CHAPTER 2 UNAFFECTED CONTRACTILITY OF DIAPHRAGM MUSCLE FIBERS IN HUMANS ON MECHANICAL VENTILATION

2. UNNAFFECTED CONTRACTILITY OF DIAPHRAGM MUSCLE FIBERS IN HUMANS ON MECHANICAL VENTILATION

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Abstract

Rationale: Several studies have indicated that diaphragm dysfunction develops in patients on mechanical ventilation (MV).

Objectives: Here, we tested the hypothesis that the contractility of sarcomeres – i.e. the smallest contractile unit in muscle - is affected in humans on MV.

Methods: We compared diaphragm muscle fibers of 9 brain-dead organ donors (cases) who had been on MV for 26 ± 5 hours with diaphragm muscle fibers from 9 patients (controls) undergoing surgery for lung cancer who had been on MV less than 2 hours. In each diaphragm specimen we determined (1) muscle fiber cross-sectional area in cryosections by immunohistochemical methods, and (2) the contractile performance of permeabilized single muscle fibers by means of maximum specific force, kinetics of cross-bridge cycling by rate of tension redevelopment, myosin heavy chain content and concentration, and calcium sensitivity of force of slow-twitch and fast-twitch muscle fibers.

Measurements and main results: Both In case subjects we noted no statistically significant decrease in outcomes compared to controls in slow-twitch or fast-twitch muscle fibers.

Conclusions: These observations indicate that 26 hours of MV of humans is not invariably associated with changes in the contractile performance of sarcomeres in the diaphragm.

Introduction

Mechanical ventilation (MV) is a life-saving treatment in modern intensive care medicine, but over the past two decades adverse effects of this therapeutic modality on the diaphragm have been indicated. Several studies showed the presence of diaphragm muscle fiber atrophy and weakness in humans on MV [62,67–69,73]. The mechanisms that underlie these diaphragmatic changes during mechanical ventilation may include unloading of the muscle, loss of trophic influences that emanate from the phrenic nerves, and contractile inactivity [4774]. Previous studies on animal models [4046], showing that controlled ventilation (nearly completely abolished diaphragm loading) induced more pronounced effects on the diaphragm than assist-mode ventilation (partially abolished diaphragm loading), support an important role for these mechanisms.

Our knowledge of the effects of MV on the functioning of sarcomeres – i.e. the smallest contractile unit in muscle - is limited to laboratory experiments in animals [75]. Several studies on small animal models indicate that MV rapidly induces atrophy of diaphragm muscle fibers [38,64,76–79]. Typically, if a muscle fiber atrophies, its force generating capacity decreases as the force a muscle fiber can generate scales with the cross sectional area of the fiber. Indeed, diaphragm fiber atrophy following mechanical ventilation rapidly induces contractile weakness [68,74,79]. However, it has consistently been reported that weakness of the diaphragm in mechanically ventilated animals persists after normalization of force to the (reduced) cross sectional area of the muscle fibers [40,64,75,80]. This reduction in so called *specific force* suggests that mechanical ventilation induces not only atrophy, but also changes in the contractile properties of the sarcomeres. To date, no studies have reported on the effects of MV on sarcomere function in diaphragm muscle fibers of humans.

In the present study, we tested the hypothesis that in the diaphragm of humans on MV the functioning of sarcomeres is affected. Studying the contractile function of sarcomeres provides not only novel insights in the pathophysiology of diaphragm weakness during MV, but may also provide direction for the development of novel treatment strategies - such as fast skeletal troponin activation [81] - to combat weaning failure in critically patients by improving sarcomere function. Similar to our previous studies [67,68] we used biopsies from the costal diaphragms of brain-dead organ donors for our experimental samples.

Specifically, we compared myosin heavy chain (MyHC)-characterized permeabilized single muscle fibers prepared from the costal diaphragms of 9 brain-dead organ donors before harvest (case subjects) and compared them with intraoperative biopsy specimens obtained from the diaphragms of 9 patients who underwent surgery for resection of lung cancer (control subjects).

We measured the cross-sectional area of diaphragm fibers using standard immunohistochemical methods [82,83]. Additionally, we used permeabilized (i.e., skinned) diaphragm fiber preparations for our contractility measurements. In these preparations, membranous structures such as the sarcolemma, sarcoplasmic reticulum, and mitochondria become highly permeable, whereas the sarcomeric proteins retain their in vivo configuration [15]. This allows the investigator to control the concentrations of calcium and other chemicals in the vicinity of the actomyosin cross-bridges.

Results

Subjects

Demographic information, reason for surgery, duration of mechanical ventilation, and relevant medical history for case and control subjects are summarized in table 1. Ventilator settings, measurement of arterial blood gases, and vital signs for case subjects are summarized in table 2. The data in table 1 indicate that case and control groups exhibited no statistically significant differences with respect to age: 57 (41, 67) years vs. 59 (54, 65) years, respectively, p=0.54. Likewise, the groups did not differ with respect to BMI: 27±1 vs. 26±1 kg/m^2, respectively, p=0.35. The case group consisted of 5 female and 4 male subjects, and the control group consisted of 3 female and 6 male subjects (p=0.64). Case subject diaphragm inactivity ranged from 6-48 hours, whereas diaphragm inactivity in controls ranged from 1.5-2 hours. The mean duration of diaphragm inactivity in cases was 14 times greater than that in controls (26±5 vs. 1.7±0.1 hours).

Table 1 Summary of Demographic Characteristics, Reasons for Surgery, Duration of Mechanical Ventilation, and Medical History for Case and Control Subjects.

(A) Case subjects

#	Age	G	BMI	MV	Cause of Brain Dead	Relevant Medical History
1	57	F	22	48	Cardiac arrest	Diabetes mellitus, hypertension, depression
2	71	F	23	44	Air embolism and myocardial infarction following surgery for mitral valve disease	Dyslipidemia, coronary artery disease, cholelithiasis and s/p cholecystectomy
3	57	F	28	16	Motor vehicle accident with intracranial hemorrhage	Excessive alcohol abuse, smoker-2 pack/yr
4	48	M	26	6	Apnea followed by cardiac arrest	Alcohol abuse, smoker
5	52	F	29	32	Cardiac arrest	Smoker, alcohol abuse, schizoaffective disorder, s/p hysterectomy
6	63	M	29	36	Head trauma with subdural hematoma	Alcohol abuse, depression, anxiety, hypertension, hyperlipidemia
7	18	M	26	18	Motor vehicle accident with intracranial hemorrhage	Seasonal allergy, s/p left shoulder surgery
8	76	F	34	12	Intracranial hemorrhage	Severe cerebral arterial disease, s/p PCT angioplasty of innominate artery and stent placement in right carotid, peripheral arterial disease, coronary artery disease with congestive heart failure
9	34	M	27	24	Intracranial hemorrhage	Hypertension, asthma, alcohol abuse, smoker-8 pack/yr

(B) Control subjects

#	Age	G	BMI	MV	Lung Cancer	Relevant Medical History
1	53	M	25	1.5	Stage 2B	Cigarette smoker 30 PY, diabetes mellitus, hypertension, COPD
2	22	M	23	2.0	Stage 1A	Cigarette smoker 3PY
3	66	M	26	2.0	Stage 2A	Cigarette smoker (stopped 10 years ago, PY N/A), hypertension, prostate cancer successfully treated 12 years ago, COPD
4	58	F	28	2.0	Stage 1A	None
5	60	M	24	1.5	Stage 2A	Cigarette smoker 9PY, s/p right upper lobectomy for Stage 1A lung cancer five years ago, pulmonary tuberculosis s/p successful drug therapy 50 years ago
6	56	M	21	2.0	Stage 3B	Cigarette smoker (stopped 15 years ago, PY N/A), COPD
7	70	M	30	1.5	Stage 2A	Cigarette smoker PY N/A, s/p basal cell cancer resection from nose three years ago
8	59	F	28	1.0	Lung cyst	Cigarette smoker 17PY
9	64	F	26	1.3	Stage 1A	Cigarette smoker 26PY, cholecystectomy, chronic bronchitis

Age in years (case and control group respectively 57 (41-67) years vs. 59 (54-65) years, respectively, p=0.5363. G=Gender, BMI=Body-Mass Index (case and control group respectively 27±1 and 26±1 kg/m², p=0.35), MV = duration of mechanical ventilation in hours (case and control group respectively 26±5 and 1.7±0.1 hours). All control patients had normal values for spirometry. N/A: information not available.

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

#	1	2	3	4	5	6	7	8	9
Ventilator settings and related measurements									
Tidal volume (ml)	700	400	600	600	650	600	750	460	650
Ventilation frequency (breaths/min)	12	12	12	18	12	10	22	21	12
PEEP (cm H_2O)	5	5	5	5	5	5	5	8	5
FiO_2 (%)	35	50	100	100	50	100	100	100	100
SaO_2 (%)	99	98	100	100	99	100	100	95	100
PaO_2 (mm H_2O)	141	116	314	207	115	296	376	77	558
$PaCO_2$ (mm Hg)	36	39	30	39	39	36	31	45	38
Arterial pH (units)	7.40	7.34	7.40	7.52	7.45	7.39	7.44	7.39	7.36
Vital signs									
Systolic pressure (mm Hg)	115	129	155	118	110	111	115	110	185
Diastolic pressure (mm Hg)	60	60	62	56	74	58	55	56	100
Heart rate (beats/min)	91	79	91	115	86	84	79	86	95
Body temperature (°C)	36.4	36.5	36.9	37.2	36.2	34.6	36.8	36.7	36.7

PEEP: positive end-expiratory pressure, FiO_2: fractional concentration of inspired oxygen, SaO_2: arterial oxygen saturation, PaO_2 arterial oxygen pressure, $PaCO_2$ arterial carbon dioxide pressure.

Histology

Figure 1 shows representative immunohistochemical sections from a control subject (figures 1a, 1c) and from a case subject (figures 1b, 1d) and suggests that the two groups exhibited no differences with respect to muscle fiber-type size. Additionally, these and

other sections show no evidence of infiltrate or edema. Figure 1e shows cross sectional area (CSA) of muscle fibers of case and control subjects, which did not differ in either slow-twitch (2876 (2476, 5989) μm^2 vs. 3194 (2988, 4154) um^2, p=0.61) or fast-twitch muscle fibers (3047 (2498, 6865) μm^2 vs. 3392 (2455, 3470) $\mu m^{2,}$ p=0.39).

We also obtained longitudinal sections of our biopsy specimens and used these to determine sarcomere length (cases: n=4; controls: n=3). Studying histological sections revealed that muscle fibers of control and case subjects did not differ in sarcomere length (case 1.84±0.07 µm and control 1.78±0.03 µm, p=0.48). Thus, assuming that the average sarcomere length in this subset of case and control subjects is representative for the whole group, the average CSA of diaphragm fibers in the case and control group was not affected by differences in the sarcomere length at which CSA was determined.

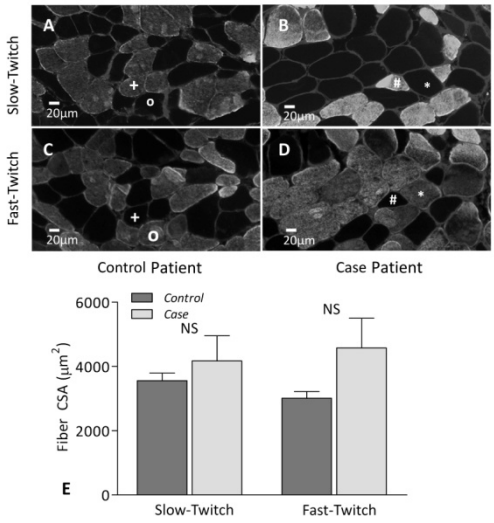

Figure 1. (A-D) typical examples (control #4, and case #9) of serial diaphragm cross-sections stained with antibodies against slow and fast MyHC (light surfaces) and against wheat germ agglutinin (thin light lines); symbols indicate same muscle fibers. Bar: 20 µm. (E) No significant differences were observed in CSA between case and control specimens in both slow- and fast-twitch diaphragm muscle fibers.

Diaphragm muscle fiber contractility

Maximum isometric force generation

Figure 2a shows the force response of a representative single permeabilized diaphragm muscle fiber to bath solutions containing different calcium concentrations. At times prior to t_1, the bath contained pre-activating solution at a pCa of 9.0, whereas at time t_1, the bath solution was changed to activating solution having a pCa of 4.5. The figure shows that from time t_1 to t_2, the bath solution at pCa of 4.5 elicited appreciable force generation. At time t_2, the bath solution is switched to relaxing solution, back to a pCa

of 9.0, and the figure shows that the force generated by the muscle fiber returns to zero. Therefore, this representative muscle fiber demonstrates that large increases in calcium concentration elicit force development that is reversible by marked lowering of the calcium concentration. This phenomenon is exhibited by both slow-twitch and fast-twitch muscle fibers.

Figure 2b compares permeabilized single muscle fibers from case and control diaphragms with respect to maximum absolute force. We observed no statistically significant difference between the two groups with respect to absolute force generated by either slow-twitch (case vs. control respectively 0.43±0.07mN vs. 0.44±0.06 mN, p=0.92) or fast-twitch muscle fibers (case vs. control respectively 0.51±0.08 mN vs. 0.53±0.06 mN p=0.89). Figure 2c compares permeabilized single muscle fibers from case and control diaphragms with respect to maximum force normalized to muscle fiber CSA; in the clinical literature, this calculation is termed maximum specific force or tension. We observed no statistically significant difference between the two groups with respect to maximum specific force of either slow-twitch (case vs. control respectively 104±13 mn/mm^2 vs. 115±7 mn/mm^2, p=0.43) or fast-twitch muscle fibers (case vs.cControl respectively 127±7 mn/mm^2 vs. 142±11 mn/mm^2 p=0.24).

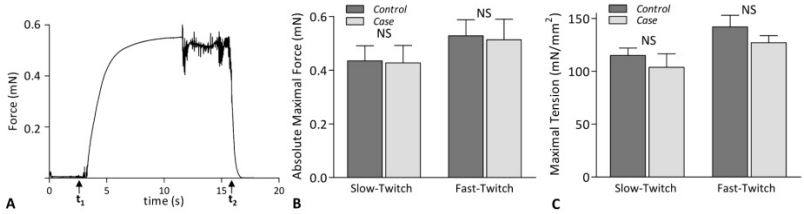

Figure 2. (A) Typical tracing showing the force response of a permeabilized diaphragm single muscle fiber (control #7, slow-twitch fiber) when exposed to a solution with saturating Ca^{2+}concentrations (t_1) and subsequently to relaxing solution (t_2). (B) absolute maximal force and (C) maximal tension (absolute force normalized to muscle fiber CSA). No statistically significant differences between case and control subjects were observed.

Determinants of maximum force.

As previously noted, in the single permeabilized muscle fiber preparation, maximum isometric force generation is determined by three factors: (a) number of available cross-bridges in parallel per half sarcomere; (b) force per cross-bridge, and (c) the fraction of cross-bridges that are in the force-generating state [84,85]. Here, we use MyHC content per half sarcomere as an index of the number of available cross-bridges, and by computing the quotient of absolute force divided by MyHC content per half sarcomere we estimated the force per cross-bridge. The rate constant of force redevelopment is an indicator of cross-bridge cycling kinetics.

Myosin heavy chain (MyHC) content and concentration.

Figure 3a illustrates the method used for quantifying MyHC content in single permeabilized muscle fibers (see figure legend for details). Figure 3b shows that MyHC content in slow-twitch muscle fibers was greater in cases than controls (respectively 452 (398,528) pg vs. 346(286,385) pg, p=0.001), and a trend towards a higher MyHC content was noted in fast-twitch muscle fibers (case vs. control respectively 442±43 pg vs. 304±49 pg, p=0.051). Figure 3c shows a trend towards a higher MyHC concentration (expressed per muscle fiber volume) in slow-twitch fibers of case subjects (case vs. control 103±15 μg/μl vs. 69±8 μg/μl, p=0.052) and a significantly higher MyHC concentration in fast-twitch muscle fibers of case subjects (case vs. control respectively 95±8 μg/μl vs. 67±810 μg/μl, p=0.043).

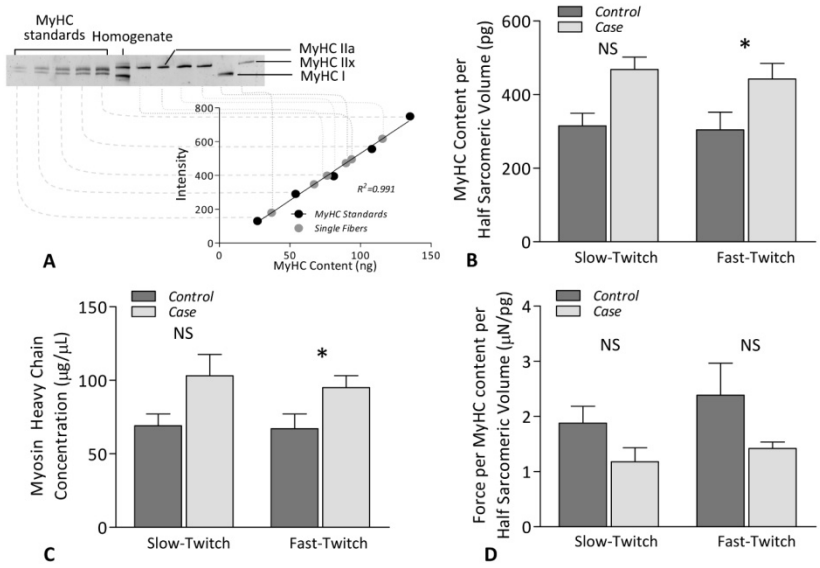

Figure 3. (A) Typical example of SDS-page gel result with five myosin heavy chain (MyHC) standards (from left to right: 27, 54, 81, 108 and 135 ng), a diaphragm homogenate and six diaphragm single muscle fiber samples. By comparing the intensity of the single muscle fiber band to that of the MyHC standard bands, we determined the amount of MyHC present in the single muscle fibers used for contractile experiments. (B) MyHC content –in pg per half sarcomeric volume– was statistically significantly higher in case subject slow-twitch muscle fibers, but not in fast-twitch muscle fibers. (C) Analysis revealed that MyHC concentration (μg/μL) was statistically significantly higher in case subjects' slow-twitch muscle fibers, but not in fast-twitch muscle fibers. (D) Force per half sarcomeric MyHC content (μN/pg), which reflects force per cross-bridge, was not statistically significantly different between case and control subjects.

Force per actomyosin cross-bridge.

Figure 3d shows the comparison of force per unit mass of myosin (i.e., μn/pg MyHC), which did not significantly differ between case vs. Control in slow-twitch (respectively

1.2±0.25 vs. 1.9±0.31 µn/pg, p=0.11) and fast-twitch muscle fibers (1.32 (1.15, 1.65) µn/pg vs. 1.72 (1.36, 3.12) µn/pg, p=0.24).

Rate constant for force redevelopment (ktr).

Figure 4a presents a typical record showing the exponential redevelopment of force after quick unloaded shortening and re-stretching of the muscle fiber to initial length. Figure 4b shows ktr per group. Ktr did not significantly differ between case and control in slow-twitch muscle fibers (respectively 5.9 (4.6, 7.2) vs. 5.5 (4.9, 6.4), p=0.75), or in fast-twitch muscle fibers (respectively 8.3±0.8 vs. 9.9±1, p=0.24).

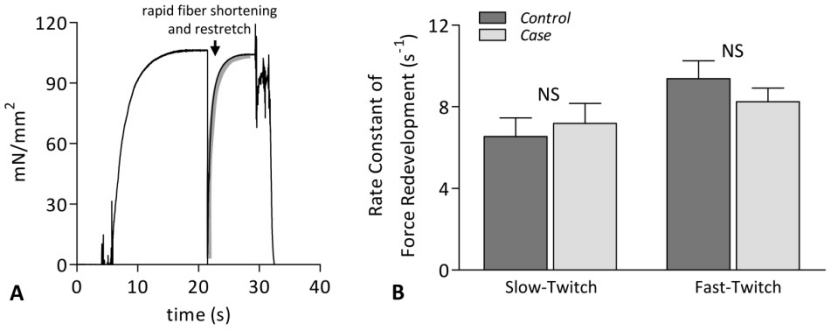

Figure 4. (A) Typical tracing recording showing the redevelopment of force (ktr) after quick unloaded shortening and restretching of the muscle fiber (control subject #7, slow-twitch fiber is shown) to initial length. The superimposed thick grey line indicates the part of the force tracing used for the determination of ktr. (B) Rate constant of force redevelopment; no significant differences between case and control subjects were observed.

Sensitivity of force generation to calcium concentration

Figure 5a shows as calcium concentration in the bath is increased from a pCa of 7.0 to a pCa of 4.5, the maximum force generated by the representative muscle fiber also progressively increases. Figure 5b illustrates the tension for all experimental concentrations of pCa normalized to the tension generated by pCa 4.5. This figure shows that the relationship between force and pCa is a sigmoid curve. A commonly used method for comparing permeabilized muscle fibers with respect to calcium-sensitivity of force generation is to compare them with respect to pCa_{50} –i.e., the pCa needed to elicit a relative force of 0.5. The solid dark line –with the arrow head– in figure b indicates that the pCa_{50} of this representative muscle fiber is approximately 5.7. Figure 5c compares slow-twitch muscle fibers from case and control permeabilized muscle fibers with respect to the relationship between relative tension and pCa. This figure shows that the two curves are virtually identical. The insert to figure 4c shows that the two groups exhibited no statistically significant difference with respect to pCa_{50} (slow-twitch fibers case vs. Control respectively 5.63±0.04 vs. 5.62±0.03, p=0.84). Figure 5d compares fast-twitch muscle fibers from case and control permeabilized muscle fibers with respect to the relationship between relative force and pCa; the two curves are very

similar. Additionally, the insert of figure 5d indicates that the two groups exhibited no statistically significant differences with respect to pCa_{50} (fast-twitch fibers case vs. Control respectively 5.70±0.02 vs. 5.75±0.03, p=0.19).

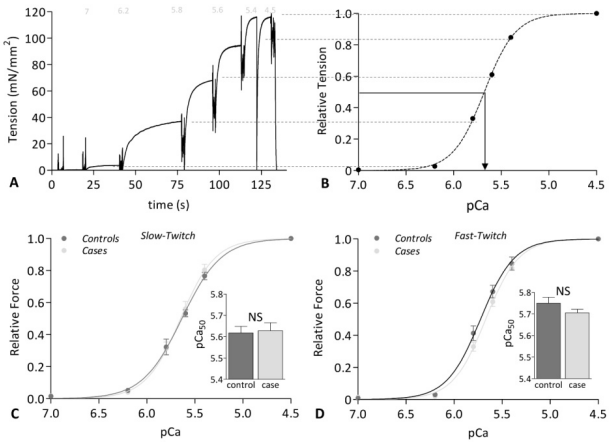

Figure 5. (A) Typical tracing showing the force response to incremental calcium concentrations of a diaphragm single muscle fiber (control subject #7, slow-twitch fiber is shown), and (B) the resulting force-calcium curves where numbers on the abscissa indicate negative logarithm of the calcium concentration and dashed lines indicate corresponding points on the force axis. (C)The sensitivity of force to calcium in slow-twitch muscle fibers. (D) Force-calcium relationship for fast-twitch fibers. Indented bar graphs show pCa_{50} values, which is the calcium concentration required to generate 50% of maximal force. No statistically significant differences between case and control subjects were observed.

The total number of fibers used per parameter per subject for the contractility measurements is shown in table 3.

Table 3 Number of fibers used for analysis of contractility parameters

Group	#	[MHC]	MHC Content	Force/ MHC-content	Absolute Maximal Force	Maximal Tension	pCa_{50}	Ktr
Slow-Twitch fibers								
Case	1	9	9	9	9	9	8	5
	2	5	5	5	5	5	4	5
	3	3	3	3	3	3	3	
	4	3	3	3	3	3	3	3
	6	4	4	4	4	4	4	4
	7	3	3	3	3	3	3	
	8	9	9	9	9	9	9	8
Sub Total		36	36	36	36	36	34	23
Control	1	5	5	5	5	5	5	3
	2	7	7	7	7	7	6	6
	3	10	10	10	10	10	6	6
	4	10	10	10	10	10	8	6
	5	10	10	10	10	10	10	9
	6	6	6	6	6	6	6	4
	7	11	11	11	11	11	9	7
	8				3	3		3
	9	8	8	8	12	12	12	11
Sub Total		67	67	67	74	74	62	55
Total Slow		103	103	103	110	110	96	78
Fast twitch fibers								
Case	1	4	4	4	4	4	4	
	2	7	7	7	7	7	6	6
	3	9	9	9	9	9	9	8
	4	10	10	10	10	10	7	9
	6	9	9	9	9	9	9	
	7	13	13	13	13	13	13	7
	8	7	7	7	7	7	5	3
	9	10	10	10	10	10	9	3
Sub Total		69	69	69	69	69	62	36
Control	1	11	11	11	11	11	9	7
	2	3	3	3	3	3	3	
	3	8	8	8	8	8	8	4
	4	7	7	7	7	7	7	4
	5	5	5	5	5	5	4	3
	6	4	4	4	4	4	4	
	7	3	3	3	3	3	3	
	8	12	12	12	14	14	11	8
Sub Total		53	53	53	55	55	49	26
Total Fast		122	122	122	124	124	111	62
Total		225	225	225	234	234	207	140

Discussion

Major findings

The data in this manuscript show that both slow-twitch and fast-twitch diaphragm muscle fibers from humans on MV (on average 26h, cases) do not differ from controls with respect to cross-sectional area. We also show that the contractility of individual permeabilized diaphragm muscle fibers from cases do not differ from the contractility of fibers from control subjects with respect to (a) specific force and (b) the calcium sensitivity of force generation. To the best of our knowledge, this is the first report on the contractility of permeabilized diaphragm single fibers in humans on mv. Our findings indicate that MV of humans is not invariably associated with changes in the contractile performance of sarcomeres.

Critique

Nearly all control patients were smokers and had lung cancer; therefore, the possibility exists that our failure to note differences between cases and controls was due to the fact that the control diaphragm biopsies were obtained from cachectic patients with diaphragm dysfunction. The term "cachexia" is used to indicate a syndrome characterized by generalized weight loss, muscle wasting, and weakness. Our control patients had a normal body mass index, no history of weight loss, no muscle wasting, and no weakness on physical examination; moreover, measures of fiber-type specific cross-sectional area in our control diaphragms are comparable with those reported in the literature [62,68,82,83,86]. Therefore, we conclude that no evidence exists that a cachectic state and associated muscle wasting was present in our control subjects.

For the contractile measurements on single diaphragm fibers, individual fibers were manually isolated from the diaphragm biopsies using micro-forceps. Therefore, we cannot rule out that a selection-bias was present, and consequently, the results might not reflect the real contractile characteristics of the average slow-twitch and fast-twitch fibers in the diaphragm. However, we and others have used this technique frequently [70,82,83,86–90], and the contractile measurements described in these studies demonstrate that this methodology is capable of revealing relevant differences between patient groups.

Additionally, the contractility data from the case subjects were from fibers isolated from snap-frozen specimens, whereas that of the control subjects was from fibers that were isolated from directly glycerinated specimens (for details, see methods). Therefore, the possibility exists that our single fiber results were affected by the discrepancy in tissue harvesting, and/or that the structure and function of case muscle fibers were compromised by the freeze-thawing cycle. Therefore, we tested our hypothesis that glycerinated and snap frozen specimens from the same biopsy will give similar results. As reported in the methods section, both approaches yielded

comparable contractility of single diaphragm fibers. Therefore, it is unlikely that the discrepancy in tissue harvesting affected our findings.

Comparison with human data in literature

A striking observation in the present study is the absence of atrophy of diaphragm muscle fibers in case subjects. This observation is not in line with previous studies on mechanically ventilated brain dead organ donors, which reported a significant reduction of diaphragm muscle fiber cross sectional area [62,68]. The apparent discrepancy between the findings from previous work and those from the present study might be a result of the following.

First, the average duration of mechanical ventilation *after* diagnosis of brain death was short in the present study (on average 26h) when compared to the duration reported in previous studies (34h in Levine et al [68] and 80h in Jaber et al [62]). As previous work indicated that the magnitude of diaphragm weakness correlates with the duration of mechanical ventilation [63], it could be speculated that 26h of mechanical ventilation was not sufficient to elicit major changes in diaphragm fiber cross sectional area in the patients studied here.

Second, the *mode* and the *duration* of mechanical ventilation *prior* to the diagnosis of brain death may contribute to the discrepancy. Different modes or durations of MV are known to differentially affect the diaphragm. For instance, earlier it was shown that assist-mode ventilation – where the diaphragm's effort to trigger the inspiratory cycle is supported rather than suppressed – largely alleviates the detrimental effect of controlled mechanical ventilation on diaphragm strength [46]. However, both in the current and in the previous studies on mechanically ventilated brain dead organ donors no information was available on the mode or duration of MV prior to brain death.

Third, we cannot completely rule out that some residual diaphragm activity persisted during mechanical ventilation of the case subjects. Although it is under debate whether inactivity *per se*, or other phenomena such as changes in neurotrophic factors are the major cause of diaphragm atrophy during MV [47,84], recent work revealed that low levels of diaphragm activity largely prevent the development of diaphragm weakness [46]. Thus, confirmation of complete inactivity of the diaphragm during controlled mv, by EMG, in the case and the control subjects would have provided valuable information. However, the brain dead organ donors in the present study showed no spontaneous ventilatory movements and had a positive apnea test (both of which are criteria for the diagnosis of brain death). The control patients were ventilated for <2 hours and showed no contractile activity based on visual inspection during the thoracotomy. Thus, based on this information, it is unlikely that diaphragm activity was present in the case and control subjects.

Fourth, the patients studied in previous work might have been exposed to higher doses of compounds that affect diaphragm protein turnover (such as corticosteroids and muscle relaxants) than the patients studied here. Unfortunately, typically no information is available on the medication used by the brain dead organ donors prior to the diagnosis of brain death.

Clearly, the limited information on brain dead organ donors complicates a comparison between the outcomes of the various studies in which diaphragm specimens from these subjects were studied. Nevertheless, based on the present findings we can conclude that 26h of controlled mechanical ventilation is not sufficient to elicit diaphragm fiber atrophy in humans.

Since the present study was the first to measure the contractile properties of muscle fibers in biopsies of human diaphragm from patients on mv, there are no data in the literature to compare our findings to. However, Jaber et al. [62] used measurements of tracheal occlusion pressure elicited by bilateral phrenic nerve stimulation as an index of diaphragm strength in patients on full support mv; they noted a statistically significant small decrease in tracheal occlusion pressure after 24 hours of full support mv, a time frame close to the average duration of MV in our case subjects. Thus, our findings of preserved muscle fiber function suggest that the mechanisms accounting for the decrease in inspiratory twitch occlusion pressure, as observed in jaber et al. [62], are unlikely to involve impaired functioning of sarcomeres, and might have been due to pathology in the phrenic nerve, in the neuromuscular junction, or in cytosolic calcium cycling. However, again, care should be taken when interpreting these findings as no information is available on the duration of mechanical ventilation prior to the diagnosis of brain death.

In a prior paper [67], we reported that biochemical measurements of cytoplasmic diaphragm homogenates indicated that the concentration of MyHC and actin were appreciably decreased in case patients; as noted above, these subjects underwent MV for 18-to-69 hours. In contrast, the results in the present manuscript show that case MyHC concentration in our permeabilized fibers were normal (or even increased above normal) while our measurements of actin and myosin function (i.e., specific force, force per unit mass of myosin and rate constant of force redevelopment) and our calculation of force per single actomyosin cross-bridge) were all "normal". We do not have a straightforward explanation for these discrepant results. However, one difference in the two measurements is that all muscle fibers in the diaphragm biopsy contributed to our biochemical measurements in the homogenates, whereas only isolated single fibers contributed to the present manuscript. Since compromised fibers with reduced MyHC content are more likely to break during the manual isolation of fibers from the biopsy for mechanical experiments, it follows that the mean of MyHC concentration in isolated fibers might be greater than the mean of all fibers in the diaphragm specimen.

Last, we comment on our findings that both case and control diaphragms showed no differences in the relationship between force and calcium concentration; indeed the pCa_{50} of case and control diaphragm permeabilized fibers exhibited no group differences in either slow- or fast-twitch muscle fibers. These findings suggest that the regulatory proteins (troponins and tropomyosin) were not rendered dysfunctional by the period of MV that our case subjects underwent.

Conclusions

The findings of the present study suggest that in humans the time of onset of diaphragm muscle fiber atrophy and dysfunction of sarcomeres elicited by MV is longer than 26 hours. Since these findings have clinical importance for the management of patients on mechanical ventilation, we believe that a prospective study should be undertaken using serial measurements of *in vivo* twitch occlusion pressure along with measurements of diaphragm biochemical, histological, and *in vitro* contractility studies performed on diaphragm biopsies. Ideally, the experimental design of such study uses smaller time windows, with bins that consist of durations of mechanical ventilation that include the duration (and mode) of ventilation prior to the diagnosis of brain death, so that the time-dependency can be studied.

Methods and materials

Ethical approval

The inclusion protocol and procedures for inclusion of brain-dead organ donors (case subjects) were approved by the Gift of Life Donor Program (http://www. donors1.org). The protocols for the inclusion of control subjects were approved by the University of Pennsylvania institutional review board and the medical ethical committee at VU University Medical Center. Biopsy specimens were obtained with appropriate written informed consent.

Subjects

Two groups of patients were studied. One group comprised brain-dead organ donors who received controlled mechanical ventilation for an average of 26 hours (range 6-48h). Since one of the criteria for brain death was lack of spontaneous (i.e., patient-generated) respiratory muscle activity during normocapnia, as well as normoxic hypercapnia, case diaphragms were completely inactive during the period of mechanical ventilation following brain-death and, therefore, our clinical observations indicate a period of diaphragm disuse in case subjects prior to diaphragm biopsy. The control group comprised patients undergoing thoracotomy for resection of lung cancer localized to the thorax (stage 1A-3B) (control subjects). Anesthesia management for control patients consisted of epidural anesthesia combined with general anesthesia by propofol and sufentanil. Rocuronium (0.6 mg/kg) was used as muscle relaxant during induction. When required, additional doses were given at the discretion of the

anesthesiologist. During resection of the cancer lung-protective mechanical ventilation was ensured. Case and controls were excluded if they suffered from chronic pulmonary obstructive disease (GOLD stages III or IV) or pulmonary hypertension (stage III or IV). Other exclusion criteria were the use of corticosteroids (>7.5mg/day, longer than three months), therapy or chronic intake of drugs known to alter muscle function and structure (including radiation and chemotherapy) and more than ten percent weight loss in the last six months. Average duration of MV of controls was less than 2 hrs.

Diaphragm biopsies

Case subject diaphragm biopsy specimens were obtained before circulatory arrest or removal of organs and frozen in liquid nitrogen and stored at -80°C as previously described [68]. From these biopsies, small sections (2x2mm) were isolated in liquid nitrogen. Subsequently, these sections were placed for 24h at -20°C in 4 mL relax-glycerol solution containing high concentrations of protease inhibitors (for the composition of solutions, see section 'Solution Composition' below). Then, the sections were placed on a roller band for 24h at 4°C. Finally, the relax-glycerol solution containing high concentrations of protease inhibitors was substituted with a relax-glycerol solution with lower concentrations of protease inhibitors (for composition, see section 'Solution Composition' below) and stored at -20°C until further use. This thawing approach greatly minimizes both freezing artifacts and protein degradation and allows for high-quality functional studies on demembranated fibers. Control subject biopsies were handled in the same manner as previously described [82,83,87,89,90].

Briefly, diaphragm biopsy specimens were obtained directly after opening of the thorax for removal of a lung tumor. The biopsy was dissected into two smaller sections. The section for histology experiments was directly frozen in liquid nitrogen and stored at -80°C, and the section for single muscle fiber contractility experiments was placed in relax-glycerol solutions containing high protease inhibitors for 24h at 4°C on a roller band and was subsequently stored at -20°C in relax-glycerol solutions containing normal levels of protease inhibitors until further use. From all specimens, single muscle fibers were isolated as described previously [91–94]. We verified within five control subjects (#2, #3, #4, #5 and #7, in total on n=108 muscle fibers), that diaphragm muscle fibers isolated from previously snap-frozen specimens had similar maximum specific force compared to diaphragm muscle fibers isolated from specimens that were directly stored in relax-glycerol solution (biopsy processing was not a significant source of variation: slow-twitch muscle fibers p=0.22; fast-twitch fibers p=0.36 (two-way ANOVA)). Force of thawed snap frozen fibers relative to the force of fibers directly stored in Relax-glycerol: 118±14% in slow-twitch fibers and 116±17% in fast-twitch fibers).

Histology experiments

To determine diaphragm muscle fiber cross-sectional area, immunohistochemical analyses with MyHC antibodies were carried out as described previously [82].

Cryosections were cut from the frozen biopsies (perpendicular to muscle fiber direction, 8µm thick) and rehydrated for 10 min in phosphate buffer (PBS) and subsequently blocked with phosphate buffer containing 0.5% (wt/vol) bovine serum albumin (BSA) (PBS-0.5% BSA, Molecular Probes). Subsequently, cryosections were incubated with primary antibodies (BAD, Radboud University Nijmegen) for slow MyHC and sc-71 (Santa Cruz Biotechnology) for fast MyHC followed by appropriate fluorescent labeled secondary antibodies (Invitrogen). Finally plasma membranes were visualized by embedding the sections in fluorescent WGA (Molecular Probes) (1:25 diluted in PBS-0.5%BSA) which selectively recognizes sialic acid and N-acetylglycosaminyl sugar residues. Following each incubation, cryosections were washed three times for 3 min with PBS. Samples were washed and protected with a layer of Vector Shield and a cover glass. Sections were analysed using an inverted digital imaging microscopy workstation [Intelligent Imaging Innovations (3i)] equipped with a motorized stage and multiple fluorescent channels. A cooled charge-coupled device camera (Cooke Sensicam; Cooke, Eugene, OR) was used to record images. Exposures, objective, montage, and pixel binning were automatically recorded and stored in memory. Dedicated imaging and analysis software (SlideBook, version 4.2, 3i) was obtained from Intelligent Imaging Innovations (Denver, CO). For each diaphragm specimen, approximately 100 diaphragm muscle fibers were analyzed per muscle fiber type. Cross-sectional areas were calculated by SlideBook software after encircling the labeled cell membranes with the optical mouse.

We considered the possibility that artifactual changes in sarcomere length –such as those due to improper freezing of the biopsies– affected our determinations of cross-sectional area, thereby masking potential differences in muscle fiber cross-sectional area between groups. To investigate this possibility, we determined sarcomere length in four case and three control diaphragms. In these experiments, cryosections of the diaphragm biopsies longitudinal to the muscle fiber were rehydrated in phosphate buffer, followed by blocking with PBS-0.5% BSA. Subsequently, the sections were incubated with the primary antibody mouse α-actinin (α-ACT, 1:40 diluted in PBS-0.5%BSA), then the sections were incubated with secondary antibody Alexa 488 anti-mouse (1:100 in PBS-0.5% BSA, Molecular Probes, Eugene, OR). The remaining protocol for determination of sarcomere length was analogous to that for muscle fiber cross-sectional area, as described above.

Solution composition

Solution composition was as described previously [91–94]. Solutions had an ionic strength of 180 mM and pH 7.1 (ionic strength was adjusted according to Fabiato and Fabiato [95]). The relaxing solution had a negative logarithm of free calcium concentration (pCa) of 9.0 and comprised of (in mM): 5.89 Na_2ATP (Sigma-Aldrich), 6.48 $MgCl_2$ (Sigma-Aldrich), 40.76 Kprop (Merck), 100 BES (Sigma-Aldrich), 6.97 EGTA (Sigma-Aldrich), 14.50 CrP (Sigma-Aldrich) and low concentration of freshly added protease inhibitors.

Pre-activating solutions consisted of (in mM): 5.87 Na_2ATP, 0.1 EGTA, 6.42 MgCl, 41.14 Kprop, 100 BES, 14.50 CrP and 6.9 HDTA (Sigma-Aldrich). Activating solutions consisted of (in mM) 5.97 Na_2ATP, 7.0 CaEGTA, 6.28 MgCl, 40.64 Kprop, 100 BES and 14.50 CrP. By accurate mixing of relaxing solutions and maximal activating solutions, sub-maximal activating solutions with pCa 7, 6.2, 5.8, 5.6 and 5.4 were made. Relax-glycerol solutions with 'normal' concentrations of protease inhibitors consisted of 50% (v/v) glycerol and relaxing solution, and in addition the following protease inhibitors (in mM) 1.0 DTT (MP Biomedicals LCC), 0.24 PMSF (Sigma-Aldrich), 0.04 leupeptin (Peptides Institute Inc.), 0.01 E64 (Peptides Institute Inc.). Relax-glycerol solution with high concentrations of protease inhibitors contained (in mM): 1.0 DTT, 0.24 PMSF, 0.4 leupeptin, 0.1 E64. Skinning solution consisted of relaxing solution with 1% Triton X-100 (Calbiochem) and protease inhibitors (in mM) 1.0 DTT, 0.24 PMSF, 0.04 leupeptin, 0.01 E64.

Diaphragm muscle fiber contractility experiments

Setup and protocol

Our protocol for the contractility experiments has been described previously [82,83,87,89,90]. Briefly, segments of single muscle fibers of approximately 1-1.5mm were isolated in a relaxing solution at 5°C. At both ends, two aluminum clips were attached. Muscle fibers were incubated for 10 minutes in cold (5°C) skinning solution to permeabilize the sarcolemma enabling activation of myofilaments with exogenous calcium (note that removal of the osmotic constraint on the fiber imposed by the sarcolemma increases the fiber cross-sectional and that this can lead to a slight underestimation of specific force; however, this 'fiber swelling' should affect fibers from both case and control subjects). Subsequently, the muscle fibers were mounted horizontally on two stainless-steel hooks in a relax solution filled chamber (200µL) with a glass coverslip-bottom on the stage of an inverted microscope (Zeiss, The Netherlands). One of the hooks was attached to a force transducer (model 403A Aurora Scientific Inc, Ontaria, Canada) with a resonance frequency of 10 kHz, whereas the other end was attached to a servo-motor (model 315C, Aurora Scientific Inc.; Aurora, Ontario, Canada) with a step time of 250 µs. Muscle fiber dimensions were measured by means of a camera device coupled to the objective. Muscle fiber length was determined with 100x magnification, depth and width were measured at the widest part of the cell with 400x magnification (an elliptical cross-section of the muscle fiber was assumed). Injury was examined microscopically; in case of severe damage, loss of striation and other irregularities the muscle fibers were excluded. Muscle fibers were stretched to optimal length by setting sarcomere length (SL) at 2.5µm with dedicated Aurora software. To ensure stable attachment of the muscle fibers in the clips throughout the mechanical protocol, the muscle fiber was briefly maximally activated prior to the experiment, and when necessary restreched to SL 2.5µm. Note that this brief activation was done before the determination of muscle fiber dimensions. Single muscle fibers were transferred from relax to pre-, sub-, and maximal activating solutions by means of an automated bath controller device. The

calcium sensitivity of force was determined as the pCa needed for half maximum force development (pCa_{50}). During the experiment, data were automatically collected by a data acquisition board (sampling rate 10,000 Hz). All measurements were performed at 20°C. From each subject a minimum of three fibers per fiber type were analyzed.

Maximal force and cross-bridge cycling kinetics

Diaphragm muscle fibers were maximally activated (pCa 4.5), and once steady-state force was reached, cross-bridge cycling kinetics (by means of rate of tension redevelopment) were studied, as described previously [89](19). Briefly, to determine the rate of tension redevelopment, the slack/restretch approach [96] was used to disengage force generating cross-bridges from the thin filaments (for details see also [85]). A slack equivalent to 30% of the muscle fiber length was rapidly induced at one end of the muscle fiber using the motor. This was followed by an unloaded shortening of the muscle fiber for 30 msec. The remaining bound cross-bridges were mechanically detached by rapidly (1 msec) restretching the muscle fiber to its original length, after which tension redevelops. The rate constant of the monoexponential tension redevelopment (ktr) was determined by fitting the rise of tension to the following equation: $F=Fss(1-e^{-ktr.t})$, where F is force at time *t, Fss* is steady state force.

Myosin heavy chain isoform composition and content determination

At the end of the contractile experiments, myosin heavy chain (MyHC) isoform composition and content per half-sarcomere in the single muscle fibers were identified by SDS-PAGE as described previously with minor modifications [9798]. Briefly, single muscle fibers were detached from the force transducer and servo-motor and placed in 25 µl of SDS sample buffer containing 62.5 mM Tris·HCL, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, and 0.001% (wt/vol) bromophenol blue at a pH of 6.8 and stored at -20°C until assayed. The stacking gel contained 4% acrylamide pH 6.8 and the separating gel 7% at pH 8.8 with 30% glycerol (v/v). Prior to gel electrophoresis, the samples were denatured by boiling for 2 min. Loaded sample volumes were 10 µl per lane.

We used human reference samples of diaphragm bundles in a 1:200 dilution of SDS sample buffer (~9.0 ng/µl) to assess migration patterns and verify isoforms of the myosin heavy chain. Note that the human diaphragm contains fibers expressing MyHC 1, 2A and 2X. However, we do not distinguish between fibers expressing MyHC IIa and IIx as typically MyHC 2X is co-expressed with MyHC 2A isoforms, with the latter isoform being dominant. Thus, fibers expressing MyHC 1 isoforms are referred to as slow-twitch fibers, whereas fibers that are referred to as fast-twitch fibers express MyHC 2A isoforms, with some of these fibers (<20%) co-expressing low levels of MyHC 2X isoforms. The Bradford method [99] was used to verify the standard concentrations of MyHC run on the gels: a standard curve of known concentrations of purified rabbit MyHC (M-3889, Sigma) was run on every gel. The gels were silver stained with alkaline phosphatase (Vectastain ABC kit, Vector Labs) according to the procedure described by

Oakley et al. [100](17) and subsequently imaged by a high-resolution scanner (600 dpi; Microtek Scan Maker 5). 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 MyHC concentration was linear across a range from 0.01 to 0.25μg/μl. The MyHC concentration in the loaded 10μl SDS buffer was determined from the standard curve. Muscle fiber MyHC concentration was computed as the quotient of total MyHC content of the muscle fiber divided by muscle fiber volume (fiber cross-sectional area × length of muscle fiber). MyHC content per half sarcomere, reflecting the number of cross-bridges in parallel in the single muscle fibers independent of cross-sectional area, was calculated as the quotient of muscle fiber MyHC concentration divided by half-sarcomeric volume at a sarcomere length of 2.5μm [90].

Statistical analysis

Outcome of continuous measurements that were normally distributed are presented as the mean ± standard error of mean (SEM) and were compared using group t-tests. Outcome of continuous measurements that were not normally distributed are presented as the median and the interquartile range (25th and 75th percentiles), and were compared using Mann-Whitney tests. Fisher's exact test (2-tail) was used to compare case and control groups with respect to categorical data. A minimum of three fibers per fiber-type, per parameter, per patient were used for statistical analyses. Data in figures are presented as the mean ± SEM, unless otherwise stated. Differences between groups were attributed to chance unless they were significant at the 0.05 level.