Respiratory Research

Research

Myosin heavy chain and physiological adaptation of the rat diaphragm in elastase-induced emphysema

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Abstract

Background: Several physiological adaptations occur in the respiratory muscles in rodent models of elastase-induced emphysema. Although the contractile properties of the diaphragm are altered in a way that suggests expression of slower isoforms of myosin heavy chain (MHC), it has been difficult to demonstrate a shift in MHCs in an animal model that corresponds to the shift toward slower MHCs seen in human emphysema.

Methods: We sought to identify MHC and corresponding physiological changes in the diaphragms of rats with elastase-induced emphysema. Nine rats with emphysema and 11 control rats were studied 10 months after instillation with elastase. MHC isoform composition was determined by both reverse transcriptase polymerase chain reaction (RT-PCR) and immunocytochemistry by using specific probes able to identify all known adult isoforms. Physiological adaptation was studied on diaphragm strips stimulated *in vitro*.

Results: In addition to confirming that emphysematous diaphragm has a decreased fatigability, we identified a significantly longer time-to-peak-tension (63.9 ± 2.7 ms versus 53.9 ± 2.4 ms). At both the RNA (RT-PCR) and protein (immunocytochemistry) levels, we found a significant decrease in the fastest, MHC isoform (IIb) in emphysema.

Conclusion: This is the first demonstration of MHC shifts and corresponding physiological changes in the diaphragm in an animal model of emphysema. It is established that rodent emphysema, like human emphysema, does result in a physiologically significant shift toward slower diaphragmatic MHC isoforms. In the rat, this occurs at the faster end of the MHC spectrum than in humans.

Introduction

Elastase-induced emphysema in rodents is the most widely studied animal model for human emphysema. Several alterations in diaphragmatic structure and function have been demonstrated in this model. Among these are diaphragm fiber shortening with a corresponding shift in the length-tension curve [1–5] and variable changes in the contractile properties of diaphragm strips *in vitro* [2,3,5,6]. Increased fatigue resistance of the muscle is the single alteration in contractile properties that has been most consistently [2,3,6], though not always [7], identified. This fatigue resistance has been accompanied by an increased oxidative capacity in the diaphragmatic muscle fibers as demonstrated by increased activities of citrate synthase [7,8] and succinate dehydrogenase (SDH) [2,3].

In contrast, fiber type has generally not been found to be significantly altered in the diaphragms of emphysematous animals [1–3,8], although in all reports addressing this issue the fibers were typed histochemically rather than by myosin heavy chain (MHC) composition. Furthermore, the kinetics of diaphragmatic muscle contraction that one would expect to result from shifts in fiber type distribution, such as time to peak tension, have also generally [2,3,6,9], but not always [7], been found to be unaltered in emphysema. A change in fiber type distribution in the diaphragm of emphysematous hamsters was shown for the first time recently [10], but again histochemical classification was employed, and no corresponding change in twitch kinetics was demonstrated. In the scalene muscle, an accessory muscle of inspiration, of emphysematous hamsters, an increase in IIa fibers and a decrease in IIx fibers has been demonstrated with the use of anti-MHC monoclonal antibodies. There was no difference, however, in contractile properties between emphysema and control muscle in that study [11].

In humans, our group and one other have demonstrated marked alterations in MHC expression in the diaphragm in severe emphysema [12,13]. This shift toward slow MHC isoforms has been proposed to subserve an adaptive resistance to fatigue in the human diaphragm in emphysema which is similar to that seen in the animal model.

Although most of the experimental work on adaptation of the diaphragm to emphysema has been performed in hamsters, rats also develop significant and often marked increases in lung volumes and compliance, and reductions in expiratory flows, after intratracheal administration of elastase [14–18]. MHC adaptation in emphysematous rat diaphragm has not been studied systematically. Because monoclonal MHC antibodies are better characterized in rat than hamster, and with the availability of a semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay for rat MHC

mRNA [19], we set out to study changes in MHC-determined fiber types and MHC mRNA expression in emphysematous rat diaphragm. To evaluate whether shifts toward more energy-efficient isoforms of the second most important ATPase in muscle might also occur in emphysematous diaphragm, we also studied changes in sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase (SERCA) expression.

Further, we sought to correlate changes in MHC and SERCA expression with the physiologic function of diaphragm strips *in vitro*. We hypothesized that we would identify significant alterations in MHC (and perhaps SERCA) expression in the emphysematous rat diaphragm similar to those identified in humans with severe emphysema, and that these changes would be accompanied by corresponding changes in contractile properties. It was expected that such findings might identify the rat model of elastase-induced emphysema as a model in which the diaphragmatic MHC changes that have been shown to result from emphysema in humans might be explored further.

Materials and methods

Induction of emphysema

Eleven 3-month-old Sprague-Dawley rats underwent emphysema induction by a single intratracheal instillation of porcine pancreatic elastase (ICN Biochemicals, Cleveland, Ohio) at 25 units per 100 g body weight, diluted in 0.60 ml of normal saline as described previously [4]. Two animals died on the night after instillation, with evidence at autopsy the following day of diffuse pulmonary hemorrhage.

Eleven control animals were instilled with an equal volume of saline without a mortality.

Animals were maintained two per cage with feeding *ad libitum* for 10 months. The protocol was approved by the Animal Care Committees of the Philadelphia Veterans Affairs Medical Center and the University of Pennsylvania.

Diaphragm strip physiology in vitro

An apparatus for the study *in vitro* of muscle contractile characteristics similar to that described previously [20] was assembled. Thirteen-month-old rats (10 months after the induction of emphysema) were killed by CO_2 inhalation and the diaphragm was quickly harvested en bloc with the ribcage intact and immersed in oxygenated Ringer's solution buffered to pH 7.4 with 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid. A muscle strip approximately 7.5 mm in width, including central tendon and rib attachments, was dissected out under magnification; care was taken to cut parallel to the muscle fibers.

Each strip was mounted horizontally in a bath of continuously circulating, oxygenated solution at 23 ± 1°C. One end of the strip was tied to a fixed post with sutures taken through the attached ribs. The central tendon at the other end was fixed to the arm of a servomotor system (motor model 6450, electronics model 300B; Cambridge Technology, Watertown, Massachusetts) on a movable platform with a single tie. Platinum electrodes (each 7 mm × 25 mm) were placed within 1 mm of the muscle strip on either side. The muscles were stimulated with a Grass S44 stimulator (Grass Instruments, Quincy, Massachusetts) with pulses 1.5 times above those needed to achieve maximal twitch force (120 V, 5 ms pulses). A series of twitches generated every 5 seconds at incrementally different muscle lengths was used to identify the point of maximal force generation (L_o). Mean muscle length at L_o was 2.68 ± 0.09 cm in control animals and 2.21 ± 0.07 cm in animals with emphysema ($P = 0.001$).

A length-tension curve was generated from the mean of five twitches at each muscle length between 70% and 120% of the previously determined L_o. Fatiguing characteristics were then determined by repeated stimulation at 100 Hz for 200 ms bursts, and 90 trains/min. Muscle length (servomotor position), stimulator pulse timing, and data collection were under computer control with custom software developed in our laboratory. A Pentium computer with a data acquisition board (DT21-EZ; Data Translation, Marlboro, Massachusetts) controlled the experiment and recorded all data to disk for later analysis.

After study, the strip was removed from the apparatus, trimmed of nonmuscle tissue, blotted dry, and weighed. Total muscle strip cross-sectional area was calculated as wet muscle mass divided by muscle length times density (taken to be 1.06 g/cm^3 [21]). Tension was calculated by dividing developed force by the calculated cross-sectional area. Study of two of the control rat strips suffered technical failure, leaving nine emphysematous and nine control animals fully studied. Mean strip wet weight was 0.092 ± 0.004 g in the control rats and 0.088 ± 0.003 g in the emphysematous rats ($P = 0.51$).

Lung volume determination
After we had dissected out the diaphragm strips for the physiologic studies described above, the animals' lungs were excised with trachea intact. The trachea was cannulated with a 16 g intravenous catheter (Angiocath; Becton Dickinson, Sandy, Utah), an airtight seal was established, and the lung was inflated to a distending pressure of 25 cmH$_2$O. This volume (at the total lung capacity) was then measured by water displacement.

MHC RT-PCR
MHC mRNA expression was analyzed by an MHC RT-PCR assay as described initially [19] and later adapted by its originators [22]. The assay is semi-quantitative, permitting accurate determination of relative amounts of mRNA of the embryonic, neonatal, I, IIa, IIx, and IIb MHC isoforms in muscle. The assay's accuracy has been confirmed against Northern blot analysis [19].

In brief, mRNA was extracted from an approximately 50 mg fragment of frozen diaphragm taken from the central portion of the strip used in the physiologic experiments described above. This was done with the Micro-fast Track 2.0 Kit (Invitrogen, Carlsbad, California) in accordance with the manufacturer's protocol. The mRNA was then suspended in 20 µl of elution buffer and the concentration was determined by measuring the attenuance at a wavelength of 260 nm. mRNA (150 ng) was reverse transcribed for each muscle sample with the First-strand cDNA Synthesis Kit (Amersham Pharmacia, Piscataway, New Jersey), and the cDNA product was then used in the PCR.

The PCR (Fig. 1) involved six reaction tubes for each muscle sample, one tube for each isoform studied. A single 5' oligonucleotide common primer, designed from a highly conserved region in all known rat MHC genes approximately 500 base pairs upstream of the stop codon, was used in every reaction tube (sequence 5'-AGAAGGAG-CAGGACACCAGC-3'). A different 3' oligonucleotide primer, designed from a divergent portion of the 3'-untranslated region of each of the different MHC genes, was used in each reaction tube to provide isoform specificity (sequences in [19,22]). Template, in addition to 5 µg of reverse transcribed cDNA, included 1 pg of an internal control fragment [22] to be coamplified with each PCR by using the same primers and target sequences. However, this control template yields a fragment of different size from that resulting from amplification of the MHC genes in each tube [22] (Fig. 1), and permits controlling for the efficiency of each PCR amplification.

The PCR was performed in a 50 µl total reaction volume. This mixture contained the two templates described above, 5 µl of 10 × PCR buffer (Promega), 4 µl of 25 mM MgCl$_2$, 5 µl dNTP, 1 µM of each of the two appropriate primers, and 0.5 units of DNA *Taq* polymerase (Promega), with water to bring it to final volume. Amplifications were performed in a thermal cycler with an initial denaturing step of 5 minutes at 96°C, followed by 25 cycles, with each cycle consisting of 1 minute at 96°C, 50 seconds at 60°C, 50 seconds at 70°C, and a final step of 3 minutes at 72°C. The number of cycles was optimized so that the amplified signal was on the linear portion of a semilogarithmic plot of the yield expressed as a function
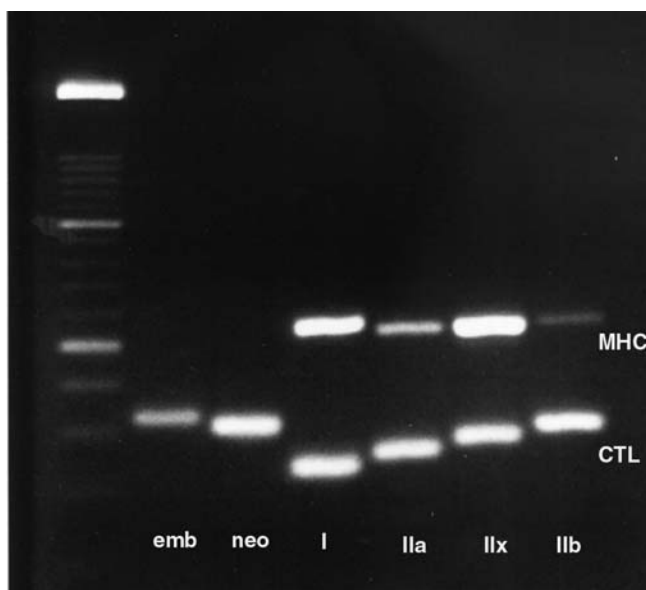
Figure 1
Representative image of myosin heavy chain (MHC) reverse transcriptase polymerase chain reaction products from the diaphragm of an emphysematous rat, separated by agarose-gel electrophoresis. The higher-molecular-mass product in each lane (labelled MHC) represents the relative amount of mRNA for that MHC isoform: emb (embryonic), neo (neonatal), I, IIa, IIx, or IIb. The lower band (labelled CTL) permits correction according to the efficiency of the amplification in each reaction tube. Note the low level of IIb expression.

of the number of cycles. The PCR products were separated on 2.0% agarose gels and stained with ethidium bromide (Fig. 1).

Negative images of the gels were produced under ultraviolet light with Polaroid film type 665, ISO 80/20, positive/negative, black and white (Polaroid, Bedford, Massachusetts). Densitometry of the bands was performed on the negatives (Personal Densitometer; Molecular Dynamics, Sunnyvale, California) with Image Quant software's 'area quantitation' paradigm. To correct for any differences in the efficiency of the reactions for each isoform, the intensity of the MHC band was divided by the intensity of the control fragment in that reaction. A correction factor was calculated for each band on the basis of its size, to normalize the intensity of the staining for the different sizes of the fragments. The final percentage content of each MHC gene in the total sample was then calculated from the fraction of a specific corrected value relative to the sum of expressed MHC mRNA isoforms in a given sample [19]. We performed the reverse transcription and the PCR in triplicate for each piece of muscle, starting with

the same mRNA sample. The final value represents the mean of these three runs.

MHC immunocytochemistry

Fiber types were determined by indirect immunofluorescence on serial frozen cross sections of rat diaphragm with monoclonal antibodies specific for the following MHCs: NOQ7.5.4D for type I [23], SC-71 for type IIa [24], and BF-F3 for type IIb [24]. The antibody useful for the identification of type IIx fibers, BF-35 [24], stains all fibers except pure IIx fibers. To aid in the visualization of the periphery of the fibers, we simultaneously co-incubated each section with a rabbit anti-rat laminin primary antibody (Sigma, St Louis, Missouri) at a dilution of 1:2500, followed by a fluorescein-conjugated sheep anti-rabbit IgG secondary antibody. Our tissue preparation and staining protocols for the type I and IIa antibodies have been described previously [20,25], with the modification that NOQ7.5.4D was used at 1:500 dilution, the section was incubated with each of these primary antibodies for 18 hours at 4°C, and rhodamine-conjugated secondary antibodies were used. For BF-F3 we used a primary antibody dilution of 1:50, an 18 hour incubation, and a donkey anti-mouse IgM rhodamine-conjugated secondary antibody. For BF-35 we used a primary antibody dilution of 1:10, a 24 hour incubation, and a goat anti-mouse IgG rhodamine-conjugated secondary antibody.

Fibers were classified on the basis of the antibody that yielded the strongest fluorescent staining (or the absence of staining with BF-35 for pure IIx fibers). Thus, we did not assess the proportions of fibers that might have coexpressed more than one MHC. Subjectively, we saw no evidence of coexpression of fiber types I and IIa, but owing to the nature of antibody BF-35, which stains all except pure IIx fibers, we are unable to rule out the coexpression of IIx with any other isoform. There were very rare fibers (less than 1%) that stained only lightly with BF-F3 and did not stain with BF-35. We interpreted these as probably representing intermediate fibers expressing both IIb and IIx. We did not count these fibers toward the totals reported in the results section.

At least 500 fibers were evaluated for each specimen in determining the percentage of each MHC-determined fiber type. Using software that had been custom-designed in our laboratory we also determined the percentage of the total surface area of each cross section of muscle occupied by each MHC-determined fiber type.

SERCA immunocytochemistry

Mouse IgG monoclonal antibodies directed against the slow and fast isoforms of SERCA, respectively, were also used on serial sections of rat diaphragm (catalogue numbers MA3-91 and MA3-911; Affinity BioReagents, Inc.,

Table 1: Relative expression of mRNA for myosin heavy chain isoforms as determined by reverse transcriptase polymerase chain reaction

Source	Expressed MHC mRNA (percentages of total)					
	Embryonic	Neonatal	I	IIa	IIx	IIb
Control (*n* = 11)	0	0	26.0 ± 0.66	20.2 ± 1.3	32.1 ± 0.32	21.6 ± 1.9
Emphysema (*n* = 9)	0	0	27.6 ± 1.2	23.6 ± 1.6	34.0 ± 1.1	14.9 ± 2.8*

*P < 0.05 versus control value.

Golden, Colorado). Each section was preincubated with 2% bovine serum albumin for 1 hour, then incubated overnight at 4°C with the appropriate primary antibody at 1:500 dilution. A goat anti-mouse IgG secondary antibody conjugated to Cy3 was used at 1:200 dilution for 1 hour, regardless of which primary antibody was used.

Statistical analysis
Data are reported as means ± SE. We used a repeated-measures analysis of variance with a Huynh-Feldt correction to compare emphysematous and control groups with respect to MHC transcript levels by PCR and MHC-determined fiber-type number by immunocytochemistry. We noted a group-by-type interaction and then performed t-tests to compare the two groups with respect to each of the MHC isoforms. Fatigue data were analyzed by t-test comparing mean values of measured to initial force ratio. A P value of 0.05 or less was considered to represent statistical significance.

Results
Lung volume
We determined H_2O displacement lung volumes at total lung capacity (25 cmH_2O airway pressure) as the simplest measure to document the hyperexpansion characteristic of emphysema in the elastase-treated animals. Because there was very little difference in body mass between control and emphysematous animals (613 ± 15 g control, *n* = 11; 629 ± 11 g emphysema, *n* = 9; *P* = 0.42), the differences in lung volume were highly significant whether or not they were normalized to body mass. Crude lung volumes were 34.2 ± 0.6 ml in emphysematous animals (*n* = 9) and 25.3 ± 0.3 ml in controls animals (*n* = 10) (*P* < 0.0001). Thus, H_2O displacement lung volumes were increased by 35% in emphysematous animals over controls.

Measurement of MHC isoform expression by RT-PCR
The mean percentage compositions of mRNA representing each MHC isoform in the diaphragms of emphysematous and control animals are listed in Table 1. Note that there was a significant decrease in the expression of MHC IIb in emphysematous versus control. Although the increase in the expression of the three slower adult isoforms did not reach statistical significance for any individual iso-

form, all demonstrated a trend to greater expression in emphysema. This trend was most marked in the IIx isoform, for which the P value for increased expression in emphysematous versus control diaphragm reached 0.06. Thus, there was modest shift toward expression of slower isoforms at the mRNA level, and this shift occurred predominantly at the faster end of the MHC spectrum.

Measurement of MHC isoform expression by immunocytochemistry
Immunocytochemical results measuring MHC protein-determined fiber type closely mirrored the PCR results at the mRNA level. Table 2 demonstrates that, as determined by immunocytochemistry, emphysematous animals had a significantly lower percentage of fibers classified as IIb and a trend toward increased numbers of IIx and slow fibers. When calculated on the basis of area occupied by each fiber type, the difference between emphysema and control did not reach significance even for the IIb antibody (*P* = 0.06). Note that although the results with RT-PCR and immunocytochemistry are qualitatively similar, they are quantitatively different. Figure 2 shows a typical panel from serial sections of one of the emphysematous diaphragm specimens stained with each of the monoclonal antibodies.

SERCA expression by immunocytochemistry
There was no significant difference in the numbers of fibers expressing either SERCA 1 or SERCA 2 between the emphysematous and control groups. For SERCA 1, 62.4% of fibers were positive in controls and 63.3% in the emphysematous animals (*P* = 57). For SERCA 2, 38.9% of fibers were positive in each group (*P* = 0.99).

In vitro *strip physiology*
The results of the diaphragm strip physiological studies are detailed in Table 3. It is notable that the physiological parameters that have previously been most strongly correlated with a shift to slower MHC isoforms (time to peak tension, and fatiguing characteristics) were significantly altered in the expected direction in the emphysematous versus control diaphragm strips. Time to peak tension was longer in the emphysematous group with a *P* value of 0.015, and the emphysematous diaphragm was less
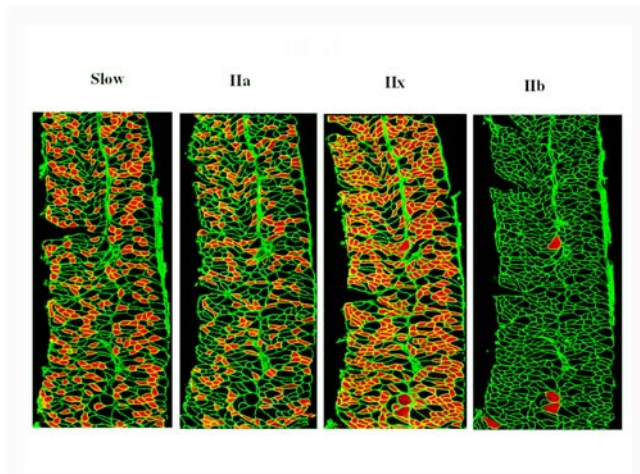
Figure 2
Representative myosin heavy chain (MHC) immunocyto-chemistry images of an emphysematous diaphragm after co-incubation with anti-laminin antibody and an antibody against one of the adult MHC isoforms, followed by a fluorescein-tagged secondary antibody against the laminin primary and a rhodamine-tagged secondary antibody against the MHC primary antibodies. Each frame shows a serial section stained with a primary antibody against a different adult MHC isoform: I, IIa, IIx, or IIb.

fatiguable with a *P* value of 0.023. There was no significant difference in specific force generated or in the half relaxation time.

Discussion

To our knowledge, these data are the first report of significant changes in MHC expression with corresponding physiologic alterations in any respiratory muscle of an animal model of emphysema. These changes are qualitatively similar to those reported in severe human emphysema [12,13]: that is, they too are manifested as a shift toward slower MHC isoforms. Quantitatively, however, the changes are far less marked than the shift demonstrated in humans.

The most studied animal model for the adaptation of respiratory muscle to emphysema has been the hamster with elastase-induced emphysema. The hamster model has probably been chosen for these studies in large part because of the impressive increases in lung volumes that can be created in hamsters, with total lung capacity sometimes reaching 200% of control values. Despite the demonstration of length adaptation [1–4,6] and fatigue resistance [2,3,6] in the diaphragms of emphysematous hamsters, not until very recently could a change in fiber

type distribution be demonstrated [10], and in that study histochemical classification was employed that could not separate out IIx from IIb fibers. Furthermore, no study in emphysematous hamster diaphragm has demonstrated a change in twitch kinetics.

Intratracheal instillation of elastase in rats creates a form of panacinar emphysema that in some ways more closely parallels the human disease than that created by instillation of elastase in hamsters. Changes in lung volumes and compliance in the rat are more similar to those seen in humans [14–18]. An electron microscopic comparison of human emphysema with elastase-induced emphysema in rats revealed remarkably similar pictures of elastin disintegration accompanied by increased collagen deposition and reorganization [26].

Our results for MHC expression in rat diaphragm from normal and emphysematous rats must be viewed first in the light of previous quantitative studies of MHC expression in normal rat diaphragm that used techniques able to separate out all known isoforms. In 7–9-week-old Sprague-Dawley rats, Kanbara *et al.* [27] found by *in situ* hybridization that 1.0% of fibers expressed only IIb and 8.8% coexpressed the IIx and IIb isoforms. This is in contrast with our RT-PCR result of 21.6% IIb in normals, but immunocytochemical results much more in line with Kanbara's findings. In that study, the values for MHCs I, IIa, and IIx were not markedly different from those reported here for normal rat diaphragm by RT-PCR. By electrophoretic separation, Sugiura *et al.* [28] found IIb MHC to represent 6.1% of all MHC protein in young rat diaphragm, and their IId/IIx result of 44.9% is also quite different from our PCR result, while their I and IIa findings are very similar to our PCR results, and their IId/IIx result closely parallels our IIx area percentage immunocytochemistry result. Okumoto *et al.* reported even less IIb and more IId protein by electrophoresis in 5-month-old rats [29].

The differences between the findings of these reports and ours might at first glance result from the age of the animals studied, as our rats were significantly older at 13 months. However, it has been reported that there is an age-related transition from fast to slow MHC in rat limb muscles [30,31] and diaphragm [32], which would render this explanation unlikely. A potential explanation of our results in relation to Kanbara's would be that there was a systematically greater concentration of IIb mRNA in IIb and IIb/IIx fibers than of other isoforms' mRNAs in their respective fibers. Sugiura *et al.* and Okumoto *et al.* used Wistar rats, although this would be unlikely to explain the difference between their findings and ours. In the final analysis, it is difficult to draw any firm conclusions from comparisons between studies addressing mRNA levels

Table 2: Relative myosin heavy chain isoform protein expression by immunocytochemistry

Source		Embryonic	Neonatal	I	IIa	IIx	IIb
Control (*n* = 11)	Fiber%	0	0	35.2 ± 1.5	29.7 ± 2.4	27.9 ± 2.2	7.2 ± 1.1
	Area%	0	0	23.8 ± 1.6	20.3 ± 1.7	43.2 ± 1.9	12.7 ± 2.0
Emphysema (*n* = 9)	Fiber%	0	0	36.5 ± 2.3	25.7 ± 2.2	30.1 ± 1.5	4.7 ± 1.0*
	Area%	0	0	25.6 ± 2.7	17.7 ± 1.3	47.7 ± 2.5	9.2 ± 1.8

Fiber% is the percentage of all fibers that were positive for the given isoform's monoclonal antibody. Area% is the percentage of total area covered by fibers positive for the given isoform's monoclonal antibody. *$P < 0.05$ versus control value.

Table 3: Diaphragm strip physiological parameters

Source	Time to peak tension (ms)	Half relaxation time (ms)	Peak twitch force (kg/cm^2)	Fatigue
Control (*n* = 9)	53.9 ± 2.4	92.3 ± 3.5	0.85 ± 0.055	0.45 ± 0.04
Emphysema (*n* = 9)	63.9 ± 2.7*	85.3 ± 5.3	0.81 ± 0.053	0.59 ± 0.04*

Half relaxation time is the time from the peak to one-half of the maximum generated force; fatigue is the ratio of force generated at 4 s after the initiation of protocol to the initial force generated. *$P \leq 0.05$ versus control values.

and those addressing protein levels; and similarly between techniques measuring protein by gel and those measuring protein by immunocytochemistry.

Although increased activities of citrate synthase [7] and SDH [2,3] have usually been demonstrated in the diaphragm in animal models of emphysema, fiber type has generally not been found to be significantly altered [1–3,7]. Only in one recent paper was a shift in MHC in emphysematous diaphragm demonstrated, but the histochemical technique used precluded the separation of IIb and IIx fibers [10]. Another study has shown increased expression of IIa at the expense of IIx MHC in emphysematous hamster scalene muscle with the use of MHC monoclonal antibodies, but there were none of the expected physiologic changes accompanying the shift [11].

We demonstrate here a shift from IIb toward IIx in emphysematous rat diaphragm at both the protein and mRNA levels. Although this shift toward a slower isoform is not as marked as that reported in humans [12,13], in which MHC type I is upregulated and both IIa and IIx are downregulated, there is precedent for restricted MHC adaptation in rats. Termin *et al.* [33] for example, have noted that whereas chronically stimulated rabbit fast-twitch muscle results in appreciable increases in slow myosin isoforms, chronic stimulation of rat muscle tends to bring about shifts toward the slower types among the type II isoforms. Other factors that might lead to less impressive MHC shifts in rodent models of emphysema than in humans include the much shorter time course over which the adaptations have a chance to occur, the greater compliance of

the rodent thorax to pulmonary hyperexpansion, and differences in the diaphragmatic load created in the human disease in comparison with the animal models that have yet to be fully worked out.

Our finding in the diaphragms of rats with emphysema is very similar to that demonstrated by Sugiura *et al.* in the diaphragms of chronically swimming rats [28]. We found IIb mRNA expression to decrease by 31% and IIb MHC-determined fiber type to decrease by 35% in emphysema. Sugiura *et al.* found that after 10 weeks of endurance swimming, IIb protein from costal diaphragm of rats fell by 54%, also without statistically significant changes in the amounts of other MHC isoforms. This suggests that the effects of emphysema on the diaphragm are, as has been suggested by others, at least in part a function of increased workload.

Other studies of MHC adaptation in animal models of increased diaphragmatic work have had conflicting results. Although various treadmill-running protocols have shown increases in the aerobic capacity of the rat diaphragm [32,34–37], running has generally not been demonstrated to cause consistent changes in the relative expression of MHC isoforms [29,38]. Although one paper has shown a decrease in type IIb fibers and an increase in type I fibers [38], and another has shown only a significant decrease in type IIb fibers [39], a third paper reported a surprising increase in type IIb fibers [40].

In contrast, inspiratory resistive loading by tracheal narrowing has consistently shown increases in the percentage

of type I fibers and the corresponding MHC isoform in the diaphragm [41–43], closely resembling the changes seen in severe human emphysema. Respiratory loading by chronic hypercapnea has also demonstrated similar changes [44].

Given these findings, one can postulate that shifts toward slower MHC isoforms in diaphragmatic muscle fibers result primarily from some combination of the degree and chronicity of the work performed by the muscle. If this is so, then one could arrange the experimental methods that have been explored that impose increased diaphragmatic work, from least imposed load to greatest imposed load, as follows: running, swimming, emphysema, hypercapnea, tracheal banding. It is possible that in emphysema, the additional impact on diaphragmatic physiology of fiber shortening resulting from pulmonary hyperexpansion [1–6] affects MHC isoform shifts beyond the influence of a pure increase in the workload on the muscle.

In addition to being the first demonstration of a decrease in the expression of the IIb MHC isoform in an animal model of emphysema, this study is the first to demonstrate that such a shift toward slower isoforms in emphysema has a significant physiologic impact on diaphragmatic function *in vitro*. We show that both twitch speed and fatiguing properties move toward slower-twitch characteristics with this decrease in IIb expression. Given previous work in this area, it is not unexpected that even a shift only between the fast isoforms but away from IIb would result in measurable physiological changes. It has been noted, for example, that IIx fibers have significantly higher SDH activity than IIb fibers [24,45] and, further, that IIb motor units are significantly more fatigue sensitive than IIx motor units [46]. Schiaffino *et al.* have also demonstrated, in rat whole muscles, a slower maximum velocity of shortening in muscles made up of predominantly IIx versus IIb fibers [47]. Further, Sant'Ana Pereira *et al.* [48] have shown a major difference in actomyosin ATPase activity in rat single IIb and IIx fibers, and Sieck *et al.* [45] have shown this in single fibers from rat diaphragm.

Finally, we examined the expression of the SERCA proteins in these animals, because these proteins are responsible for a significant fraction of energy consumption in skeletal muscle, second only to the energy consumed by the ATPase responsible for movement of the myosin head. Further, diaphragmatic fatigue might be related to SERCA function [49] and, as discussed above, our emphysematous animals showed decreased diaphragm strip fatigability. Previous work has suggested that although SERCA expression in limb muscles seems to respond to increases in functional load by upregulating SERCA 2 (the slow SERCA isoform) and downregulating

SERCA 1 (the fast isoform) [50], similar responses might not occur in diaphragm [51]. Our data showing no difference in the expression of SERCA 1 and SERCA 2 between emphysematous and control animals are consistent with this finding. We did not measure SERCA activity or phospholamban phosphorylation. It is certainly possible that the overall activities of one or both of the SERCA isoforms are, in fact, different between emphysematous and control diaphragm, although the numbers of fibers expressing each isoform are not different.

Because mechanical indices of relaxation reflect the function of SERCA in sequestering calcium in the sarcoplasmic reticulum [49], we measured half relaxation times on our diaphragmatic strips. It is not surprising that, with no change in SERCA 1 and SERCA 2 expression detected by immunocytochemistry, we found no difference in the relaxation times between control and emphysematous diaphragm strips.

Conclusion
In sum, we have demonstrated a significant decrease in IIb MHC expression at both the protein and mRNA levels in the diaphragmatic muscle of rats with elastase-induced emphysema. In concert with this shift away from the fastest MHC isoform, we have demonstrated 'slower' physiological characteristics *in vitro*, including a longer time to peak tension and a greater resistance to fatigue in diaphragm strips. These findings suggest that earlier reports of physiological adaptation within the diaphragm in animal models of emphysema without the demonstration of concomitant shifts in MHC expression toward slower isoforms were limited by the technologies then available, which did not allow the evaluation of the expression of each of the type II MHC isoforms with specific probes.

A physiologically significant shift toward slower MHC isoforms does occur in elastase-induced emphysema in rodents as it does in severe human emphysema, but in the animal model the shift occurs at the faster end of the isoform spectrum, primarily between the type II isoforms.

Abbreviations
MHC = myosin heavy chain; RT-PCR = reverse transcriptase polymerase chain reaction; SDH = succinate dehydrogenase; SERCA = sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase.

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