DIAPHRAGM DYSFUNCTION IN

CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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Online data supplement

METHODS

Diaphragm biopsies

The muscle sample for ubiqituin-conjugation analysis was rapidly frozen in liquid nitrogen-cooled isopentane and stored at -80°C. From the muscle sample for determination of single fiber contractile properties muscle bundles were dissected parallel to the longitudinal axis of the muscle fibers, pinned to cork and stored at 4˚C in a relaxing solution (for composition of relaxing solution, see below). After 24 hours, the muscle strip was transfered to relaxing solution containing 50% glycerol (vol/vol) and stored at -20˚C for later analysis.

Composition of solutions for single fiber measurements

Composition of activating and relaxing solutions used for contractile measurements were reported previously [1]. Briefly, the relaxing solution consisted of 1.0 mM $MgCl_2$, 4.0 mM Na_2ATP, 5 mM EGTA, 10 mM imidazole and sufficient KCL to adjust the total ionic strength to 150 mM at pH 7.0. The negative logarithm of the free Ca^{2+} concentration (pCa) of the relaxing solution was 9.0 while in activating solutions the pCa ranged from 7.0 to 4.5 (with maximum activation at pCa 4.5). To achieve appropiate pCa in activating solutions, sufficient $CaCl_2$ was added to solutions. The composition of the solution for maximum rigor activation was the same as that of the pCa 4.5 solution, except that Na_2ATP was omitted. Ionic strength of activating solutions was kept was 150 mM at pH 7.0.

Single fiber contractile measurements

Single fiber contractile measurements and experimental protocol were performed according to previously described methods [2;3] with minor modifications. Fibers were mounted in a temperature-controlled flow-through acrylic chamber (120 μl volume), with a glass cover-slip bottom, on the stage of an inverted microscope (model IX-70, Olympus, Zoeterwoude, The Netherlands). Two stainless-steel hooks were used to mount the fiber horizontally in the chamber. One end of the fiber was attached to a force transducer (model AE-801, SensoNor; Horten, Norway) with a resonance frequency of 10 kHz, whereas the other end was attached to a servo-motor (model 308B, Aurora Scientific Inc.; Aurora, Ontario, Canada) with a step time of 250 µs. Sarcomere length was set at 2.5 µm. During experiments, sarcomere length was stabilized with the Brenner cycling method [4] as modified by Sweeney et al. [5]. MIDAC software (Radboud University Medical Centre Nijmegen, The Netherlands) and a data-acquisition board were used to record signals. Muscle fiber length (1 ~ 1.5 mm) was measured using a reticule in the microscope eyepiece [x10 Olympus Plan 10, 0.30 numerical aperature (NA)]. The *XY* fiber diameter (width) was measured with a x40 objective [x40 Olympus Plan 40, 0.60 NA]. The x40 objective was also used to measure the *XZ* fiber diameter (depth) by noting the displacement of the microscope's objective while focusing on the top and bottom surfaces of the fiber. The fiber cross-sectional area was calculated from width and depth measurements made along the length of the fiber while mounted in relaxing solution at a sarcomere length of 2.4 µm, assuming that the fiber was ellipsoid in shape.

Maximum force per cross sectional area was determined by dividing the isometric force generated at pCa 4.5 by fiber cross sectional area.

To determine force-pCa relationships, the isometric force generated in response to incubation with incremental [Ca^{2+}] (pCa 9, 7, 6.5, 6.2, 6.0, 5.8, 5.6, 5.0, and 4.5) was recorded. Graphpad software (Graphpad Software Inc., San Diego, CA, USA) was used to

calculate the [Ca^{2+}] required for half-maximum activation (pCa_{50}), as an index of Ca^{2+} sensitivity.

The rate constant of force redevelopment was measured during maximum activation as described by Brenner and Eisenberg [6].

Fiber stiffness was determined from sinusoidal length oscillations of 0.4% at 1 kHz and the force was normalized to cross sectional area. Stiffness provides an estimation of the number of strongly bound cross bridges. Stiffness was measured when fibers were in relaxing solution, and during activation at pCa 4.5 in the presence and absence (rigor solution) of ATP. Assumed that, during rigor activation, all cross bridges are in the strongly bound state, the ratio of stiffness during Ca^{2+} activation to rigor activation provides an estimate of the fraction of cross bridges in the strongly attached state.

Single fiber experimental protocol

Maximum force per cross sectional area was determined by perfusing the experimental chamber with successively pCa 9 and pCa 4.5. After a plateau was reached, again, pCa 9 was perfused through the chamber to relax the fiber. During the plateau phase of maximum activation (pCa 4.5), rate constant of force redevelopment was determined. Then fiber stiffness was determined at pCa 9. Subsequently, fibers were perfused with solutions containing incremental Ca^{2+}- concentrations. Each time a plateau was reached, the next pCa solution was perfused through the experimental chamber. When maximum force was reached at pCa 4.5 a final rate constant of force redevelopment was determined followed by a determination of fiber stiffness. Subsequently, the fiber was perfused with rigor pCa 4.5 to determine stiffness during rigor activation. Finally, the fiber was perfused with pCa 9 to verify baseline force. To maintain sarcomeric order, the fiber underwent Brenner cycling [4]

throughout the experimental protocol. During the experimental protocol functional stability of the fibers was maintained as force generated during the second maximum activation was ~ 96% of force generated during the first maximum activation.

Myosin heavy chain isoform composition determination

Myosin heavy chain isoform composition of the fiber was identified by SDS-PAGE as described previously [1] with minor modifications [7]. Briefly, single fibers were detached from the force transducer and servo-motor and placed in 25 µl of SDS sample buffer containing 62.5 mM Tris·HCL, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, and 0.001% (wt/vol) bromophenol blue at a pH of 6.8. The samples were stored at -80°C until assayed. The samples were denaturated by boiling for 2 min. The stacking gel contained a 4% acrylamide concentration (pH 6.8), and the separating gel contained 7% acrylamide (pH 8.8) with 30% glycerol (v/v). Control samples of human diaphragm bundles were run on the gels for comparison of migration patterns of the MHC isoforms. Sample volumes of 10 µl were loaded per lane. The gels were silver stained according to the procedure described by Oakley et al. [8].

Myosin heavy chain content per half sarcomere measurements

Our methodology for determining myosin heavy chain content per half sarcomere in single fibers is reported previously [9]. In Short, a modification of the procedure of Suguira and Murakami [10] was used to prepare the gradient gels. The stacking gel contained 3.5% acrylamide (pH 6.8), and the separating gel contained 5–8% acrylamide (pH 8.8) with 25% glycerol (8 × 10 cm, 0.75 mm thick; SE250, Hoefer). Control samples of diaphragm bundles

in a 1:200 dilution of SDS sample buffer (~9.0 ng/µl myosin heavy chain concentration determined by the Bradford method [11] were used to compare migration patterns of the myosin heavy chain isoforms and to verify the myosin heavy chain isoform composition of the single muscle fibers as determined previously (see *Myosin heavy chain isoform composition determination*). Sample volumes of 10 µl were loaded per lane. The gels were silver stained according to the procedure described by Oakley et al. [8]. Identification of myosin heavy chain isoforms by migration patterns was confirmed by Western blot analysis, as previously described [2;12]. One of the following mouse monoclonal or polyclonal antibodies was used to identify myosin heavy chain isoform expression: NCL (IgG; Novocastra), which reacts with myosin heavy chain type slow; SC.71 (IgG; American Type Culture Collection), which reacts with myosin heavy chain type 2A; and BF-35 (IgG; Schiaffino), which reacts with all but the myosin heavy chain 2X isoform. Isoform specificity of these antibodies was previously determined [13;14]. A biotinylated secondary antibody specific to IgG (SC.71 and BF-35; NCL) or IgM (BF-F3) was used, and bands were visualized with alkaline phosphatase (Vectastain ABC kit, Vector Labs). A standard curve of known concentrations of purified rabbit myosin heavy chain (M-3889, Sigma) was run on every gel to determine myosin heavy chain concentration in human diaphragm muscle fibers. The Bradford method [11] was used to verify the standard concentrations of myosin heavy chain run on the gels. This technique has been previously described [12]. After silver staining, a high-resolution scanner (600 dpi; Microtek ScanMaker 5) was used to image the gels. Background staining was subtracted from the density of the electrophoretic bands to determine the brightness-area product for each diaphragm muscle fiber. The relationship between the brightnessarea product and myosin heavy chain concentration was linear across a range from 0.01 to 0.25 µg/µl. The myosin heavy chain concentration in the loaded 10 µl SDS buffer was determined from the standard curve. Fiber myosin heavy chain concentration was determined through dividing total myosin

heavy chain content of the fiber by fiber volume (fiber cross-sectional area × length of fiber). Myosin heavy chain content per half sarcomere, at sarcomere length of 2.5 μm, was calculated through dividing fiber myosin heavy chain concentration by half-sarcomere volume.

Assay of ubiquitin-protein conjugates

The content of ubiquitin-protein conjugates in diaphragm muscle was assayed after homogenization of muscle samples in a buffer containing 0.25 M sucrose, 50 mM Tris, pH 7.4, 2 mM ATP, 10 mM $MgCl_2$, and 1 mM DTT. The crude extract was boiled in Laemmli sample buffer, and the proteins present in the crude extract were separated by SDS-PAGE (8 % gel). Each lane was loaded with 20 μg protein. Proteins were subsequently transferred to nitrocellulose membrane for Western blotting. The blots were blocked with 5 % milk in TBS/Tween, and incubated with anti-ubiquitin (rabbit polyclonal, Sigma) at 1:100 dilution in TBS/Tween. After incubation with peroxidase-labelled goat anti rabbit IgG (Nordic Immunology) at 1:12500 dilution in TBS/Tween, the ubiquitinated proteins on the blot were visualized by enhanced chemiluminescence detection. The blots were scanned with an imaging densitometer and optical densities of ubiquitin-protein conjugate bands were quantified with GeneTools software (Syngene). Total ubiquitin-protein conjugate optical density was calculated for each diaphragm muscle homogenate by adding optical density of individual ubiquitin-protein conjugate bands.

Statistical methods

Differences within parameters describing single fiber characteristics and contractile properties were tested using a random intercept (mixed) linear model. This statistical approach was chosen as different cells within the same patient can be regarded as repeated measurements, being correlated within that patient. When the patient is regarded as random (from the population of patients), a random intercept (mixed) linear model is a natural way to model these measurements.

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