Diaphragm single-fiber weakness and loss of myosin in congestive heart failure rats

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Departments of 1Pulmonary Diseases, 2Intensive Care Medicine, 3Cardiology and 4Cardio-Thoracic Surgery and 5Institute for Fundamental and Clinical Human Movement Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; and 6Department of Veterinary and Comparative Anatomy, Pharmacology, and Physiology, Washington State University, Pullman, Washington

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van Hees HW, van der Heijden HF, Ottenheijm CA, Heunks LM, Pigmans CJ, Verheugt FW, Brouwer RM, Dekhuijzen PN. Diaphragm single-fiber weakness and loss of myosin in congestive heart failure rats. Am J Physiol Heart Circ Physiol 293: H819–H828, 2007. First published April 20, 2007; doi:10.1152/ajpheart.00085.2007.—Diaphragm weakness commonly occurs in patients with congestive heart failure (CHF) and is an independent predictor of mortality. However, the pathophysiology of diaphragm weakness is poorly understood. We hypothesized that CHF induces diaphragm weakness at the single-fiber level by decreasing myosin content. In addition, we hypothesized that myofibrillar Ca2+ sensitivity is decreased and cross-bridge kinetics are slower in CHF diaphragm fibers. Finally, we hypothesized that loss of myosin in CHF diaphragm weakness is associated with increased proteolytic activities of caspase-3 and the proteasome. In skinned diaphragm single fibers of rats with CHF, induced by left coronary artery ligation, maximum force generation was reduced by ~35% (P < 0.01) compared with sham-operated animals for slow, 2a, and 2x fibers. In these CHF diaphragm fibers, myosin heavy chain content per half-sarcomere was concomitantly decreased (P < 0.01). Ca2+ sensitivity of force generation and the rate constant of tension redevelopment were significantly reduced in CHF diaphragm fibers compared with sham-operated animals for all fiber types. The cleavage activity of the proteolytic enzyme caspase-3 and the proteasome were ~30% (P < 0.05) and ~60% (P < 0.05) higher, respectively, in diaphragm homogenates from CHF rats than from sham-operated rats. The present study demonstrates diaphragm weakness at the single-fiber level in a myocardial infarct model of CHF. The reduced maximal force generation can be explained by a loss of myosin content in all fiber types and is associated with activation of caspase-3 and the proteasome. Furthermore, CHF decreases myofibrillar Ca2+ sensitivity and slows cross-bridge cycling kinetics in diaphragm fibers.

skinned muscle single fibers; cross-bridge cycling kinetics; Ca2+ sensitivity; proteasome; caspase-3

THE MOST IMPORTANT CLINICAL manifestations of congestive heart failure (CHF) include impaired exercise capacity and dyspnea. Yet the severity of these symptoms poorly correlates with measures of left-ventricular performance in CHF patients (21). Interestingly, the degree of exercise intolerance is significantly correlated with peripheral muscle mass and function (11, 38, 59). Likewise, the sensation of dyspnea is closely related to respiratory muscle dysfunction (32). Moreover, maximal inspiratory pressure is reduced in patients with mild-to-moderate CHF (28) and has been identified as an independent predictor of survival in these patients (35). Although peripheral skeletal muscle alterations in CHF have been investigated in detail (11, 38, 59), relatively little is known about the pathophysiology of diaphragm weakness in CHF.

In line with reduced maximal inspiratory pressure in CHF patients, several animal studies of CHF described decreased specific force generation of diaphragm muscle bundles in vitro (29, 49, 50). Recently, Coirault et al. (12) found reduced in vitro motility of isolated myofilaments from the diaphragm of CHF rats, suggesting slower cross-bridge cycling kinetics. In addition, a rightward shift of the force-frequency relationship of CHF diaphragm bundles could involve a decreased Ca2+ sensitivity of force generation (52). Contractile properties are known to differ among fiber types in the diaphragm muscle (24, 25, 47). Because CHF has been shown to induce a fiber-type shift in the diaphragm (16, 27), changes in contractile properties could be ascribed to this fiber-type shift. However, to improve understanding of the pathophysiology of diaphragm weakness in CHF, diaphragm muscle function should be evaluated independently of a fiber-type shift, which has not been done yet. Skinned diaphragm single fibers provide an excellent model for studying myofilament function in the CHF diaphragm for every fiber type separately. The first hypothesis of the present study is that CHF decreases maximum force generation, slows cross-bridge cycling kinetics, and reduces Ca2+ sensitivity in skinned diaphragm single fibers.

Force-generating capacity of skinned muscle fibers depends on contractile protein content per half-sarcomere of the muscle fiber (6, 24). CHF is associated with peripheral skeletal muscle wasting (19), and, more particularly, loss of myosin is related to decreased leg muscle strength in CHF patients (57). In patients with chronic obstructive pulmonary disease, in experimentally induced hypothyroidism, and in unilateral denervation, decreased force generation of diaphragm fibers was explained by reduced myosin content per half-sarcomere (22, 23, 40). Myosin content in CHF diaphragm fibers has not been studied. The second hypothesis of the present study is that impaired force generation of skinned fibers is associated with reduced myosin content per half-sarcomere in CHF diaphragm fibers.

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Selective degradation of myosin through the ubiquitin-proteasome pathway seems to play an important role in the loss of muscle mass in various diseases (1, 10). Caspase-3 activation has been proposed to be an initial step in the cleavage of the myofilaments, yielding fragments that are subsequently degraded by the ubiquitin-proteasome pathway (18). Our group has recently shown (40) that loss of myosin is a key event in the pathophysiology of diaphragm muscle weakness in patients with chronic obstructive pulmonary disease. Loss of myosin is associated with activation of the ubiquitin-proteasome pathway and the proteolytic enzyme caspase-3 in the diaphragm of these patients (39). It is yet unknown if activation of the proteasome and caspase-3 also occurs in the CHF diaphragm. The third hypothesis of the present study is that CHF enhances the proteolytic activity of caspase-3 and the proteasome in the diaphragm.

MATERIALS AND METHODS

Experimental model. Adult male Wistar rats (260–300 g) were anesthetized by inhalation of an isoflurane-oxygen mixture (2–5% isoflurane), intubated, and mechanically ventilated. Ligation of the left coronary artery or sham operation were executed as described previously (44). In short, a left thoracotomy between the fourth and fifth rib was performed, exposing the left-ventricular wall. The left coronary artery was ligated at its origin by a 5-0 silk suture. Subsequently, the thorax was closed in three layers with 3-0 vicryl sutures. Sham operations were performed identically, without ligating the coronary artery. After the operation and during the following 2 days, buprenorphine (20 μg/kg sc) was administered daily for postoperative analgesia. Fifty coronary artery ligations and ten sham operations were performed. Perioperative survival was 42% in the ligated group and 100% in the sham-operated group and was comparable with previous studies using this model of heart failure (31, 56). At 14 wk after ligation or sham operation, the rats were anesthetized with pentobarbital (70 mg/kg ip) and were mechanically ventilated. Aortic and left-ventricular pressures were measured by a micromanometer-tipped catheter (SPC 530; Millar Instruments, Houston, TX) inserted through the right carotid artery. At the end of the hemodynamic measurements, a combined thoracotomy/laparotomy was performed, and the diaphragm, lungs, and heart were quickly excised. Heart weight was determined, and the heart was fixated in formalin for >72 h. Subsequently, the left ventricle of each heart was cut into three transverse sections in parallel with the atrioventricular groove. Transverse sections (7 μm thick) were cut and stained with Masson’s trichrome stain. The size of the infarct areas was determined by planimetry, as described previously (44). Only data from rats with infarcts >35% of the left ventricle (n = 14) were analyzed in this study. Lungs were cleaned of fat and weighed before and after desiccation (3 days at 40°C). The diaphragm was divided in two parts: the right hemidiaphragm was used for determination of single-fiber contractile properties, and the left hemidiaphragm was used for caspase-3 and proteasome analysis. From seven CHF and six sham-operated rats, a part of the right hemidiaphragm was cut and weighed before and after desiccation (3 days at 40°C) to measure water content in the diaphragm muscle. This study was approved by the Animal Ethics Committee at Radboud University (Nijmegen, The Netherlands).

Diaphragm muscle single-fiber contractile measurements. Single-fiber contractile measurements and experimental protocol were performed according to previously described methods (40), with minor modifications, as described below.

Composition of solutions for single-fiber measurements. Relaxing solution consisted of (in mM) 1.0 MgCl2, 4.0 Na2ATP, 5 EGTA, 10 imidazole, and 15 creatine phosphate with sufficient KCl to adjust the total ionic strength to 150 mM at pH 7.0. The negative logarithm of the free Ca2+ concentration (pCa) of the relaxing solution was 9.0, whereas in activating solutions the pCa ranged from 7.0 to 4.0 (with maximal activation at pCa 4.0). To achieve appropriate pCa in activating solutions, sufficient CaCl2 was added to solutions. The composition of the solution for maximal rigor activation was the same as that of the pCa 4.0 solution, except that NaATP was omitted.

Diaphragm muscle single-fiber preparation and experimental setup. After excision, the diaphragm and adherent lower ribs were immediately submerged in cooled oxygenated (95% O2–5% CO2) Krebs solution at pH 7.4. This Krebs solution consisted (in mM) of 137 NaCl, 4 KCl, 2 CaCl2, 1 MgCl2, 1 KH2PO4, 24 NaHCO3, and 7 glucose, with 25 μM l-tubocurarine (Sigma Aldrich, Bornem, Belgium). From the central costal region of the right hemidiaphragm, a rectangular bundle was dissected parallel to the long axis of the muscle fibers for single-fiber contractile measurements. The muscle bundle was pinned to cork and stored at 4°C in a relaxing solution containing 50% glycerol (vol/vol). After 24 h, the muscle strip was stored at −20°C for later analysis.

Approximately 1 h before determination of single-fiber contractile properties, the muscle bundle was transferred to relaxing solution (5°C) containing 1% Triton X-100 to permeabilize lipid membranes. From the muscle bundle, ~2-mm segments of single fibers were isolated by using microforceps. Subsequently, the fiber ends were attached to aluminum-foil clips and mounted on the single-fiber apparatus. Fibers were mounted in a temperature-controlled (20°C) flow-through acrylic chamber (120-μl volume) with a glass coverslip bottom on the stage of an inverted microscope (model IX-70; Olympus, Amsterdam, 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 servomotor (model 308B; Aurora Scientific, Aurora, ON, Canada) with a step time of 250 μs. In relaxing solution, sarcomere length was set at 2.4 μm as the optimal length for force generation (9, 61) with the use of a calibrated eyepiece micrometer. During experiments, sarcomere length was stabilized with the Brenner cycling method (5) as modified by Sweeney et al. (54). MIDAC software (Radboud University) and a data-acquisition board were used to record signals. Muscle-fiber length (~1.5 mm) was measured by using a reticule in the microscope eyepiece (×10 Olympus Plan 10, 0.30 numerical aperture (NA)). The x-y fiber diameter (width) was measured with a ×40 objective (×40 Olympus Plan 40, 0.60 NA). The ×40 objective also was used to measure the x-z fiber diameter (depth) by noting the displacement of the microscope’s objective while focusing on the top and bottom surfaces of the fiber. Three width and depth measurements were made along the length of the fiber, and the average values were used to calculate the fiber cross-sectional area, assuming that the fiber was ellipsoid in shape. Similar incidences of fibers that appeared injured (i.e., sarcolemmal damage, loss of cross- striation, or other irregularities) during microscopic examination were found in both groups, and those fibers were excluded from the study.

Diaphragm muscle single-fiber contractile determinations. Maximum specific force was determined by dividing the isometric force generated at pCa 4.0 by cross-sectional area. Maximum force at pCa 4.0 was also divided by the estimated value of myosin heavy chain content per half-sarcomere (see Myosin heavy chain isoform composition and content per half-sarcomere determination) to determine the force per half-sarcomere myosin heavy chain content.

The rate constant for tension redevelopment (kD) was measured as described by Brenner and Eisenberg (7) during activation at pCa 4.0. In short, fibers were rapidly released by ~15% and then, ~50 ms later, restretched to their original length. During the rapid release and restretch, cross-bridges detach and force drops to zero. The cross-bridges then reattach, and force redevelops. The kD value was determined by using a computer algorithm for least-squares fit of a first-order exponential.
Fiber stiffness was determined during sinusoidal length oscillations of 0.4% at 1 kHz. Stiffness provides an estimation of the number of strongly attached cross bridges (20). Stiffness was measured during Ca\textsuperscript{2+} activation at pCa 4.0 in the presence and absence (rigor solution) of ATP. Assuming that, during rigor activation, all cross-bridges are in the strongly attached state, the ratio of stiffness during Ca\textsuperscript{2+} activation to that during rigor activation (\(\alpha_{\text{tr}}\)) provides an estimate of the fraction of cross-bridges in the strongly attached state.

To determine force-pCa relationship, the isometric force generated in response to incubation with incremental Ca\textsuperscript{2+} concentrations (pCa 9.0, 7.0, 6.3, 6.0, 5.8, 5.5, 5.2, 5.0, 4.7, 4.5, and 4.0) was recorded. Graphpad Prism (GraphPad Software, San Diego, CA) was used to calculate the Ca\textsuperscript{2+} concentration required for half-maximum activation (pCa\textsubscript{50}), as an index of Ca\textsuperscript{2+} sensitivity of force generation, and the Hill coefficient, as a measure of myofilament cooperativity.

**Diaphragm muscle single-fiber experimental protocol.** Maximal specific force was determined by perfusing the experimental chamber at, successively, pCa 9 and 4.0. During the plateau phase of maximal activation at pCa 4.0, \(k_{\text{tr}}\) was determined. Then pCa 3.0 solution was perfused through the chamber to relax the fiber.

Subsequently, fibers were perfused with solutions containing incremental Ca\textsuperscript{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 4.0, \(k_{\text{tr}}\) was determined, followed by a determination of fiber stiffness. Subsequently, the fiber was perfused with rigor solution of pCa 4.0 to determine stiffness during rigor activation. Finally, the fiber was perfused at pCa 9 to verify baseline force.

Structural and functional instability of skinned single muscle fibers, reflected by an increased heterogeneity of sarcomere spacing during activation and reduced force generation over time, is a well-recognized problem (4). To improve functional stability of muscle fibers, we applied Brenner cycling (5) throughout the experimental protocol, except during determination of \(k_{\text{tr}}\), stiffness, and rigor. Accordingly, no functional decline in maximal force generation and \(k_{\text{tr}}\) over time was observed.

**Myosin heavy chain isoform composition and content per half-sarcomere determination.** Determination of myosin heavy chain isoform composition and content by SDS-PAGE was adapted from Geiger et al. (24). Single fibers were detached from the force transducer and servomotor and were placed in 25 \(\mu\)l 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 −20°C until they were assayed. The samples were denatured 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 (vol/vol). Control samples of rat diaphragm bundles were run on the gels for comparison of migration patterns of the myosin heavy chain isoforms. Volume samples of 8 \(\mu\)l were loaded per lane. The gels were silver stained according to the procedure described by Oakley et al. (37). A standard curve of known contents of purified rabbit myosin heavy chain (M-3889; Sigma, Zwijndrecht, The Netherlands) was run on every gel to determine myosin heavy chain content in rat diaphragm muscle fibers.

After silver staining, the gels were scanned with an imaging densitometer and optical densities of the electrophoretic bands were quantified with GeneTools software (Syngene, Cambridge, UK). 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 brightness-area product and myosin heavy chain content was linear across a range from 0.01 to 0.25 \(\mu\)g. The myosin heavy chain content in the loaded 8 \(\mu\)l SDS buffer was determined from the standard curve. Subsequently, the total myosin heavy chain content of the fiber (in 25 \(\mu\)l SDS buffer) was determined. Myosin heavy chain concentration was determined by dividing total myosin heavy chain content by fiber volume. Myosin heavy chain content per half-sarcomere, at a sarcomere length of 2.4 \(\mu\)m, was calculated through dividing fiber myosin heavy chain content by the amount of half-sarcomeres (2 \times length of fiber/2.4).

**Measurement of caspase-3 activity.** Caspase-3 activity was determined as described previously (39). Frozen diaphragm samples were pulverized and homogenized on ice in a buffer containing 100 mM HEPES (pH 7.5), 10% sucrose, 0.1% Nonidet P-40, 10 mM dithiothreitol, and protease inhibitor cocktail (Sigma). Homogenates were subjected to three cycles of freeze-thaw before centrifugation at 18,000 \(\times\) g for 30 min. The supernatant (92.5 \(\mu\)g protein) was added to a reaction buffer consisting of 100 mM HEPES (pH 7.5), 10% sucrose, and 10 mM dithiothreitol. The fluorogenic substrate N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin was then added, and the reaction was performed at 30°C for 60 min. The caspase-3 activity was determined by measuring the generation of the fluorogenic cleavage product (methylcoumarylamidate) at 360 nm excitation wavelength and 460 nm emission wavelength with a spectrophotometer. Amido-4-methylcoumarin standards were used to quantify activity levels. Results were expressed as picomoles per minute per milligram protein. Addition of N-acetyl-Asp-Glu-Val-Asp-CHO (a caspase-3 inhibitor) to the reaction resulted in inhibition of methylcoumarylamide production, indicating the specificity of the assay for measuring caspase-3 activity.

**Isolation of 20S proteasomes and measurement of proteasome activity.** The 20S proteasome isolation and proteolytic activities were determined as described previously (39). To isolate 20S proteasomes, diaphragm samples frozen in liquid nitrogen were homogenized in ice-cold buffer (pH 7.5) containing (in mM) 50 Tris-HCl, 5 MgCl\textsubscript{2}, 250 sucrose, 1.1 4-dithiothreitol, and 0.2 PMSF, with protease inhibitor cocktail (Sigma) by means of a Dounce homogenizer. Subsequently, the proteasomes were isolated from the homogenates by three sequential centrifugation steps; the first centrifugation was at 10,000 \(\times\) g for 20 min. The obtained supernatant was then centrifuged at 100,000 \(\times\) g for 1 h. The final pellet containing the 20S proteasomes was resuspended in buffer (pH 7.5) containing 50 mM Tris-HCl, 5 mM MgCl\textsubscript{2}, and 20% glycerol. The protein content of the proteasome preparation was determined on the basis of a Bio-Rad protein assay (Bio-Rad, Veenendaal, The Netherlands). The proteolytic activity of the 20S proteasomes was determined by measuring the activity against the fluorogenic substrates succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarylamide (LLVY) and benzoyl-Leu-Leu-Glu-7-amido-4-methylcoumarylamide (LE) (Sigma). These substrates are preferentially hydrolyzed by the chymotrypsin-like and peptidylglutamyl peptidase activities of the 20S proteasome, respectively (15). To measure proteolytic activity, 15 \(\mu\)g proteasome extract was added to 60 \(\mu\)l medium containing 62.5 mM Tris-HCl, 12.5 mM MgCl\textsubscript{2}, 1.25 mM 1.4-dithiothreitol, 0.01 units apyrase, and 100 \(\mu\)l LLVY or 375 \(\mu\)l LE. The reaction took place at 37°C for 45 min. The peptide activity was determined by measuring the generation of the fluorogenic cleavage product (amido-4-methylcoumarylamide) at 380 nm excitation wavelength and 440 nm emission wavelength with a spectrophotometer. Standard curves were established for the fluorogenic product, and peptide activity was expressed as picomoles per microgram protein per minute. Addition of MG-132 (a specific proteasome inhibitor) to the reaction resulted in complete inhibition of amido-4-methylcoumarylamide production, indicating successful isolation of proteasomes without the presence of significant amounts of other proteases.

**Proteasome content analysis.** For determining the proteasome content in the diaphragm muscle, a Western blot was performed by using an antibody against the 20S proteasome subunit C8 (MCP72; mouse monoclonal; 1:10,000; Affiniti, Gorinchem, The Netherlands). In short, frozen diaphragm samples were homogenized in ~200 \(\mu\)l ice-cold buffer (pH 7.5) containing (in mM) 50 Tris, 1 EDTA, 1 dithiothreitol, 1 PMSF, and protease inhibitor cocktail (Sigma) by...
Diaphragm weakness in CHF rats

Means of a Dounce homogenizer. Homogenates were centrifuged at 10,000 g at 4°C for 10 min, and the protein concentration of the resulting supernatants was determined by using the Bio-Rad protein assay. Soluble proteins (10 μg) were subjected to routine Western blotting by using 10% polyacrylamide SDS gels and the MCP72 antibody. After being washed, the blot was incubated with a horseradish peroxidase-conjugated goat anti-mouse Ig (1:10,000; Pierce, Ettten-Leur, The Netherlands). Chemiluminescence was performed by using an ECL Western-blotting analysis system (Amersham Biosciences, Roosendaal, The Netherlands). Protein bands were quantified by using optical densitometry software (GeneTools). An isolated proteasome fraction from a sham-operated rat was used as a positive control, and for the negative control a homogenate of a sham-operated rat was stained without addition of the primary (anti-C8) antibody. Equal loading was controlled by Coomassie blue staining.

Data treatment and statistical methods. Contractile properties of 82 diaphragm muscle single fibers from 14 CHF rats and 70 single fibers from 6 sham-operated rats were measured. These fibers expressed a single myosin heavy chain isoform, and contractile data were grouped per fiber type: slow, 2a, and 2x. Every rat was equally represented in the fiber-type groups. None of the fibers solely expressed myosin heavy chain 2b isoform. Fibers that coexpressed myosin heavy chain isoforms (n = 3 in CHF fibers and n = 4 in sham-operated rat fibers) were excluded from further analysis. Due to technical circumstances, in some fibers not all contractile parameters could be measured. Therefore, the number of single fibers analyzed differs per parameter.

Differences in animal characteristics, caspase-3, and proteasome activity between groups were analyzed with a Student’s t-test. Differences in parameters describing single-fiber characteristics and contractile properties between groups were tested with one-way ANOVA, followed by the Student-Newman-Keuls post hoc test. Because of a lack of experimental data, the number of cross-bridges in single fibers was determined from myosin heavy chain concentration and half-sarcomere. Myosin heavy chain concentration in single fibers from CHF rats was significantly reduced compared with fibers from sham-operated rats for all fiber types. Numbers above bars represent number of single fibers analyzed. Data are presented as means ± SE. *P < 0.01 vs. sham-operated group. Fa,max, maximum force.

RESULTS

CHF indexes. All rats with infarct sizes >35% of the left ventricle (n = 14) showed clinical signs of severe heart failure, including pulmonary congestion and cardiomegaly, as supported by increased wet-to-dry lung-weight ratio and heart weight, respectively (Table 1). Elevated left-ventricular end-diastolic pressure, decreased left-ventricular peak systolic pressure, and decreased aortic pressures indicate impaired left-ventricular function (Table 1).

Table 1. Animal characteristics

<table>
<thead>
<tr>
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<th>Sham, n = 10</th>
<th>CHF, n = 14</th>
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<tr>
<td>Body weight before operation, g</td>
<td>282 ± 6</td>
<td>291 ± 4</td>
</tr>
<tr>
<td>Body weight after 5 wk, g</td>
<td>382 ± 10</td>
<td>350 ± 6*</td>
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<tr>
<td>Body weight after 10 wk, g</td>
<td>426 ± 11</td>
<td>395 ± 6*</td>
</tr>
<tr>
<td>Body weight after 14 wk, g</td>
<td>444 ± 7</td>
<td>435 ± 9</td>
</tr>
<tr>
<td>Aorta systolic pressure, mmHg</td>
<td>112 ± 8</td>
<td>87 ± 5*</td>
</tr>
<tr>
<td>Aorta diastolic pressure, mmHg</td>
<td>94 ± 8</td>
<td>72 ± 5*</td>
</tr>
<tr>
<td>LV peak systolic pressure, mmHg</td>
<td>114 ± 7</td>
<td>91 ± 6*</td>
</tr>
<tr>
<td>LV end-diastolic pressure, mmHg</td>
<td>1 ± 1</td>
<td>11 ± 1*</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>1.4 ± 0.03</td>
<td>2.2 ± 0.1*</td>
</tr>
<tr>
<td>Infarct size, macro, %</td>
<td>34 ± 2</td>
<td>43 ± 2</td>
</tr>
<tr>
<td>Lung weight wet-to-dry ratio</td>
<td>4.2 ± 0.1</td>
<td>4.6 ± 0.1*</td>
</tr>
<tr>
<td>Diaphragm weight wet-to-dry ratio</td>
<td>3.5 ± 0.2</td>
<td>3.6 ± 0.3</td>
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Values are means ± SE. *P < 0.05 vs. sham group. CHF, congestive heart failure; LV, left ventricle.

Maximum force per cross-sectional area. Single-fiber cross-sectional area did not differ significantly between fibers from CHF and sham-operated rats (1.0 ± 0.1 × 10⁻³ vs. 1.1 ± 0.1 × 10⁻³ mm² for slow fibers; 2.5 ± 0.5 × 10⁻³ vs. 2.4 ± 0.5 × 10⁻³ mm² for type 2a fibers, and 3.3 ± 0.3 × 10⁻³ vs. 3.5 ± 0.4 × 10⁻³ mm² for type 2x fibers, respectively). Cross-sectional areas of slow fibers were significantly smaller compared with 2a and 2x fibers for both groups (P < 0.05). Maximum force per cross-sectional area of single fibers from the diaphragm of CHF rats was significantly reduced compared with fibers from sham-operated rats for all fiber types (P < 0.01, Fig. 1). Maximum force per cross-sectional area did not significantly differ between fiber types within groups (Fig. 1).

Diaphragm muscle fiber myosin heavy chain concentration. Myosin heavy chain concentration in single fibers from CHF rats was significantly lower than in sham-operated rats for all fiber types (P < 0.05, Fig. 2A).

Diaphragm muscle fiber myosin heavy chain content per half-sarcomere. Myosin heavy chain content per half-sarcomere reflects the number of cross-bridges in parallel and was determined from myosin heavy chain concentration and half-sarcomere volume. Myosin heavy chain content per half-sarcomere is reduced in single fibers from CHF rats compared with sham-operated rats for all fiber types (P < 0.01, Fig. 2B). Type 2x fibers contain more myosin heavy chain per half-sarcomere than slow fibers (P < 0.001 for both CHF and sham-operated rats; Fig. 2). Figure 2C shows a representative silver-stained gel.

Maximum force per half-sarcomere myosin heavy chain content. To evaluate the effect of the number of cross-bridges in parallel on the maximum force per cross-sectional area, maximal force generated by single fibers was normalized to myosin heavy chain content per half-sarcomere volume. Maximum force per myosin heavy chain content per half-sarcomere was not significantly different between CHF and sham-operated diaphragm for all fiber types (Fig. 3). Maximum force per myosin heavy chain content per half-sarcomere was lower in slow fibers than in 2a and 2x fibers for both groups, but this difference did not reach significance (Fig. 3).

Fraction of cross-bridges in strongly attached state during maximal activation. Maximum force also depends on the fraction of all available cross-bridges that are in the strongly

Fig. 1. Maximum force per cross-sectional area (CSA) in slow, type 2a, and type 2x diaphragm muscle single fibers. Maximum force per CSA of single fibers from the diaphragm of congestive heart failure (CHF) rats was significantly reduced compared with sham-operated rats for all fiber types. Numbers above bars represent number of single fibers analyzed. Data are presented as means ± SE. *P < 0.01 vs. sham-operated group. Fmax, maximum force.

Fig. 2. Maximum force per half-sarcomere myosin heavy chain concentration. Myosin heavy chain concentration in single fibers from CHF rats was significantly lower than in sham-operated rats for all fiber types (P < 0.05, Fig. 2A).

Fig. 2A. Maximum force per CSA in slow, type 2a, and type 2x diaphragm muscle single fibers. Maximum force per CSA of single fibers from the diaphragm of congestive heart failure (CHF) rats was significantly reduced compared with sham-operated rats for all fiber types. Numbers above bars represent number of single fibers analyzed. Data are presented as means ± SE. *P < 0.01 vs. sham-operated group. Fmax, maximum force.

Fig. 2B. Diaphragm muscle fiber myosin heavy chain content per half-sarcomere. Myosin heavy chain content per half-sarcomere reflects the number of cross-bridges in parallel and was determined from myosin heavy chain concentration and half-sarcomere volume. Myosin heavy chain content per half-sarcomere is reduced in single fibers from CHF rats compared with sham-operated rats for all fiber types (P < 0.01, Fig. 2B). Type 2x fibers contain more myosin heavy chain per half-sarcomere than slow fibers (P < 0.001 for both CHF and sham-operated rats; Fig. 2). Figure 2C shows a representative silver-stained gel.
attached state during maximal activation. The fraction of cross-
bridges in the strongly attached state during maximal activation
was not significantly different between diaphragm muscle
single fibers from CHF and sham-operated rats (Fig. 4).

Cross-bridge cycling kinetics. The $k_{tr}$ was slower in dia-
phragm muscle fibers from CHF rats than sham-operated rat
fibers ($P < 0.05$, Fig. 5). As expected, $k_{tr}$ of type 2a and 2x
fibers was significantly faster than in slow fibers in both groups
($P < 0.01$, Fig. 5). Figure 5B shows representative force
redevelopment signals for the different fiber types.

$Ca^{2+}$ sensitivity of force generation. The force-pCa curves
for slow and 2x fibers of CHF rats are shifted rightward
compared with sham-operated rats (Fig. 6). Consequently, the
negative logarithm of pCa50 is significantly reduced for slow
and 4.91 ± 0.06 vs. 5.16 ± 0.04, $P < 0.05$ for type 2x,
respectively). The pCa50 of type 2x fibers was significantly
lower compared with slow fibers in both groups ($P < 0.001$).
Hill coefficients were not significantly different between fibers
from CHF and sham-operated rats (1.9 ± 0.2 vs. 1.8 ± 0.2 for
slow and 2.3 ± 0.2 vs. 2.6 ± 0.3 for type 2x, respectively). The
Hill coefficients for type 2x fibers were significantly higher
than for slow fibers in the sham group ($P < 0.01$). Due to a
limited presence of 2a fibers in the rat diaphragm, we were
unable to measure the force-pCa relation of a sufficient amount
of 2a fibers in both CHF and sham-operated groups.

Caspase-3 activity. Caspase-3 activity of diaphragm homog-
enates was ~1.3-fold higher for CHF compared with sham-
operated rats ($P < 0.05$, Fig. 7).

Proteasome activity and content. The proteasome activity
against both LLVY and LLE was ~1.6-fold higher in dia-

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Fig. 2. A: myosin heavy chain concentration in slow, type 2a,
and type 2x fibers from the diaphragm of CHF rats are
decreased compared with sham-operated rats for all fiber types.
B: myosin heavy chain content per half-sarcomere in slow, type
2a, and type 2x fibers from the diaphragm of CHF are reduced
compared with sham-operated rats for all fiber types. Type 2x
fibers contained more myosin heavy chain per half-sarcomere
than slow fibers for both the CHF and sham-operated groups.
Numbers above bars represent number of single fibers ana-
yzed. C: representative silver-stained gel loaded with rat
diaphragm homogenate, single fibers from the diaphragm of
CHF and sham-operated rats, and myosin standards. Data are
presented as means ± SE. *$P < 0.05$ vs. sham-operated group.
†$P < 0.001$ compared with slow fibers within group. MHC,
myosin heavy chain.
phragm from CHF rats compared with sham-operated rats ($P < 0.05$, Fig. 8A). To investigate if changes in proteasome activity are the result of changes in the number of proteasomes, we determined the protein level of the C8 subunit of the 20S proteasome. Figure 8B demonstrates that the protein levels of the 20S proteasome C8 subunit were not significantly different between the diaphragm from CHF and sham-operated rats.

**DISCUSSION**

The present study is the first to investigate the effects of CHF on diaphragm skinned single fiber function. The data from our study show that single fibers from the diaphragm of CHF rats generate less force per cross-sectional area for every fiber type. In these fibers, myosin heavy chain content per half-sarcomere was concomitantly reduced. Therefore, when normalized for myosin content per half-sarcomere, maximum force of diaphragm fibers was not different between CHF and sham-operated rats for every fiber type. In addition to reduced maximal force generation, CHF decreased Ca$^{2+}$ sensitivity of force generation and slowed cross-bridge cycling kinetics in every fiber type. Finally, CHF enhanced caspase-3 and proteasome activity in the diaphragm.

**Comparisons of control data with other studies.** Single fiber-specific force in sham-operated rats was not different between fiber types, which is in line with previous publications.
from other groups (42, 51), although some controversy exists (3, 24). In agreement with the first report on diaphragm fiber-type differences in cross-bridge cycling kinetics (47), the present study shows that $k_o$ of slow fibers is lower than for fast (2x and 2a) fibers. Differences in myosin heavy chain content per half-sarcomere, calcium sensitivity, and Hill coefficients between fiber types are also in accordance with previous studies on rat diaphragm fibers (8, 23, 25). However, absolute values of myosin content per half sarcomere and pCa50 in sham-operated diaphragm single fibers were slightly different from reports by other groups (23, 25, 51). These differences could possibly have arisen from the use of dissimilar compositions of activating solutions, animal race, and age. Nevertheless, it is unlikely that the discrepancies can explain the significant differences between sham-operated and CHF diaphragm fiber contractile properties presented in this study. Values of cross-sectional area and the fraction of all available cross-bridges that are in the strongly attached state ($\alpha_{fs}$) of sham-operated diaphragm fibers were comparable with results of previous studies on rat diaphragm fibers (22, 23).

Causes of impaired diaphragm muscle single-fiber contractility in CHF. Diaphragm muscle bundle isometric contractility has been evaluated in several animal models for CHF (29, 49, 50). However, no previous studies have investigated the contractile performance of diaphragm muscle single fibers in CHF. This study shows that maximal force-generating capacity of skinned single fibers from the diaphragm of CHF rats is reduced in all fiber types. This suggests that the reduced maximal force generation of the diaphragm in CHF patients (28) and animal models (29, 49, 50) is not merely attributable to a fiber-type shift but rather to a decrease of maximal force generation of individual fibers.

Maximal force ($F_{max}$) generation of skinned muscle fibers is determined by the sum of forces generated by individual cross-bridges (6, 24). Therefore

$$F_{max} = n \times f_m \times \alpha_{fs}$$

where $n$ is the number of available cross-bridges in parallel per half-sarcomere and $f_m$ is the mean force per attached cross-bridge in the force-generating state. The reduced maximal force generation of the diaphragm muscle fibers from CHF rats should be reflected by a reduction in one or more of these determinants. Indeed, we showed that myosin heavy chain concentration was decreased in all fiber types in the diaphragm from CHF rats (Fig. 2A). Because fiber cross-sectional areas were not different between groups, this indicates that myosin heavy chain content per half-sarcomere was significantly reduced in CHF fibers compared with sham-operated rats (Fig. 2B). This reduction causes a proportional decrease in $n$. The $\alpha_{fs}$ (Fig. 4) was not different between

diaphragm fibers from CHF and sham-operated rats. Also, the $f_m$, which can be derived from $F_{max}$ (Fig. 3) and $\alpha_{fs}$ (see Eq. 1), did not differ between both groups. Therefore, the reduction in maximal force generation of diaphragm fibers from CHF rats can be mainly explained by the loss of myosin heavy chain content ($n$) in these fibers, although the involvement of other processes cannot be excluded.

A recent study (58) showed no differences of myosin heavy chain content between diaphragm homogenates of Dahl salt-sensitive rats with and without heart failure. Next to a different animal model of heart failure that was used in that study, another reason for dissimilar results lies in the difference in reliability of the methods that were used to measure myosin heavy chain content. Measuring myosin content in diaphragm homogenates is sensitive for differences in the amount of connective tissue and fiber-type distribution, whereas this variance is omitted in myosin content determinations on single fibers, as was done in the present study. Moreover, correcting myosin content for the total amount of protein in diaphragm homogenates underestimates a loss of myosin, because myosin constitutes a significant fraction of the total protein amount. Correcting myosin content for fiber volume in single fibers is therefore more accurate.

The most important findings of this study include decreased maximal force generation together with a loss of myosin in CHF diaphragm fibers. However, in vivo the diaphragm does not perform maximal isometric contractions during normal breathing but shortens against a certain load. Therefore, submaximal and kinetic parameters of single-fiber measurements provide more relevant physiological information.

![Fig. 5. Rate constant of force redevelopment ($k_o$) was used as a measure for cross-bridge cycling kinetics. A: $k_o$ in slow, type 2a, and type 2x fibers from the diaphragm of CHF was slower than in diaphragm fibers from sham-operated rats. $k_o$ at maximal activation was significantly faster in type 2a and 2x fibers than in slow fibers for both groups. Numbers above bars represent total number of single fibers analyzed. B: representative plots of force vs. time during the $k_o$ protocol for slow fibers (B1), 2a fibers (B2) and 2x fibers (B3). After rapid detachment of all cross-bridges, force redevelops from 0 according to a first-order exponential curve. $k_o$ value represents time constant of this curve. Data are presented as means ± SE. *P < 0.05 vs. sham group. †P < 0.01 compared with slow fibers within group.](http://ajpheart.physiology.org/)

![Fig. 6. Force-pCa relation of single fibers from the diaphragm of CHF and sham-operated rats. Isometric force generated in response to incubation of single fibers with incremental Ca$^{2+}$ concentrations was determined. Force-pCa relations of CHF diaphragm slow and 2x fibers are shifted to the right compared with sham-operated rats. Data are presented as means ± SE. pCa, negative log of Ca$^{2+}$ concentration.](http://ajpheart.physiology.org/)
ward shift of the pCa-force curve displays that at a certain Ca$^{2+}$/H$^{11001}$ concentration, a smaller percentage of maximal force is generated in diaphragm fibers from CHF rats compared with sham-operated rats. In other words, Ca$^{2+}$/H$^{11001}$ sensitivity of force generation is reduced. This implies that force generation in the CHF diaphragm is even more affected at submaximal than at maximal activation, which is in line with a previously observed rightward shift of the force-frequency relation in CHF diaphragm bundles (52). The decreased rate constant of force redevelopment shows that cross-bridge cycling kinetics are slower in CHF diaphragm fibers compared with sham-operated fibers. This observation is in accordance with the decreased in vitro sliding velocities of myosin, isolated from the diaphragm of CHF rats, recently described by Coirault et al. (12). However, by using an in vitro motility assay, Coirault et al. could not exclude a possible effect of fiber-type shift on the decreased sliding velocities of myosin. Our single-fiber data unambiguously show that slower cross-bridge kinetics is an intrinsic phenomenon of all fiber types in the CHF diaphragm.

The causes of slower cross-bridge cycling kinetics and decreased Ca$^{2+}$/H$^{11001}$ sensitivity in the CHF diaphragm remain speculative. Previous studies showed that increased filament-lattice spacing results in slower rates of tension redevelopment (33) and decreased Ca$^{2+}$/H$^{11001}$ sensitivity (26). Indeed, in our study myosin content per half-sarcomere is reduced in the CHF diaphragm (Fig. 2) without alterations of cross-sectional area, indicating increased filament-lattice spacing. Thus myosin loss in CHF diaphragm fibers could not only result in decreased maximal force generation but may also affect submaximal and kinetic parameters of diaphragm contractility. Besides, modification of the myosin head or the Ca$^{2+}$/H$^{11001}$-binding protein complex troponin could also account for slower cross-bridge kinetics and reduced Ca$^{2+}$/H$^{11001}$ sensitivity (41, 43, 45). For example, in their in vitro motility study, Coirault et al. (12) showed that the decreased sliding velocities of myosin are associated with increased levels of myosin oxidation in the diaphragm of CHF rats. However, further studies on filament-lattice spacing and posttranslational modifications of contractile proteins are needed to establish the precise origin of slower cross-bridge kinetics and decreased Ca$^{2+}$/H$^{11001}$ sensitivity in CHF diaphragm fibers.

**Pathways toward a loss of myosin heavy chain content in CHF.** The loss of myosin heavy chain in the CHF diaphragm suggests an imbalance between the synthesis and degradation of this contractile protein. In peripheral skeletal muscles of CHF patients, rates of myosin heavy chain synthesis were not different from control patients, whereas the decrease in muscle strength was closely related to the loss of myosin (57). This suggests that enhanced degradation of myosin needs to be involved. Indeed, recent studies have indicated that the degradation of myosin plays an important role in diseases associated with diaphragm (39) and peripheral muscle wasting (1, 30), including CHF (46). It is well recognized that myofibrillar proteins such as myosin are degraded by the ubiquitin-proteasome-
some pathway (34, 36, 55). The present study shows that the activity of the ubiquitin-proteasome pathway is increased in the CHF diaphragm. More specifically, these data indicate that the proteolytic activity per proteasome is increased rather than the number of proteasomes. Thus in the CHF diaphragm, the loss of myosin heavy chain is associated with an increased activity of the ubiquitin-proteasome pathway.

Because the ubiquitin-proteasome pathway is unable to degrade intact actomyosin complexes (48), preceding cleavage of these proteins from the sarcomere is necessary for subsequent degradation. The exact mechanisms for cleaving contractile proteins from the sarcomere are unknown, but an important role is ascribed to proteolytic enzymes, such as calpains (2, 60) and caspase-3 (18, 53). Previously, a twofold increase of calpain activity in the diaphragm of CHF rats has been reported (17). This study demonstrates that caspase-3 activity is enhanced in the CHF diaphragm and is associated with loss of myosin and activated proteasomes. However, further studies are needed to elucidate the exact role for caspase-3 activation in CHF diaphragm weakness.

Conclusion. This study shows diaphragm weakness at the single-fiber level in a myocardial infarction model of CHF. Maximal force generation of diaphragm muscle single fibers from CHF rats is reduced and can be explained by a decreased myosin heavy chain content in all fiber types. Additionally, cross-bridge kinetics are slower and Ca\(^{2+}\) sensitivity is decreased in the diaphragm fibers of CHF rats. Finally, CHF diaphragm weakness and the loss of myosin heavy chain are associated with an increased activation of the ubiquitin proteasome pathway and its upstream proteolytic enzyme caspase-3.

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