	Cofilin-1 (Cfl1)	147	7.23	5.26	1.00:1.72	31.7
Q6P8I7	Creatine kinase S-type, mitochondrial (Ckmt2)	317	30.69	30.36	1.00:0.64	16.04

Accession	Protein	Redox cys	Adult red/ox	old red/ox	Adult vs. old	(-10logP
P07310	Creatine kinase M-type (Ckm)	254	54.98	46.91	1.00:0.63	68.46
Q9CV13	Cytochrome b-c1 complex subunit 1, mitochondrial (Uqcrcl)	380	7.59	8.08	1.00:1.05	2.35
P99028	Cytochrome b-c1 complex subunit 6, mitochondrial (Uqcrrh)	51	0.12	0.11	1.00:1.12	3.6
O35215	D-dopachrome decarboxylase (Ddt)	57	0.51	0.56	1.00:1.12	2.87
Q99LC5	Electron transfer flavoprotein subunit alpha, mitochondrial (EtfA)	53	4.73	3.90	1.00:1.00	1.75
P1782/P21580	Alpha-enolase (Eno1)	337	2.48	2.58	1.00:0.90	7.87
		357	9.41	11.12		
		389	9.74	11.73		
		399	0.65	0.78		
P04117	Fatty acid-binding protein, adipocyte (Fabp4)	118	7.54	8.95	1.00:1.15	5.94
P97447	Four and a half LIM domains protein 1 (Fhl1)	71	7.44	7.15	1.00:0.62	43.48
		89	6.50	6.54		
		255	1.36	2.09		
		273/76	1.77	2.26		
P05064	Fructose-bisphosphate aldolase A (Aldoa)	178	2.06	1.35	1.00:0.58	62.34
		339	9.51	9.91		
P16858	Glyceraldehyde-3-phosphate dehydrogenase (Gapdh)	22	12.81	12.05	1.00:0.60	63.76
		150/54	1164.5	471.3		
Q9WUB3	Glycogen phosphorylase, muscle form (Pygm)	172	11.36	12.34	1.00:0.69	33.84
P15626	Glutathione S-transferase Mu 2 (Gstm2)	87	0.53	0.88	1.00:1.46	14.86
P63017	Heat shock cognate 71 kDa protein (Hspa8)	603	7.52	6.29	1.00:0.94	1.8
P54071	Isocitrate dehydrogenase [NADP], mitochondrial (Idh2)	113	0.11	0.09	1.00:1.07	5.04
		308	59.88	45.45		
		392	9.37	9.15		
P70404	Isocitrate dehydrogenase [NAD] subunit gamma 1, mitochondrial (Idh3g)	235/36	25.03	18.14	1.00:0.79	11.21
Q9JH15	Isovaleryl-CoA dehydrogenase, mitochondrial (Ivd)	252	8.62	6.53	1.00:1.20	5.66
P06151	L-lactate dehydrogenase A chain (Ldha)	84	16.84	15.84	1.00:0.73	20.17
P16125	& LDHB	163	11.73	11.83	1.00:0.92	3.58
P51174	Long-chain specific acyl-CoA dehydrogenase, mitochondrial (Acadl)	166	5.74	5.65	1.00:1.24	14.76
		342	9.36	11.79		
		351	4.41	5.32		

Accession	Protein	Redox cys	Adult red/ox	old red/ox	Adult vs. old	(-10logP
P08249	Malate dehydrogenase, mitochondrial (Mdh2)	93	6.69	7.62	1.00:1.06	4.34
		212	10.73	9.67		
		285	7.83	10.69		
P14152	Malate dehydrogenase, cytoplasmic (Mdh1)	137	9.61	9.88	1.00:0.94	3.84
		154	9.36	9.86		
P04247	Myoglobin (Mb)	67	48.08	51.68	1.00:1.02	1.05
P97457	Myosin regulatory light chain 2, skeletal muscle isoform (MyIpf)	128	37.29	37.72	1.00:01.04	1.3
		157	25.39	20.91		
Q01768	Nucleoside diphosphate kinase B (Nme2)	109	2.88	2.06	1.00:0.69	17.23
P99029	Peroxiredoxin-5, mitochondrial (Prdx5)	96	10.50	7.98	1.00:1.05	2.1
O08709	Peroxiredoxin-6 (Prdx6)	47	0.96	1.60	1.00:0.90	2.88
O70250	Phosphoglycerate mutase 2 (Pgam2)	23	11.53	10.69	1.00:0.67	39.65
		153	21.41	20.78		
Q9D0F9	Phosphoglucosmutase-1 (Pgm1)	374	22.25	23.58	1.00:0.71	21.73
Q9QYG0	Protein NDRG2 (Ndr2)	255	39.33	10.47	1.00:0.94	5.1
P35486	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial (Pdha1)	41	3.81	3.11	1.00:0.80	11.94
Q9D051	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial (Pdhb)	263	26.22	27.19	1.00:0.87	7.74
P52480	Pyruvate kinase PKM (Pkm)	49	16.40	17.90	1.00:0.69	33.86
		152	7.66	7.88		
		358	14.78	13.25		
		474	70.16	68.01		
Q8R429	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (Atp2a1)	674/75	40.53	52.44	1.00:0.66	37.47
P07759	Serine protease inhibitor A3K (Serpina3k)	260	8.63	18.18	1.00:0.52	60.81
Q92111	Serotransferrin (Tf)	156	0.031	0.023	1.00:2.97	89.76
P07724	Serum albumin (Alb)	58	2.83	2.16	1.00:1.28	28.13
		77	1.38	1.02		
		289	0.012	0.012		
Q9ZZ19	Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial (Succla2)	152	0.90	0.84	1.00:0.70	17.35
Q9D0K2	Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial (Oxct1)	456	6.29	6.61	1.00:1.09	2.96
P08228	Superoxide dismutase [Cu-Zn] (Sod1)	7	0.95	0.94	1.00:1.06	2.54
		147	0.65	0.56		

Accession	Protein	Redox cys	Adult red/ox	old red/ox	Adult vs. old	(-)10logP
P17751	Triosephosphate isomerase (Tpi1)	117	13.52	14.21	1.00:0.95	1.43
P58774	Tropomyosin beta chain (Tpm2)	268	15.57	15.53		
P13412	Troponin I, fast skeletal muscle (Tnni2)	190	8.59	10.17	1.00:1.21	6.41
		134	8.94	8.90	1.00:1.14	6.76