CHAPTER 4 DIAPHRAGM FIBER STRENGTH IS REDUCED IN CRITICALLY ILL PATIENTS AND RESTORED BY A TROPONIN ACTIVATOR

4. DIAPHRAGM FIBER STRENGTH IS REDUCED IN CRITICALLY ILL PATIENTS AND RESTORED BY A TROPONIN ACTIVATOR

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Abstract

Rationale: Diaphragm weakness acquired in the intensive care unit plays an important role in difficult weaning from mechanical ventilation. However, whether contractile weakness of diaphragm muscle fibers develops in mechanically ventilated critically ill patients is unknown. Objective: To determine (1) the contractile strength of diaphragm muscle fibers from mechanically ventilated critically ill patients, and (2) test the ability of the fast skeletal troponin activator, CK-2066260, to improve contractile strength.

Methods: Diaphragm biopsy specimens were obtained from mechanically ventilated critically ill patients (n=10) undergoing laparotomy or thoracotomy for various reasons. Patients undergoing elective lung surgery served as controls (n=10). We performed immunohistochemical analyses on diaphragm cryosections and contractility experiments on isolated single diaphragm muscle fibers.

Measurements and Main Results: Diaphragm fiber cross sectional area was smaller and force generating capacity of single diaphragm muscle fibers was markedly lower in mechanically ventilated critically ill patients when compared to controls, and remained lower after normalization to their cross sectional area. CK-2066260 improved the force response to calcium such that the force produced by diaphragm fibers from critically ill patients at physiological calcium concentrations was restored to control levels.

Conclusions: Diaphragm muscle fibers of mechanically ventilated critically ill patients (1) are severely weakened due to atrophy and to dysfunction of the remaining contractile proteins, and (2) regain normal strength when exposed to the fast troponin activator CK-2066260. Fast troponin activators may offer an appealing and novel therapeutic approach to improve diaphragm muscle strength in critically ill patients and to facilitate weaning from mechanical ventilation.

Introduction

Diaphragm weakness in the intensive care unit (ICU) plays an important role in difficult weaning from mechanical ventilation. Diaphragm strength in mechanically ventilated (MV) critically ill patients has been assessed indirectly using phrenic nerve stimulation, which demonstrated that the pressure generating capacity of the diaphragm was reduced in these patients [62,63,123]. However, this technique cannot distinguish between impaired phrenic nerve function, abnormal neuromuscular transmission, or intrinsic abnormalities in the diaphragm muscle itself. Consequently, it is unknown whether intrinsic contractile weakness of diaphragm muscle fibers occurs in MV critically ill patients. If so, targeted treatment strategies that enhance contractility may improve the success of weaning. Such treatment strategies may include the administration of a novel class of small molecule drugs named fast skeletal troponin activators, which improve the contractile strength of skeletal muscle fibers [81]. In this study, we obtained diaphragm biopsy specimens from critically ill patients (n=10; mechanically ventilated for 28-603 hours) undergoing laparotomy or thoracotomy, and compared them with control patients undergoing elective lung surgery (n=10; mechanically ventilated 1-2 hours, Table 1). The size and the contractile performance of isolated diaphragm muscle fibers were determined. In addition, we tested the ability of the fast skeletal troponin activator, CK-2066260, to improve contractile strength.

Results

Patients

The demographic and clinical characteristics of critically ill and control patients are shown in Table 1 and 2. Control and critically ill patients were mechanically ventilated for, on average, 1.6±0.4 and 210±185 hours, respectively. The control and critically ill group did not significantly differ with respect to age (57±13 vs. 52±13 years, respectively, p=0.47), body mass index (25±3 vs. 24±5 kg/m^2, respectively, p=0.72) or gender (male/female: 4/6 vs. 6/4, p=0.66)

Table 1. Critically ill patient characteristics

Nr	Ag	G	BMI	Relevant Medical History	Reason admission ICU	Reason for Surgery	SOA	APII	Sep	MV Tot	MV PC	MV PSV	FiO_2	PaO_2 FiO_2	PEEP
1	48	F	18	COPD	Respiratory failure after VATS lobectomy	Re-thoracotomy: lobectomy necrotic middle lobe	7	22	N	30	30	0	0.58	162	9
2	67	M	28	Concentric LV hypertrophy with impaired function, biventricular ICD, DMII, hypertension	Hemorrhagic shock due to retroperitoneal hematoma	Final closure of abdominal wound after re-re-laparotomy	12	38	N	60:	200	403	0.57	174	10
3	67	F	22	Rheumatoid Arthritis, CVA	Septic shock due to intestinal perforation. Small bowel resection	Re-laparotomy: 2nd look, drainage abdomen	8	18	Y	28	228	0	0.40	246	5
4	53	M	30	None	Thoracic endovascular aortic repai for type B dissection, hemorrhagic shock	Thoracotomy with surgical re-evacuation hematothorax	1C	29	N	31:	255	58	0.72	135	17
5	47	F	22	Depression	Severe trauma	Re-laparotomy: removal of gauze	12	28	N	38	35	3	0.62	216	13
6	67	F	23	Hypertension, cigarette smoker, thyroid dysfunction	Gastro-enteric ischemia, thrombosi of celiac trunk and AMS/ endovascular treatment	Re-laparotomy for drainage abdominal abscess	7	28	Y	33ł	316	22	0.44	160	6
7	25	F	21	Thalassemia	Severe trauma	Re-Re-laparotomy: removal gauze	16	44	N	22(	139	81	1	136	17
8	51	M	29	None	Severe trauma and traumatic shocl	Re-re-re laparatomy for removal gauze	11	30	N	79	77	2	0.44	220	12
9	46	F	19	Asthma, dysrhythmia, cigarette smoker	Hemorrhagic shock from abdomina origin	Re-re-laparotomy fo removal of gauze	8	37	N	14:	101	41	0.40	147	9
10	52	M	29	None	Severe trauma	Relaparotomy for closing abdomen	3	20	Y	30!	135	170	0.40	268	8

*G=Gender, Age in years, BMI= body-mass index kg/m², AP II= APACHE II=Acute Physiology and Chronic Health Evaluation II score at first 24h at ICU; SOFA=Sequential Organ Failure Assessment score at day of biopsy; DM= Diabetes Mellitus; Sep= sepsis yes/no; MV total = sum of hours on mechanical ventilation specified to hours on PC(pressure control) and PSV (pressure support ventilation); FiO_2= Fraction of Inspired Oxygen, PaO_2 = Partial Arterial Oxygen Pressure mmHg; PEEP= Positive End Expiratory Pressure in cmH_2O. Ventilatory settings are averaged from moment of intubation until biopsy.*based on post-operative staging*

Table 2. Control patient characteristics

Nr	Age	G	BMI	Relevant Medical History	TNM-stage of removed tumor*
I	52	M	25	Cigarette smoker, diabetes mellitus II, hypertension	pT3N0M0
II	22	M	23	Cigarette smoker	pT1bN0M0
III	66	M	26	Cigarette smoker, hypertension, prostate cancer, COPD	pT2aN1M0
IV	58	F	28	None	pT1aN0M0
V	60	M	24	Cigarette smoker, s/p right upper lobectomy for T1 N0 M0 lung cancer, pulmonary tuberculosis s/p successful drug therapy	pT1aN1M0
VI	55	M	21	Cigarette smoker, COPD	pT4N2M0
VII	70	M	20	Cigarette smoker, s/p basal cell cancer skin	pT1bN1M0
VIII	60	F	26	Hypertension	pT2aN0M0
IX	59	F	28	Cigarette smoker	Suspected for tumor, turned out to be lung cyst
X	64	F	26	Cigarette smoker, cholecystectomy	pT1bN0M0

Histology

Diaphragm fiber cross sectional area (CSA) was determined by means of immunohistochemical analyses with myosin heavy chain antibodies performed on cryosections of the biopsy specimens [83,102]. Figure 1a demonstrates atrophy of slow- and fast-twitch diaphragm fibers in critically ill patients. Quantification of in total 4686 fibers revealed that compared to controls, fiber CSA is 29% smaller in slow twitch (controls vs critically ill: 3284±793 μm^2 vs. 2328±763 μm^2, p=0.004) and 34% smaller in fast twitch (controls vs ICU: 2766±606 μm^2 vs. 1819±527 μm^2, p<0.0001) diaphragm muscle fibers of critically ill patients (right panel).

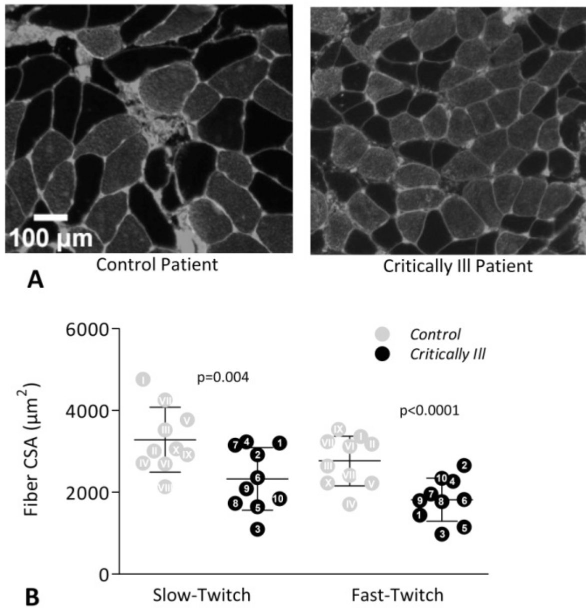

Figure 1. (A) Severe diaphragm muscle fiber atrophy in mechanically ventilated critically ill patients. Typical examples of serial diaphragm cross sections stained with antibodies against slow-twitch myosin heavy chain (light surfaces); Wheat germ agglutinin (WGA) staining (grey thin lines) was used to demarcate the muscle fibers; Bar: 100 µm (left panel). (B) Quantification of fiber cross sectional areas revealed that compared to controls, fiber CSA is 29% smaller in slow twitch (left panel) and 34% smaller in fast twitch diaphragm muscle fibers of critically ill patients (right panel). Each dot with corresponding patient number indicates the mean CSA per patient (● control, ● critically ill). Horizontal bars indicate group mean, error bars indicate ± SD.

We measured the contractile performance of permeabilized single diaphragm fibers isolated from the biopsy specimens. Fibers were mounted between a force transducer and a length motor, and exposed to activating calcium solutions. Compared to controls, the maximal absolute force– determined at maximally activating calcium concentrations (pCa 4.5) – was reduced by 56% in slow-twitch (controls vs ICU 0.44±0.16 mN vs. 0.19±0.07 mN, $p<0.0001$) and by 52% in fast-twitch (controls vs ICU 0.49 ±0.21 mN vs. 0.24 ± 0.09 mN, $p=0.0002$) diaphragm fibers from critically ill patients. In addition, we measured the sensitivity of force to calcium. The pCa_{50} (i.e. the negative logarithm of the calcium

concentration needed to obtain 50% of maximal force) was unaffected in slow-twitch fibers (control 5.64±0.03 vs. critically ill 5.61±0.08, $p=0.30$), whereas in fast-twitch fibers the pCa_{50} was significantly lower in critically ill patients (controls 5.76±0.07 vs. critically ill 5.70±0.06, $p=0.036$).

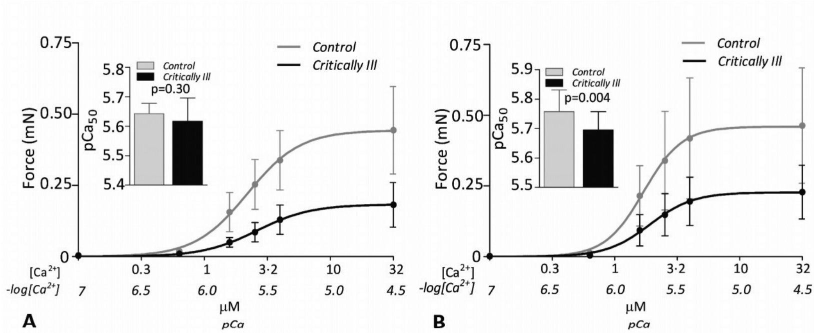

Figure 2. (A) Severe diaphragm muscle fiber weakness in mechanically ventilated critically ill patients. Curves indicate the absolute force-calcium relation of diaphragm fibers of control and MV critically ill patients. Compared to controls, the maximal absolute force was reduced by 56% in slow-twitch and by 52% in fast-twitch diaphragm fibers of critically ill patients (B) Indents show calcium sensitivity (pCa50). In slow-twitch fibers, pCa50 is not affected (left panel), whereas in fast-twitch fibers pCa50 was significantly lower in critically ill patients (right panel). Bullets and column bars indicate group mean, error bars indicate ± SD.

By normalizing absolute force to the cross sectional area of each fiber, maximal specific force was obtained (figure 3). Compared to controls, slow-twitch fibers of critically ill patients had 17% lower specific force, control vs. critically ill respectively 114±21 mN/mm2 vs. 95±26 mN/mm2, p=0.049 and fast-twitch fibers of critically ill patients were 20% lower, respectively 147±28 mN/mm2 vs. 118±38 mN/mm2, p=0.030. These findings suggests that in these critically ill patients there is not only a loss of contractile proteins, but also dysfunction of the remaining ones and that fast-twitch diaphragm fibers also require more calcium to generate force.

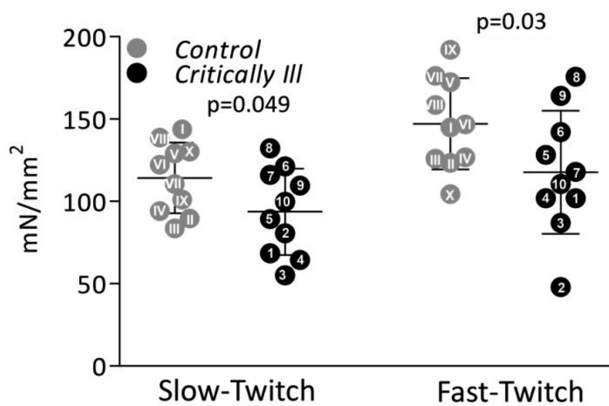

Figure 3. By normalizing absolute force to the cross sectional area of each fiber, maximal specific force was obtained; compared to controls, slow-twitch fibers of critically ill patients had 17% lower

specific force and fast-twitch fibers had 20% lower specific force. Horizontal bars indicate group mean, error bars indicate ± SD, ● *control,* ● *critically ill.*

We exposed diaphragm fibers of a representative subset of control (#I, IV, VI) and critically ill patients (#1, 3, 4, 5) to the fast skeletal troponin activator CK-2066260, which improves the sensitivity of the calcium sensor in the muscle sarcomere. We verified that the subset of patients were representative for the group by means of comparing the force responses to calcium in the diaphragm fibers from the subset of control patients to all control patients and by comparing the subset of critically ill patients to all critically ill patients: the force response to calcium in the subset of control patients was not significantly different from all control patients (p=0.9994). Also the force response to calcium in the subset of critically ill patients was not different in from all critically ill patients (p= 0.9833) (see figure 4a). To determine a concentration-force response curve for the fast skeletal troponin activator CK-2066260, single diaphragm fibers from control patients were exposed to pCa solutions of 5.8 – this pCa typically yielded ~40% of maximal active force – with increasing concentrations (1, 2, 5, 10 and 20 μM) of CK-2066260 dissolved in 1% dimethylsulfoxide (DMSO) as vehicle (see figure 4b). CK-2066260 enhanced submaximal force generation with a maximal effect at 5 μM therefore we used a concentration of 5μM CK-2066260 to determine the potential of CK-2066260 to improve force generation in diaphragm fibers. Note that 1% DMSO did not affect muscle fiber contractility (data not shown). Note that no difference in fiber type proportion (percentage slow-twitch fibers in control patients 49±10 % vs. critically ill patients 48±6 %, p=0.825) or in fiber type area fraction (percentage slow-twitch in control patients 53±12 % vs. critically ill patients 54±10 %, p=0.875) was observed (see figure 5).

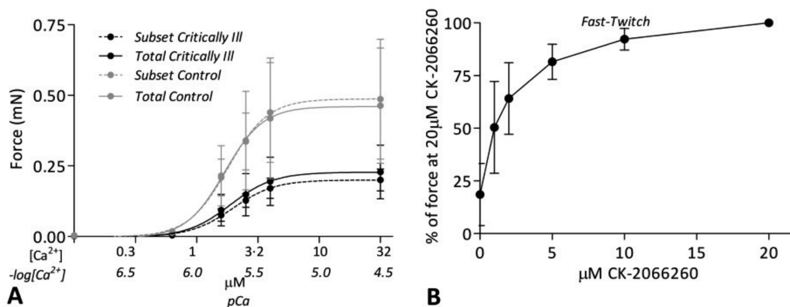

Figure 4 (A) Curves indicate the absolute force-calcium relation of diaphragm fibers of the subset of control and MV critically ill patients (dotted lines) and all control and MV critically ill patients (solid lines). The force response to calcium was significantly different neither in the subset of control patients vs. all controls, nor in the subset of critically ill patients vs. all critically ill patients. (B) Concentration-force response relation of the fast skeletal troponin activator CK-2066260 conducted at pCa 5.8 in seven fast-twitch diaphragm fibers from two critically ill patients. Note that [CK-

2066260] >5μM did not further augment the force response. Bullets indicate group mean, error bars indicate ± SD.

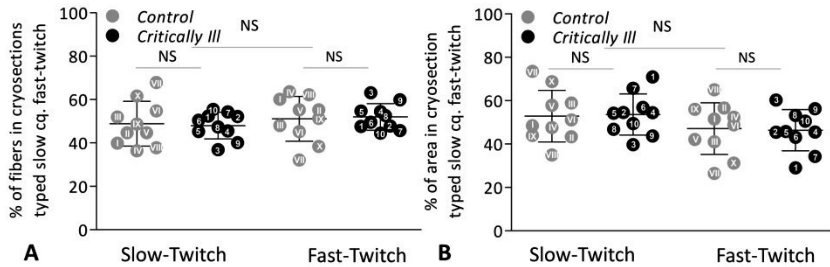

Figure 5. The percentages of fibers in cryosections typed slow-twitch or fast-twitch did not significantly differ between the critically ill and control patients (B) The percentages of total fiber area in cryosections typed slow-twitch or fast-twitch did neither significantly differ between critically ill and control patients, nor within groups. Horizontal bars indicate group mean, error bars indicate ± SD, ● control, ● critically ill.

Compared to vehicle, 5μM of CK-2066260 significantly increased the calcium sensitivity of diaphragm fibers both in controls (pCa_{50}: 5.75±0.04 vs. 6.18±0.1, respectively, $p<0.001$) and in critically ill patients (5.70±0.07 vs. 6.00±0.13, respectively, $p<0.01$) (see figure 6). Importantly, at physiological calcium concentrations, CK-2066260 restored the contractile force of fast-twitch diaphragm fibers of critically ill patients back to levels observed in untreated fibers from controls (force at pCa 5.8: untreated controls 0.22±0.05 vs. treated critically ill 0.22±0.07 mN, p=0.954). The magnitude of increase in pCa_{50} upon exposure to 5μM CK-2066260 was comparable in both groups.

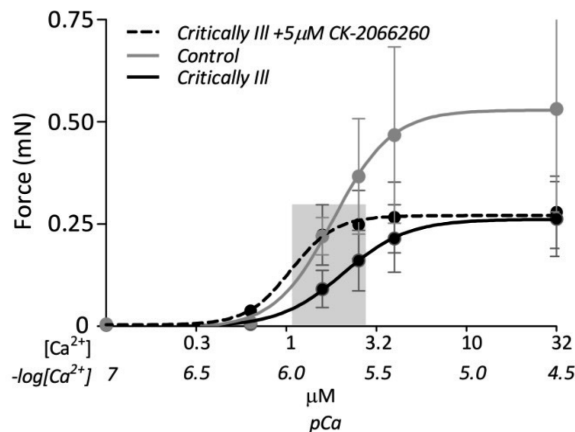

Figure 6. The curves show force response of fast-twitch fibers of a subset of patients to incremental calcium concentrations when exposed to vehicle (DMSO) (control grey solid line, critically ill black solid line). Fibers from critically ill patients show a marked leftward shift of the force-calcium curve

when treated with 5µM CK-2066260 (dotted black line), such that at physiological calcium concentration (indicated by grey bar) force is restored to levels observed in untreated fibers from controls.. Bullets indicate group mean, error bars indicate ± SD.

Discussion

The current study is the first to show that atrophy and contractile weakness of diaphragm muscle fibers develop in a clinically relevant group of mechanically ventilated critically ill patients. Interestingly, the reduction in the contractile force of diaphragm fibers of these critically ill patients is comparable to the reduction in diaphragm strength estimated previously by phrenic nerve pacing [62,63], indicating that the reduction in diaphragm strength in these patients largely results from muscle fiber weakness. To date, no drug is approved to improve respiratory muscle function in MV critically ill patients. We made a step towards such a strategy by testing the ability of the fast skeletal troponin activator, CK-2066260, to restore diaphragm fiber strength. We observed that upon exposure to CK-2066260, fast-twitch diaphragm fibers from critically ill patients regained strength at calcium concentrations which reflect activation during daily live activities to levels found in untreated fibers from control patients (Fig.1c). Since ~50% of fibers and total fiber area in the human diaphragm consists of fast-twitch fibers (eFig.3), fast skeletal troponin activators might significantly improve *in vivo* diaphragm strength. The potential of fast troponin activators is further strengthened by the notion that these drugs do not affect cardiac function [81], which would be an undesirable side effect in critically ill patients. The analogue of CK-2066260, *tirasemtiv* (formerly CK-2017357) is currently under study in patients with amyotrophic lateral sclerosis [NCT01709149].

What causes weakness of diaphragm muscle fibers in critically ill patients? It seems plausible that the observed diaphragm weakness was acquired during ICU-stay, as we used strict exclusion criteria to rule out that our study patients had pre-existing diaphragm weakness. Also, during stay in the ICU, patients received nutrition according to an optimized nutrition algorithm [124]. A commonly suggested concept is that mechanical ventilation *per se* rapidly induces weakness and atrophy of muscle fibers due to contractile inactivity of the diaphragm [40,56,64,68,125]. The critically ill patients we studied received MV for 28-603 hours prior to biopsy, a time frame that was associated with significant reductions in the CSA of diaphragm fibers in brain dead organ donors [68,69]. Thus, the diaphragm muscle fiber atrophy and weakness that we observed may, at least partly, be explained by mechanical ventilation *per se.* Other ICU related phenomena that could contribute to diaphragm muscle weakness include underlying disease such as sepsis [126,127]. Clearly, to elucidate the main factors that contribute to the observed diaphragm muscle fiber weakness requires studies with larger cohorts of various patient groups.

Methods

Patients
Diaphragm biopsy specimens were obtained from mechanically ventilated critically ill patients (n=10) undergoing laparotomy or thoracotomy for various reasons. Diaphragm biopsies obtained from patients undergoing thoracotomy for removal of a primary lung tumor served as controls (n=10). Exclusion criteria were COPD (GOLD II, III, IV), CHF, neuromuscular disease, chronic metabolic disorders, pulmonary hypertension, chronic use of corticosteroids (>7.5 mg/day for at least 3 months), and >10% weight loss within the last 6 months. The biopsy protocol was approved by the institutional review board at VU University Medical Center (#2010/69) and written informed consent was obtained from the patient, next of kin, or a legal representative.

Biopsy handling
A small part of the fresh biopsy was directly frozen in liquid nitrogen and stored at -80°C for histology experiments. The other part of the biopsy – destined for contractility experiments - was placed for 24h at -20°C in 4 mL relax-glycerol solution containing high concentrations of protease inhibitors ($RxGlyc_{high}$); for the composition of solutions, see below. Subsequently, the biopsy was placed on a roller band for 24h at 4°C. Finally, $RxGlyc_{high}$ was substituted with a relax-glycerol solution with lower concentrations of protease inhibitors (RxGlyc, for composition, see below) and stored at -20°C until further use.

Solution composition
Composition of solutions for contractility experiments was as described previously [82,92,94]. All solutions had an ionic strength of 180mM and pH 7.1. The relaxing solution had a negative logarithm of free calcium concentration (pCa) of 9.0 and comprised of (in mM): 5.89 Na_2ATP, 6.48 $MgCl_2$, 40.76 Kprop, 100 BES, 6.97 EGTA, 14.50 CrP 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. 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 solution (RxGlyc) consisted of 50% (v/v) glycerol and relaxing solution, and in addition the following protease inhibitors (in mM) 1.0 DTT, 0.24 PMSF, 0.04 leupeptin, 0.01 E64. $RxGlyc_{high}$ contained higher concentrations of leupeptin and E64 (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 and protease inhibitors (in mM) 1.0 DTT, 0.24 PMSF, 0.04 leupeptin, 0.01 E64.

Contractility experiments

For contractility experiments an adapted protocol from Ottenheijm et al. was used, as described previously.[90] Segments of single diaphragm fibers of approximately 1-1.5 mm were isolated in a relaxing solution at 5°C. At both ends, two aluminium clips were attached. The diaphragm fibers were incubated for 10 minutes in cold (5°C) skinning solution to permeabilize the plasma membrane enabling activation of myofilaments with exogenous calcium. Subsequently, the fibers were mounted horizontally on two stainless-steel hooks in a relax solution filled chamber (200 μL) with a glass coverslip-bottom on the stage of an inverted microscope (Zeiss, The Netherlands). One of hooks was attached to a force transducer (model 403A Aurora Scientific Inc, Ontario, Canada) which has a resonance frequency of 10 kHz, whereas the other end was attached to a servo-motor (model 315C, Aurora Scientific Inc.; Aurora, Ontario, Canada) which has a step time of 250 μs. Diaphragm fiber dimensions were measured by means of a camera device coupled to the objective. Diaphragm fiber length was determined with 100x magnification, depth and width were measured with 400x magnification (an elliptical cross section of the diaphragm muscle fiber was assumed). Injury was examined microscopically; in case of severe damage, loss of striation and other irregularities the fibers were excluded. Diaphragm fibers were stretched to optimal length by setting sarcomere length at 2.5 μm with dedicated Aurora software. To ensure stable attachment of the diaphragm fiber in the clips throughout the mechanical protocol, the fiber was briefly maximally activated prior to the experiment, and when necessary restreched to sarcomere length 2.5μm. Note that this brief activation was done before the determination of diaphragm fiber dimensions. To determine the force response of single diaphragm fibers to calcium (force-pCa relation (pCa = -log of molar free Ca^{2+} concentration), fibers were sequentially transferred from relax, to pre-activation solutions and finally to solutions with pCa values ranging from 4.5 to 9.0 and the steady-state force was measured. The Hill-equation was fit to the obtained force-pCa data, providing the pCa_{50}.During the experiment, data were automatically collected by a data acquisition board (sampling rate 10000 Hz). All measurements were performed at 20°C.

Myosin Heavy Chain Isoform Composition

At the end of the contractile experiments, Myosin Heavy Chain (MHC) isoform composition of the single diaphragm fiber were identified by SDS-PAGE as described previously with minor modifications [90]. Briefly, single diaphragm fiber 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 denaturated 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 asses migration patterns and verify isoforms of the myosin heavy chain. The

gels were silver stained with alkaline phosphatase (VectastainABC kit, Vector Labs) according to the procedure described by Oakley et al[E100] and subsequently imaged by a high-resolution scanner (600 dpi; Microtek Scan Maker 5). Fibers expressing the slow isoform were classified as slow-twitch fibers and fibers expressing the 2a and 2x isoform were classified as fast-twitch fibers.

Immunohistology

To determine diaphragm fiber cross sectional area, immunohistochemical analyses with myosin heavy chain antibodies were performed on cryosections of the biopsy specimens, as decscrebid previously [83,102]. Cryosections were cut from the frozen biopsies (perpendicular to diaphragm fiber direction, 8μm thick), rehydrated for 10 min in phosphate buffer (PBS) and subsequently blocked with phosphate buffer containing 0.5% (wt/vol) bovine serum albumin (PBS-0.5%BSA, Molecular Probes). Subsequently, cryosections were incubated with primary antibodies for slow MHC (1:100; NOQ7.5.4D, Abcam) and for fast MHC (1:50 MY-32, Abcam) followed by appropriate fluorescent labelled secondary antibodies (Molecular Probes, Eugene, OR). Finally plasma membranes were visualized by embedding the sections in fluorescent WGA (1:25 diluted in PBS-0.5%BSA, Molecular Probes) 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 with use of 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). Per patient approximately 130 diaphragm fibers were analyzed per fiber type. Cross sectional areas were calculated by ImageJ software.

Statistical analysis

Fisher's exact test and unpaired t-test were used for patient characteristics when appropriate. Comparisons of contractile parameters and fiber cross sectional areas between groups were performed using multilevel analysis with correction for non-independence of successive measurements per subject (MLwiN, 2.22)[87,128,129]. We performed two-way repeated measurement ANOVA to study fiber type proportion, and fiber type area and in CK-2066260 experiments to study pCa_{50} and whether the subset of patients was representative. To compare the force response at pCa5.8 in CK-2066260 experiments unpaired t-test was used. Differences were attributed to chance unless they were significant at the 0.05 level. Data in text and in figures are represented as mean±SD.