Experimental Gerontology 48 (2013) 881–887

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Diaphragm muscle sarcopenia in aging mice

Sarah M. Greising [a], Carlos B. Mantilla [a,b], Britney A. Gorman [a], Leonid G. Ermilov [a], Gary C. Sieck [a,b,*]

[a] Department of Physiology and Biomedical Engineering, College of Medicine, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA
[b] Department of Anesthesiology, College of Medicine, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA

ARTICLE INFO

Article history:
Received 11 March 2013
Received in revised form 31 May 2013
Accepted 11 June 2013
Available online 19 June 2013

Section Editor: Christiaan Leeuwenburgh

Keywords:
Atrophy
Contractile properties
Cross-sectional area
Fiber type
Myosin heavy chain
Respiratory muscles

ABSTRACT

Sarcopenia, defined as muscle weakness and fiber atrophy, of respiratory muscles such as the diaphragm (DIAm) has not been well characterized. The DIAm is the main inspiratory muscle and knowledge of DIAm sarcopenia is important for establishing the effects of aging on respiratory function. We hypothesized that aging is associated with a loss of DIAm force and reduced fiber cross-sectional area (CSA), and that these changes vary across fiber types. DIAm sarcopenia was assessed in young (5 month; n = 11) and old (23 month; n = 12) wild-type mice reflecting ~100 and 75% survival, respectively. In addition, DIAm sarcopenia was evaluated in *BubR1*$^{H/H}$ mice (n = 4) that display accelerated aging (~60% survival at 5 months) as a result of expression of a hypomorphic allele (*H*) of the mitotic checkpoint protein BubR1. Maximum specific force (normalized for CSA) of the DIAm was 34% less in old mice and 57% lower in *BubR1*$^{H/H}$ mice compared to young mice. Mean CSA of type IIx and/or IIb DIAm fibers was 27% smaller in old wild-type mice and 47% smaller in *BubR1*$^{H/H}$ mice compared to young mice. Mean CSA of type I or IIa fibers was not different between groups. Collectively these results demonstrate sarcopenia of the DIAm in aging wild-type mice and in *BubR1*$^{H/H}$ mice displaying accelerated aging. Sarcopenia may limit the ability of the DIAm to accomplish expulsive, non-ventilatory behaviors essential for airway clearance. As a result, these changes in the DIAm may contribute to respiratory complications with aging.

1. Introduction

The physiological process of aging significantly impacts an individual's quality of life, as consequence of the gradual functional, structural, and biochemical changes due to aging. The age-related decline in skeletal muscle function is defined as sarcopenia, which is characterized by muscle weakness and fiber atrophy (Cesari et al., 2012; Fielding et al., 2011). Persons with sarcopenia have a greater incidence of functional disability including loss of independence and higher risks of falling (Landers et al., 2001) and sarcopenia is a substantial predictor of mortality in aging individuals (Fielding et al., 2011).

Respiratory complications, such as pneumonia and respiratory infections, are common in aging individuals (Fein and Niederman, 1994; Houston et al., 1997) and are a common cause of death in this population (Heron, 2011). Decreased function of the diaphragm muscle (DIAm), the main inspiratory muscle, may contribute significantly to the increased susceptibility to respiratory complications during aging. In aging adults, respiratory muscle force is decreased by 13–25% as measured by the maximal sniff and Mueller maneuvers (Polkey et al., 1997; Tolep et al., 1995). Thus, DIAm sarcopenia may compound age-related lung and chest wall changes reducing an individual's ability to perform the large forces generated during expulsive, non-ventilatory behaviors important for airway clearance.

While sarcopenia of limb muscles is well characterized and defined (Brooks and Faulkner, 1988), the presence of DIAm sarcopenia remains controversial. Previous research in rodent models of aging has been both limited and conflicting in regards to age-related changes in the DIAm. For example, cross-sectional area (CSA) of rat DIAm fibers reportedly does not change (Kavazis et al., 2012) or increases ~28% (in type IIa fibers) (Kawai et al., 2012) with age. In aging mice, DIAm force shows either no difference (Lynch et al., 1997) or a trend for a reduction (Dupont-Versteegden and McCarter, 1992; Faulkner et al., 2008). None of these studies, however, comprehensively examined the DIAm in aging animals to confirm sarcopenia.

Rodent models are useful to examine genetic and molecular determinants of aging. Characterizing aging effects on specific muscle groups, and in particular respiratory muscles such as the DIAm, is important. The goal of this project was to systematically determine the effects of aging on the DIAm, specifically those properties defining sarcopenia (i.e., specific force and CSA). Additionally, we sought to determine if sarcopenia is fiber-type specific, i.e., more pronounced in type IIx and/or IIb DIAm fibers (fiber type classification based on myosin heavy chain — MyHC isoform expression). We hypothesized that the DIAm is susceptible to sarcopenia, resulting in a reduction in both force and fiber atrophy, with greater effects at type IIx and/or IIb fibers.

Additionally, we examined a recently developed mouse model of accelerated aging that exhibits physiologically relevant reductions in

* Corresponding author at: 200 First Street SW, SMH Jo 4-184, Rochester, MN 55905, USA. Tel.: +1 507 284 6850; fax: +1 507 255 7300.
E-mail address: sieck.gary@mayo.edu (G.C. Sieck).

0531-5565/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.exger.2013.06.001

the mitotic checkpoint protein kinase BubR1 (budding uninhibited by benzimidazole-related 1). This novel mouse model expresses a hypomorphic allele (*H*) of BubR1 (*BubR1*$^{H/H}$) and displays many aging-related phenotypes such as failure to grow, lipodystrophy, alopecia, decreased dermal thickness, cataracts, and atherosclerotic changes in addition to a shortened lifespan (Baker et al., 2004). Decreasing BubR1 is thought to play a relevant role in aging since protein levels of BubR1 decrease naturally in healthy tissues with age, including skeletal muscles (Baker et al., 2004, 2008).

2. Methods

2.1. Animals

All wild-type (strain background C57BL/6 × 129) and *BubR1*$^{H/H}$ mice were bred at colonies maintained at the Mayo Clinic. Mice were group housed by genotype and maintained on a 12 h light–dark schedule under specific pathogen-free conditions with ad libitum access to food and water. Wild-type mice were examined at ~5 and 23 months of age (mo), representing 100% and ~75% survival (young and old), respectively (Flurkey, 2009; Turturro et al., 1999). *BubR1*$^{H/H}$ mice were used at 5 mo representing ~60% survival (Baker et al., 2004, 2008). Considerations for animal use in gerontological research were followed (Miller and Nadon, 2000). Subsets of mice were used for analysis of in vitro force and histo-morphological analysis of the DIAm muscle. At the terminal experiment, mice were anesthetized with an intramuscular injection of ketamine (90 mg/kg) and xylazine (10 mg/kg) and euthanized by exsanguination. Harvested midcostal DIAm segments were stored at −80 °C until further analysis. All protocols and animal care guidelines were approved by the Institutional Animal Care and Use Committee at the Mayo Clinic, in compliance with National Institute of Health guidelines.

2.2. Diaphragm muscle in vitro contractility

Isometric force was measured using DIAm strips from a subset of mice, as previously described (Ameredes et al., 2000; Gosselin et al., 1996; Lewis et al., 1986; Miyata et al., 1995; Sieck et al., 2012). Briefly, ~3 mm midcostal DIAm segments were carefully dissected, with the rib origin of the muscle stabilized by minutien pins and the central tendon insertion attached to a force transducer (model 6350, Cambridge Technology, Cambridge, MA). Muscle segments were incubated in Reese-Simpson buffer (pH 7.4) with 95% O_2 and 5% CO_2 and maintained at 26 °C. Muscles were then set to optimal length, at which all contractile measurements were conducted. Maximum twitch force was elicited by stimulating the muscle with a 0.5-ms pulse (Grass S88 stimulator delivered through a SIU5D stimulus isolation unit; Grass Telefactor) via plate electrodes. Isometric tetanic force was elicited by stimulating muscles for 1000 ms and 120 Hz. The relationship between force and stimulation frequency of DIAm segments was determined with a protocol of 1-s trains of 0.5-ms stimuli at 10, 25, 50, 75, 100, 125, and 150 Hz. The stimulator and servomotor system were controlled by computer using a data acquisition interface board and custom-developed LabView software (National Instruments). Specific force was analyzed as maximal isometric force normalized to physiological CSA defined as DIAm mass/(optimal length × muscle density).

2.3. Contractile protein analyses

Midcostal DIAm sections were homogenized on ice in 10 mM phosphate buffer (pH 7.0). Homogenates were assayed to determine noncollagenous protein content using the Lowry method (Bio-Rad, Hercules CA). Homogenate samples were further electrophoresed on SDS-polyacrylamide gels to determine total contractile protein content (myosin and actin), as previously reported (Geiger et al., 2003; Greising et al., 2011).

2.4. Diaphragm muscle histo-morphological analysis

Strips of DIAm were dissected and saved for histological analysis of MyHC isoform classification, fiber type-specific CSA, and fraction of total muscle CSA comprised by interstitial space. Samples were serially cut into 10 μm thick cross-sections for analysis. Gross histologic examination of DIAm cross-sections was conducted on hematoxylin and eosin-stained sections. Muscle sections were labeled using primary antibodies for MyHC isoforms: anti-$MyHC_{Slow}$ (Vector Labs VP-M667) and anti-$MyHC_{2A}$ (SC-71 obtained from Developmental Studies Hybridoma Bank, Iowa City IA) and laminin (Sigma L9393) to visualize the sarcolemma. Sections were treated with appropriate fluorescently-conjugated secondary antibodies. Individual muscle fibers were classified as type I, type IIa and type IIx and/or IIb, based on expression of $MyHC_{Slow}$, $MyHC_{2A}$, and the absence of staining, respectively. A single image of each DIAm section was obtained via confocal microscopy using a Nikon Eclipse C1 laser scanning confocal microscope system (Nikon Instruments Inc., Melville NY) equipped with Argon (488 nm) and solid state (405 and 561 nm) lasers capable of simultaneous multi-label fluorescence imaging. Confocal images were saved separately for each fluorescence channel using Nikon C1 software as 8-bit grayscale-TIFF files. All images were pseudo-colored and merged in NIS-Elements software (Nikon Instruments) for analysis. As previously described (Sieck et al., 2012), ~400 type-identified fibers per DIAm section were randomly sampled to determine fiber type counts and measure fiber CSA using the morphometric tool in NIS-Elements software. The total number of DIAm fibers was then used to determine the proportion of fibers of each type.

Separate muscle sections were labeled with wheat germ agglutinin to determine the fraction of total muscle CSA comprised by interstitial space, with modification of a previous methodology (Gosselin et al., 1993). Briefly, DIAm sections were incubated with 1 μg/ml of wheat germ agglutinin conjugated to Alexa Fluor 488 (Molecular Probes W11261). Single confocal images were obtained for each DIAm section (as above) and were analyzed using the threshold, binarize and morphometric analysis functions in MetaMorph (Universal Imaging Corp.). Briefly, wheat germ agglutinin fluorescence was thresholded manually such that all DIAm fibers were clearly outlined. Images were binarized and both the total muscle CSA and area comprised by interstitial space were determined. On average, four non-continuous sections of DIAm were analyzed per animal and the fraction of total muscle CSA comprised by interstitial space was determined as the ratio of the sum of areas comprised by interstitial space and the total muscle CSA for each animal.

For the purpose of image presentation, individual images were exported into Adobe Photoshop (Adobe Systems Inc., San Jose CA) as TIFF files and cropped to highlight representative areas. Fluorescence images were pseudocolored by changing the color gamut (RGB) while preserving original contrast and brightness.

2.5. Statistical analysis

All data were analyzed using JMP (JMP version 8.0; SAS Institute Inc., Cary NC). Data for body mass, DIAm contractility, and DIAm fiber CSA and fraction of total muscle CSA comprised by interstitial space were analyzed using one-way ANOVA comparing across groups (young, old, and *BubR1*$^{H/H}$ mice). Force–frequency relationships were compared using a one-way ANOVA with Bonferroni correction. The proportion of DIAm fiber types and the relative fiber type contribution to total DIAm CSA were analyzed by two-way ANOVA (group x fiber type). When appropriate, post hoc analyses were conducted using Tukey–Kramer's honestly-significant difference test. Chi-squared was used to analyze the distribution of DIAm fiber CSAs. Unless otherwise specified, all data are reported as mean ± standard error (SE). Significance was accepted at the $\alpha < 0.05$ level.