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Impaired Calcium Signaling in Muscle Fibers from Intercostal and Foot Skeletal Muscle in a Cigarette Smoke-Induced Mouse Model of COPD

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Abstract

Introduction—Respiratory and locomotor skeletal muscle dysfunction are common findings in chronic obstructive pulmonary disease (COPD), however, the mechanisms that cause muscle impairment in COPD are unclear. Because Ca^{2+} signaling in excitation-contraction (E-C) coupling is important for muscle activity, we hypothesized that Ca^{2+} dysregulation could contribute to muscle dysfunction in COPD.

Methods—Intercostal and flexor digitorum brevis muscles from control and cigarette smoke-exposed mice were investigated. We used single cell Ca^{2+} imaging and western blot assays to assess Ca^{2+} signals and E-C coupling proteins.

Results—We found impaired Ca^{2+} signals in muscle fibers from both muscle types, without significant changes in releasable Ca^{2+} or in the expression levels of E-C coupling proteins.

Discussion— Ca^{2+} dysregulation may contribute or accompany respiratory and locomotor muscle dysfunction in COPD. These findings are of significance to the understanding of the pathophysiological course of COPD in respiratory and locomotor muscles.

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Competing Interests

The author(s) indicated below declare that they have no competing interests.

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Author Contributions

P.R., M.F.S. and E.H. designed research; P.R. and E.H. performed research and data analysis; T.E.S. and S.B. generated and contributed the cigarette smoke-exposed mice and controls; H.C. performed biostatistical data analysis; P.R., T.E.S., H.C., S.B., M.F.S. and E.H. edited and approved the manuscript; P.R., M.F.S. and E.H. wrote the paper.

Keywords

COPD; cigarette smoke; mice; respiratory muscle; locomotor muscle; excitation-contraction coupling; Ca^{2+} transients

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is characterized by airflow limitation associated with chronic inflammation in the airways and the lung in response to particulate or gaseous triggers (i.e., cigarette smoke)¹. The airflow limitation and inflammatory response are progressive and are associated with a complex array of co-morbidities². Patients with COPD lose muscle strength and mass, become physically inactive³, exhibit increased COPD-related mortality⁴, and accrue disproportionate health care costs⁵.

Muscle dysfunction in COPD affects respiratory and locomotor muscles^{6–11}. It seems to be caused by a complex interaction of systemic and local factors¹². Hypoxemia, loss of capillary capacity¹³, production of excessive reactive oxygen species (ROS)¹⁴, changes in the metabolism, muscle fiber type conversion^{10,15–17}, and inflammation¹⁸, have all been implicated in the development of muscle dysregulation in COPD¹². These alterations are not uniform among the respiratory and limb muscles. Numerous studies of the impact of COPD on muscle function have focused on the diaphragm and large lower limb muscles (quadriceps, vastus lateralis, tibialis anterior, and gastrocnemius)^{7,6,12}. Single cell-level studies from accessory respiratory muscles (i.e., intercostal) and foot muscles (i.e., flexor digitorum brevis muscles) are limited¹⁶.

While it is well documented that COPD affects both respiratory and limb muscle function, the underlying cellular mechanisms involved in COPD muscle dysfunction remain unclear. One muscle cell function that could be affected in COPD is excitation-contraction (E-C) coupling. In skeletal muscle, E-C coupling involves a series of sequential molecular steps that link muscle excitability to muscle contraction¹⁹. These steps include initiation and propagation of the electrical impulse through the muscle fiber, electrochemical transduction via protein-protein interactions between surface sarcolemma and t-tubule Cav1.1 channels and internal sarcoplasmic reticulum (SR) RyR1 channels, which elicit SR Ca^{2+} release²⁰. Once in the myoplasm, the released Ca^{2+} binds to actomyosin filaments, the contractile machinery, to produce muscle contraction, a process that is followed by active SR Ca^{2+} reuptake and muscle relaxation²⁰. Ca^{2+} release from the SR is a key Ca^{2+} signaling mechanism between incoming action potential signals and force output in skeletal muscle^{21,20}. Previous studies of COPD patients have shown mainly an indirect association with deficits in both diaphragm and leg muscles^{10,9,22}. The vast majority of these studies were performed using whole muscle tissues and analyzing histological and biochemical changes^{7,23,24,11}. Some studies have investigated the impact of COPD on muscle contractility^{8,23,11} and the final step(s) of E-C coupling at the single cell level; this neglects the impact of COPD on early and intermediate E-C coupling steps.

The aim of this study was to investigate whether COPD affects cellular processes that precede muscle contraction (i.e., intermediate E-C coupling steps). Specifically, this study

was intended to test the hypothesis that abnormal function of intercostal and foot muscles is associated with abnormal SR Ca^{2+} release.

Methods

Animals and cigarette smoke exposure

Ten week old male C57BL/6 mice were exposed to cigarette smoke (whole-body) for 6 months, using the TE-10 whole-body cigarette smoking machine (Teague Enterprises, Inc). Mice were exposed to the smoke of 3R4F reference cigarettes (University of Kentucky) for 5 hours per day, 5 days per week, and the concentration of total suspended particulates was maintained at 150 mg/m^3 as previously described²⁵. All mice were housed in appropriate animal facilities under controlled conditions for temperature and humidity, with food and water provided *ad libitum* with a 12 h light/dark cycle. Control mice of the same strain were housed in similar conditions, but they received no exposure to cigarette smoke. All experimental procedures were revised and approved by the corresponding Institutional Animal Care and Use Committees, and in accordance with the standards established by the US Animal Welfare Acts, as set forth in NIH guidelines and in the Policy and Procedures Manual of both the Johns Hopkins University and the School of Medicine, University of Maryland.

Single muscle fiber preparation

We isolated intercostal and flexor digitorum brevis (FDB) muscles. The intercostals are accessory respiratory muscles that are repetitively activated during normal respiration and are important for forced respiration²⁶. The FDB muscles in humans and small mammals are locomotor intrinsic foot muscles critical for balance and postural stability during walking, running, and jumping^{27–30}. Muscle tissues were isolated from control and 6 month whole-body cigarette smoke-exposed mice. This 6-month whole-body cigarette smoke exposure is known to cause muscle dysfunction in different species including mice^{31–33}. The procedures for isolating single muscle fibers were previously described in detail³⁴. In brief, intercostal (combined internal and external) and FDB muscles were dissected and enzymatically digested, then mechanically triturated with a polished glass pipette³⁴. Resulting fibers were suspended in minimum essential media (MEM) supplemented with 10% fetal bovine serum (FBS) for 12 to 24 hours until they were plated on laminin-coated dishes or used in releasable Ca^{2+} experiments. In all cases isolated fibers were kept in an incubator at 37°C under 5% CO_2 until dye loading. Whole muscles for tissue lysates were frozen in liquid N_2 and then stored at -80°C until ready for use.

Depolarization-induced Ca^{2+} Transients

Isolated fibers were maintained for 12–48 hours in plain MEM after plating on laminin coated dishes. Prior to imaging, fibers were loaded with the acetoxymethyl ester form of indo-1 or mag-fluo-4 (Life Technologies, Carlsbad, CA, Cat. No. I-1223 and M-14205) at $1\text{--}2 \mu\text{M}$ in L-15 media (supplemented with 0.25% bovine serum albumin)³⁵. Loading was allowed to proceed at room temperature for 30 minutes followed by dye washout and 30 minutes of conversion time to allow the dye signal to stabilize. Where indicated, fibers loaded with mag-fluo-4 were imaged on a Zeiss LSM 5 Live confocal microscope (Carl

Zeiss, Jena, Germany) operating in line-scan mode with a sampling rate of 10 kHz using a 63× water immersion objective and excited with a 488nm laser. Raw intensity mag-fluo-4 profiles were background and bleach corrected. Fibers loaded with indo-1 were recorded on an Olympus IX71 inverted microscope (Olympus, Center Valley, PA, USA) and viewed with an Olympus 60× water immersion objective. Fluorescence emission was detected at 405 and 485nm simultaneously, using 2 parallel PMTs with a sampling rate of 2 kHz. Excitation light from a broadband UV source was filtered through a 380 ± 10 nm bandpass filter, and emission was split using a dichroic mirror and passed through either a 405 ± 10 or 485 ± 10 nm bandpass filter. Electrical field stimulation was provided in 1ms pulses through platinum electrodes.

Estimation of releasable Ca^{2+}

Releasable Ca^{2+} was measured in grease (Chemplex 825, Lubritech, Harvey, IL) embedded fibers with ends protruding into the experimental solution (below) using a method modified from earlier studies^{36,37}. Because long-term fiber survival in grease is poor, isolated fibers were maintained for 12–48 hours in suspension in MEM supplemented with 10% FBS rather than plating. Fibers were then transferred in batches to L-15 supplemented with 0.25% BSA and 2 μM fluo-4 FF AM, a lower affinity Ca^{2+} dye (Life Technologies, Carlsbad, CA, Cat. No. F-23980). Prior to recording, single fibers were transferred to grease-coated channel-well plates and partially embedded using a 20% FBS/grease mixture in a method modeled after grease gap experiments³⁶ under $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hanks balanced salt solution (HBSS) containing 100 μM N-benzyl-*p*-toluene sulphonamide (BTS). Plates were then transferred to a Zeiss LSM 5 Live confocal microscope equipped with a gravity-fed semi-local perfusion apparatus. During image recording, the exposed segment of the fiber was constantly perfused locally (1–2 ml/min) with the appropriate solution. Solution reservoirs were selected by means of solenoid valves, and solution changes were accomplished in <10 sec (AutoMate Scientific, Inc., Berkeley, CA). Imaging of the exposed portion of the fiber began under perfusion with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS containing 100 μM BTS with *x-y* image acquisition at 1Hz in-frame mode and continued for a 30 second control period, followed by a switch to perfusion with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS containing 100 μM BTS, 1mM 4-chloro-m-cresol (4CMC, a RyR Ca^{2+} release agonist³⁸) and 5 μM cyclopiazonic acid (CPA, a SERCA pump inhibitor³⁷). Releasable Ca^{2+} was recorded as the change in fluorescence from the control level to the first peak following administration of 4CMC.

Western Blots

Frozen muscles were ground with a pestle under tissue protein extraction reagent (T-PER) lysis buffer (Thermo Scientific, Rockford, IL) supplemented with protease inhibitors (Complete-Mini EDTA free; Roche Diagnostics, Indianapolis, IN) and kept on ice with periodic agitation for up to 3 hours until tendons were clean. Insoluble debris was removed by centrifuging samples at 4°C for 10 minutes at 5000 RPM. The supernatant was removed and concentration was estimated by Nanodrop-1000 spectrophotometer (Thermo Scientific; Wilmington, DE). Approximately 30 μg per lane were denatured at 74°C for 10 minutes and loaded onto precast 3–8% polyacrylamide gels under reducing conditions. After transfer, membranes were blocked in 5% milk with 0.1% Tween and cut according to molecular weight so that samples could be normalized to proteins run in the same lane. Membrane

sections were incubated overnight in primary antibodies. Primary antibodies were washed out, then secondary fluorescent antibody was applied for 1 hour and washed out. All antibodies used were commercially available as follows: α -actin (Santa Cruz Inc, Dallas, TX, Cat. No. sc-58671), RyR1 (Thermo Fisher, Rockford, IL, Cat. No. MA3-925), S100A1 (Thermo Fisher, Cat. No. MA1-23594); CaM (Developmental Studies Hybridoma Bank, Iowa City, IA, Cat. No. 23-132-27-s); Secondary antibody (Thermo-Fisher, Rockford, IL, Cat. No. A21235). Membranes were imaged on a Typhoon FLA 9500 biomolecular imager (GE Healthcare Bio-Sciences, Pittsburgh, PA). Bands were measured using ImageJ, following automated background subtraction. RyR1/S100A1/CaM bands were normalized to the skeletal muscle (α) actin band from the same lane and to the average of the control samples.

Data and Statistical Analysis

After initial processing in Excel (Microsoft) and/or ImageJ (National Institutes of Health; <http://rsb.info.nih.gov/ij/>), data were analyzed in OriginPro-v8 (OriginLab, Northampton, MA) and SPSS-v24 (IBM Corp, Armonk, NY). Normality was assessed by the Shapiro-Wilk test. A linear mixed model was used for statistical analyses of all Ca^{2+} imaging studies. The model accounted for multiple fibers derived from the same animal by including mice as a random effect in the model. To examine differences between groups (control vs. cigarette smoke-exposed), the linear mixed model with condition (control and cigarette smoke-exposed) as a fixed effect and mice as a random variable was used to analyze this data set. For western blot results, statistical analysis was performed using a 2-factor (2×3) analysis of variance, followed by *post hoc* test (Scheffé F test) to evaluate the expression levels of proteins critical for SR Ca^{2+} release (RyR1, CaM and S100A1) on control and cigarette smoke-exposed mice. The 2 independent variables in this study were condition (control and cigarette smoke exposure) and SR Ca^{2+} release proteins (RyR1, CaM and S100A1). The dependent variable was the densitometry measurement on the western blot assays. Adjusted *P*-value thresholds are indicated as *P*< or *P*> the corrected value. The word “significant” refers only to a statistical difference (*P* < 0.05).

Results

Both intercostal and FDB fibers exhibit depressed action potential-induced Ca^{2+} transients

We first sought to evaluate the consequences of chronic exposure to cigarette smoke on Ca^{2+} homeostasis and action potential-induced Ca^{2+} transients, a metric of SR Ca^{2+} release, in muscle fibers isolated from intercostal and FDB muscles. Individual muscle fibers were assayed for both resting and evoked changes in fluorescence emission intensity using fluorometric quantitation of indo-1 signals by fluorescent microscopy. Ca^{2+} signals at rest were measured as the mean of the indo-1 signal during a 10 ms time interval, evaluated 20 ms before application of electrical field stimulation (brackets in Fig.1A and C). In the intercostal fibers from cigarette smoke-exposed mice, mean ($\pm$ S.D.) resting fluorescence of the indo-1 ratio signal was 0.53 ± 0.09 arbitrary units (a.u.) vs. 0.56 ± 0.1 a.u. for control mice. In FDB fibers from the cigarette smoke-exposed mice mean ($\pm$ S.D.) resting indo-1 was 0.43 ± 0.13 a.u. vs. 0.49 ± 0.09 a.u. in control mice. Using a mixed effect linear model, we found no significant differences in the mean resting myoplasmic Ca^{2+} levels in

intercostal and FDB fibers derived from cigarette smoke-exposed mice compared to control counterparts [Fig. 1B and D; intercostal: $R(1,21) = 0.83$, $P = 0.37$; FDB: $R(1,23) = 1.4$, $P = 0.24$).

Electrical field stimulation delivered at time zero elicits action-potential-induced Ca^{2+} transients (Fig. 1A and C). In the intercostal fibers from cigarette smoke-exposed mice, mean ($\pm$ S.D.) peak fluorescence of the indo-1 ratio signal was: 0.95 ± 0.26 a.u. vs. 1.21 ± 0.21 a.u. in control mice. In FDB fibers from the cigarette smoke-exposed mice mean ($\pm$ S.D.) peak indo-1 was 1.19 ± 0.34 a.u. vs. 1.43 ± 0.33 a.u. for control mice. These results showed a Ca^{2+} transient peak amplitude reduction of 36% and 16% for intercostal and FDB fibers, respectively, from cigarette smoke-exposed mice. The mixed effect linear model shows that the peak indo-1 amplitudes of the Ca^{2+} transient elicited by electrical stimulation were significantly reduced in both intercostal [Fig. 1B, D, $F(1, 21) = 9.49$, $P = 0.0057$] and FDB [Fig. 1C, D, $F(1,24) = 14$, $P < 0.001$] fibers derived from cigarette smoke-exposed mice when compared to corresponding control counterparts.

Next, we used high-speed confocal microscopy and the non-ratiometric fluorescent Ca^{2+} indicator mag-fluo-4 to increase the temporal resolution of the assayed Ca^{2+} transient and to examine possible kinetic differences. Figure 2A shows transmitted light and confocal images of muscle fiber loaded with mag-fluo4. A representative line-scan ($x-t$) image, and the corresponding time course of a Ca^{2+} transient imaged at high speed is illustrated in Fig. 2B. The differences in the peak amplitude of the Ca^{2+} transients seen in Fig. 1 are preserved using F/F_0 signals from these mag-fluo-4 recordings from both intercostal (Fig. 2C) and FDB fibers (Fig. 2D). In intercostal fibers from cigarette smoke-exposed mice, the mean ($\pm$ S.D.) time required to decay to half peak fluorescence of the mag-fluo-4 signal was 1.54 ± 0.33 ms vs. 1.7 ± 0.37 ms for control mice. In FDB fibers from cigarette smoke-exposed mice, the mean ($\pm$ S.D.) time required to decay to half peak fluorescence of the mag-fluo-4 signal was 1.77 ± 0.39 ms vs. 2.07 ± 0.76 ms for control mice. Using the mixed effects linear model we found no significant change in the decaying phase of the Ca^{2+} transients in cigarette smoke-exposed vs. control mice: intercostal [Fig. 2C right: $F(1,30) = 1.552$, $P = 0.223$] or FDB fibers [Fig. 2D right: $F(1,27) = 1.883$, $P = 0.181$].

Releasable Ca^{2+} in locomotor and respiratory muscle fibers is similar between control and cigarette smoke-exposed mice

The observed reduction in the amplitude of the Ca^{2+} transient in both respiratory and locomotor muscle fibers from cigarette smoke-exposed mice might result from either a deficit in the amount of Ca^{2+} available for release^{21,39} or decreased activation of the SR RyR1 Ca^{2+} release channels. To determine whether chronic cigarette smoke exposure results in reduced Ca^{2+} pools in SR, we assessed maximal Ca^{2+} release after stimulation with the RyR agonist 4CMC (Fig. 3A–C). In the intercostal fibers challenged with 4CMC from cigarette smoke-exposed mice, mean ($\pm$ S.D.) maximal amplitude of fluo-4 FF signal was 4.39 ± 2.38 a.u. vs. 3.46 ± 2.18 a.u. in control mice. In FDB fibers challenged with 4CMC from cigarette smoke-exposed mice, mean ($\pm$ S.D.) maximal amplitude of fluo-4 FF signal was 3.52 ± 1.91 a.u. vs. 5.19 ± 2.17 a.u. in control mice. Using a mixed effect linear model, neither FDB [$F(1,45) = 2.727$, $P = 0.106$] nor intercostal [$F(1,37) = 2.339$, $P = 0.135$] fibers

showed significant differences in releasable Ca^{2+} in cigarette smoke-exposed fibers compared to control fibers (Fig. 3D). These results indicate that the reduced Ca^{2+} transients in intercostal and FDB muscle fibers from the cigarette smoke-exposed mouse model are not the result of deficits in SR releasable Ca^{2+} .

RyR1, S100A1, and CaM expression in muscle tissue from control and cigarette smoke-exposed mice

RyR1 channels are essential for voltage-dependent Ca^{2+} release from SR^{40} , and Ca^{2+} binding proteins S100A1 and calmodulin (CaM) modulate the activity of the RyR1 Ca^{2+} release channel⁴¹. Consequently, the reduced magnitude of the Ca^{2+} transient seen in fibers from cigarette smoke-exposed mice might be explained by changes in expression levels of the RyR1 or its regulatory proteins. We compared the expression levels of RyR1, CaM, and S100A1 in intercostal and FDB muscles from cigarette smoke-exposed mice and corresponding controls by western blotting analysis. Figure 4A shows representative blots from intercostal and FDB muscles isolated from control and cigarette smoke-exposed mice. Summary plots of quantification of band intensities for expression levels of RyR1, S100A1, and CaM on intercostal and FDB muscles from the cigarette smoke-exposed mice and control counterparts are shown in Figure 4B–C. Two-way ANOVA revealed that there was a non-significant main effect of the condition (control vs. cigarette smoke exposure) on the protein expression levels in intercostal muscle [$F(1,48) = 0.08$, $P = 0.76$]. Also, there was a non-significant main effect of E-C coupling proteins on the protein expression levels [$F(2,48) = 0.21$, $P = 0.807$]. There was a non-significant interaction between cigarette smoke exposure and the E-C coupling proteins we examined in the intercostal muscles [$F(2,48) = 0.21$, $P = 0.807$]. A similar analysis in FDB muscle revealed that there was a non-significant main effect of the condition (control vs. cigarette smoke exposure) on the protein expression levels [$F(1,44) = 3.84$, $P = 0.056$]. There was a significant main effect of E-C coupling proteins on the protein expression levels [$F(2,44) = 0.604$, $P = 0.048$]. Also, there was a significant interaction between cigarette smoke exposure and the E-C coupling proteins we examined in the FDB muscles [$F(2,44) = 6.17$, $P = 0.004$]. However, *post hoc* (Scheffé test) pairwise comparisons of the individual proteins examined in cigarette smoke-exposed mice and control animals did not reach the adjusted significance threshold at 0.05 level.

Discussion

Although pulmonary abnormalities could explain physical limitations in patients with COPD, many studies also point to the contributing role of respiratory and locomotor muscle dysfunction^{9,11}. The diaphragm and major leg muscles have been the focus in characterization of skeletal muscle alterations in COPD^{9,10}. However, accessory respiratory muscles and foot muscles, such as the intercostal and FDB, respectively, may also be affected in COPD. Moreover, the cellular mechanisms that lead to respiratory and locomotor skeletal muscle dysfunction have not been elucidated¹².

Our results demonstrate E-C coupling deficits, mainly impairment of the action potential-evoked Ca^{2+} release, in intercostal and FDB muscle fibers, without significant changes in

basal Ca^{2+} signaling, the amplitude of the releasable Ca^{2+} pool, or the expression levels of RyR1, S100A1, and CaM.

Several mechanisms might explain the differences in action potential-evoked Ca^{2+} signals found in both intercostal and FDB between fibers from cigarette-exposed mice and controls. These include changes in the ability of the fibers to release and buffer/remove Ca^{2+} ⁴² and alterations in the function of proteins involved in E-C coupling^{43,20}. Compromised internal Ca^{2+} stores are a possible explanation for reduced Ca^{2+} transient magnitude^{44,39}, however, the lack of change in releasable Ca^{2+} under pharmacological stimulation indicates that the Ca^{2+} stores of fibers from the cigarette smoke-exposed mice remain intact. This is supported by the close similarity in the decay phase of the Ca^{2+} transients in both groups and the absence of any change in the resting Ca^{2+} levels.

The reduced Ca^{2+} transient could account for the reduced force seen in respiratory and locomotor skeletal muscle in humans with COPD and in a mouse model of chronic cigarette smoke exposure^{7,33,23}. Ca^{2+} signaling in skeletal muscle also controls, among many other processes, gene expression and metabolism^{42,45}. One common finding in respiratory and skeletal muscle in COPD is the transformation of fiber types from fast to slow, consistent with an adaptation for enhanced endurance capacity^{46,10}. The amplitude and frequency of the Ca^{2+} signals controls the activity of the Ca^{2+} -dependent transcription factor NFATc1^{47,48}, which in turn controls fiber type transition in different cell types, including skeletal muscle⁴⁷. The observed reduction in amplitude of the Ca^{2+} signals in respiratory muscle fibers from cigarette smoke-exposed mice could favor this plastic transition in respiratory muscle tissues. Future investigations will be designed to explore whether the activity of NFATc1 transcription factor is modified in respiratory skeletal muscle in response to chronic cigarette exposure.

Several reports indicate that, in contrast to respiratory muscles, locomotor skeletal muscle in human with COPD and cigarette smoke-exposed models does not exhibit transformation of fiber types from fast to slow^{12,46}. However, locomotor muscle displays reduced strength and endurance, reduced oxidative metabolism, and enhanced muscle atrophy^{15,49,50}. This study also shows that cigarette exposure in mice results in reduced Ca^{2+} transients in locomotor muscle fibers. Ca^{2+} signaling from the SR regulates metabolism, cell survival, and cell death⁵¹. Basal myoplasmic Ca^{2+} concentration and physiological Ca^{2+} oscillations (i.e., Ca^{2+} transients elicited by trains of action potential during muscle activity) regulate cell survival by promoting mitochondria bioenergetics^{52,51}. We hypothesize that depressed Ca^{2+} transients, as observed in muscle from cigarette-exposed mice, could contribute to muscle atrophy by affecting cell survival/death balance. In support of this idea, several studies have shown that COPD affects the metabolism of locomotor muscle in cigarette smoke-exposed animals³³. Whether Ca^{2+} dysregulation is cause or consequence of the muscle dysfunction seen in COPD remains to be explored, as do its mechanisms and relevance.

The absence of significant changes in releasable Ca^{2+} suggests that the defect in the Ca^{2+} transient could derive from abnormal function of RyR1s, the SR Ca^{2+} release channels that control release of Ca^{2+} from the SR stores^{53,54}. RyR1 interacts with multiple intracellular and SR intraluminal proteins to form a large and intricate protein assembly with at least 22

interacting/regulatory members^{53,55}. The mere presence of the core proteins of the complex (i.e., RyR1) does not demonstrate a functional Ca^{2+} release apparatus.

While we did not observe significant changes in the levels of RyR1, S100A1, or CaM in either intercostal or FDB tissue (Fig. 4), there is a significant interaction in the response of RyR1, S100A1, and CaM examined by cigarette smoke treatment in the FDB muscles, likely driven by the reduction trend observed in the RyR1. While this trend is not itself significant, it suggests that the reduction of RyR1 expression plays some role in the dysfunction of skeletal muscle E-C coupling. This reduction in RyR1 protein expression may result from protein degradation. Indeed, several reports have confirmed that enhanced protein degradation and atrophy of limb muscles of COPD patients is mediated in part through activation of the ubiquitin-proteasome pathway and that this activation is triggered by enhanced ROS production^{8,56,57}. This differs from the results observed in the intercostal muscles, where we found no significant interaction in the response of the RyR1, CaM, and S100A1 expression levels examined with cigarette smoke treatment. This suggests that, while intercostal and FDB muscles share a functional defect in COPD, the underlying mechanisms may be different.

Finally, it is possible that multiple mechanisms, occurring with different time courses, contribute to the differences in Ca^{2+} signaling seen when comparing intercostal and FDB muscle fibers from the cigarette smoke-exposed mice. Regardless of the underlying mechanism(s), our data reveal that the amplitude of the action potential-evoked Ca^{2+} transient is significantly reduced in intercostal and FDB muscle fibers. Given the critical role of Ca^{2+} signaling in many aspects of muscle function (such as contraction, gene expression, and metabolism)⁴², remodeling of the action potential-evoked Ca^{2+} transient and consequent generation of inappropriate cellular responses⁴⁵ could dramatically affect the overall function of the intercostal and FDB fibers, contributing to the progressive muscle weakness seen in COPD.

Limitations of the mouse model of cigarette smoke exposure

In the cigarette smoke exposure model, the susceptibility to develop emphysema is variable between mouse strains and does not reproduce chronic bronchitis or severe disabling disease as observed in humans⁵⁸. Also, whole-body cigarette smoke exposure, in contrast to only-nose exposure, might include systemic effects due to smoke particle uptake through the skin. Nevertheless, whole-body cigarette smoke exposure has been previously demonstrated to produce emphysematous mice^{59,60,25}, with alveolar destruction and airspace enlargement^{59,61,60,25,58,62}, the hallmarks of emphysema. Importantly, this mouse model is a valuable tool for further examination of the role of smoking in the systemic pathogenesis in COPD, such as respiratory and skeletal muscle atrophy and dysfunction^{31–33}.

Conclusions

In this report we demonstrate the effects of chronic cigarette exposure on action-potential induced Ca^{2+} transients on intercostal and intrinsic foot muscles. The intrinsic muscles of the foot are important for ambulation and balance in bipeds and quadrupeds, including humans and small mammals^{27,28}. We hypothesize that intrinsic foot muscle dysfunction

could contribute to the increased susceptibility to falls in individuals with chronic COPD. In support of this hypothesis, recent reports have shown that COPD in advanced stages is associated with an increased history of falls, balance impairment, and lower extremity muscle weakness^{65–68}.

Our results highlight that intercostal and intrinsic foot muscle Ca^{2+} signaling dysregulation occurs in cigarette smoke exposure. Understanding in more detail the pathophysiology of COPD and its effects in different skeletal muscles groups could help further understanding of the natural history of the disease and to develop targeted treatments aimed to improve muscle performance of specific muscle groups.

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Abbreviations list

AM	acetoxymethylester
BSA	bovine serum albumin
BTS	N-benzyl- <i>p</i> -toluene sulphonamide
CaM	calmodulin
Ca_v1.1	voltage gated Ca^{2+} type 1.1
COPD	Chronic Obstructive Pulmonary disease
CPA	cyclopiazonic acid
E-C	excitation-contraction
FBS	fetal bovine serum
FDB	flexor digitorum brevis
HBSS	Hank's balanced salt solution
L-15	Leibovitz (L-15) media
MEM	minimum essential media
PMT	photomultiplier tube
ROS	reactive oxygen species
RyR1	ryanodine receptor type-1
SR	sarcoplasmic reticulum
T-PER	Tissue Protein Extraction Reagent

UV	ultraviolet
4-CMC	4-chloro-m-cresol

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Figure 1.

Averaged action potential-evoked Ca^{2+} transients in intercostal (A) and FDB (C) muscle fibers from cigarette smoke-exposed (black) and control mice (gray). Fibers loaded with the ratiometric Ca^{2+} indicator indo-1 were subjected to single (15–20 V; 1 ms, arrowheads) electrical field stimuli, resulting in a single Ca^{2+} transient. Intercostal (B, right bars) and FDB (D, right bars) fibers maintain resting Ca^{2+} levels after smoke treatment, but the peak of the Ca^{2+} transient is significantly reduced in both intercostal and FDB fibers. Intercostal, n= 14 fibers from 6 animals (Control) and 22 fibers from 9 animals (Smoke); FDB, n= 15 fibers from 5 animals (Control) and 20 fibers from 6 animals (Smoke). Error bars show standard error of the mean (SEM). * $P < 0.05$.

Figure 2.

Action potential evoked- Ca^{2+} transient decay phase in muscle fibers from control and cigarette smoke-exposed mice. (A) Transmitted light (*left*) and confocal x-y (*right*) images of a FDB fiber loaded with mag-fluo-4. Dashed line in A indicates the location of the line-scan used to measure the Ca^{2+} transient. (B) Line-scan confocal image (B, *left*) showing the fast change in mag-fluo-4 fluorescence profile (B, *right*) in response to electrical field stimulation (gray mark). Dashed rectangle shows example of a region of interest (ROI, box) used to measure the time course of the Ca^{2+} transient. Average mag-fluo-4 Ca^{2+} transients from intercostal (C, *left*) and FDB (D, *left*) muscle fibers from control (gray traces) and cigarette smoke-exposed mice (black traces). Traces were normalized to peak intensity and had pre-peak period removed before generating average traces of the decay phase (C, D, *middle*). Measurements of decay time from peak to half-peak showed no significant differences due to cigarette smoke in either intercostal (C, *right*) or FDB (D, *right*). Intercostal, n= 14 fibers from 3 animals (Control) and 18 fibers from 4 animals (Smoke); FDB, n= 13 fibers from 3 animals (Control) and 15 fibers from 4 animals (Smoke). Error bars show SEM.

Figure 3.

Evaluation of releasable Ca^{2+} . Individual muscle fibers were embedded in grease (A) and loaded with the Ca^{2+} indicator fluo-4 FF (B). Partial embedding in grease (dashed areas in A and B) prevents movement out of frame during direct perfusion with RyR1 Ca^{2+} release agonist 4CMC. Fluorescence intensity was recorded during perfusion in a single region of interest (ROI) within the fiber, and the peak response for each fiber was recorded (C). There were no significant differences due to cigarette smoke exposure in either intercostal or FDB fibers (D) when compared to corresponding controls. Intercostal, n= 19 fibers from 3 animals (Control) and 20 fibers from 3 animals (Smoke); FDB, n= 19 fibers from 3 animals (Control) and 28 fibers from 2 animals (Smoke). Error bars show SEM.

Figure 4.

Representative western blots (A) of RyR1, S100A1 calmodulin, and skeletal muscle actin for intercostal and FDB muscles from control and cigarette smoke-exposed mice (Smoke). Quantification (B, C) of each band after background subtraction was normalized to skeletal muscle actin from the same lane, then to control samples in the same blot. A 2-way ANOVA of the intercostal samples (B) did not detect any main effects or significant interactions between cigarette smoke and changes in the level of E-C coupling proteins. Similar analysis of the FDBs detected an interaction between cigarette smoke treatment and changes in the levels of RyR1, but pairwise testing of each protein did not reveal any significant differences after adjusting threshold for multiple comparisons. Intercostal, n= 9 muscles (Control and Smoke); FDB, n= 6 muscles (RyR1 Control and Smoke) or 9/10 muscles (S100 and CaM, Control/Smoke). Error bars show SEM.