Diaphragm and ventilatory dysfunction during cancer cachexia

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ABSTRACT  Cancer cachexia is characterized by a continuous loss of locomotor skeletal muscle mass, which causes profound muscle weakness. If this atrophy and weakness also occurs in diaphragm muscle, it could lead to respiratory failure, which is a major cause of death in patients with cancer. Thus, the purpose of the current study was to determine whether colon-26 (C-26) cancer cachexia causes diaphragm muscle fiber atrophy and weakness and compromises ventilation. All diaphragm muscle fiber types were significantly atrophied in C-26 mice compared to controls, and the atrophy-related genes, atrogin-1 and MuRF1, significantly increased. Maximum isometric specific force of diaphragm strips, absolute maximal calcium activated force, and maximal specific calcium-activated force of permeabilized diaphragm fibers were all significantly decreased in C-26 mice compared to controls. Further, isotonic contractile properties of the diaphragm were affected to an even greater extent than isometric function. Ventilation measurements demonstrated that C-26 mice have a significantly lower tidal volume compared to controls under basal conditions and, unlike control mice, an inability to increase breathing frequency, tidal volume, and, thus, minute ventilation in response to a respiratory challenge. These data demonstrate that C-26 cancer cachexia causes profound respiratory muscle atrophy and weakness and ventilatory dysfunction.—Roberts, B. M., Ahn, B., Smuder, A. J., Al-Rajhi, M., Gill, L. C., Beharry, A. W., Powers, S. K., Fuller, D. D., Ferreira, L. F., Judge, A. R. Diaphragm and ventilatory dysfunction during cancer cachexia. FASEB J. 27, 000–000 (2013). www.fasebj.org

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Cancer cachexia is characterized by whole body wasting due to a continuous loss of muscle and fat mass (1). Cachexia is associated with most advanced-stage cancers, but is particularly prevalent in patients with gastrointestinal, pancreatic, lung, and colorectal cancers (2). The significant loss of muscle mass in patients with cancer is believed to cause muscle weakness, thereby compromising physical function, functional independence, and quality of life (3). Moreover, cancer cachexia accounts for over a quarter of cancer-related deaths, which is believed to be due, at least in part, to the wasting of skeletal muscle (2). Experimental evidence in support of this can be found in two landmark studies. First, skeletal muscle-specific inhibition of nuclear factor-kB (NF-kB) activation in mice inoculated with Lewis lung carcinoma (LLC) cells attenuated body weight loss and muscle fiber atrophy, and extended life span compared to wild-type mice inoculated with LLC cells, despite the same tumor burden (4). Second, colon 26 (C-26) tumor-bearing mice injected with a soluble activin receptor IIB (sActRIIB) to antagonize the ActRIIB pathway show no loss of muscle mass or strength and a significantly extended life span compared to untreated C-26 mice, despite similar tumor burden and serum proinflammatory cytokine levels (5). Although other systemic effects cannot be ruled out as the explanation for extended life span in this second study, combined, these two studies strongly suggest that preservation of skeletal muscle mass and, presumably, function is critical to survival during cancer cachexia. In this regard, sparing of muscle mass and function in relation to survival is presumed to be related to the sparing of the respiratory muscles.

Although significant evidence exists showing that cancer cachexia causes locomotor muscle atrophy (4–8), to our knowledge there are only 3 studies that have measured diaphragm mass, muscle fiber size, and/or function during cancer cachexia, 2 of which are from the same research group. Tessitore et al. (9) reported no change in diaphragm muscle mass of Yoshida ascites hepatoma AH-130 tumor-bearing rats 4 and 10 d

Abbreviations: C-26, colon 26; CSA, cross-sectional area; H&E, hematoxylin and eosin; ktr, rate of tension redevelopment; LLC, Lewis lung carcinoma; MAFbx, muscle atrophy F box (atrogin-1); MyHC, myosin heavy chain; nH, slope of the force-pCa relationship; MuRF1, muscle RING-finger 1; sFm, max, maximum specific calcium activated force

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postinoculation, despite there being significant atrophy of locomotor muscles. In agreement with this finding, in 2011 Murphy et al. (10) found no change in diaphragm muscle fiber cross-sectional area (CSA) and no change in diaphragm-specific force (force normalized to mass) in LLC tumor-bearing mice compared to controls. However, the degree of cachexia in this latter study was only moderate, with a 9% decrease in tumor-free body mass compared to controls. If muscle wasting associated with cancer cachexia decreases life span and this is related to respiratory muscle weakness, one might expect diaphragm muscle function to be compromised in more severe stages of cachexia. Indeed, a 2012 study by Murphy et al. (11) reported a 10% lower maximum specific force and lower absolute force during a fatigue protocol in diaphragm strips of C-26 tumor-bearing mice that lost 22% tumor-free body mass. This recent study was the first evidence of diaphragmatic isometric contractile dysfunction in cancer cachexia. However, it is unclear whether these effects were due to alterations in membrane excitability, Ca2+
cachexia. Yet, to our knowledge, there are still no data to demonstrate whether severe cancer cachexia causes diaphragm muscle fiber atrophy, impairs diaphragm isotonic contractile properties, or compromises ventilation. Therefore, the aim of the current study was to determine whether severe C-26 cancer cachexia causes significant diaphragm muscle fiber atrophy and isotonic contractile dysfunction, and whether cachexia compromises ventilation. To gain further insight into the mechanisms of contractile dysfunction, we also examined chemically skinned diaphragm fibers.

MATERIALS AND METHODS

Animals

CD2F1 mice weighing ~20 g were purchased from Charles River Laboratories (Wilmington, MA, USA) and were used for all animal experiments, which were approved by the University of Florida Institutional Animal Care and Use Committee. Mice were maintained in a temperature- and humidity-controlled facility with a 12-h light-dark cycle. Water and standard diet were provided ad libitum.

Cancer cachexia

C-26 cells were obtained from the National Cancer Institute Tumor Repository (Frederick, MD, USA). Cells were cultured in RPMI 1640 (Mediatech, Herndon, VA, USA) supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a 5% CO2 humidified atmosphere. To induce cancer cachexia, 5 × 10^5 cells were injected subcutaneously into each flank, and muscles were harvested when the largest tumor diameter reached 1.5 cm (~25–27 d postinoculation).

Muscle preparation and analysis

Mice were acutely anesthetized with an intraperitoneal injection of pentobarbital sodium (70 mg/kg body weight). Once a surgical level of anesthesia was reached, the costal diaphragm was divided into segments, with a strip of the medial costal diaphragm used immediately for in vitro contractile measurements. For histological analysis, a separate segment of the costal diaphragm was embedded in freezing medium in a tissue-embedding cassette prior to freezing in liquid-nitrogen-cooled isopentane, and stored at −80°C. For mRNA analysis, a separate segment of the costal diaphragm was processed immediately, as described below.

Histology

For histological and immunohistochemical analysis, 10-µm sections were taken from the diaphragm using a Microm HM 550 cryostat (Microm International, Walldorf, Germany) and transferred to positively charged glass slides. Sections were placed at room temperature for 1 h and then either stained immediately with hematoxylin and eosin (H&E) or frozen at −80°C until further immunohistochemical processing.

H&E staining

Muscle cross sections were H&E stained as described previously (13). Briefly, slides were sequentially submerged in the following solutions: 100% ethanol for 1 min, 70% ethanol for 1 min, dH2O for 2 min, and Gill’s hematoxylin for 2 min. Sections were subsequently washed in dH2O and then sequentially submerged in the following solutions: Scott’s solution for 15 s, dH2O for 2 s, 70% ethanol for 1 min, eosin for 2 min, 95% ethanol with gentle shaking for 1 min, 100% ethanol for 30 s, and xylene for 3 min. Slides were dried at room temperature for 30 min and then mounted with glass coverslips using Permount.

Immunohistochemistry

To measure the muscle fiber CSA of the different muscle fiber types, sections were permeabilized for 5 min in 0.1% Triton-X-100, washed 2 × 5 min in PBS, and blocked in Pierce Superblock (Pierce, Rockford, IL, USA) for 15 min. Sections were subsequently incubated in primary antibody diluted in blocking buffer at 4°C overnight in a humid chamber. Primary antibodies for the following proteins were used: myosin heavy chain (MyHC) type I (A4.840, 1:15; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) and MyHC type IIα (GC-71, 1:50; Developmental Studies Hybridoma Bank), and laminin (1:200; Sigma-Aldrich, St. Louis, MO, USA). Sections were washed 3 × 5 min in PBS prior to incubation with the appropriate fluorescently conjugated secondary antibodies from Invitrogen (Carlsbad, CA, USA) in blocking solution for 1 h at room temperature. Sections were then washed 3 × 5 min in PBS and mounted with coverslips using Vectorshield fluorescence mounting medium (Vector Laboratories, Burlingame, CA, USA).

Images were visualized with a Leica DM5000B microscope (Leica Microsystems, Bannockburn, IL, USA) using DAPI (blue), GFP (green), and rhodamine (red) filter cubes to visualize MyHC type I fibers, MyHC type IIα fibers, and laminin, respectively. Images were captured and merged...
using Leica Application Suite 3.5.0 software. This same software was used to measure the CSA of each fiber type.

**mRNA levels**

Costal diaphragm segments for RNA isolation were processed immediately using a TRIzol-based method, as described previously (14). cDNA was generated from 1 μg of RNA using an Ambion RETROscript first-strand synthesis kit (Life Technologies, Grand Island, NY, USA) and was used as a template for quantitative RT-PCR using primers for atrogin-1 (GenBank NM_026346.2), MuRF-1 (GenBank NM_001039048.2), cathepsin L (GenBank NM_0019084.3), Bnip3 (GenBank NM_009761.3), and 18S S (GenBank X03205.1).

**In vitro muscle contractile properties**

The solutions and methods used for studies of diaphragm muscle isometric function were described in detail previously (15, 16). Briefly, mice were anesthetized using isoflurane (5%, induction; 3%, maintenance), and the diaphragm was excised and immediately placed in a dissecting chamber containing a Krebs-Henseleit solution equilibrated with 95% O₂-5% CO₂ gas. A strip of the medial costal diaphragm was dissected, and one end of the muscle was tied to a Dual-Mode Muscle Lever System (300C-LR; Aurora Scientific, Aurora, ON, Canada) and the other end was tied to a secured glass rod using a 4.0-gauge silk suture. Muscles were placed at optimal length (Lₒ) and allowed 20 min of theremoequilibration at 25°C. Thereafter, measurements of force-frequency or force-velocity were initiated. In all electrical stimulations, a supramaximal current (600–800 mA) and 0.25-ms pulse duration were delivered through a stimulator (701C; Aurora Scientific), while train duration for isometric contractions was 300 ms. For comparative purposes, force production from diaphragm strips was normalized to the total CSA of the muscle strip, as specific force. To calculate the strip CSA, the following algorithm was used: total muscle CSA (cm²) = [muscle mass/(fiber length × 1.056)], where 1.056 is muscle density (g/cm³), and fiber length (cm) is measured at Lₒ (17, 18). To study diaphragm isotonic contractile properties, an afterloaded isotonic release protocol was used, as described by others (19). The diaphragm was stimulated at 300 Hz for 200 ms to contract against a force clamped to 4–80% of the maximal active tetanic tension after theremoequilibration at 37°C (20, 21). Velocity was measured ~20 ms after the onset of shortening in a region corresponding to the linear phase of length changes and relatively constant force. All data were recorded and analyzed using commercial software (DCC and DMA; Aurora Scientific). The force-velocity was analyzed using the Hill equation, as described previously (20).

**Diaphragm-permeabilized single-fiber mechanics**

Our preparation for single-fiber mechanics is consistent with that used in previous studies (22, 23), with slight modifications. The diaphragm was removed and immediately immersed in ice-cold relaxing solution (in mM: 100 KCl, 20 imidazole, 4 ATP, 2 EGTA, 7 MgCl₂; pH adjusted to 7.0 using KOH) for bundle dissections. Diaphragm fiber bundles (~2 mm width) were tied to glass capillary tubes and placed in permeabilizing solution (relaxing solution with 1% Triton X-100) for 4 h at 4°C. Permeabilized fiber bundles were stored in 50% v/v glycerol/relaxing solution at −20°C, and single fibers were tested within 3 wk of diaphragm harvest. At the time of experiments, single fibers were isolated from permeabilized bundles in ice-cold relaxing solution and tied to a force transducer (403A; Aurora Scientific) and a motor arm (315G4; Aurora Scientific). The temperature of the apparatus was kept at 15°C during fiber mounting and raised to 22°C for mechanical studies. When the apparatus reached 22°C, fibers were positioned at a sarcomere length of ~2.60 μm in relaxing solution. Fiber length and diameter were measured using computerized video microscopy. The fiber was placed in pCa 9.0 and allowed ≥3 min for equilibration. A quick-release step was subsequently performed by rapidly shortening the fiber by 20% of the segment length for 20 ms before returning to the initial length. This step was repeated by exposing the fiber to pCa solutions (22°C) ranging from 6.4 to 4.5 to determine calcium-activated force. In each solution, force was allowed to plateau before starting the quick-release step. The force response to this procedure was fitted using a single exponential equation starting at the residual force (24) to determine the rate of tension redevelopment (kᵣ). Experiments were performed and analyzed using SLControl software (25). The force-pCa relationship of individual fibers was analyzed using a 4-parameter Hill equation (Prism 5.0b; GraphPad Software, La Jolla, CA, USA):

\[ F = \frac{F_{\text{pas}} + F_{\text{max}} \cdot 10^{\text{pCa} \cdot k_{\text{pas}}}}{[10^{\text{pCa} \cdot k_{\text{pas}}} + 10^{\text{pCa} \cdot k_{\text{pas}}}]^{H}} \]

where \( F_{\text{pas}} \) is passive force, \( F_{\text{max}} \) is maximal active force, \( k_{\alpha} \) is the Hill coefficient, and pCa₀ is the pCa that elicits half-maximal activation. Maximal active force was also calculated by subtracting baseline force (pCa 9.0) from maximal Ca²⁺-activated force (pCa 4.5). Fiber “run-down” was determined as the decrease in force at pCa 4.5 from the beginning to the end of the experiment. Three to 5 fibers were studied per diaphragm fiber bundle, from 5 mice/group. Fibers with rundown > 15% were not included in analysis of force-pCa relationship. Consistent with our previous investigation (22), data from individual fibers were fitted using the Hill equation, and parameters from fibers of the same bundle were averaged to yield a mean value for each animal.

**Muscle homogenization**

To solubilize myosin and actin proteins, diaphragm samples were homogenized in a high-salt lysis buffer, previously shown to be optimal for myosin and sarcomeric actin extraction and solubility (26). The buffer consisted of 300 mM NaCl, 0.1 M NaH₂PO₄, 0.05 M Na₂HPO₄, 0.01 M Na₃P₂O₇, 1 mM MgCl₂, 10 mM EDTA, and 1 mM DTT (pH 6.5) and complete protease inhibitor cocktail (Sigma-Aldrich). Tissue lysates were subsequently centrifuged at 16,000 g for 3 min at 4°C, and the supernatant was collected. For measurement of troponin and tropomyosin protein, diaphragm samples were homogenized in RIPA buffer with protease inhibitor (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor (Sigma-Aldrich). Protein concentration was determined using the RC-DC Assay (Bio-Rad, Hercules, CA, USA).

**Myofibrillar gels and Western blots**

Samples were mixed in Laemmli buffer (Bio-Rad) and boiled before loading. To determine MyHC abundance, we loaded 0.6 μg protein/lane (which was within the linear range determined in our laboratory) into a 10% polyacrylamide gel (Criterion precast gels; Bio-Rad) and ran the gel electrophoresis at 200 V for 50 min at 4°C. The gel was stained with Coomassie blue for 3 h (Thermo Fisher Scientific, Waltham, MA, USA) and washed with dH₂O 3 times. We quantified the optical density corresponding to MyHC using an Odyssey Infrared Imaging system (LI-COR, Lincoln, NE, USA). For measurement of actin, troponin, and tropomyosin levels, Western blot analysis was performed according to standard...
procedures that have been described previously (14). Primary antibodies for sarcomeric actin (1:1000 dilution, JLA20; Developmental Studies Hybridoma Bank), tropomyosin (1:500 dilution, CH1; Developmental Studies Hybridoma Bank), and troponin T1, T2, and T3 (1:500 dilution, ab66183; Abcam) were used according to the manufacturer’s directions. Alexa Fluor 680 fluorescent dye-conjugated secondary antibodies (1:10,000 dilution, Invitrogen, Carlsbad, CA, USA) were used for visualization with the Li-Cor Odyssey fluorescent detection system, as described previously (14).

**Ventilation measurements**

A commercially available barometric plethysmograph (Buxco, Wilmington, NC, USA) with a continuous airflow design (0.5 L/min) was used to measure the pattern of breathing in control and C-26 mice, as described previously (27, 28). A clear Plexiglas chamber (diameter 3.5 inches, height 5.75 inches) was calibrated with known airflow and pressure signals prior to data collection. Signals were analyzed using the Drorbaugh and Fenn equation (29) to provide a breath-by-breath display of ventilation. Ventilation data were collected and analyzed in 10-s bins. A minimum of 30 min was allowed for acclimation to the chamber, and then a stable 5-min period of breathing was used for baseline measurements. During both acclimation and baseline, mice were breathing normoxic air (21% O₂, 79% N₂). Following baseline, ventilation was stimulated by a brief hypoxic ventilatory challenge (5 min, 10% O₂, balance N₂).

**Statistical analysis**

All data were analyzed using a Student’s *t* test or a 2-way ANOVA followed by Bonferroni *post hoc* comparisons (GraphPad). All data are expressed as means ± se, and significance was set at *P* ≤ 0.05.

**RESULTS**

**Diaphragm muscle fiber atrophy in C-26 mice**

Mice were euthanized once the largest tumor reached 1.5 cm in diameter (~25 to 27 d postinoculation), at which point tumor-free body weight was decreased by 26% compared to preinoculation body weight (control initial body weight, 26.3 g; control final body weight, 26.8 g; C-26 initial body weight, 26.7 g; C-26 final tumor free body weight, 19.8 g; Fig. 1A), which is reflective of severe cachexia (7). To determine the extent to which

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**Figure 1.** Diaphragm fiber size, fiber type distribution, and atrophy-related gene expression in control and C-26 tumor-bearing mice. *A* Body weight of control mice and tumor-free body weight of C-26 mice at study endpoint (when largest tumor reached 1.5 cm in diameter). *B, C* Representative cross sections taken from the diaphragm of control and C-26 mice. Sections were H&E stained (*B*) or incubated with an anti-laminin antibody to allow for visualization of muscle fibers (red) and anti-MyHC type I (blue) and anti-MyHC type IIa (green) antibodies; black fibers represent type IIb/x fibers (*C*). *D* Mean CSA of all fibers and of each muscle fiber type. *E* Percentage of each diaphragm muscle fiber type in control and C-26 mice. *F* Relative mRNA levels of atrogin-1, MuRF1, Bnip3, and cathepsin L (CthL) from the diaphragm of control and C-26 mice. Bars represent means ± se for 6 muscles/group. *P* < 0.05 vs. control.
severe cancer cachexia causes diaphragm muscle fiber atrophy, we stained muscle cross sections using H&E (Fig. 1B) and measured the mean muscle fiber CSA of all fibers. The mean CSA of all fibers was decreased by 26% in C-26 mice compared to controls (Fig. 1D). To more specifically determine the effect of cancer cachexia on diaphragm muscle fiber atrophy, we subsequently performed immunohistochemistry on muscle cross sections to identify type I, IIA, and Iib/x myofibers (Fig. 1C) and calculated the mean CSA of each fiber type. The mean CSA of type I muscle fibers was decreased by 24%, type IIA by 22%, and type Iib/x by 29% in C-26 mice compared to controls (Fig. 1D). To our knowledge, this is the first evidence demonstrating that severe cancer cachexia causes significant diaphragm muscle fiber atrophy. We next determined whether the diaphragm undergoes a fiber type shift by calculating the percentage of each muscle fiber type in the diaphragm of control and C-26 mice (Fig. 1E). Although not statistically significant, there was a 14% decrease in the percentage of type I fibers ($P = 0.054$) and a 13% increase in the percentage of type IIA fibers ($P = 0.085$).

Two commonly used biomarkers of muscle atrophy and, specifically, biomarkers of the ubiquitin proteasome pathway of protein degradation are the muscle-specific E3 ligases, atrogin-1/muscle atrophy F-box (MAFbx), and muscle-specific RING finger 1 (MuRF1). The use of these ligases as biomarkers is based, in part, on their up-regulation in multiple models of muscle atrophy (30, 31). Atrogin-1 and MuRF1 mRNA levels were increased 2.2- and 2.8-fold, respectively, in the diaphragm of severely cachectic C-26 tumor-bearing mice compared to controls. In addition, the mRNA levels of cathepsin L and Bnip3 were increased 5.3- and 3.5-fold, respectively, in C-26 mice compared to control. Cathepsin L is a component of the lysosomal pathway, which degrades membrane proteins, and Bnip3 a central mediator of autophagy. Both markers are consistently up-regulated during muscle wasting (Fig. 1F and refs 30, 32–34).

To determine whether diaphragm fiber atrophy during severe cancer cachexia is associated with diaphragm contractile dysfunction, a strip of the medial costal diaphragm was used for in vitro contractile measurements. Measurement of the in vitro force-frequency response of diaphragm strips clearly demonstrated that severe C-26 cancer cachexia causes a significant decrease in diaphragm muscle force production at all stimulation frequencies $\geq 60$ Hz (Fig. 2A). Maximal specific force was decreased by 30% in response to severe C-26 cancer cachexia (control, 20.74 N/cm$^2$; C-26, 14.48 N/cm$^2$; Fig. 2B). These findings clearly demonstrate that severe cancer cachexia decreases both submaximal and maximal diaphragm specific force production.

This decrease in diaphragm-specific force in C-26 mice could be due to compromised excitability of the sarcolemma, calcium release, and/or function of myofibrillar proteins. We therefore subsequently measured the absolute maximal calcium-activated force, maximal specific calcium-activated force ($sF_{\text{v}}$ max), calcium sensitivity (pCa50), slope of the force-pCa relationship ($n_H$), and rate of tension redevelopment ($k_{\text{tr}}$) in permeabilized single fibers from the diaphragm of control and C-26 mice to eliminate any role of the sarcolemma or calcium release and focus on the contractile apparatus. The absolute maximal force was 33% lower in permeabilized fibers from C-26 mice compared to fibers from control mice (Fig. 2C). To determine $sF_{\text{v}}$ max, we first determined single-fiber CSA and found an 18% decrease in fibers from C-26 mice compared to controls (Fig. 2D). Subsequent determination of $sF_{\text{v}}$ max revealed an 18% lower force in fibers from C-26 mice compared to fibers from control mice (Fig. 2E), suggesting that sarcomeric dysfunction is a primary cause of diaphragm muscle weakness in C-26 mice. We also found a significant decrease in the contractile apparatus calcium sensitivity in C-26 fibers compared to controls (Fig. 2F). This effect appears small when examined as pCa50 but considering that pCa50 is the $-\log[^{+}\text{Ca}^2+]$, the difference seen in Fig. 2F reflects an $\sim 20$% decrease in calcium sensitivity. Similarly, cancer decreased the slope of the force-pCa relationship as represented by $n_H$ (Fig. 2G). The $k_{\text{tr}}$ value, which is an index of cross-bridge kinetics (35, 36), was decreased by 30% in fibers from C-26 mice compared to controls (Fig. 2H). Overall, these functional data in intact bundles and permeabilized fibers of diaphragm clearly demonstrate significant contractile dysfunction, the latter of which suggests impaired sarcomere contractile function. To determine whether this impairment is related to alterations in myofibrillar protein expression, we quantified MyHC and actin protein levels. Samples were processed in a high-salt lysis buffer since a recent study clearly demonstrated their significantly greater solubility, and, therefore, more accurate quantification, when processed in this buffer (26). Normalized to total protein within the solubilized fraction, MyHC levels were decreased by 27% in the diaphragm of C-26 mice compared to controls (Fig. 3). However, the protein levels of sarcomeric actin remained unchanged (Fig. 3A). We also measured troponin and tropomyosin protein levels in samples processed in conventional RIPA buffer and found no differences in their protein levels between control and C-26 (data not shown). These findings suggest that MyHC is targeted for degradation in the diaphragm during severe cancer cachexia, which could explain, in part, the impaired contractile function.

Isotonic contractile properties of the diaphragm were affected to a greater extent than isometric function. Indeed, although the shape of the force-velocity relationship ($a/P_o$) was unchanged in C-26 mice compared to controls (Fig. 4A), C-26 cancer cachexia decreased maximum shortening velocity in diaphragm strips by 39% (Fig. 4B). Further, peak power output was decreased by 51% in diaphragm strips of C-26 mice compared to controls (Fig. 4C, D). Interestingly, C-26 cancer cachexia did not alter the relative...
force (in $P/P_o$; control, 0.29±0.04; C26, 0.31±0.04) or velocity (in $V/V_{\text{max}}$; control, 0.39±0.05; C-26, 0.34±0.07), where peak power was developed. Because cancer decreased power in almost all loads tested, we hypothesized that cancer would impair ventilation and thus subsequently tested this hypothesis.

Ventilation in C-26 mice is impaired in response to a challenge

Representative airflow traces obtained in unanesthetized mice using whole body plethysmography are shown in Fig. 5A. This representative figure illustrates that C-26 tumor-bearing mice developed a shallow pattern of breathing compared to control mice during normoxic baseline conditions and had a blunted ability to increase ventilation when the respiratory system was challenged, which we did in this study via hypoxia. During the normoxic baseline period, tidal volume was significantly decreased in C-26 mice (Fig. 5C), and the rapid-shallow breathing index, (RSBI; breath frequency/tidal volume), was significantly greater in the C-26 mice compared to control mice (16.0±7.0 vs. 10.6±9.1, respectively; $P=0.021$). The RSBI is used clinically as an indicator of respiratory difficulty, and larger numbers are associated with an inability to wean from respiratory support in intensive care patients (37). Even though baseline tidal volume was decreased for C-26 mice, baseline breathing frequency and ventilation were not significantly different between control and C-26 mice (Fig. 5B, D).
DISCUSSION

Cancer cachexia accounts for more than a quarter of cancer-related deaths (2), and preservation of skeletal muscle mass during experimental cancer cachexia significantly prolongs life span (4, 5), demonstrating that muscle wasting during cancer contributes significantly to mortality. Patients with cancer have respiratory muscle dysfunction, documented by a diminished ability to generate inspiratory pressure (and presumably volume; ref. 12). The consequence is that, with decreased airflow and inability to inflate the lungs and cough effectively, airway clearance is impaired, facilitating the development of pneumonia (41), a major cause of respiratory failure and death in critically ill patients with cancer (39, 42). It is therefore presumed that respiratory muscle atrophy and weakness enhance morbidity and mortality in patients with cancer. In support of this presumption, the current study provides the first evidence to clearly demonstrate that severe cancer cachexia causes diaphragm muscle fiber atrophy, and compromises isotonic diaphragm contractile properties and ventilatory function. Our studies in permeabilized single fiber also revealed that cancer impairs sarcomeric contractile function.

Our finding of decreased isometric diaphragm specific force in C-26 mice compared to controls is consistent with very recently published data from Murphy et al. (11). Although it is not possible to measure “absolute” diaphragm force production, it is likely to be decreased in the C-26 mice compared to controls. This is because absolute force is a function of specific force and muscle mass, and both specific force and muscle fiber CSA were significantly decreased in C-26 mice. This overall diaphragm weakness will likely impair the generation of airway pressure and lung volume and may explain the ventilatory dysfunction measured in the current study. Indeed, during baseline conditions, the rapid shallow breathing index, which is a clinically used measure for predicting readiness for extubation from ventilator support (37), was significantly increased in C-26 mice compared to controls. Although minute ventilation was not significantly different between control and C-26 mice during resting/baseline conditions, it has been suggested that only a small fraction of the phrenic motor pool is required to maintain adequate alveolar ventilation during quiet breathing (43). Therefore, a respiratory challenge is commonly used to identify ventilatory deficits (39, 40). Indeed, during a brief hypoxic exposure, control mice, but not cachectic mice, significantly increased their breathing frequency and minute ventilation. These findings clearly demonstrate that severe experimental cancer cachexia causes profound ventilatory deficits, which parallels that reported in patients with advanced cancer (44). Transitionally, the lack of ability of cancer mice to increase ventilation during a ventilatory challenge could suggest that patients with cancer cachexia may have a compromised ability to increase ventilation when required, such as during physical activity. Although the ventilatory deficits are likely due, at least in part, to the diaphragm muscle fiber atrophy and weakness, it is also possible that the neural control of breathing is altered during cancer cachexia. How-
ever, although this warrants investigation, measurement of neural motor output was beyond the scope of the current study.

Several potential explanations exist for the impairments in isometric and isotonic contractile properties of the diaphragm during cancer cachexia. One is through disruption to the sarcolemma, thus disrupting membrane excitability and/or intracellular signaling events that regulate muscle contraction. Indeed, both sarcolemmal morphology and permeability are altered in the skeletal muscle of cachectic mice (45), thus supporting this possibility. A second possibility is disruption to calcium homeostasis and release, which could be affected by structural disorganization of the sarcoplasmic reticulum, as has previously been shown in skeletal muscle during cancer cachexia (46). A disruption in calcium homeostasis could also explain the increased activation of calcium-dependent proteolysis that has been reported in skeletal muscle during cancer cachexia (47).

In addition to these possibilities, the data in the current study demonstrate that dysfunction of the contractile apparatus explains a significant proportion of the force deficits during cancer cachexia, which could be related to the selective degradation of MyHC. Indeed, we found a 27% decrease in MyHC expression, but no decrease in actin, troponin, or tropomyosin. This finding is in close agreement with several studies that have shown targeted degradation of MyHC in limb muscle during experimental cancer cachexia (7, 8) and in the rectus abdominis muscle of patients with pancreas carcinoma (48) and patients with esophagogastric cancer (49). However, our findings are in contrast with a recent paper showing no differences in MyHC protein levels in the tibialis anterior muscle of C-26 and control mice (26). This latter study demonstrated that myosin does not solubilize well in low-salt lysis buffers, potentially compromising the interpretations made in the studies showing targeted MyHC degradation that used low-salt buffers (7, 8, 48, 49). Indeed, lysis in a low-salt buffer resulted in 94% less MyHC in the soluble fraction compared to lysis in a high-salt buffer. The researchers also found 70% higher levels of actin when tissue was processed in the high-salt vs. the low-salt lysis buffer (26). Therefore, in the current study, we processed diaphragm tissue in the same high-salt lysis buffer used by Cosper and Leinwand (26) and found significant targeted degradation of MyHC. Given that the myosin-actin interaction determines muscle contractile function, the preferential loss of MyHC could explain, at least in part, the contractile deficits. Although further mechanistic experiments would need to be conducted, it is interesting to speculate that the loss of MyHC in the diaphragm of C-26 mice could be related to the up-regulation of MuRF1 given that MuRF1 ubiquitiniates MyHC and is required for MyHC degradation, at least in response to the glucocorticoid dexamethasone (50).

Our studies of permeabilized single-fiber mechanics lend further insights into potential molecular mechanisms of contractile dysfunction in cancer. Specifically, maximal calcium-activated specific force in diaphragm-permeabilized single fibers was decreased 18% in C-26 mice compared to controls. This may be a consequence of a reduced number of strongly bound force generating cross bridges and/or less force per cross bridge (51, 52). To this end, $k_e$ during maximal calcium activation was 30% slower in C-26 mice compared to controls.

**Figure 5.** Breathing pattern in control and C-26 tumor-bearing mice. All data were collected during baseline conditions (21% O$_2$) and challenge (hypoxia, 10% inspired O$_2$) in unanesthetized mice at study endpoint. A) Representative airflow traces to demonstrate the pattern of breathing. B) Breathing frequency. C) Tidal volume. D) Minute ventilation in control and C-26 mice. Bars represent means ± se for 8 mice/group. *$P < 0.05$ vs. control baseline; †$P < 0.05$ vs. control challenge.
Values of $k_v$ measured during maximal calcium activation reflect the rate of cross-bridge transition from weakly bound, non-force-generating state, to a strongly bound, force-generating state (36, 53). A reduced rate of weak to strong binding transition will contribute to decrease the fraction of force-generating cross bridges, and, therefore, maximal force. C-26 cancer cachexia also decreased the calcium sensitivity ($pC_{50}$) and cooperativity (Hill coefficient) of the contractile apparatus. A potential mechanism for the decrease in calcium sensitivity and cooperativity is a decrease in thin filament activation due to a decrease in the number of bound cross bridges (54).

Since breathing requires diaphragm shortening, changes in isotonic contractile properties are of primary relevance for in vivo muscle function. In this regard, we found that cancer impaired both $V_{\text{max}}$ and peak power in the diaphragm. Given that the curvature of the force-velocity relationship was unchanged, the depression of peak power must be caused by the compounded decrease in $V_{\text{max}}$ and $P_o$. Although $V_{\text{max}}$ underestimates the unloaded shortening velocity of the muscle (55, 56), or shortening velocity measured during isotonic release (19), $V_{\text{max}}$ correlates with unloaded shortening velocity and provides insight into cross-bridge kinetics in the intact muscle (55, 56). Notably, we studied the force-velocity relationship during afterloaded isotonic contractions, the advantage of which is that it better represents muscle contraction in vivo (19).

In our protocol, muscle shortening during low-force contractions occurs when the muscle is not fully activated. Hence, the decrease in $V_{\text{max}}$ we found can reflect reductions in the level of activation and cross-bridge cycle speed (19, 57, 58). Our findings from single-fiber studies (diminished Ca$^{2+}$ sensitivity, cooperativity, and slowing of $k_v$) suggest that both mechanisms explain the decrease in $V_{\text{max}}$, $P_o$, and peak power. Therefore, decreased level of activation and slowing of cross-bridge kinetics will play an important role on ventilatory function.

Another physiological variable that can alter muscle functional properties is the fiber-type distribution, and this has previously been addressed in limb muscles of C-26 mice. Interestingly, although the soleus muscle of C-26 mice shows a significant decrease in the percentage of type I fibers and increase in the percentage of type IIb fibers, the plantaris, gastrocnemius, and tibialis anterior muscles do not show a fiber type shift (8, 59). In the current study, alterations in the percentage of each muscle fiber type in the diaphragm were not statistically significant, but we did detect a 14% decrease in the percentage of type I fibers and a 13% increase in the percentage of type IIa fibers. This statistically nonsignificant shift in fiber type, however, would not explain the contractile changes reported in the current study. Indeed, specific force, shortening velocity, and peak power were diminished in C-26 mice, yet these variables are higher in type II fibers than type I.

CONCLUSIONS

Patients with severe cancer cachexia present with considerable skeletal muscle weakness that significantly impairs physical functional, independence, quality of life, and ultimately causes respiratory muscle failure and death. Therefore, understanding the contributing factors to skeletal muscle weakness and associated clinical outcomes is extremely important. In the current study, we show significant changes in the breathing pattern of cachectic cancer mice and a significantly blunted response to a respiratory challenge. We speculate that these ventilatory deficits are due, at least in part, to the significant diaphragm atrophy and weakness. This muscle weakness is not entirely due to muscle atrophy since isotonic contractile properties, and specific force in isolated muscle and permeabilized single fibers was significantly decreased. Thus, other contributing factors intrinsic to the contractile apparatus also play a role in the muscle weakness. These should be further explored, such that potential countermeasures can be tested on these variables in addition to the muscle atrophy.

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