

Diaphragm Atrophy and Weakness in the Absence of Mitochondrial Dysfunction in the Critically Ill

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Abstract

Rationale: The clinical significance of diaphragm weakness in critically ill patients is evident: it prolongs ventilator dependency and increases morbidity, duration of hospital stay, and health care costs. The mechanisms underlying diaphragm weakness are unknown, but might include mitochondrial dysfunction and oxidative stress.

Objectives: We hypothesized that weakness of diaphragm muscle fibers in critically ill patients is accompanied by impaired mitochondrial function and structure, and by increased markers of oxidative stress.

Methods: To test these hypotheses, we studied contractile force, mitochondrial function, and mitochondrial structure in diaphragm muscle fibers. Fibers were isolated from diaphragm biopsies of 36 mechanically ventilated critically ill patients and compared with those isolated from biopsies of 27 patients with suspected early-stage lung malignancy (control subjects).

Measurements and Main Results: Diaphragm muscle fibers from critically ill patients displayed significant atrophy and contractile weakness, but lacked impaired mitochondrial respiration and increased levels of oxidative stress markers. Mitochondrial energy status and morphology were not altered, despite a lower content of fusion proteins.

Conclusions: Critically ill patients have manifest diaphragm muscle fiber atrophy and weakness in the absence of mitochondrial dysfunction and oxidative stress. Thus, mitochondrial dysfunction and oxidative stress do not play a causative role in the development of atrophy and contractile weakness of the diaphragm in critically ill patients.

Keywords: mechanical ventilation; critically ill; diaphragm weakness; mitochondrial dysfunction; oxidative stress

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At a Glance Commentary

Scientific Knowledge on the

Subject: The clinical significance of diaphragm weakness in critically ill patients is evident: it prolongs ventilator dependency and increases morbidity, duration of hospital stay, and healthcare costs. Diaphragm weakness in critically ill patients is caused largely by atrophy and contractile weakness of individual diaphragm muscle fibers. The mechanisms underlying diaphragm muscle fiber atrophy and contractile weakness are unknown, but they might include mitochondrial dysfunction.

What This Study Adds to the

Field: We tested the hypothesis that weakness of diaphragm muscle fibers in critically ill patients is accompanied by impaired mitochondrial function and structure and by increased markers of oxidative stress. Our findings show that diaphragm muscle fibers from critically ill patients display significant atrophy and contractile weakness, but lack both dysfunctional mitochondrial respiration and increased levels of oxidative stress markers. Thus, mitochondrial dysfunction and oxidative stress do not play a causative role in the development of atrophy and contractile weakness of the diaphragm in critically ill patients.

Critically ill patients develop weakness of the diaphragm, the main inspiratory muscle, as indicated by a reduced pressure-generating capacity, and a decreased diaphragm thickness and thickening fraction (1–6). Weakness of the diaphragm contributes to prolonged weaning from mechanical ventilation, and increases morbidity and mortality in critically ill patients (7–9).

Diaphragm weakness in mechanically ventilated critically ill patients is largely caused by atrophy and contractile weakness of individual diaphragm muscle fibers (10, 11). Fiber atrophy is accompanied by activation of the ubiquitin–proteasome pathway, indicating a role for proteolytic pathways in diaphragm weakening in critically ill patients (11). The mechanisms that trigger proteolysis in diaphragm

muscle fibers of critically ill patients are unknown.

Studies on animal models and on brain-dead organ donors suggest that these mechanisms include oxidative stress, induced by mitochondrial alterations during mechanical ventilation (12–18). Mitochondria adapt to energetic stress by changing their structure and function either through fusion with adjacent mitochondria or through division, also referred to as *fission*. This process of fusion and fission is referred to as *mitochondrial dynamics* (19). Fusion allows sharing of mitochondrial DNA (20) and matrix contents (21) for optimal quality and function, whereas impaired fusion leads to fragmentation of the mitochondrial network, mitochondrial dysfunction, and the production of reactive oxygen species (22, 23). Studies suggest that influx of energetic substrates, such as lipid and glucose, exceeds the demand of diaphragm muscle fibers during a sudden drop in substrate utilization rate during mechanical unloading of the diaphragm in brain-dead organ donors and mechanically ventilated rodents (14, 24). This state of metabolic oversupply might promote mitochondrial fission (25) and mitochondrial dysfunction (23), and increase oxidative stress (16).

However, the clinical features of critically ill patients greatly differ from those of controlled ventilated animals and brain-dead organ donors. Therefore, it is currently unknown, yet important to establish, whether mitochondrial dysfunction and oxidative stress develop in the diaphragm of critically ill patients. In the case that mitochondria play a key role in development of diaphragm weakness in these patients, modulation of mitochondrial function might prove an important strategy to attenuate the development of respiratory muscle weakness.

The present study aimed to establish that contractile weakness of the diaphragm in critically ill patients is associated with mitochondrial dysfunction. We hypothesized that weakness of diaphragm muscle fibers of these patients was accompanied by impaired mitochondrial respiration and structure, and increased markers of oxidative stress. To test these hypotheses, we studied diaphragm biopsies of 36 critically ill patients and compared the findings with those obtained from biopsies of 27 patients with suspected early-stage lung malignancy who served as control

subjects. Some of the results of this study have been previously reported in the form of an abstract (26).

Methods

Additional details are in the online supplement.

Patients

Diaphragm muscle biopsies were obtained from mechanically ventilated critically ill patients undergoing abdominal or thoracic surgery for clinical reasons (critically ill group). As a control group, diaphragm biopsies were obtained from patients undergoing lung surgery for suspected early-stage lung malignancy. Exclusion criteria are listed in the online supplement. The Medical Ethics Committee of the Vrije Universiteit (VU) University Medical Center approved the protocol. Patients were recruited in the VU University Medical Center, Netherlands Cancer Institute–Antoni van Leeuwenhoek Hospital (both in Amsterdam, the Netherlands), and Medisch Spectrum Twente (Enschede, the Netherlands), and written informed consent was obtained from each patient and/or legal representative.

Biopsy Handling

Diaphragm biopsies were handled as described in the online supplement. Because of the limited biopsy size, not all experiments could be performed on all biopsies; see Tables E1.1 and E1.2 in the online supplement.

Contractile Force Measurements

Contractility of single diaphragm muscle fibers was measured as described previously (11). Some of the contractility data have been published previously (11); see Tables E1.1 and E1.2.

Mitochondrial Function

Mitochondrial respiration and succinate dehydrogenase activity were determined as described previously (27, 28).

Electron Microscopy

Micrographs were assessed for mitochondrial abnormalities, lipid droplets, and glycogen deposits and quantified for mitochondrial size, number, and density.

Protein and mRNA Analyses

The content of mitochondrial complexes I–V, markers for mitochondrial biogenesis

and dynamics, and oxidative stress were measured by Western blotting, and described previously (29–34). Nuclear factor erythroid 2–related factor 2 (Nrf2) was measured by real-time PCR, as described previously (30).

Bioluminescence

ATP concentration was measured by bioluminescence.

Statistical Analysis

Normally distributed data are represented as the mean \pm SEM, and not normally

distributed data are shown as median and interquartile range. Data were analyzed with GraphPad Prism version 6.07 (GraphPad Software, San Diego, CA). Differences between groups were considered significant if $P < 0.05$.

Results

Patient Characteristics

The demographic and clinical characteristics of control patients ($n = 27$) and critically ill patients ($n = 36$) are

described in Tables 1, 2, and E2. Control and critically ill patients were mechanically ventilated for 1.5 (1.0–2) hours and 76.5 (34–247) hours, respectively ($P < 0.0001$). Specifications of mechanical ventilation duration and arterial blood gas analysis are shown in Tables E3 and E4.

Atrophy and Contractile Weakness of Diaphragm Muscle Fibers

To investigate whether diaphragm muscle fibers from critically ill patients show atrophy and contractile weakness, we measured the cross-sectional area and

Table 1. Characteristics of Control Patients

Control Patient (No.)	Age (yr)	Sex (M/F)	BMI (kg/m ²)	Relevant Medical History	TNM Classification	MV (h)
1	52	M	25	T2DM, HT, COPD GOLD II, ex-smoker	pT3N0M0	1.5
2	22	M	23	Smoker	pT1bN0M0	2
3	66	M	24	HT, prostate cancer, COPD GOLD I, ex-smoker	pT2aN1M0	2
4	58	F	28	None	pT1aN0M0	2
5	60	M	24	Lobectomy for T1N0M0 lung tumor, COPD GOLD I, ex-smoker	pT1aN1M0	1.5
6	56	M	21	COPD GOLD II, ex-smoker	pT4N2M0	2
7	70	M	30	HT, COPD GOLD I, ex-smoker	pT1bN1M0	1.5
8	60	F	26	HT	pT2aN0M0	0.75
9	59	F	28	Chronic bronchitis, smoker	Benign, cyst	0.75
10	64	F	26	COPD GOLD II, smoker	pT1bN0M0	1.25
11	59	F	21	Breast carcinoma, cervix carcinoma, chronic bronchitis, COPD GOLD II, smoker	Adenocarcinoma CT2BN2M1A	0.75
12	52	M	23	Smoker, alcohol abuse	pT2N0M0	1
13	64	M	31	HT, MI, T2DM, smoker	Benign, bronchiectasis and inflammation	1
14	73	F	21	Bronchiectasis, allergic bronchopulmonary aspergillosis, COPD GOLD I	Benign, inflammation with central necrosis	2.5
15	74	M	23	Colon cancer, alcohol abuse, COPD GOLD I, ex-smoker	Chondrosarcoma	1
16	75	M	26	T2N2C tongue base carcinoma, COPD GOLD I	Benign, inflammation	0.5
17	55	F	27	Allergic asthma, recurrent pneumonia, smoker	Carcinoid T1bN0	1
18	35	M	26	Smoker	Benign, hamartoma	0.5
19	40	M	24	None	Carcinoid pT1aN0R0	1.5
20	72	M	28	AF, non-small cell lung cancer cT3TN0M0, COPD GOLD I, ex-smoker	pT2N0M0	1.5
21	68	F	29	HT, T2DM, ex-smoker	pT2N2M0	0.75
22	67	M	27	HT, T2DM, renal cell carcinoma, prostate cancer	Metastasis of clear cell renal cell carcinoma	1.25
23	50	M	24	COPD GOLD I, smoker	Mucinous adeno-carcinoma T1aN0M0	1.25
24	58	F	23	Chronic bronchitis, COPD GOLD I, ex-smoker	Adenocarcinoma pT0N0M0	2.5
25	61	M	33	Testis seminoma, COPD GOLD II, ex-smoker	pT1aN0M0	2
26	69	M	33	HT, ex-smoker	Adenocarcinoma pT3N1M0	1.5
27	58	M	28	HT, ex-smoker	pT3N1	2.4

Definition of abbreviations: AF = atrial fibrillation; BMI = body mass index; COPD = chronic obstructive pulmonary disease (GOLD classification); GOLD = Global Initiative for Chronic Obstructive Lung Disease; HT = hypertension; MI = myocardial infarction; MV = mechanical ventilation; T2DM = type 2 diabetes mellitus; TNM = classification of tumors based on tumor size (T), lymph nodes involved (N), and metastases (M).

Table 2. Characteristics of Critically Ill Patients

Critically Ill Patient (No.)	Age (yr)	Sex (M/F)	BMI (kg/m ²)	Relevant Medical History	Reason for Admission to ICU	Surgery in Which Biopsy Was Obtained	MV (h)	Septic	Died in ICU	APACHE II Score
1	48	F	18	COPD GOLD I	Respiratory failure after VATS lobectomy	Rethoracotomy: lobectomy necrotic middle lobe	31	Y	N	22
2	67	M	28	Concentric LV hypertrophy with decreased LVEF	Hemorrhagic shock due to retroperitoneal hematoma	Third relaparotomy: closure abdomen	574	N	N	38
3	67	F	19	CVA	Abdominal sepsis due to intestinal perforation	Relaparotomy: drainage abscess	28	Y	Y	18
4	53	M	30	None	Hemorrhagic shock after thoracic endovascular aortic repair	Rethoracotomy: evacuating hemothorax	315	N	N	29
5	47	F	22	None	Severe trauma	Relaparotomy: gauze removal	40	N	N	35
6	67	F	23	HT, CVA, hypothyroidism, smoker	Abdominal sepsis due to intestinal necrosis by mesenteric ischemia	Relaparotomy: drainage abscess	337	Y	Y	28
7	25	F	33	None	Severe trauma	Second relaparotomy: closure abdomen	219	Y	N	45
8	66	M	29	HT	Abdominal sepsis due to perforated appendicitis	Relaparotomy: rinsing abdominal cavity	28	Y	N	11
9	69	M	22	None	Esophageal rupture	Thoracotomy: esophageal repair	17	Y	N	27
10	51	M	29	Hypothyroidism	Severe trauma	Fourth relaparotomy: closure abdomen	85	N	N	30
11	46	F	19	Asthma, smoker	IHCA due to hemorrhagic shock of abdominal origin	Third relaparotomy: gauze removal	61	N	Y	37
12	52	M	29	None	Severe trauma	Relaparotomy: closure abdomen	287	Y	N	20
13	81	F	24	HT	Severe trauma	Thoracotomy: reconstruction of two ribs	43	N	Y	19
14	77	F	28	T2DM, AF	Abdominal sepsis due to cecal perforation	Second relaparotomy: rinsing abdominal cavity	32	Y	Y	19
15	72	M	20	T2DM, colon carcinoma, chronic bronchitis, COPD GOLD II	Abdominal sepsis due to duodenal perforation	Relaparotomy: cholecystectomy	20	Y	Y	19
16	79	F	23	Breast carcinoma, L4-L5 paraplegia	Abdominal sepsis due to gastro-intestinal perforations	Third relaparotomy: colostomy	48	Y	Y	22
17	74	M	25	HT, AF	Respiratory failure due to bilateral pneumonia	Relaparotomy: evacuation empyema	275	Y	Y	19
18	83	M	24	HT, rectum carcinoma	Abdominal sepsis due to intestinal perforation	Relaparotomy: hemicolectomy	49	Y	Y	35
19	68	M	24	Prostate carcinoma, COPD GOLD II, smoker	IHCA after Bentall procedure because of aortic dissection	Thoracotomy: fixation rib fractures	350	N	N	NA
20	68	F	27	HT, T2DM, CVA, smoker	Respiratory failure due trachea deviation by struma	CABG and hemistrumectomy	90	N	Y	18
21	69	F	34	HT, T2DM, chronic kidney disease, smoker	Heart failure after aortic valve replacement	Relaparotomy: drainage ascites	255	N	Y	16
22	22	F	20	None	Severe trauma	Second relaparotomy: closure abdomen	203	N	N	31

(Continued)

Table 2. (Continued)

Critically Ill Patient (No.)	Age (yr)	Sex (M/F)	BMI (kg/m ²)	Relevant Medical History	Reason for Admission to ICU	Surgery in Which Biopsy Was Obtained	MV (h)	Septic	Died in ICU	APACHE II Score
23	72	M	26	HT, chronic kidney disease, secondary hyperparathyroidism	OHCA with traumatic CPR	Second relaparotomy: removal gauze	61	N	N	42
24	55	F	21	Lower leg amputation, right kidney infarction, mild infarction	Abdominal sepsis due to perforations by intestinal ischemia	Relaparotomy: closure abdomen	69	Y	N	19
25	78	M	18	HT, PAD, stenosis AMS, ex-smoker	Postoperative care after endovascular treatment because of mesenteric ischemia	Third relaparotomy: closure abdomen	122	Y	N	27
26	75	M	22	HT, chronic coronary artery disease, chronic kidney disease, smoker	Postoperative care after thoracoabdominal aortic aneurysm repair	Fourth relaparotomy: Hartmann procedure	290	Y	Y	22
27	67	F	29	HT, hypothyroidism, chronic sensory idiopathic axonal polyneuropathy	Postoperative care after thoracoabdominal aortic aneurysm repair	Relaparotomy: stent replacement	20	Y	Y	30
28	54	M	26	None	Postoperative care after ruptured abdominal aortic aneurysm repair	Fourth relaparotomy: sigmoid resection with placement colostomy	50	N	N	40
29	21	M	33	None	Severe trauma	Relaparotomy: cecal and transcolon suture	222	Y	N	26
30	66	F	21	None	Severe trauma with intestinal damage	Fourth relaparotomy: rinsing abdominal cavity	134	Y	N	24
31	55	M	28	HT, nephrectomy	Intestinal ischemia after nephrectomy	Relaparotomy: resection part of duodenum and jejunum	21	Y	N	19
32	61	M	29	HT, T2DM	Postoperative care after ruptured abdominal aortic aneurysm repair	Third relaparotomy: closure abdomen	298	Y	N	27
33	69	M	29	HT, hypercholesterolemia, PAD, ex-smoker	IHCA after myocardial infarction	Laparotomy: resection necrotic ileum	225	N	N	30
34	71	M	28	HT, aortic valve sclerosis	Abdominal sepsis after hemicolectomy	fifth relaparotomy: resection sigmoid and part of small intestine, replacement colostomy	84	Y	N	31
35	60	F	20	HT, CVA, COPD GOLD II, smoker	Postoperative care after endovascular treatment because of mesenteric ischemia	Third relaparotomy: resection terminal ileum	42	N	N	17
36	34	M	28	None	Severe trauma	Relaparotomy: closure abdomen	18	N	N	16

Definition of abbreviations: AF = atrial fibrillation; AMS = arteria mesenterica superior; APACHE = Acute Physiology and Chronic Health Evaluation; BMI = body mass index; CABG = coronary artery bypass grafting; COPD = chronic obstructive pulmonary disease; CPR = cardiopulmonary resuscitation; CVA = cerebrovascular accident; GOLD = Global Initiative for Obstructive Lung Disease; HT = hypertension; ICU = intensive care unit; IHCA = in-hospital cardiac arrest; LV = left ventricle; LVEF = left ventricular ejection fraction; MV = mechanical ventilation; N = no; NA = not available; OHCA = out-of-hospital cardiac arrest; PAD = peripheral artery disease; T2DM = type 2 diabetes mellitus; VATS = video-assisted thoracoscopic surgery; Y = yes.

maximal absolute force of individual diaphragm muscle fibers. Compared with control patients, the cross-sectional area in critically ill patients was significantly smaller in both the slow-twitch fibers (3,490 [2,763–4,595] vs. 2,461 [1,608–3,037] μm^2 ; $P < 0.0001$) (Figure 1A) and fast-twitch fibers (3,275 [2,473–4,064] vs. 2,235 [1,570–3,550] μm^2 ; $P = 0.008$) (Figure 1D). Moreover, maximal absolute force was lower in both slow-twitch fibers (0.40 [0.35–0.55] vs. 0.21 [0.14–0.30] mN; $P < 0.0001$) (Figure 1B) and fast-twitch fibers (0.56 [0.33–0.68] vs. 0.26 [0.17–0.56] mN; $P = 0.01$) (Figure 1E) of critically ill patients versus control patients. Next, we examined whether this reduction in force was proportional to the reduction in cross-sectional area by normalizing maximal force to cross-sectional area. This examination revealed that in critically ill patients normalized maximal force was significantly reduced in slow-twitch fibers (122 ± 5 vs. 96 ± 5 mN/mm 2 ; $P = 0.001$) (Figure 1C) but

not in fast-twitch fibers (145 ± 9 vs. 137 ± 8 mN/mm 2 ; $P = 0.52$) (Figure 1F). Taken together, these results indicate that critically ill patients develop significant atrophy resulting in contractile weakness of the diaphragm, exacerbated by a reduction in the intrinsic force-generating capacity of slow-twitch fibers.

Preserved Mitochondrial Respiration in Diaphragm Muscle Fibers

To examine whether atrophy and contractile weakness of diaphragm fibers of critically ill patients were associated with mitochondrial dysfunction, we measured mitochondrial respiration in the diaphragm biopsies. Figure 2A shows a typical example of the respirometry recordings. Leak respiration, reflecting proton leak, was not different in critically ill than in control patients (6.7 [5.5–11.7] vs. 6.1 [2.6–7.2] pmol s $^{-1}$ mg $^{-1}$; $P = 0.31$) (Figure 2B). Also, maximal complex I-stimulated respiration (19.4 [13.4–30.1] vs. 27.6 [9.6–31.9] pmol s $^{-1}$ mg $^{-1}$;

$P = 0.78$) (Figure 2C), maximal uncoupled respiration (48.9 [25.4–58.3] vs. 52.7 [30.6–66.2] pmol s $^{-1}$ mg $^{-1}$; $P = 0.56$) (Figure 2D), and uncoupled complex II-stimulated respiration (22.6 [16.6–37.9] vs. 32.4 [16.0–38.9] pmol s $^{-1}$ mg $^{-1}$; $P = 0.58$) (Figure 2E) did not differ between control and critically ill patients, respectively. *Post-hoc* analysis revealed that mitochondrial respiration was also not different in nonseptic versus septic critically ill patients, patients with a low versus high Acute Physiology and Chronic Health Evaluation (APACHE) II score, and survivors versus nonsurvivors (see Figure E1).

To assess mitochondrial complex II function at the single-cell level, we measured succinate dehydrogenase (SDH) activity in cryosections of the diaphragm biopsies. Figure 3A shows representative examples of cryosections stained for SDH activity. Compared with control patients, the SDH activity in both slow-twitch fibers ($1.4 [1.1–1.7] \times 10^{-5}$ vs. $1.3 [1.1–1.8] \times 10^{-5}$

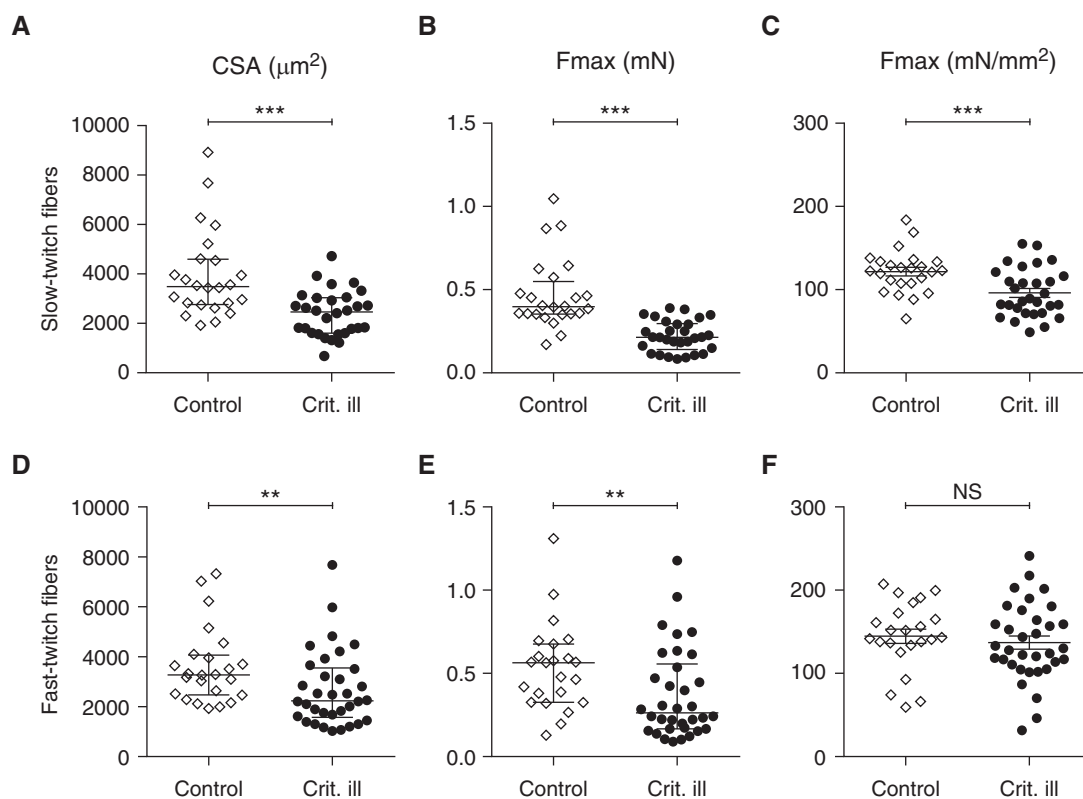


Figure 1. Atrophy and contractile weakness of diaphragm muscle fibers in critically ill patients. (A and D) The cross-sectional area of diaphragm muscle fibers was significantly smaller for both slow- and fast-twitch fibers in critically ill patients than in control patients, indicating atrophy. (B and E) Maximal absolute force was lower in both slow- and fast-twitch fibers from critically ill patients, indicating contractile weakness. (C and F) Maximal normalized force, that is, maximal absolute force normalized to fiber cross-sectional area, was lower in slow-twitch but not in fast-twitch diaphragm muscle fibers from critically ill patients. Each data point represents the mean of all slow-twitch or fast-twitch muscle fibers measured from a diaphragm biopsy of one patient. Data in A, B, D, and E are presented as median and interquartile range, and data in C and F are presented as mean \pm SEM. Crit. ill = critically ill; CSA = cross-sectional area; Fmax = maximal force; NS = not significant. ** $P \leq 0.01$, *** $P \leq 0.001$.

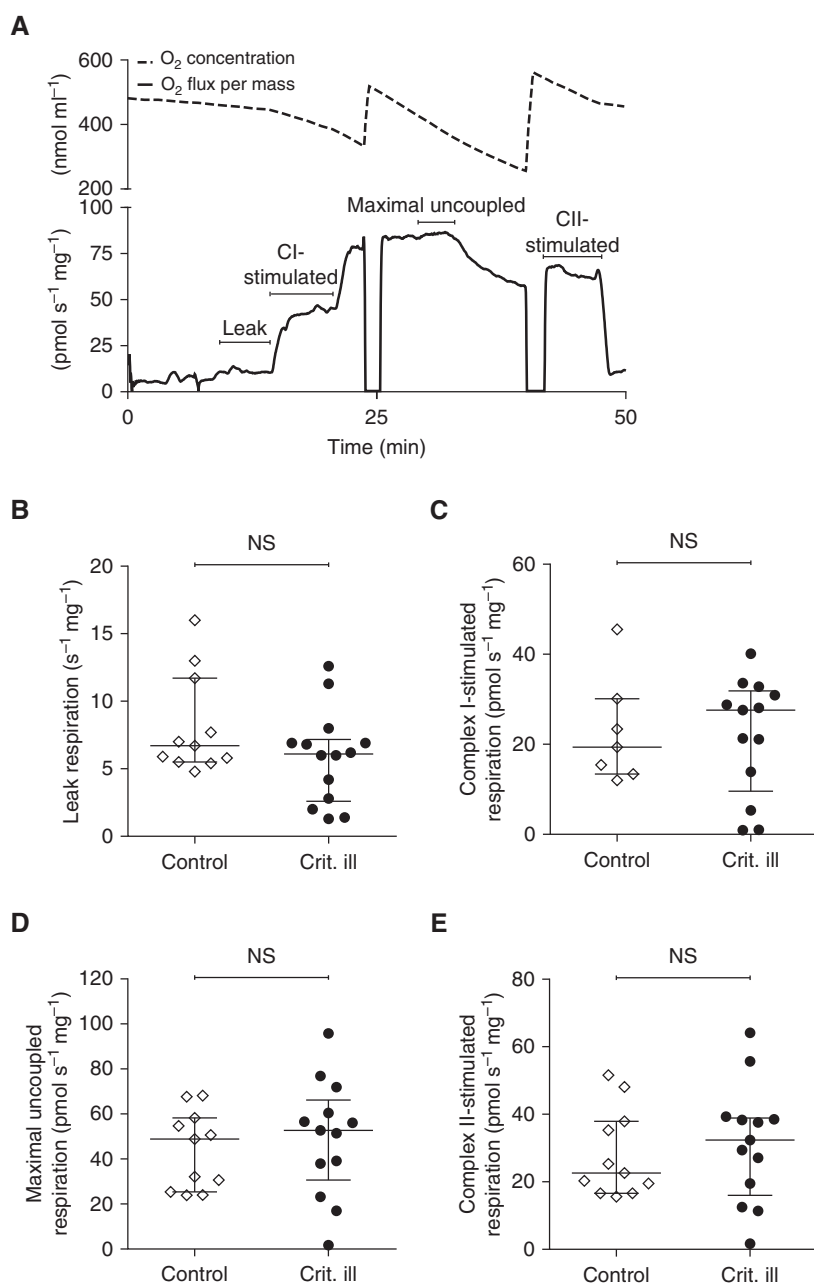


Figure 2. Maintained mitochondrial respiration in diaphragm muscle fibers. (A) Example of experimental recording of mitochondrial respiration in diaphragm muscle fibers. (Top) Oxygen concentration, which was maintained above 300 μM to avoid limitations in oxygen supply. (Bottom) Oxygen consumption per milligram wet weight muscle mass. Leak respiration is a measure of oxygen flux compensating for proton leakage from the intermembranous space through the inner membrane to the matrix. Maximal complex I-stimulated respiration is measured after addition of glutamate, malate and pyruvate, and ADP. Maximal uncoupled respiration is measured after adding succinate and FCCP [carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone] to dissipate the mitochondrial membrane potential. Uncoupled complex II-stimulated respiration is the oxygen consumption of complex II by blocking complex I with rotenone. No differences were observed in (B) leak respiration, (C) complex I-stimulated respiration, (D) maximal uncoupled respiration, and (E) uncoupled complex II-stimulated respiration between critically ill patients and control patients. Each data point represents the mean of two mitochondrial respiration measurements in a diaphragm biopsy of one patient. Data in B–E are presented as median and interquartile range. CI = complex I; CII = complex II; Crit. ill = critically ill; NS = not significant.

$A_{660} \mu\text{m}^{-1} \text{s}^{-1}$; $P = 0.96$) (Figure 3B) and fast-twitch fibers ($1.2 [1.0\text{--}1.5] \times 10^{-5}$ vs. $1.2 [0.9\text{--}1.4] \times 10^{-5} A_{660} \mu\text{m}^{-1} \text{s}^{-1}$; $P = 0.64$) (Figure 3C) of critically ill patients was not different. These findings are in line with the respirometry results, and suggest that mitochondrial capacity and function are preserved in diaphragm fibers from critically ill patients.

To verify that the preserved mitochondrial respiration was not a reflection of a fiber type switch toward slow-twitch muscle fibers (which typically have a higher mitochondrial density) in critically ill patients, we measured the fiber area fraction and fiber type fraction in diaphragm cryosections. Compared with control patients, the fiber area fraction (fast/slow-twitch, 48/52 vs. 44/56%; $P = 0.24$) (Figure 3D) and fiber type fraction (fast/slow-twitch, 49/51 vs. 47/53%; $P = 0.59$) (Figure 2E) in critically ill patients were not different.

Furthermore, the protein contents of mitochondrial complex I–V in the diaphragm biopsies were determined by Western blotting (see Figure 4A and Figure E3 for typical examples). The protein content of the complexes was not significantly different in critically ill patients compared to control subjects ($P = 0.56$; Figure 4B), a finding that is in line with the maintained mitochondrial respiration in diaphragm fibers of critically ill patients.

No Major Alterations in Mitochondrial Structure in Diaphragm Muscle Fibers

We obtained electron micrographs from six control and seven critically ill patients. Mild abnormalities of mitochondrial shape and internal complexity were observed in three of six control subjects versus six of seven critically ill patients ($P = 0.27$). Misalignment of mitochondria was observed in zero of six control subjects versus three of seven of the critically ill patients ($P = 0.19$; Figures 5A and 5B). Furthermore, compared with control patients, mitochondria of critically ill patients did not significantly differ with respect to size ($0.10 [0.09\text{--}0.013]$ vs. $0.15 [10\text{--}18] \mu\text{m}^2$; $P = 0.097$) (Figure 5C), number ($0.53 [0.37\text{--}0.79]$ vs. $0.50 [0.40\text{--}0.54]$ mitochondria/ μm^2 ; $P = 0.52$) (Figure 5D), and volume density ($5.24 [3.25\text{--}7.44]$ vs. $6.29 [4.22\text{--}7.33]\%$ mitochondrial area/total area; $P = 0.36$)

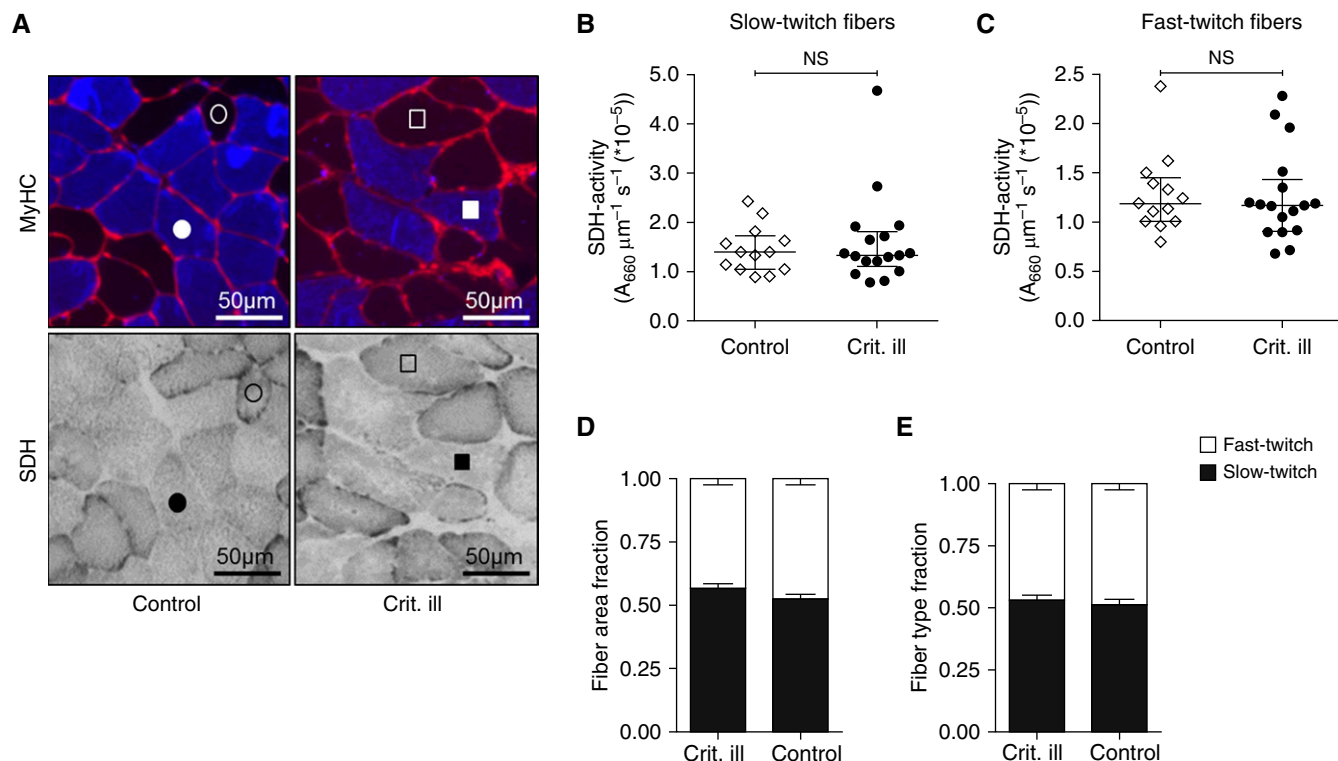


Figure 3. Maintained succinate dehydrogenase (SDH) activity in diaphragm muscle fibers. (A) Typical examples of diaphragm cryosections stained for fast-myosin heavy chain (MyHC, top) and for SDH activity (bottom). Blue, fast-MyHC; red, wheat germ agglutinin, indicating cell membranes. The circles represent corresponding slow-twitch (open) and fast-twitch (solid) fibers in control patients, and the squares represent corresponding slow-twitch (open) and fast-twitch (solid) fibers in critically ill patients. (B and C) SDH activity of diaphragm muscle fibers was comparable in both slow- and fast-twitch fibers in critically ill patients compared with control patients. Each data point represents the mean SDH activity of all slow-twitch or fast-twitch fibers measured in cryosections of a diaphragm biopsy of one patient. Data are presented as median and interquartile range. (D and E) The area of the cryosections covered by slow- and fast-twitch fibers (fiber area fraction) and the distribution of slow- and fast-twitch fibers (fiber type fraction) were preserved, suggesting that maintained respiration in critically ill patients was not caused by a fiber type shift toward slow-twitch muscle fibers. Data are presented as mean \pm SEM. Crit. ill = critically ill; NS = not significant.

(Figure 5E). Thus, no significant alterations in mitochondrial structure were observed in diaphragm fibers from critically ill patients.

Mitochondrial structure is regulated by mitochondrial dynamics, which in turn are under the control of peroxisome proliferator-activated receptor γ

coactivator 1 α (PGC-1 α), the master regulator of mitochondrial biogenesis. Overexpression of PGC-1 α stimulates up-regulation of pro-fusion proteins mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic atrophy 1 (OPA1) (35). We measured the contents of PGC1- α , the pro-fusion proteins Mfn1 (0.45 [0.44–0.50] vs. 0.40 [0.37–0.44]; $P = 0.026$) (Figure 6C), Mfn2 (0.075 [0.070–0.082] vs. 0.067 [0.063–0.068]; $P = 0.007$) (Figure 6D), and OPA1 (0.084 [0.076–0.087] vs. 0.073 [0.070–0.076]; $P = 0.015$) (Figure 6E) were also significantly lower in critically ill

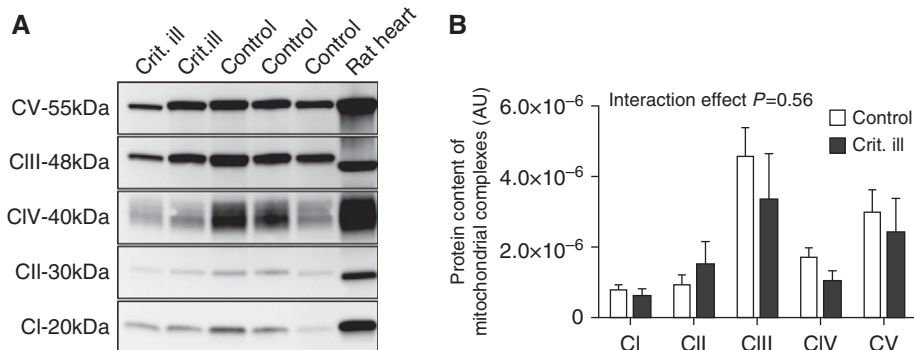


Figure 4. Preserved content of mitochondrial complexes in diaphragm muscle fibers. (A) Typical example of a Western blot with MitoProfile antibody cocktail of the five complexes. Lanes 1 and 2, diaphragm homogenates of critically ill patients; lane 3, rat heart homogenate (loading control); lanes 4 and 5, diaphragm homogenates of control patients. (B) Quantification of protein content of mitochondrial complexes. Protein content was not lower in diaphragm muscle fibers of critically ill patients (analysis of variance, interaction effect $P = 0.56$). Data are presented as mean \pm SEM. AU = arbitrary units; CI = complex I; CII = complex II; CIII = complex III; CIV = complex IV; Crit. ill = critically ill; CV = complex V.

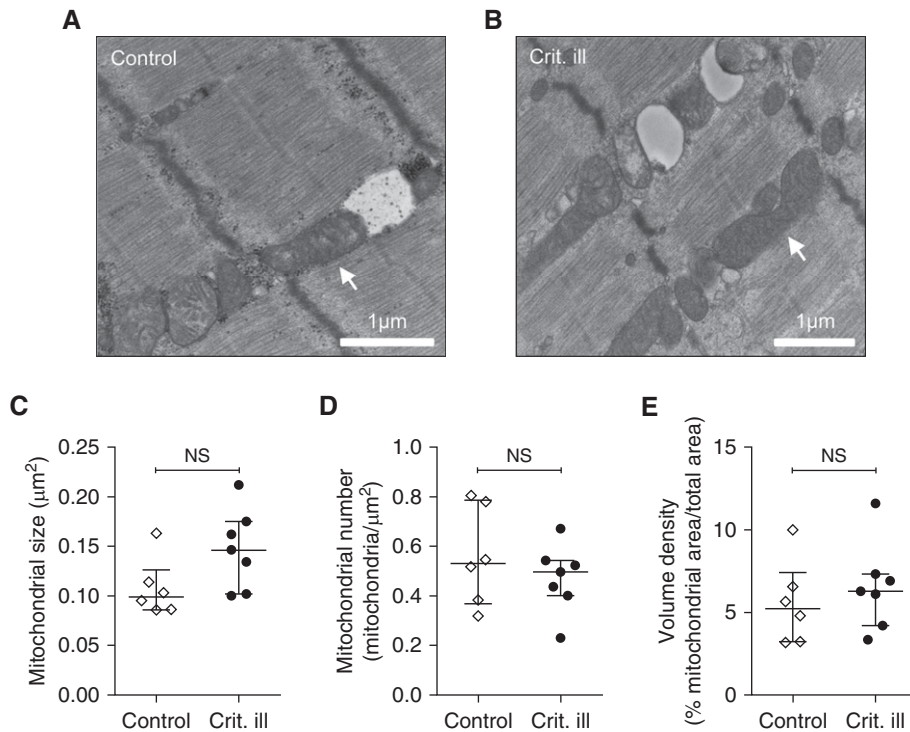


Figure 5. No major alterations of mitochondrial structure in diaphragm muscle fibers. (A) Electron micrograph of diaphragm biopsy from a control patient. Mitochondria are well aligned and have a normal general appearance. (B) Electron micrograph of a diaphragm biopsy from a critically ill patient. In A and B, the arrow indicates an example of a mitochondrion with a normal appearance. (C–E) Quantification of mitochondria in the micrographs revealed no differences in mitochondrial size, number, and density. Each data point represents the mean mitochondrial size/number/volume density as measured or counted in micrographs of a diaphragm biopsy of one patient. Data are presented as median and interquartile range. Crit. ill = critically ill; NS = not significant.

patients than in control patients. However, the content of fission protein DRP1 was comparable in critically ill and control patients (0.47 [0.43–0.54] vs. 0.53 [0.52–0.67]; $P = 0.13$) (Figure 6F). These results indicate that mitochondria in diaphragm fibers have impaired fusion dynamics; however, this was not accompanied by major changes in mitochondrial function and structure (based on electron micrographs; Figure 5).

Absence of Metabolic Oversupply in Diaphragm Muscle Fibers

In mechanically ventilated brain-dead organ donors and rodents, a relative state of energetic oversupply has been observed in the diaphragm, which inhibits the mitochondrial electron flow and thereby reduces ATP synthesis rates (14). The preserved mitochondrial respiration in the diaphragm of critically ill patients suggests that ATP synthesis is unaffected, and we verified this by measuring the ATP concentration by bioluminescence. Compared with

control patients, the ATP concentration in critically ill patients was not different (0.42 [0.18–0.85] vs. 0.25 [0.10–0.71] AU; $P = 0.45$) (Figure 7A). These findings suggest that metabolic oversupply does not occur in diaphragm fibers from critically ill patients.

To further investigate this, we determined AMP-activated protein kinase (AMPK) activity, which increases if the AMP-to-ATP ratio increases (36, 37). AMPK is activated by phosphorylation. Therefore, we measured the ratio of phosphorylated AMPK relative to total AMPK (pAMPK/AMPK) in the diaphragm biopsies (see Figure 7B and Figure E5 for typical examples of the Western blots). Compared with control patients, the pAMPK-to-AMPK ratio was significantly higher in critically ill patients (1.04 [0.92–1.09] vs. 1.65 [1.29–2.01]; $P = 0.002$) (Figure 7C). Thus, in diaphragm fibers from critically ill patients the ratio of pAMPK to AMPK is elevated without changes in ATP concentration.

Decreased Oxidative Stress Levels in Diaphragm Muscle Fibers

We hypothesized that oxidative stress levels are increased in the diaphragm of critically ill patients. However, no evidence supporting metabolic oversupply or mitochondrial dysfunction in diaphragm muscle fibers from critically ill patients was observed in the present study, suggesting that oxidative stress levels are comparable between control and critically ill patients. To verify this, we determined nitrosative stress marker nitrotyrosine, oxidative stress marker 4-hydroxy-2-nonenal (HNE), and the protein oxidation index in the diaphragm biopsies (see Figures 8A, 8E, and 8I, and Figures E6 and E7A for typical examples of Western blots and OxyBlots). Outcomes of nitrotyrosine and HNE assays were strongly correlated ($\rho = 0.824$; Figure E2A). Against our expectations, nitrotyrosine content (1.3 [0.7–1.8] vs. 0.9 [0.4–1.1]; $P = 0.037$) (Figure 8B) and HNE content (12.5 [6.5–17.2] vs. 4.5 [2.4–8.7]; $P < 0.001$) (Figure 8F) were lower in critically ill patients than in control patients, whereas the oxidation index was comparable (0.7 [0.5–0.8] vs. 0.6 [0.6–0.7]; $P = 0.18$) (Figure 8J). Because decrease in diaphragm thickness, as measured by ultrasound, is most pronounced during the first 72 hours of mechanical ventilation (38), we anticipated that oxidative stress would be increased during this period. Therefore, we compared nitrotyrosine, HNE, and oxidation indices of patients mechanically ventilated less than 72 hours with those of patients mechanically ventilated for more than 72 hours, but no differences were found (nitrotyrosine: 0.9 [0.5–1.2] vs. 0.8 [0.2–1.2] AU; $P = 0.34$; HNE: 4.7 [3.6–9.2] vs. 3.1 [1.9–9.0] AU; $P = 0.37$; oxidation index: 0.57 [0.55–0.67] vs. 0.59 [0.55–0.73]; $P = 0.80$) (Figures 8C, 8G, and 8K). Also, no correlation between nitrotyrosine content, HNE content, oxidation index, and the duration of mechanical ventilation was observed (Figures 8D, 8H, and 8L), further indicating that oxidative stress is not an early phenomenon in the diaphragm of mechanically ventilated critically ill patients. Finally, oxidative stress levels were not increased in critically ill patients ventilated with a controlled mode compared with those ventilated with an assist mode, and there was no difference between nonseptic and septic critically ill patients, patients with a low versus high disease severity (APACHE II score), or

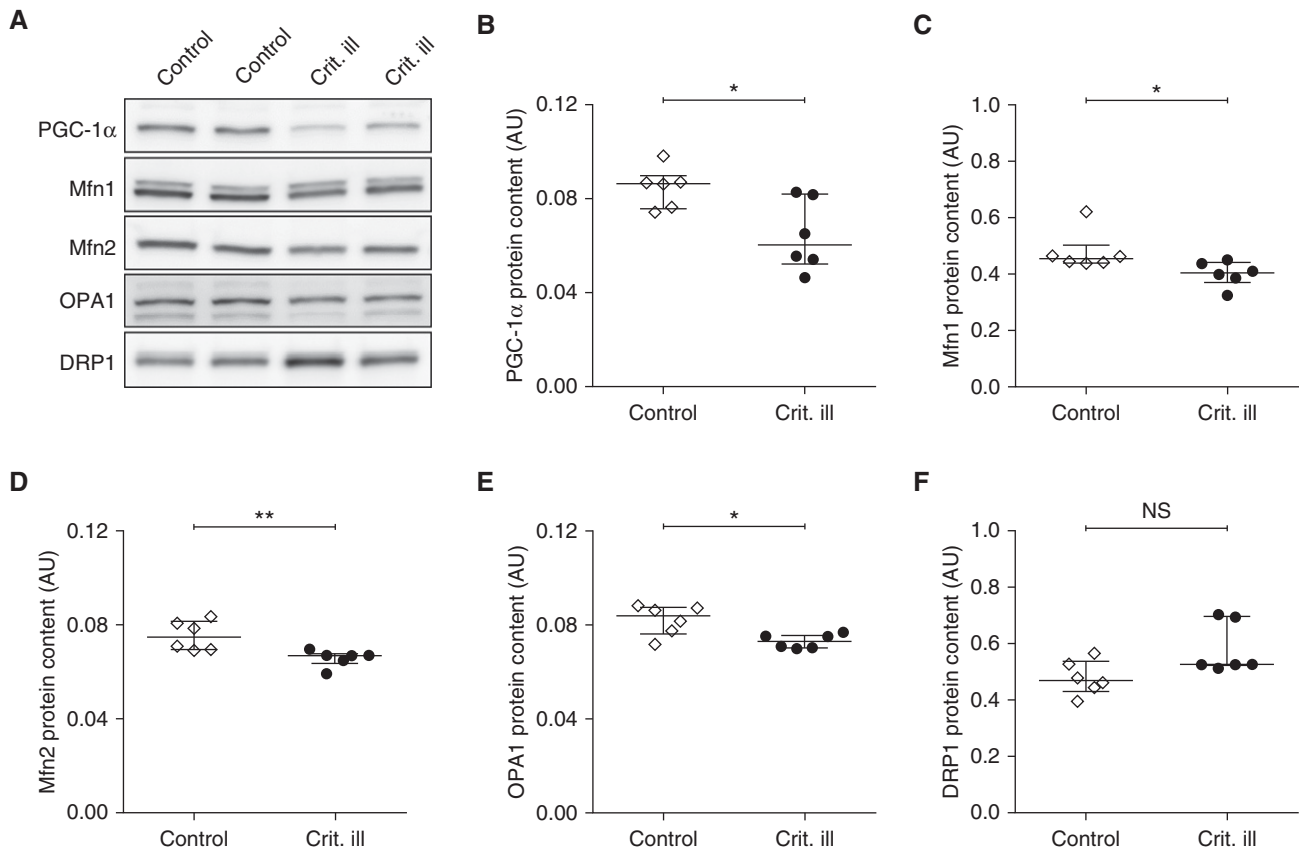


Figure 6. Down-regulation of master regulator of biogenesis PGC-1 α in diaphragm muscle fibers from critically ill patients. (A) Typical example of Western blot with antibodies for master regulator of biogenesis PGC-1 α ; fission proteins Mfn1, Mfn2, and OPA1; and fusion protein DRP1 from control and critically ill patients. (B) Compared with control subjects, PGC-1 α protein content was lower in critically ill patients. (C–E) In line with lower PGC-1 α , pro-fusion proteins Mfn1, Mfn2, and OPA1 were also lower in critically ill patients. This suggests that mitochondrial fusion dynamics are impaired in diaphragm fibers of critically ill patients, but without major changes in mitochondrial structure and function (see Figure 5). (F) Finally, content of pro-fusion protein DRP1 was comparable in critically ill patients and control patients. Each *data point* represents the mean protein content per group of diaphragm biopsies of three to four patients. Data are presented as median and interquartile range. AU = arbitrary units; Crit. ill = critically ill; DRP1 = dynamin-related protein 1; Mfn1 = mitofusin 1; Mfn2 = mitofusin 2; NS = not significant; OPA1 = optic atrophy 1; PGC-1 α = peroxisome proliferator-activated receptor γ coactivator 1 α . * $P \leq 0.05$, ** $P \leq 0.01$.

survivors versus nonsurvivors (see Figures E2B and E2C).

Finally, we determined the levels of superoxidase dismutase-1 (SOD1) and catalase, two antioxidant enzymes (see Figure 9B and Figure E7B for typical examples of the Western blots), as well as the expression level of nuclear erythroid 2-related factor 2 (Nrf2), a redox balance sensor and master regulator of antioxidant pathways. Nrf2 expression was increased in critically ill patients compared with control patients (1.0 [0.9–1.1] vs. 1.7 [1.3–2.8]; $P < 0.001$) (Figure 9A), whereas SOD1 and catalase protein contents were comparable in both groups (SOD1: 2.3 ± 0.1 vs. 2.5 ± 1.1 ; $P = 0.47$; catalase: 0.16 [0.07–0.38] vs. 0.23 [0.11–0.87]; $P = 0.09$) (Figures 9C and 9D). These data suggest that in diaphragm fibers of critically ill patients a

redox imbalance might be developing, triggering Nrf2 expression.

Discussion

The aim of this study was to establish that contractile weakness of the diaphragm in mechanically ventilated critically ill patients is associated with mitochondrial dysfunction. Our findings reveal that diaphragm muscle fibers from these patients display significant atrophy and contractile weakness in the absence of major mitochondrial alterations and oxidative stress. Thus, mitochondrial alterations and oxidative stress do not play a causative role in the development of atrophy and contractile weakness of diaphragm muscle fibers in critically ill patients.

Preserved Mitochondrial Function in Diaphragm Muscle Fibers of Critically Ill Patients

Contractile weakness of diaphragm fibers in critically ill patients can be largely explained by fiber atrophy and activation of proteolytic pathways (10, 11). The mechanisms underlying the activation of proteolysis are unknown. Studies on brain-dead organ donors and mechanically ventilated animals have suggested a major role for mitochondrial dysfunction: diaphragm unloading during mechanical ventilation causes a state of energetic oversupply (14, 24), promoting fission (25), leading to mitochondrial dysfunction (22, 25) and oxidative stress (16), and hence activation of proteolytic pathways (13–15, 17, 18).

Surprisingly, in the present study in mechanically ventilated critically ill patients

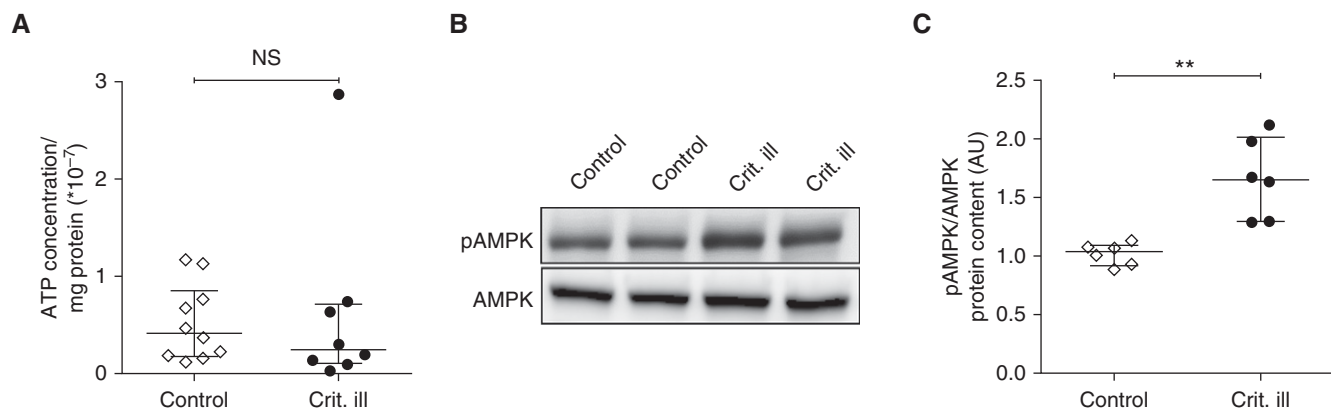


Figure 7. Absence of metabolic oversupply in diaphragm muscle fibers. (A) Compared with control patients, the ATP concentration was not changed in diaphragm muscle fibers of critically ill patients, suggesting the absence of metabolic oversupply in critically ill patients. Each *data point* represents the mean ATP concentration of a diaphragm biopsy of one patient. (B) Typical example of Western blot with antibodies for metabolic stress markers pAMPK and AMPK. (C) The ratio of pAMPK to AMPK was higher in diaphragm muscle fibers of critically ill patients than in those of control patients. Each *data point* represents the mean protein content per group of diaphragm biopsies from three to four patients. Data are presented as median and interquartile range. AMPK = AMP-activated protein kinase; AU = arbitrary units; Crit. ill = critically ill; NS = not significant; pAMPK = phosphorylated AMPK. $**P \leq 0.01$.

we observed atrophy and contractile weakness of diaphragm muscle fibers, in the absence of mitochondrial dysfunction, oxidative stress, and metabolic oversupply. Impaired fusion dynamics were present in critically ill patients (Figure 6), similar to findings in ventilated animals (14, 24). Typically, impaired mitochondrial fusion leads to impaired mitochondrial function (22), as occurs during a state of metabolic oversupply. However, the normal ATP concentration does not support such a state in the diaphragm of critically ill patients (Figure 7), and electron microscopy images do not show major morphological changes in mitochondria. In line with these findings, mitochondrial respiration was not impaired in critically ill patients, and also not in those who were septic (Figure E1). The latter findings corroborate data from Fredriksson and colleagues, which indicated normal ATP levels, and mitochondrial respiration and morphology, in the intercostal muscles of septic intensive care patients (39). Note that we cannot rule out that, eventually, the imbalance in mitochondrial dynamics impairs mitochondrial respiration and increases levels of oxidative stress in the diaphragm of critically ill patients. However, as the patients already displayed manifest diaphragm fiber atrophy and weakness at the time of biopsy (Figure 1), changes in mitochondrial dynamics cannot be the cause of diaphragm weakness.

Furthermore, the present findings do not support a concept in which

mitochondrial dysfunction and oxidative stress peak during the first days of ICU admission to initiate proteolytic pathways, after which they rapidly normalize: markers of oxidative stress did not correlate with duration of mechanical ventilation (Figures 8D, 8H, and 8L). Patients who received less than 72 hours of mechanical ventilation before biopsy (a duration that has been associated with the most prominent diaphragm thinning on ultrasound [38]) had similar levels of oxidative stress markers as patients who were ventilated for longer durations. It should be noted that biopsies were obtained after at least 17 hours of mechanical ventilation, and thus we cannot rule out that oxidative stress levels were elevated during the first 17 hours.

In fact, increased Nrf2 expression and a trend toward increased catalase levels in the diaphragm of critically ill patients (Figures 9A and 9D) may reflect a (developing) redox imbalance. This is not unlikely, given the infiltration of neutrophils and macrophages into the diaphragm of critically ill patients (11). Clearly, the absence of increased oxidative stress markers indicates an adequate antioxidant response in the diaphragm, a response that is known to be well developed in respiratory muscles (39, 40). Indeed, catalase and levels of oxidative stress markers showed a significant negative correlation (HNE: $r = -0.558$, $P = 0.01$; nitrotyrosine: $r = -0.456$, $P = 0.04$; data not shown)

Catalase decomposes hydrogen peroxide, and its induction in diaphragm fibers of critically ill patients is apparently

sufficient to prevent the development of oxidative stress. A redox imbalance due to hydrogen peroxide generation, independent of changes in ATP levels, can explain the activation of AMPK (Figure 7C) (41). AMPK activation induces FOXO (Forkhead box, class O) via the AMPK-FOXO pathway (42), which activates the ubiquitin-proteasome pathway. The latter pathway was shown to be activated in diaphragm fibers of critically ill patients from a similar cohort as studied in the present study (11). Thus, we propose that a redox imbalance triggers atrophy of diaphragm muscle fibers in critically ill patients, in the absence of mitochondrial dysfunction and oxidative stress.

The discrepancy between our findings and those from mechanically ventilated animal models and brain-dead organ donors can be explained by differences in their clinical features. For instance, critically ill patients can have sepsis, and do not have the excessive catecholamine release and hormonal disturbances that characterize brain-dead organ donors (43, 44). Another clinical feature that differs between critically ill patients and brain-dead organ donors relates to arterial blood gases. Hypercapnia can attenuate diaphragm contractile dysfunction (45), whereas hyperoxia contributes to oxidative stress formation (46) and impairs contractile function in skeletal muscle (47). The Pa_{CO_2} levels in our study (42 ± 5.6 mm Hg) were significantly higher than the levels in two other studies on brain-dead organ donors

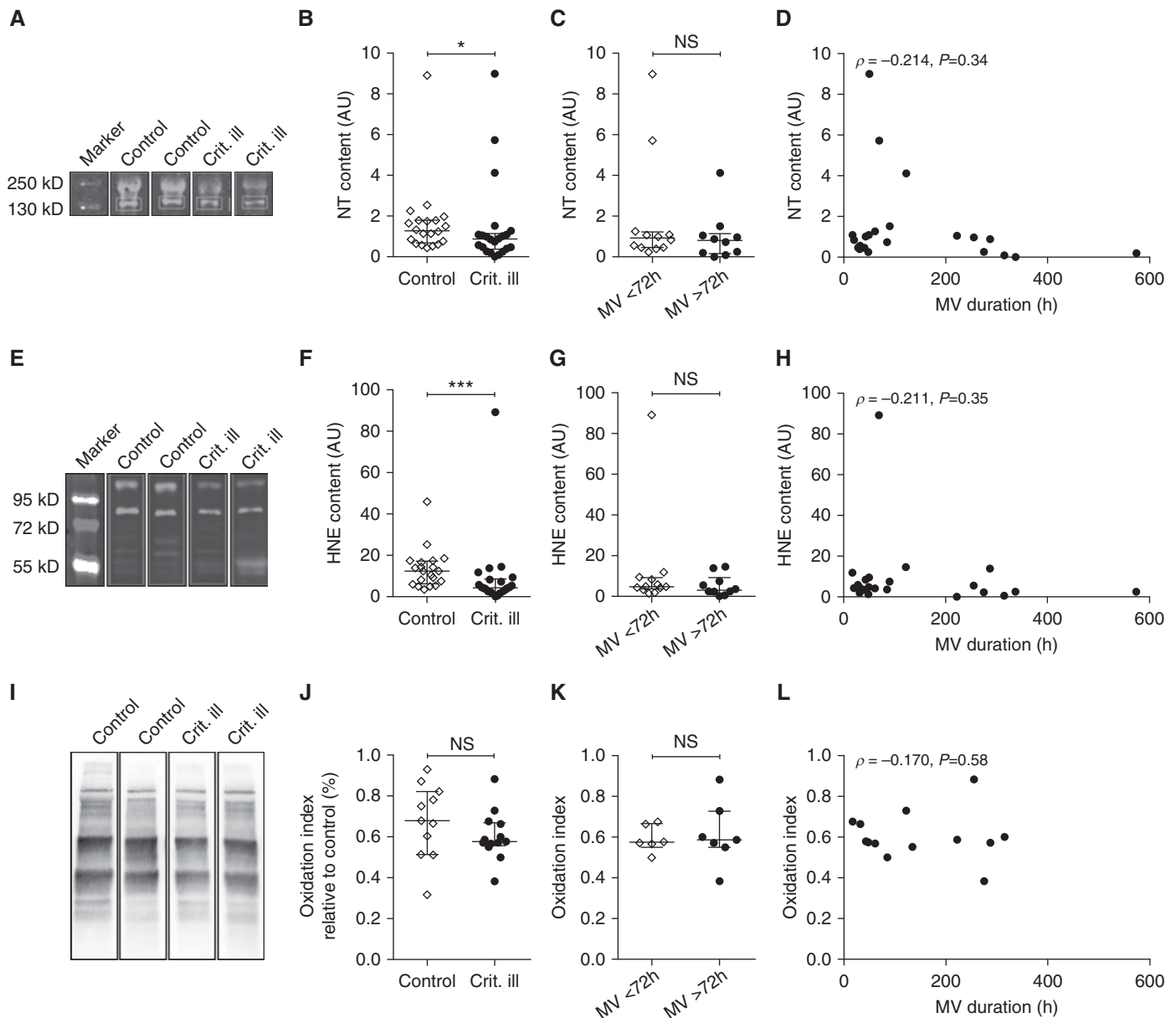


Figure 8. Lower oxidative stress levels in diaphragm muscle fibers from critically ill patients. (A) Typical example of a Western blot stained for nitrosative stress marker nitrotyrosine (NT)-specific antibodies, indicating a footprint of nitrosative stress. (E) Typical example of a Western blot with 4-hydroxy-2-nonenal (HNE)-specific antibodies, a measure for lipid peroxidation. (I) Typical example of an OxyBlot, showing carbonylated proteins. (B, F, and J) NT and HNE contents were lower in diaphragm muscle fibers of critically ill patients than in those of control subjects, whereas the oxidation index was comparable. (C, G, and K) NT content, HNE content, and the oxidation index were not higher in critically ill patients mechanically ventilated for less than 72 hours than in critically ill patients mechanically ventilated longer than 72 hours. (D, H, and L) No correlation between the duration of ventilation and NT content, HNE content, and oxidation index was observed, suggesting that oxidative stress was not an early phenomenon in critically ill patients. Each *data point* represents the NT content, HNE content, or oxidation index in a diaphragm biopsy of one patient. Data in B, C, F, G, J, and K are presented as median and interquartile range. AU = arbitrary units; Crit. ill = critically ill; MV = mechanical ventilation; NS = not significant. * $P \leq 0.05$, *** $P \leq 0.001$.

(Levine and colleagues [12]: 34 ± 6 mm Hg [$P = 0.0004$], and Hussain and colleagues [13]: 37 ± 1.8 mm Hg [$P = 0.04$]). In addition, those brain-dead organ donors had significantly higher PaO_2 levels (Levine and colleagues [12]: 147 ± 88 mm Hg [$P = 0.02$]; Hussain and colleagues [13]:

260 ± 53 mm Hg [$P < 0.0001$]; Picard and colleagues [14]: 273 ± 132 mm Hg [$P < 0.0001$]) than did the critically ill patients in the present study (average pressure and pressure at biopsy, respectively; 100 ± 14 and 96 ± 26 mm Hg). Thus, it is conceivable that lower

PaCO_2 and extremely high PaO_2 might have contributed to the elevated levels of oxidative stress markers in the diaphragm of brain-dead organ donors.

Furthermore, brain-dead organ donors and controlled mechanically ventilated animals completely lack diaphragm activity

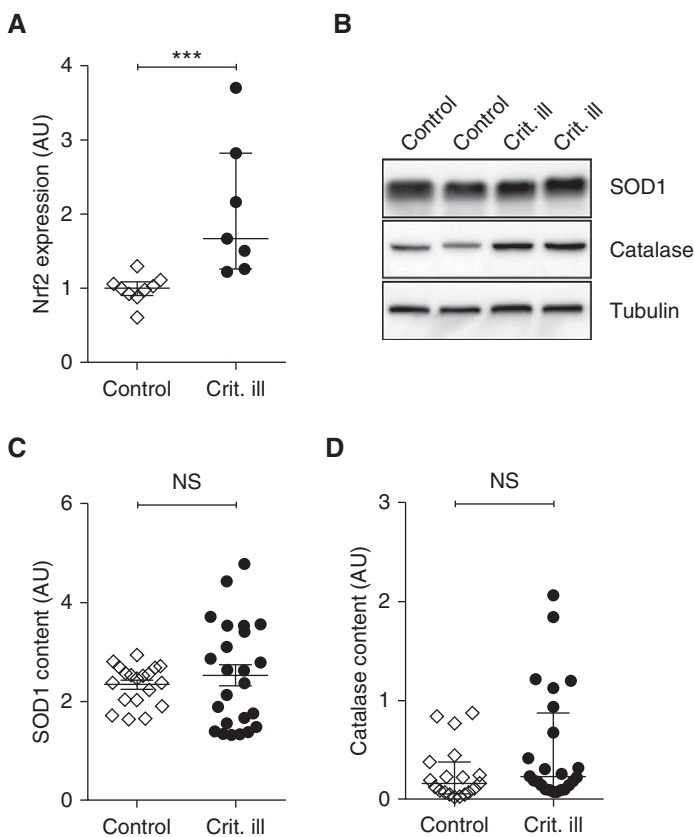


Figure 9. Antioxidant response in diaphragm muscle fibers from critically ill patients. (A) Expression of Nrf2, a master regulator of antioxidant pathways, was increased in critically ill patients, indicating activation of antioxidant pathways. (B) Typical example of a Western blot stained for antioxidant enzymes SOD1 and catalase, and loading control tubulin. (C and D) Antioxidant enzymes SOD1 and catalase were comparable in critically ill patients and control patients. Each *data point* represents the Nrf2 expression or SOD1 or catalase content in a diaphragm biopsy of one patient. Data in A and D are presented as median and interquartile range, and data in C are presented as mean \pm SEM. AU = arbitrary units; Crit. ill = critically ill; Nrf2 = nuclear erythroid 2-related factor 2; NS = not significant; SOD1 = superoxide dismutase-1. $***P \leq 0.001$.

(48, 49), whereas in critically ill patients the ventilation mode—and thus diaphragm activity—depends on respiratory drive, level of sedation, the use of neuromuscular blocking agents, and clinician preference. Studies have shown that even low levels of diaphragm activity are sufficient to improve mitochondrial function (50, 51) and decrease oxidative stress levels (50, 51). In these studies, the diaphragm of patients undergoing cardiothoracic surgery was activated by applying a magnetic stimulus to one phrenic nerve. The stimulus was applied every 30 minutes for 1 minute, which is a low frequency compared with the normal breathing frequency. Thus, even low levels of diaphragm activity in critically ill patients receiving controlled ventilation might be sufficient to protect against

mitochondrial dysfunction and oxidative stress (see also Figures E2B and E2C).

Study Limitations

First, we did not determine whether all critically ill patients had *in vivo* diaphragm weakness, although it seems likely that the majority had, given the markedly reduced force-generating capacity of slow-twitch diaphragm fibers (fibers that are predominantly recruited during inspiration). Second, the patients in this study represent a subgroup of critically ill patients: they required abdominal or thoracic surgery, and had an average APACHE II score of 26 ± 1.4 , which is at the high end of the ICU population. Surprisingly, even in this subgroup of severely ill patients, mitochondrial function in the diaphragm was preserved and

oxidative stress levels were not increased. Thus, it is unlikely that in patients with less severe illness mitochondrial dysfunction develops and triggers diaphragm weakening. Third, our control group consisted of patients with lung cancer undergoing surgery for tumor removal. Lung cancer, a history of smoking, and surgery have been associated with systemic oxidative stress, and we cannot rule out that these systemic factors have negatively affected mitochondrial function and oxidative stress levels in diaphragm muscle fibers of our control patients (52–54). However, the control patients in the present study underwent surgery at an early stage of lung cancer, and they were not cachectic (Table 1). Furthermore, several control patients were diagnosed with COPD (Global Initiative for Chronic Obstructive Lung Disease [GOLD]) stage I or II, which has been associated with impaired diaphragm fiber contractility (55). However, in the present study we observed no difference in diaphragm fiber cross-sectional area and contractility between control patients with or without COPD (data not shown), indicating that COPD did not confound our results. Note that studies on brain-dead organ donors (12–14) with comparable control groups did show significant differences in mitochondrial function and oxidative stress in the diaphragm. This suggests that absence of mitochondrial dysfunction and oxidative stress in the present study was not due to our selection of control patients. Finally, we acknowledge that, because of size limitations of these human biopsies, not all experiments could be performed on all patients (for an overview of the number of biopsies studied per parameter, see Table E1).

Clinical Implications

Diaphragm weakness is an important factor in the development of weaning failure in critically ill patients. The results of the present study suggest that treatment aimed at targeting mitochondrial function may not be beneficial for critically ill patients with clinical features comparable to those of the patients studied here.

Conclusions

Diaphragm weakness in critically ill patients is not caused by mitochondrial dysfunction and oxidative stress. Future exploration of alternative pathways is necessary to understand the development of

diaphragm weakness and to combat weaning failure. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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