Rapid Disuse Atrophy of Diaphragm Fibers in Mechanically Ventilated Humans

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ABSTRACT

BACKGROUND

The combination of complete diaphragm inactivity and mechanical ventilation (for more than 18 hours) elicits disuse atrophy of myofibers in animals. We hypothesized that the same may also occur in the human diaphragm.

METHODS

We obtained biopsy specimens from the costal diaphragms of 14 brain-dead organ donors before organ harvest (case subjects) and compared them with intraoperative biopsy specimens from the diaphragms of 8 patients who were undergoing surgery for either benign lesions or localized lung cancer (control subjects). Case subjects had diaphragmatic inactivity and underwent mechanical ventilation for 18 to 69 hours; among control subjects diaphragmatic inactivity and mechanical ventilation were limited to 2 to 3 hours. We carried out histologic, biochemical, and gene-expression studies on these specimens.

RESULTS

As compared with diaphragm-biopsy specimens from controls, specimens from case subjects showed decreased cross-sectional areas of slow-twitch and fast-twitch fibers of 57% (P=0.001) and 53% (P=0.01), respectively, decreased glutathione concentration of 23% (P=0.01), increased active caspase-3 expression of 100% (P=0.05), a 200% higher ratio of atrogin-1 messenger RNA (mRNA) transcripts to *MBD4* (a housekeeping gene) (P=0.002), and a 590% higher ratio of MuRF-1 mRNA transcripts to *MBD4* (P=0.001).

CONCLUSIONS

The combination of 18 to 69 hours of complete diaphragmatic inactivity and mechanical ventilation results in marked atrophy of human diaphragm myofibers. These findings are consistent with increased diaphragmatic proteolysis during inactivity.

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MECHANICAL VENTILATION IS A CRITI-cal component of modern intensive care medicine, but the process of discontinuing mechanical ventilation can be difficult.[1,2] Laboratory studies have shown that the combination of diaphragmatic inactivity and mechanical ventilation for prolonged periods (more than 18 hours) is associated with atrophy of myofibers in the rat diaphragm.[3-5] We hypothesized that similar changes occur in the human diaphragm and that disuse atrophy of human diaphragm myofibers could be a major contributor to the weaning problems that occur in some of our patients.

We evaluated the diaphragms of brain-dead organ donors, who show respiratory-muscle inactivity and undergo mechanical ventilation for prolonged periods, to determine whether disuse atrophy of the diaphragm occurs in ventilated humans. We compared intraoperative biopsy specimens obtained from the costal diaphragms of 14 brain-dead organ donors before harvest (case subjects) and compared them with intraoperative biopsy specimens obtained from the diaphragms of 8 patients who were undergoing surgery for either benign lesions or stage 1 lung cancer (control subjects). Case subjects with diaphragmatic inactivity underwent mechanical ventilation for 18 to 69 hours, whereas in control subjects, the combination of diaphragm inactivity and mechanical ventilation was limited to 2 to 3 hours.

METHODS

SUBJECTS

Our protocol for case subjects was approved by the Gift of Life Donor Program (http://www.donors1.org), and our protocol for control subjects was approved by the University of Pennsylvania institutional review board; the protocols appear in the Supplementary Appendix, available with the full text of this article at www.nejm.org. All biopsy specimens were obtained with appropriate written informed consent.

BIOPSIES

Full-thickness biopsy specimens (about 20 to 24 mm by 6 to 8 mm in size) were obtained from the same region of the right anterior costal diaphragm in all case and control subjects, frozen in isopentane after 3 to 5 minutes for length equilibration, and then transferred to liquid nitro-gen and stored at −80°C until used. Specimens from case subjects were obtained before circulatory arrest or removal of any organs, and specimens from control subjects were obtained during the surgery for their lung lesions. In addition, to determine whether our hypothesis regarding atrophy was limited to the diaphragm or primary respiratory muscles, we obtained specimens of the pectoralis major muscle at the level of the third interspace in six subjects from each group. (These subjects were the only ones for whom appropriate consent was obtained for these biopsies.) To avoid surgical trauma to this superficial muscle, these specimens were obtained immediately after the skin incision and processed in the same manner as the diaphragm specimens.

MEASUREMENTS

We carried out histologic, biochemical, and gene-expression measurements on diaphragm specimens. Only histologic data were obtained from the pectoralis specimens.

We measured fiber-type proportions, fiber-type cross-sectional areas, and area fractions to characterize fiber atrophy in diaphragm-biopsy specimens. We also measured the concentrations of glutathione, active caspase-3, and procaspase-3. Glutathione-concentration analyses were used to assess the presence of oxidative stress[6]; we used active caspase-3 and procaspase-3 as indicators of caspase activity. Active caspase is known to dissociate proteins from the myofibrillar lattice,[7] which is a critical step in muscle proteolysis.

We quantitatively assessed the number of messenger RNA (mRNA) transcripts for atrogin-1 and MuRF-1 relative to *MBD4*, a housekeeping gene, using real-time reverse-transcriptase polymerase chain reaction.[8] Atrogin-1 and MuRF-1 are ubiquitin ligases that are key components of the ubiquitin–proteasome pathway for proteolysis.[7,9]

Histologic studies were carried out using previously described immunohistologic methods,[10] and a minimum of 400 fibers were studied in each specimen. Biochemical and gene-expression studies were performed in triplicate on each specimen. Glutathione was measured using an enzyme-recycling assay kit (glutathione assay kit, Cayman Chemicals). Caspase measurements were conducted using sodium dodecyl sulfate–polyacrylamide-gel electrophoresis, followed by immunoblotting with monoclonal antibodies specific for the 32- and 17-kD fragments. We used the

relative standard curve method to compute the gene-expression level in each of our diaphragm samples.[8] Complete details for all methods are available in the Supplementary Appendix.

STATISTICS

Means (±SD) and medians are presented for all continuous data. Demographic, histologic, biochemical, and gene-expression variables were compared between case and control groups using t tests for normally distributed continuous data, Mann–Whitney tests for non-normally distributed data, and Fisher's exact tests for categorical variables.[11,12]

RESULTS

CHARACTERIZATION OF EXPERIMENTAL COHORT

Demographic information, reason for inclusion in the study, and medical history for case and control subjects are summarized in Table 1; ventilator settings, measurements of arterial blood gases, and vital signs are summarized in Table 2. Clinical data for each of the case subjects are presented in the Supplementary Appendix in Table S1; Tables S2 and S3 contain histologic data for case and control subjects; and Table S4 contains usual laboratory measurements. Case subjects were younger than control subjects (mean age, 35±16 years vs. 57±18; P=0.008). The two groups did not differ with respect to proportions of men and women or to body-mass index. After brain death, case subjects' diaphragms were inactive for 18 to 69 hours, whereas inactivity in control subjects' diaphragms was limited to 2 to 3 hours; the mean inactivity time for case subjects' diaphragms was appreciably greater — more than 10 times that of control subjects' diaphragms (2.4±0.5 vs. 34±16, P<0.001).

ANALYSIS OF DIAPHRAGM-BIOPSY SPECIMENS

Histology

A comparison of Figures 1A and 1B indicates that the fibers in the diaphragm-biopsy specimens from case subjects were appreciably smaller than those from control subjects. Figures 1C, 1D, 1E, and 1F show that both slow-twitch and fast-twitch fibers in the case specimens were affected by atrophy. Importantly, all panels in Figure 1 indicate that fiber atrophy in case specimens was not accompanied by an inflammatory-cell infiltrate.

In case specimens, the mean cross-sectional areas of slow-twitch and fast-twitch fibers were 2025±745 and 1871±589 μm^2, respectively, whereas in control specimens these cross-sectional areas were 4725±1547 and 3949±1805 μm^2, respectively. Therefore, in case specimens, the cross-sectional area of slow-twitch fibers decreased 57% (P=0.001) as compared with control values, and the cross-sectional area of fast-twitch fibers decreased 53% (P=0.01) (Fig. 2A). Case and control specimens did not differ with respect to the numerical proportions or area fractions of slow-twitch and fast-twitch fibers (Fig. 2B and 2C). In addition, in an age-matched subgroup of five case and five control subjects, the cross-sectional areas of both slow-twitch and fast-twitch fibers did not differ statistically from those of the full groups; in this subgroup, slow-twitch and fast-twitch fibers in case specimens exhibited mean decreases in cross-sectional areas of 39% (P=0.004) and 41% (P=0.02), respectively, as compared with controls.

Biochemistry

Total glutathione concentration in diaphragm-biopsy specimens from case subjects was 1.03±0.17 mM, whereas that in control specimens was 1.35±0.21 mM; therefore, case specimens exhibited a decrease of 23% (P=0.01) from that noted in controls (Fig. 3A). Figures 3B and 3C show immunoblots and a quantitative comparison of case and control specimens with respect to the expression of the active 17-kD caspase-3 fragment and the 32-kD inactive procaspase fragment. Figure 3C shows that active caspase-3 in case specimens had a value of 1.52±1.15 optical-density units, whereas that in controls had a value of 0.66±0.45 optical-density unit; therefore, the diaphragm-biopsy specimens from case subjects showed an increase of 154% above controls (P=0.05). In addition, Figure 3C shows that procaspase in case specimens measured 0.72±0.40 optical-density unit, whereas that in control specimens was 1.13±0.51 optical-density units; this higher value of procaspase in the control specimens approached but did not reach statistical significance (P=0.07).

Gene Expression

Expression of *MBD4* was used to normalize the number of transcripts of atrogin-1 and MuRF-1 (the two ubiquitin ligases of interest), since its expression in the diaphragm-biopsy specimens

Table 1. Summary of Demographic Characteristics, Reason for Surgery, and Medical History for Control and Case Subjects.*

Subject No.	Age (yr)	Sex	BMI	Reason for Surgery or Cause of Brain Death	Relevant Medical History
Control subjects					
1	79	M	31	Stage 1A adenocarcinoma of the lung	Prostate carcinoma, nonsmoker, farmer
2	64	M	36	Stage 1A adenocarcinoma of the lung	Peripheral arterial disease, rheumatoid arthritis, hypertension, coronary artery disease, smoked 90 pack/yr
3	55	F	23	Stage 1A benign fatty tumor	Hypercholesterolemia, osteoarthritis, smoked 10 pack/yr
4	76	M	27	Stage 1A adenocarcinoma of the lung	Coronary artery disease with history of myocardial infarction, macular degeneration, prostate carcinoma (radiation therapy, 1999), coronary-artery bypass graft (1988), pipe smoker (quit 30 yr ago)
5	58	F	30	Stage 1 carcinoid tumor	Hypercholesterolemia, primary hyperparathyroidism, kidney stones, smoked 40 pack/yr
6	25	F	27	Ganglioneuroma	Gallstones, nonsmoker
7	54	F	23	Ganglioneuroma	Glaucoma, seasonal allergies, nonsmoker
8	41	F	26	Hamartoma	Herniated lumbar disks, dysfunctional uterine bleeding, smoked 24 pack/yr (quit 2 yr ago)
Case subjects					
1	18	F	24	Motor vehicle accident	None
2	21	F	29	Drug overdose	Drug abuse
3	18	M	25	Gunshot wound to head	None
4	19	M	24	Respiratory arrest secondary to seizure	Seizure disorder with implanted pacemaker
5	49	M	24	Motor vehicle accident	Hypertension, peptic ulcer disease, depression, hypogonadism, smoker
6	33	F	44	Drug overdose	Drug and ethyl alcohol abuse, metronidazole and ceftriaxone for vaginitis
7	25	F	21	Motor vehicle accident	Pregnant
8	50	M	21	Stroke	Hypertension, ethyl alcohol abuse, smoked 30 pack/yr
9	23	M	20	Motor vehicle accident	Hypertension, ethyl alcohol abuse, marijuana abuse
10	53	F	45	Stroke	Hypertension, type 2 diabetes mellitus, gastresophageal reflux disease, atrial fibrillation (new onset)
11	45	M	32	Stroke	Hypertension, ethyl alcohol abuse, drug abuse
12	26	M	28	Cardiac arrest	Seizure disorder
13	56	F	26	Stroke	Smoked 80 pack/yr
14	58	F	36	Stroke	Hypertension, chronic obstructive pulmonary disease, hypothyroidism, schizoaffective disorder, bipolar disorder, smoked 25 pack/yr, obesity, oral corticosteroid prescription

* All control subjects had normal values for spirometry. BMI denotes body-mass index (defined as the weight in kilograms divided by the square of the height in meters).

from case and control subjects did not differ (data not shown). Control specimens contained 72±19 arbitrary normalized copy units (ANCU) of atrogin-1, whereas case specimens contained 216±67 ANCU. In addition, control specimens contained 128±51 ANCU of MuRF-1, whereas case specimens contained 885±294 ANCU. Therefore, the case specimens showed 3.0 times as much expression of atrogin-1 mRNA transcripts (P=0.002) and 6.9 times as much expression of MuRF-1 mRNA transcripts (P=0.001) as control specimens (Fig. 4).

Table 2. Summary of Ventilator Settings, Arterial Blood Gas Measurements, and Vital Signs for Control and Case Subjects.*

Measurement	Control Subjects (N = 8)	Case Subjects (N = 14)	P Value
Ventilator settings and related measurements			
Tidal volume (ml/kg of body weight)	7.5±1.3	8.0±2.0	0.47
Ventilation frequency (breaths/min)	11±1.7	14±3.0	0.02
PEEP (cm H_2O)	0.0±0.0	6.0±1.0	<0.001
FIO_2 (%)	—	52±0.11	—
SaO_2 (%)	99±2.0	—	—
PaO_2 (mm H_2O)	—	147±88	—
$PETCO_2$ (mm Hg)	31±3.8	—	—
$PaCO_2$ (mm Hg)	—	34±6.0	—
Arterial pH (units)	—	7.39±0.05	—
PaO_2/FIO_2†	—	412±167	—
Vital signs			
Systolic pressure (mm Hg)	115±10	125±20	0.20
Diastolic pressure (mm Hg)	62±6.0	70±10	0.08
Heart rate (beats/min)	72±9.0	105±18	<0.001
Body temperature (°C)	35.7±0.3	36.4±1.1	0.06

* Plus–minus values are means ±SD. FIO_2 denotes fractional concentration of inspired oxygen, $PaCO_2$ arterial carbon dioxide pressure, PaO_2 arterial oxygen pressure, PEEP positive end-expiratory pressure, $PETCO_2$ end-tidal carbon dioxide pressure, and SaO_2 arterial oxygen saturation.
† These measurements were made at an FIO_2 of 1.0.

## HISTOLOGY OF PECTORALIS MAJOR—BIOPSY SPECIMENS

The cross-sectional areas of slow-twitch and fast-twitch fibers in the biopsy specimens from the pectoralis major in case subjects were 3084±796 and 2933±1343 μm^2, respectively, whereas the cross-sectional areas of these fiber-types in the control specimens were 3325±1256 and 3418±1281 μm^2, respectively. These data show that the pectoralis fibers from case and control subjects did not differ with respect to cross-sectional area of any fiber type (see Fig. S1 in the Supplementary Appendix). Likewise, these case and control specimens did not differ with respect to the numerical proportion or area fractions of slow-twitch and fast-twitch fibers (Table S7 in the Supplementary Appendix).

DISCUSSION

In our case subjects, the combination of 18 to 69 hours of diaphragmatic inactivity and mechanical ventilation was associated with marked atrophy of both slow-twitch and fast-twitch fibers of the diaphragm. Muscle inactivity is known to effect oxidative stress and increase cytosolic calcium concentration, perturbations known to elicit increases in the activity of proteases (e.g., caspases) that cause increased dissociation of the myofibrillar lattice, the critical initial step in proteolysis.[7,16] In case subjects, the decrease in diaphragmatic glutathione concentration is consistent with oxidative stress,[6,17] and the increases in active caspase-3 suggest an increased rate of protein release from the myofibrillar lattice.[16]

After release from the lattice, the major route of proteolysis for muscle proteins is the ubiquitin–proteasome pathway, which consists of the following sequential steps: activation of the small protein cofactor ubiquitin (76 amino acid residues), formation of activated ubiquitin-chain moieties catalyzed by specific ubiquitin-conjugase enzymes (i.e., E2 enzymes), attachment of ubiquitin chains to specific proteins by ubiquitin-ligase enzymes (i.e., E3 enzymes such as atrogin-1 and MuRF-1), and recognition of specific ubiquitin-protein chains by the 26S proteasome, followed by release of ubiquitin residues and deg-
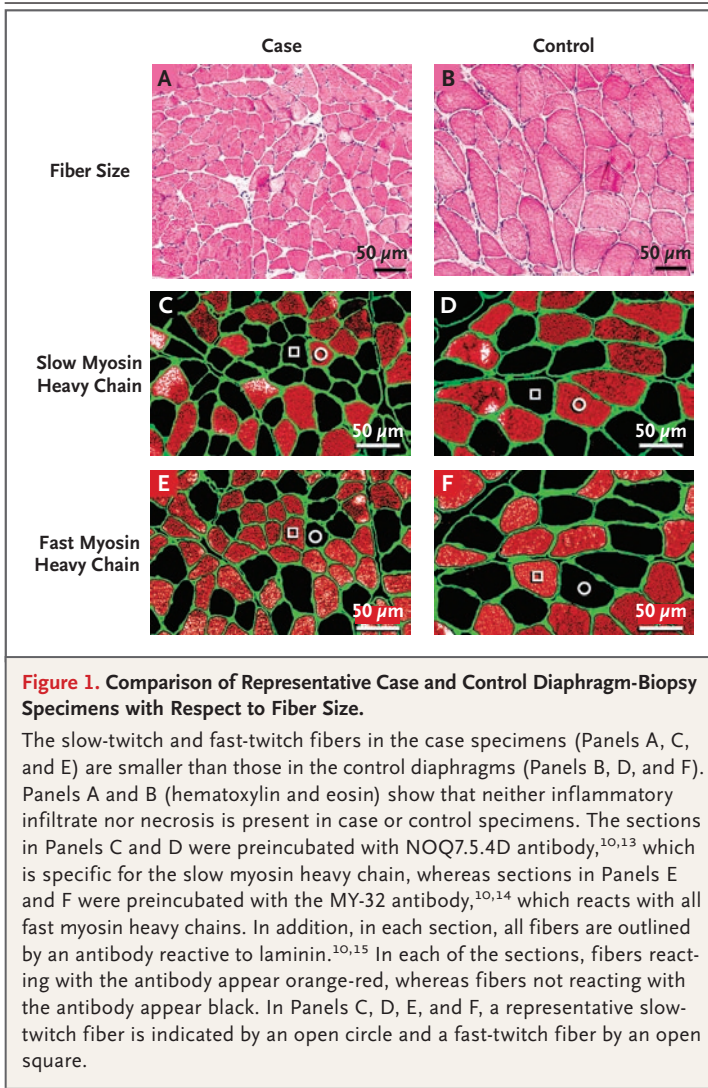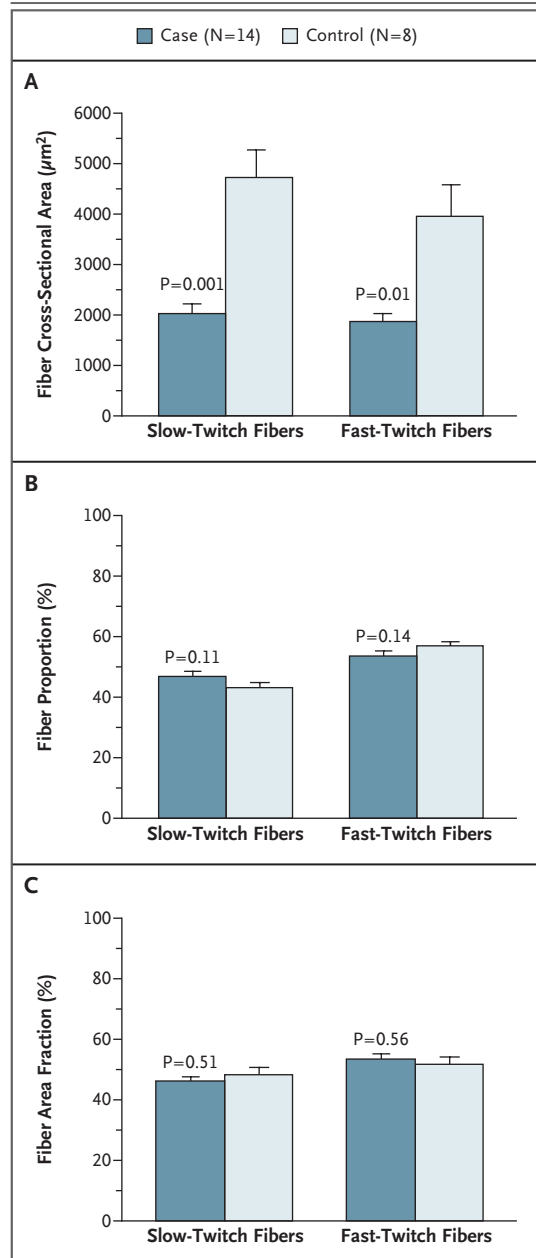
Figure 1. Comparison of Representative Case and Control Diaphragm-Biopsy Specimens with Respect to Fiber Size.

The slow-twitch and fast-twitch fibers in the case specimens (Panels A, C, and E) are smaller than those in the control diaphragms (Panels B, D, and F). Panels A and B (hematoxylin and eosin) show that neither inflammatory infiltrate nor necrosis is present in case or control specimens. The sections in Panels C and D were preincubated with NOQ7.5.4D antibody,[10,13] which is specific for the slow myosin heavy chain, whereas sections in Panels E and F were preincubated with the MY-32 antibody,[10,14] which reacts with all fast myosin heavy chains. In addition, in each section, all fibers are outlined by an antibody reactive to laminin.[10,15] In each of the sections, fibers reacting with the antibody appear orange-red, whereas fibers not reacting with the antibody appear black. In Panels C, D, E, and F, a representative slow-twitch fiber is indicated by an open circle and a fast-twitch fiber by an open square.
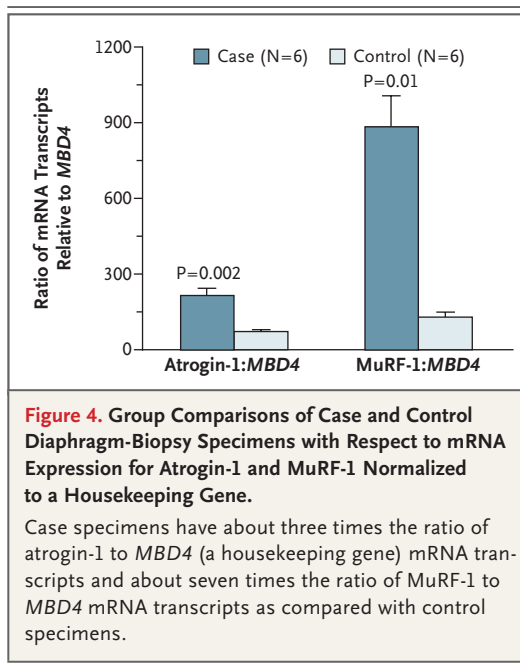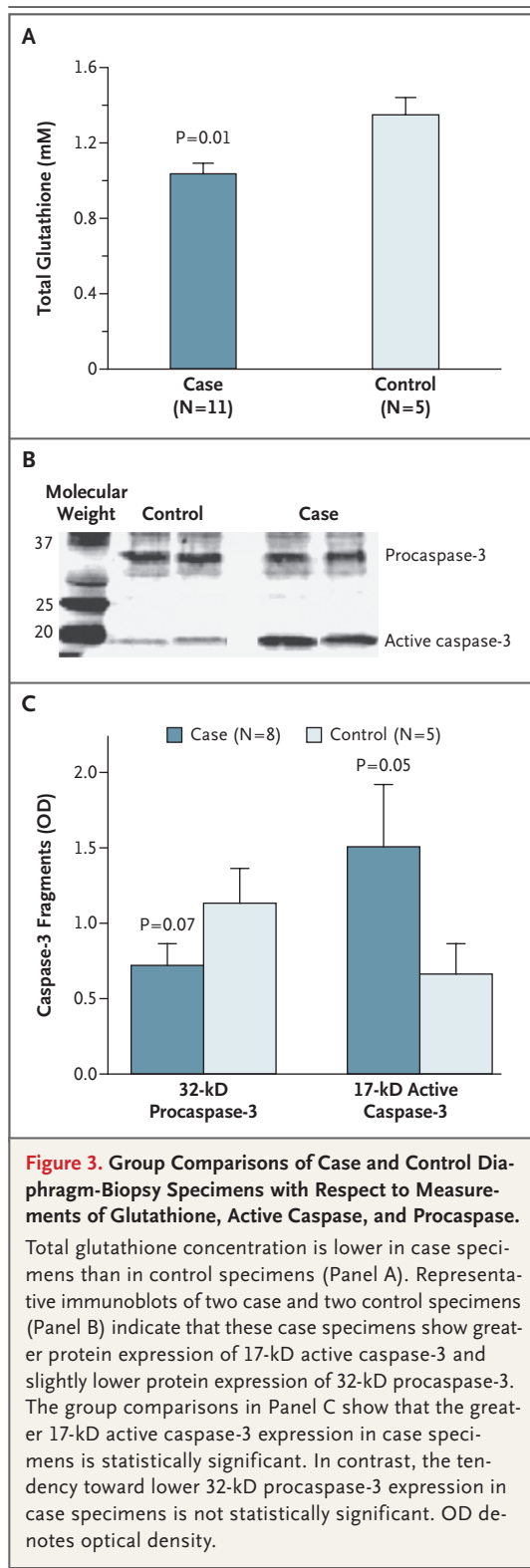
radation of proteins to small peptides (8 to 11 amino acid residues) by the 20S catalytic core of the proteasome.[18] In conditions characterized by degradation of muscle protein, there is an up-regulation of mRNAs coding for atrogin-1 and MuRF-1[18,19]; therefore, the marked increases in these transcripts noted in the diaphragm-biopsy specimens from case subjects are consistent with increased proteolysis.

Since our data are histologic or biochemical, we can only speculate on the functional significance of our findings. One report re-emphasized[20] the idea that weaning patients from ventilators is closely linked to diaphragm force generation (usually assessed clinically as transdiaphragmatic pressure). The question then becomes how mea-

Figure 2. Group Comparisons of Case and Control Diaphragm-Biopsy Specimens with Respect to Histologic Features.

Both the slow-twitch fibers and the fast-twitch fibers in the case specimens are appreciably smaller than those in the control specimens (Panel A). However, the case and control specimens do not differ with respect to proportions of slow- and fast-twitch fibers (Panel B), nor do they differ with respect to area fractions of slow- and fast-twitch fibers (Panel C) (i.e., the proportion of diaphragm-fiber cross-sectional area accounted for by slow- or fast-twitch fiber types).

A

B

C

Figure 3. Group Comparisons of Case and Control Diaphragm-Biopsy Specimens with Respect to Measurements of Glutathione, Active Caspase, and Procaspase.

Total glutathione concentration is lower in case specimens than in control specimens (Panel A). Representative immunoblots of two case and two control specimens (Panel B) indicate that these case specimens show greater protein expression of 17-kD active caspase-3 and slightly lower protein expression of 32-kD procaspase-3. The group comparisons in Panel C show that the greater 17-kD active caspase-3 expression in case specimens is statistically significant. In contrast, the tendency toward lower 32-kD procaspase-3 expression in case specimens is not statistically significant. OD denotes optical density.

Figure 4. Group Comparisons of Case and Control Diaphragm-Biopsy Specimens with Respect to mRNA Expression for Atrogin-1 and MuRF-1 Normalized to a Housekeeping Gene.

Case specimens have about three times the ratio of atrogin-1 to *MBD4* (a housekeeping gene) mRNA transcripts and about seven times the ratio of MuRF-1 to *MBD4* mRNA transcripts as compared with control specimens.

surements of fiber atrophy in our case subjects relate to force generation. If the findings in our samples occurred throughout the diaphragm, the degree of atrophy we observed would predict an approximately 55% decrease in transdiaphragmatic pressure (i.e., to 45% of control values). Therefore, we believe that fiber atrophy of the magnitude noted in case specimens could have clinical significance.

There are limitations to our study. We recognize that the decreases in fiber cross-sectional area — noted in the diaphragm-biopsy specimens from case subjects — can be explained by either artifactual increases in mean sarcomere length due to improper fixation or actual functional decreases in mean fiber volume. To distinguish between these possibilities, we used both longitudinal and transverse sections of case specimens and determined a mean sarcomere length of 2.0 ± 0.2 μm for both slow-twitch and fast-twitch fibers. This value is very similar to that noted for the diaphragm fibers from control subjects.[21] On the basis of these observations, we conclude that the decrease in cross-sectional area of diaphragm fibers from case subjects could be attributed entirely to atrophy.

Another limitation is that our case subjects were younger than the control subjects. Although

the preferred method for analyzing this type of data is a matched-pair design,[11] we lacked a sufficient number of subjects to allow matching for both age and sex. The results from our previous study[22] as well as the present data strongly suggest that sex is not a determinant of cross-sectional area in diaphragm fibers. Therefore, since we found no statistically significant differences in cross-sectional area between the age-matched (5 subjects) and full case (14 subjects) groups, we reason that the difference in age between case and control subjects does not account for the atrophy of diaphragm fibers from case subjects.

There are other possible causes of atrophy in the diaphragm fibers from case subjects. One or more of the following conditions may have played some role in eliciting the atrophy: systemic inflammatory response syndrome (SIRS) or sepsis,[23] barotraum–volutrauma,[24] brain death,[25] or unmeasured humoral substances. Current concepts suggest that diaphragm atrophy associated with SIRS, sepsis, or barotrauma–volutrauma should be associated with an inflammatory-cell infiltrate or increased proinflammatory cytokines. Neither of these findings, however, was evident in the diaphragm fibers from case subjects (Fig. 1A, and Table S5 in the Supplementary Appendix).

To assess the possibility that noncytokine humoral substances or other factors associated with brain death accounted for the diaphragm-fiber atrophy, we compared biopsy specimens from the pectoralis major muscle of six case subjects and six control subjects. The specimens from the two groups did not differ with respect to cross-sectional area of either slow-twitch or fast-twitch fibers (Fig. S1 in the Supplementary Appendix). These observations suggest that neither brain death nor unmeasured humoral factors played a role in effecting fiber atrophy in case subjects' diaphragms.

There are factors affecting fiber atrophy in disuse states that could influence our results. Our data do not elucidate the complex relationships between conditions present in the diaphragms of case subjects — inactivity, level of phrenic motoneuron activity, diaphragm muscle lengths, and perhaps additional factors — and the marked atrophy of both slow-twitch and fast-twitch fibers in the diaphragms. The orthopedic literature indicates that limb muscles that are used frequently show appreciably more inactivity-associated fiber atrophy than muscles used less frequently.[26] Because the diaphragm is active in most people 24 hours a day, one might expect it to show a greater rate of atrophy than limb muscles that are rendered inactive by interventions such as spinal cord injury,[27] microgravity,[28] or bed rest.[29]

In summary, our study indicates that the combination of 18 to 69 hours of diaphragm inactivity and mechanical ventilation is associated with marked atrophy of both slow-twitch and fast-twitch fibers in the human diaphragm. Since our observations strongly suggest that increased proteolysis accounts for the fiber atrophy noted in the diaphragm-biopsy specimens from case subjects, we speculate that blocking or attenuating diaphragm proteolytic pathways in patients on mechanical ventilation might mitigate the weaning problems that occur in some patients.

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