Levosimendan Enhances Force Generation of Diaphragm Muscle from Patients with Chronic Obstructive Pulmonary Disease

Hieronymus W. H. van Hees[1,3], P. N. Richard Dekhuijzen[1,3], and Leo M. A. Heunks[1,2,3]

[1]Department of Pulmonary Diseases, [2]Intensive Care Medicine, and [3]Institute for Fundamental and Clinical Human Movement Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Rationale: Levosimendan is clinically used to improve myocardial contractility by enhancing calcium sensitivity of force generation. The effects of levosimendan on skeletal muscle contractility are unknown. Patients with chronic obstructive pulmonary disease (COPD) suffer from diaphragm weakness, which is associated with decreased calcium sensitivity.

Objectives: To investigate the effects of levosimendan on contractility of diaphragm fibers from patients with COPD.

Methods: Muscle fibers were isolated from diaphragm biopsies obtained from thoracotomized patients with and without COPD (both groups n = 5, 10 fibers per patient). Diaphragm fibers were skinned and activated with solutions containing incremental calcium concentrations and 10 μM levosimendan or vehicle (0.02% dimethyl sulfoxide). Developed force was measured at each step and force versus calcium concentration relationships were derived. Results were grouped per myosin heavy chain isoform, which was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Measurements and Main Results: At sub-maximal activation levosimendan improved force generation of COPD and non-COPD diaphragm fibers by approximately 25%, both in slow and fast fibers. Levosimendan increased calcium sensitivity of force generation ($P < 0.01$) in both slow and fast diaphragm fibers from patients with and without COPD, without affecting maximal force generation.

Conclusions: Levosimendan enhances force generating capacity of diaphragm fibers from patients with and without COPD patients by increasing calcium sensitivity of force generation. These results provide a strong rationale for testing the effect of calcium sensitizers on respiratory muscle dysfunction in patients with COPD.

Keywords: diaphragm; chronic obstructive pulmonary disease; calcium sensitization; levosimendan

Weakness of the diaphragm frequently occurs in patients with chronic obstructive pulmonary disease (COPD) (1, 2). Inspiratory muscle weakness in these patients is associated with prolonged mechanical ventilation and increased mortality (3, 4). The pathophysiology of inspiratory muscle dysfunction in COPD is complex (1). Studies have shown that, in patients with COPD, impaired contractility of muscle fibers from the diaphragm, the most prominent inspiratory muscle, results from a loss of contractile protein and impaired function of remaining contractile proteins (5–7). Diaphragm muscle fibers from these patients exhibit reduced ability to respond to calcium, indicating reduced

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Loss of contractile protein and impaired calcium sensitivity of force generation contribute to respiratory muscle dysfunction in patients with chronic obstructive pulmonary disease (COPD). No specific treatment exists to improve respiratory muscle function in these patients.

What This Study Adds to the Field

The calcium sensitizer levosimendan improves contractile protein function in diaphragm muscle fibers of patients with COPD.

calcium sensitivity of force generation. Based on these findings, we proposed that pharmacological modulation of the regulatory proteins is an effective strategy to improve diaphragm contractility in patients with COPD.

Calcium sensitizers enhance the binding of calcium to regulatory proteins of the contractile apparatus. This improves the responsiveness of myofilaments to calcium, resulting in a greater amount of force for the same amount of cytosolic calcium. An advantage of this mechanism of action is that force generation is improved without increased adenosine triphosphate (ATP) consumption (8). Currently, levosimendan is the only calcium sensitizer approved for use in human (∼40 countries worldwide). Levosimendan has been shown to improve cardiac contractility *in vivo* (9) and *in vitro* (10), by enhancing calcium sensitivity of force generation through binding with cardiac troponin C (11). The effects of calcium sensitizers on human peripheral or respiratory skeletal muscle have not been investigated. Nevertheless, cardiac troponin C isoform is expressed in human diaphragm muscle. Moreover, Levine and colleagues have recorded increased expression of this isoform in the diaphragm of patients with COPD (12). Therefore, we hypothesize that levosimendan improves calcium sensitivity of muscle fibers isolated from the diaphragm of patients with COPD.

METHODS

Subjects

Diaphragm muscle biopsies (∼100 mg) were obtained from five patients with COPD (four males) and five subjects without COPD (two males). Biopsies from the right anterior costal diaphragm were obtained during thoracotomy for lung cancer (stage $T_{1-3}N_{0-1}M_x$). Stages of lung cancer did not significantly differ between patients with COPD and those without COPD. Patients with chronic corticosteroid use, primary myopathies, endocrine diseases, and more than 10% weight loss in the last 6 months before surgery were excluded from the study. General characteristics and pulmonary function data are shown in Table 1. Informed consent was obtained from each subject. The

study was approved by the Ethics Committee of Radboud University Nijmegen Medical Centre.

Single Fiber Contractile Measurements

According to previously described methods (5, 13), single fiber contractile measurements and experimental protocol were performed as follows.

Single Fiber Preparation

A bundle was dissected from the central part of the fresh muscle biopsy. The muscle bundle was pinned to cork and stored at 4°C in a relaxing solution, containing 50% glycerol (vol/vol). After 24 hours, the muscle strip was stored at −20°C for later analysis. Approximately 1 hour before determination of single fiber contractile properties, the muscle bundle was transferred to relaxing solution (5°C) containing 1% Triton X-100 to permeabilize lipid membranes. From the muscle bundle, approximately 2-mm segments of single fibers were isolated using micro-forceps. Subsequently, the fiber ends were attached to aluminum foil clips and mounted on the single fiber apparatus. Fibers were mounted in a temperature-controlled (20°C) flow-through acrylic chamber (120-μl volume), with a glass coverslip bottom, on the stage of an inverted microscope (model IX-70; Olympus, Amsterdam, The Netherlands).Two stainless steel hooks were used to mount the fiber horizontally in the chamber. One end of the fiber was attached to a force transducer (model AE-801; SensoNor, Horten, Norway) with a resonance frequency of 10 kHz, whereas the other end was attached to a servomotor (model 308B; Aurora Scientific, Aurora, ON, Canada) with a step time of 250 μs. In relaxing solution, sarcomere length was set at 2.4 μm as the optimal length for force generation (14, 15) with the use of a calibrated eyepiece micrometer. Muscle fiber length ($\sim$1–1.5 mm) was measured using a reticule in the microscope eyepiece ($\times$10 Olympus Plan 10,0.30 numerical aperture [NA]). The *XY* fiber diameter (width) was measured with a $\times$40 objective ($\times$40 Olympus Plan 40, 0.60 NA). The $\times$40 objective also was used to measure the *XZ* fiber diameter (depth) by noting the displacement of the microscope's objective while focusing on the top and bottom surfaces of the fiber. Three width and depth measurements were made along the length of the fiber, and the average values were used to calculate the fiber cross-sectional area, assuming that the fiber was ellipsoid in shape.

Composition of Solutions for Single Fiber Measurements

Concentrated stock solutions of levosimendan (a kind gift from Orion Pharma, Espoo, Finland) were prepared in dimethyl solfoxide (DMSO) and stored at −20°C. Before use, levosimendan stock solutions were diluted with experimental solutions. The final concentration of DMSO did not exceed 0.02%. Control experimental solutions contained 0.02% DMSO (vehicle). Relaxing solution consisted of 1.0 mM $MgCl_2$, 4.0 mM Na_2ATP, 5 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), 10 mM imidazole, 15 mM creatine phosphate, and

sufficient KCL to adjust the total ionic strength to 150 mM at pH 7.0. The negative logarithm of the free Ca^{2+} concentration (pCa) of the relaxing solution was 9.0, whereas in activating solutions the pCa ranged from 7.0 to 4.5 (with maximal activation at pCa 4.5). Sufficient $CaCl_2$ was added to achieve appropriate pCa in activating solutions.

Diaphragm Muscle Single Fiber Experimental Protocol

The response of five COPD diaphragm fibers to different doses of levosimendan was determined during submaximal activation (at pCa 5.8). Figure 1 shows that levosimendan enhanced submaximal force generation with a maximal effect at 10 μM, which is in accordance with previously reported dose–response curves for cardiac fibers (10). Accordingly, further experiments were conducted with either levosimendan (10 μM) or vehicle. Maximal force was determined by perfusing the experimental chamber with solutions of, successively, pCa 9 and pCa 4.5. Then pCa 9.0 solution was perfused through the chamber to relax the fiber. Subsequently, fibers were perfused with solutions containing incremental Ca^{2+} concentrations. Each time a plateau was reached, the next pCa solution was perfused through the experimental chamber. To improve functional stability of muscle fibers, we applied Brenner cycling (16), as modified by Sweeney and colleagues (17), throughout the experimental protocol. Accordingly, no functional decline in maximal force generation over time was observed. MIDAC software (Radboud University, Nijmegen, The Netherlands) and a data-acquisition board were used to record signals.

Diaphragm Muscle Single Fiber Contractile Determinations

Maximum specific force was determined by dividing the isometric force generated at pCa 4.5 by cross-sectional area (CSA). The force–pCa relationship was derived by plotting recorded force against pCa of the corresponding activating solution. Graphpad software (Graphpad Software Inc., San Diego, CA) was used to calculate the Ca^{2+} concentration required for half-maximum activation, pCa_{50} (pCa = $-^{10}log[Ca^{2+}]$), as an index of Ca^{2+} sensitivity of force generation and the Hill coefficient, as a measure of myofilament cooperativity (18).

Myosin Heavy Chain Isoform Composition Determination

Because pCa_{50} is significantly different between slow and fast diaphragm fibers (19), diaphragm fibers were designated as slow or fast on the basis of myosin heavy chain isoform determination. Determination of myosin heavy chain isoform composition by SDS-PAGE was described previously (13).

TABLE 1. PATIENT CHARACTERISTICS

Patients	Age (yr)	BMI (kg/m²)	FEV$_1$ (% predicted)	VC (% predicted)	FEV$_1$/VC (%)	TLC (% predicted)	D$_{LCO}$/VA (% predicted)
Non-COPD							
Female	59	23	83	102	74	88	87
Male	61	27	106	110	73	118	98
Female	50	21	98	120	72	115	45
Female	63	23	126	139	74	108	87
Male	66	21	83	79	78	77	113
COPD							
Male	73	24	86	125	68	112	102
Male	62	24	60	114	52	111	73
Female	61	21	54	105	42	124	64
Male	60	27	85	104	61	106	91
Male	44	23	29	90	26	133	51

Definition of abbreviations: BMI = body mass index; D$_{LCO}$/VA = carbon monoxide transfer coefficient per alveolar volume.
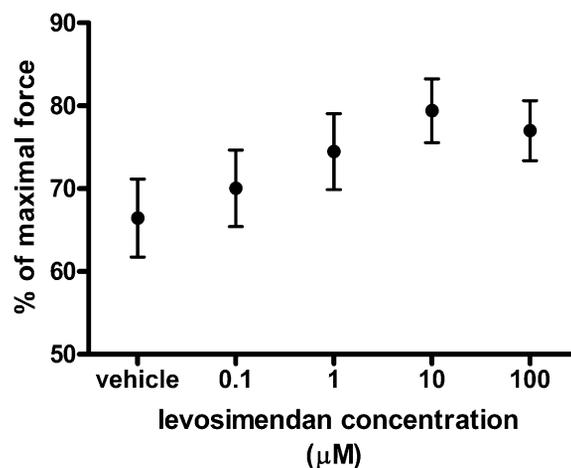

Figure 1. Dose–response effects of levosimendan on diaphragm fiber submaximal force-generating capacity. Incremental levosimendan concentrations were added to diaphragm fibers (n = 5) from patients with chronic obstructive pulmonary disease during sub-maximal activation at pCa 5.8. Force generation increases with rising levosimendan concentration, with a maximal effect at 10 μM.

Data Treatment and Statistical Methods

Differences regarding force–pCa relations between groups were statistically analyzed with a two-way repeated measures analysis of variance (ANOVA) test with pCa as the repeated measures factor and the presence of levosimendan as the second factor. One-way ANOVA with *post hoc* Student-Newman-Keuls testing was performed to evaluate the statistical significance of differences between groups of other contractile characteristics. A probability level of $P < 0.05$ was considered significant. Mean $\pm$ SEM values are presented in text, tables, and figures.

Sample size of 25 fibers (i.e., 12–13 per treatment group), calculated *a priori*, was based upon the assumption to detect an increase of submaximal force of at least 15% by 10 μM levosimendan (10) with a standard deviation of 15% (5), 80% probability, and an α level of 0.05. Because we determined the effect in both slow and fast fibers, a total of 100 diaphragm fibers obtained from five patients with COPD and five non-COPD patients were measured (i.e., 10 fibers per patient; five with 10 μM levosimendan and five with vehicle).

RESULTS

Patient Characteristics

Patient characteristics and pulmonary function data are shown in Table 1. Based on Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification, two patients had mild COPD (stage I), two patients had moderate COPD (stage II) and one patient was classified as very severe COPD (stage IV). One patient without COPD had a mild restriction, and another patient without COPD had a reduced lung diffusion capacity.

Diaphragm Fiber Contractility

Levosimendan increased calcium sensitivity of force generation in diaphragm fibers from patients with and without COPD, which is indicated by a leftward shift of the force–pCa relation in levosimendan-exposed fibers (Figure 2). During submaximal activation, for instance at pCa 6.0, levosimendan increased diaphragm fiber force by 20 to 30% compared with vehicle-exposed fibers. Accordingly, the pCa_{50}, a measure for calcium sensitivity of force generation, is higher in levosimendan-treated fibers than in vehicle-treated fibers for both COPD and non-COPD diaphragm fibers (Figure 3). Separation of the results into fibers expressing slow or fast myosin heavy chain showed that levosimendan's effect was more pronounced in slow fibers (Figure 3A) than in fast fibers (Figure 3B). Myofilament cooperativity, indicated by the Hill coefficient, did not differ between vehicle and levosimendan-treated fibers; for slow non-COPD fibers (1.47 ± 0.11 vs. 1.77 ± 0.22), for slow COPD fibers (1.55 ± 0.16 vs. 1.30 ± 0.13), for fast non-COPD fibers (2.85 ± 0.18 vs. 3.09 ± 0.73) and for fast COPD fibers (3.18 ± 0.56 vs. 2.02 ± 0.18). Also, levosimendan did not significantly affect diaphragm fiber maximal force generation in both COPD and non-COPD fibers (Figure 4).

COPD Versus Non-COPD

The effect size of levosimendan on calcium sensitivity did not differ between COPD and non-COPD diaphragm fibers, for both slow and fast typed fibers (Figure 3). Vehicle-treated COPD fibers displayed lower calcium sensitivity and maximal force-generating capacity compared with vehicle-treated fibers from patients without COPD (Figures 3 and 4), which confirms our previously published data (5).

DISCUSSION

The present study is the first to show that pharmacological modulation of the troponin complex improves human diaphragm muscle fiber contractility. Recently, we have shown that calcium sensitivity of force generation in the diaphragm of patients with COPD is impaired (5). Data of the present study confirm these findings and show that levosimendan improves calcium sensitivity in diaphragm muscle fibers of these patients. Interestingly, levosimendan also improved calcium sensitivity of diaphragm fibers from the diaphragm of patients without COPD. Under certain conditions, calcium sensitization offers a promising mechanism for the treatment of diaphragm weakness, especially in patients with COPD.

Respiratory failure is a prominent manifestation in COPD and is characterized by an increased energy demand on weakened respiratory muscles (1). Increasing the workload on weakened respiratory muscle leads to reduced efficiency of respiratory muscles, which further restricts the limited energy reserves of the diseased body (20, 21). To break this viscous circle it is therefore clinically relevant to seek therapies that improve the efficiency of respiratory muscles. Levosimendan has been developed in attempts to find solutions for similar questions regarding cardiac muscle (22). The positive inotropic effects of levosimendan are thought to result mainly from calcium sensitization of cardiac muscle fibers (23). *In vivo*, both the heart and diaphragm muscle undergo repetitive cycles of contraction and relaxation. This requires continuous sequestration of calcium at the muscle fiber level, which is an energy-consuming process that comprises 30 to 40% of total muscle energy consumption during normal contractions (24). Current and previous (5) data show that more calcium is needed in diaphragm fibers from patients with COPD to generate the same amount of force as patients without COPD. Accordingly, the stimulation frequency of diaphragm fibers should be increased. Indeed, discharge frequency of motor neurons that activate the diaphragm is twofold higher in patients with COPD during resting breathing (25). This will however impose a higher burden on the calcium sequestration, rendering diaphragm performance less efficient. Conversely, improvement of diaphragm efficiency could be established by generating the same amount of force with less calcium (i.e., increasing the sensitivity of myofilaments for calcium). The present study demonstrates that levosimendan does increase the calcium sensitivity of diaphragm fibers from patients with COPD.

Mechanisms of Action

In cardiac muscle, levosimendan is presumed to increase calcium sensitivity by stabilizing the conformation of the troponin complex, which triggers muscle contraction (26). Indeed, troponin C has been shown to interact with levosimendan in cardiac muscle (11). Two isoforms of troponin C exist in muscle tissue: the cardiac/slow isoform present in cardiac and slow-type skeletal muscle, and the fast isoform present in fast-type skeletal muscles. The present finding, that levosimendan improves contractility of slow diaphragm fibers, can be explained by its interaction with cardiac/slow troponin C. In addition, we observed that levosimendan enhances fast diaphragm fiber contractility. Fast skeletal and slow skeletal troponin C are approximately 70% identical (27), but whether levosimendan can also bind to fast troponin C has, as far as we know, never been described. Our observations in fast diaphragm fibers suggest a possible interaction of levosimendan with fast troponin C. Another possibility is that some fibers express fast myosin heavy chain with slow troponin C. Increased expression of slow troponin C has previously been demonstrated in the diaphragm of patients with COPD (12). It was postulated that this results from a reduction of fast fibers rather than an altered isoform expression within fast fibers. Our results support this postulation, because the effect size of levosimendan on COPD fibers was similar to its effect size on non-COPD fibers. Furthermore, adult skeletal muscle generally coexpresses fast
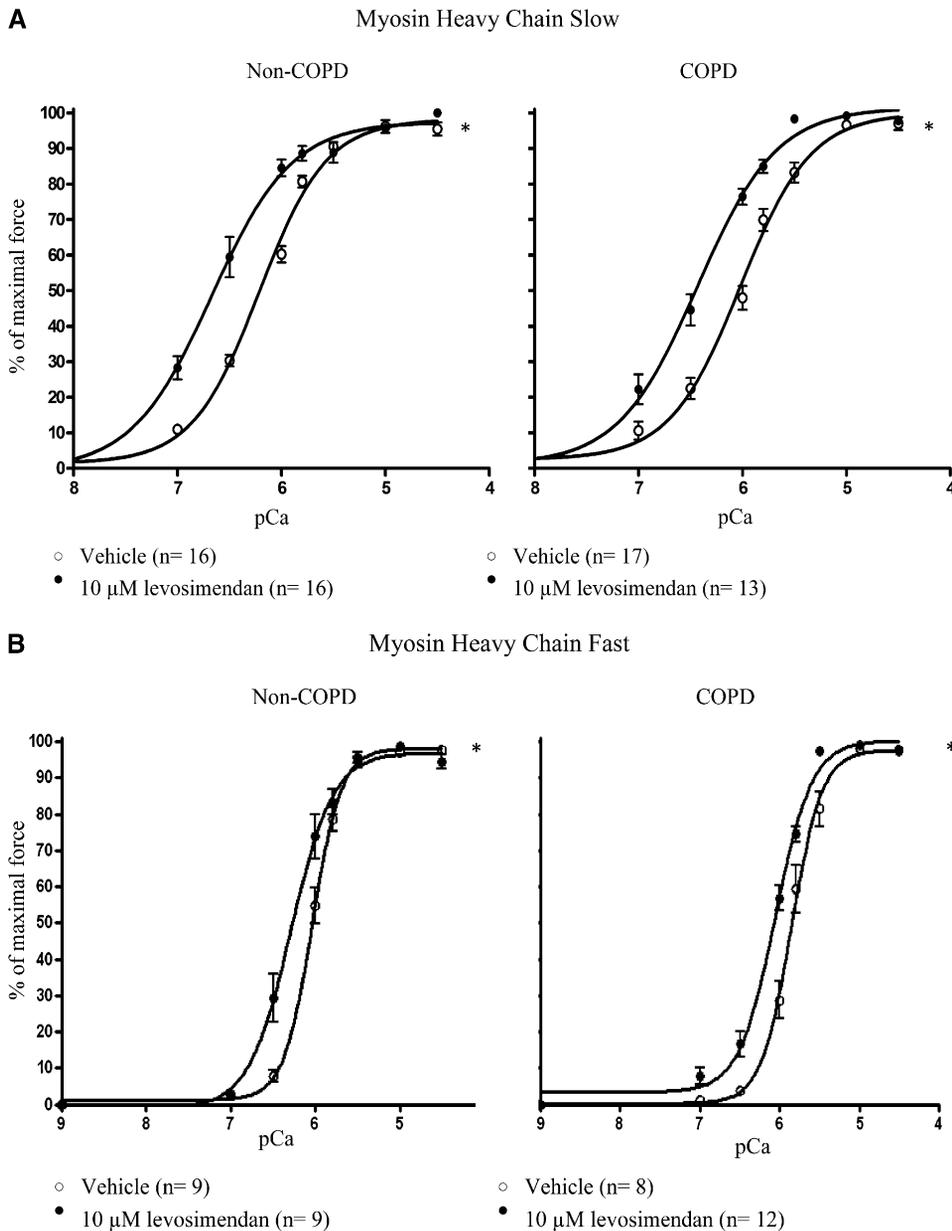
A   Myosin Heavy Chain Slow

○ Vehicle (n= 16)   ○ Vehicle (n= 17)
● 10 μM levosimendan (n= 16)   ● 10 μM levosimendan (n= 13)

B   Myosin Heavy Chain Fast

○ Vehicle (n= 9)   ○ Vehicle (n= 8)
● 10 μM levosimendan (n= 9)   ● 10 μM levosimendan (n= 12)

Figure 2. Force-calcium characteristics of diaphragm fibers from patients with and without chronic obstructive pulmonary disease in the presence of 10 μM levosimendan or vehicle activating solutions. Isometric force generation in response to solutions with incremental calcium concentrations was determined. Submaximal force generation is higher in diaphragm fibers treated with levosimendan when compared with vehicle. *Two-way repeated measures analysis showed that force-pCa relations of fibers treated with levosimendan were significantly different from force-pCa relations of fibers treated with vehicle, for both slow and fast fibers (*A* and *B*, respectively). Data are presented as means ± SEM.

myosin heavy chain with fast troponin C (28), so we do not expect that expression of slow troponin C explains the effect of levosimendan in fast fibers. Nevertheless, the effect of levosimendan on pCa_{50} was more profound in slow fibers compared with fast fibers (Figure 2), indicating that the effect size of levosimendan depends on the isoform expression of myofibrillar proteins. A recent study showed that specific structures in troponin I influence the binding of levosimendan to the troponin complex. It could therefore be that the effect size of levosimendan is modulated by isoforms of different myofibrillar proteins. As expression of type I fibers in the diaphragm of patients with COPD is increased (12) levosimendan treatment may be more effective in patients with COPD than in healthy subjects. Levosimendan did not affect maximal force generation, which implies that the maximal amount of attached cross-bridges as well as force per cross-bridge was not affected by levosimendan.

Study Limitations

The effect of levosimendan on diaphragm muscle fibers was tested *in vitro*, which brings several advantages. Using skinned

muscle fibers we were able to specifically study the effects of levosimendan on contractile protein function. This would not be possible *in vivo* because measured force *in vivo* depends on many other factors including patient motivation, neuromuscular transmission, and intracellular calcium signaling. In addition, the cardiovascular effects of levosimendan may affect respiratory muscle function in patients with COPD. Thus, skinned diaphragm fibers provide an excellent model to study the effects of levosimendan on diaphragm contractility. The data of the present study provide a strong rationale to test the effects of calcium sensitizers on diaphragm muscle function *in vivo* under appropriate conditions.

The levosimendan concentration used in the present study (10 μM) was derived from dose–response study and was in line with previous data from cardiac muscle fibers (10). Although 0.1 μM levosimendan resulted in enhanced force generation, the dose–response curve on COPD diaphragm fibers revealed that 10 μM levosimendan induces a maximal effect on force improvement. This concentration is slightly higher than plasma concentrations measured after infusion using clinically recom-
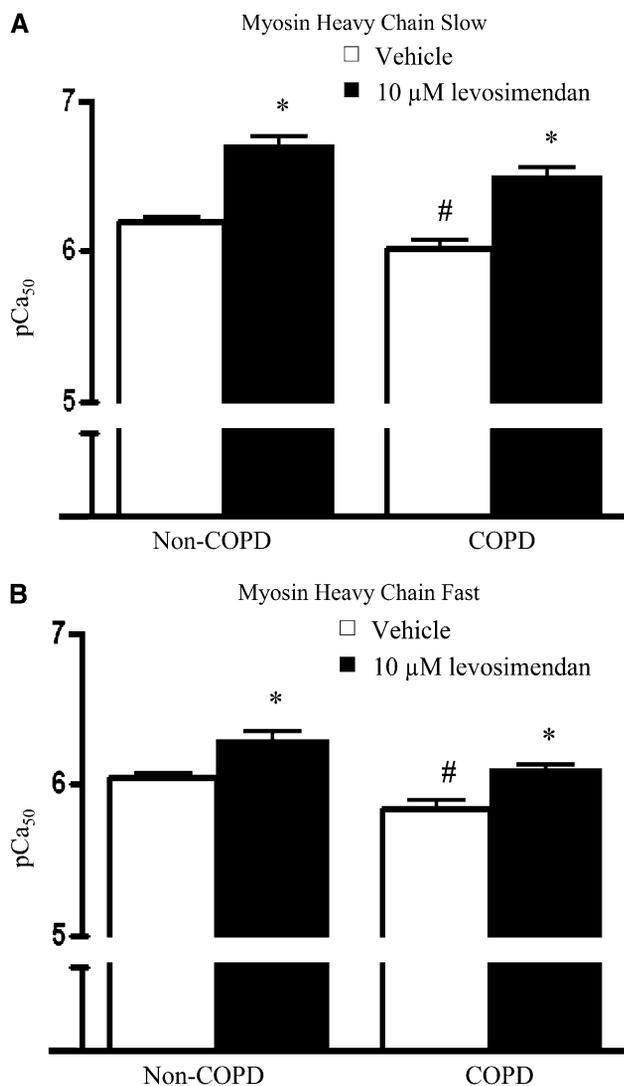
Figure 3. Values of pCa$_{50}$, an index for calcium sensitivity of force generation, from diaphragm fibers of patients with and without chronic obstructive pulmonary disease (COPD) in the presence of 10 μM levosimendan or vehicle solution. Calcium sensitivity is lower in COPD fibers than in non-COPD fibers. Diaphragm fibers treated with levosimendan display higher calcium sensitivity than fibers treated with vehicle, for both slow and fast fibers (A and B, respectively). Data are presented as means ± SEM. *P < 0.01 compared with vehicle-treated fibers within subject group; #P < 0.05 compared with vehicle treated non-COPD fibers within fiber-type group. pCa$_{50}$ = $-^{10}$log[Ca^{2+}] at which 50% of maximal force is generated.

mended dosages (24-h infusion with 0.1–0.4 μg/kg/min) (29). Furthermore, intracellular levosimendan concentrations seem to be more relevant and could exceed blood values. Local concentrations are, however, difficult to determine because *in vivo* levosimendan has a short half-life (∼1 h) as it is reduced to OR-1855 by intestinal bacteria (30). OR-1855 is further metabolized by acetylation to OR-1896, which has similar calcium sensitizing properties as levosimendan (23), but a much longer half-life (30).

Clinical Implications

The pathophysiology of weaning failure from mechanical ventilation is complex and multifactorial (31). However, it has been
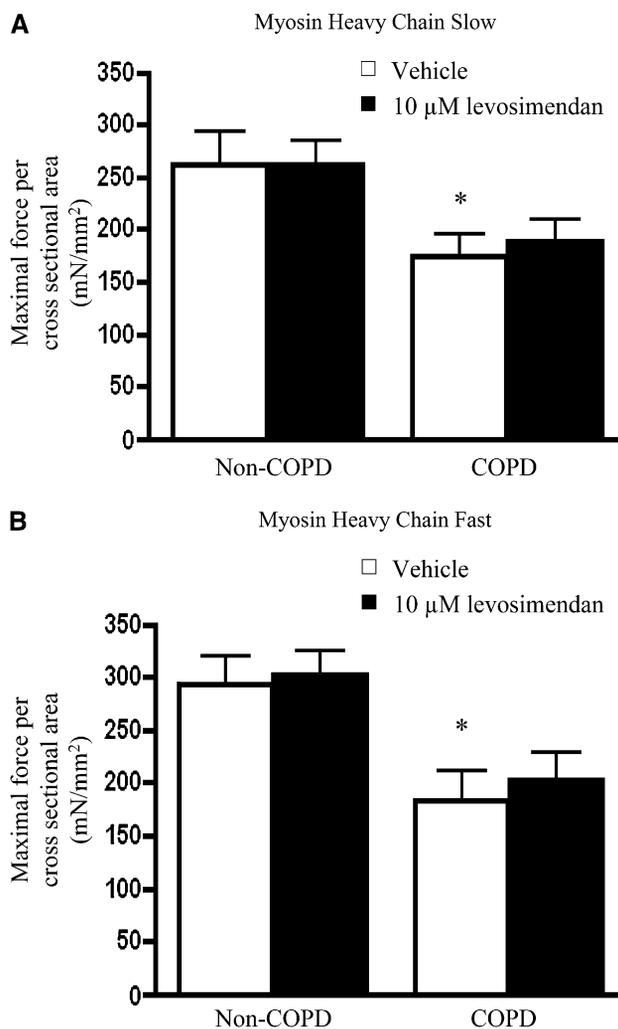

Figure 4. Maximal force generation of diaphragm fibers from patients with and without chronic obstructive pulmonary disease (COPD) in the presence of 10 μM levosimendan or vehicle solution. COPD diaphragm fibers generated lower maximal forces than non-COPD fibers, in both slow and fast fibers (Figures 4A and 4B, respectively). Maximal force generation is not significantly different between levosimendan and vehicle-treated fibers within patient groups. Data are presented as means ± SEM. *P < 0.05 compared with vehicle-treated non-COPD fibers within fiber-type group.

recognized that, in patients with COPD, maximal inspiratory pressure is significantly lower at the time of weaning failure than at the time of successful weaning (32). In addition, patients with severe COPD failing to wean from mechanical ventilation generate lower inspiratory transdiaphragmatic pressure than patients without severe COPD (33). Accordingly, improving respiratory muscle contractility may contribute to successful weaning from mechanical ventilation in these patients. Moreover, as levosimendan also improves contractility in diaphragm fibers from patients without COPD, this agent may appear beneficial to other patients difficult to wean from mechanical ventilation. The results of the present study provide an excellent rationale to test the efficacy of levosimendan in this clinical setting. However, it should be kept in mind that *in vivo* levosimendan affects other cell processes besides calcium sensitivity. For instance, opening of ATP-sensitive potassium channels in vascular smooth muscle affects pulmonary and systemic vascular

resistance, resulting in reduction in mean arterial blood pressure (34). The final effects of levosimendan on cardiac contractility, respiratory muscle function, and oxygen transport in non–heart-failure patients are difficult to predict but should be answered by clinical studies. By increasing pCa_{50} levosimendan could improve respiratory muscle function during weaning without increasing energy expenditure. The latter is important because respiratory muscles could fatigue during weaning trails (32, 35). A major cause of fatigue is the accumulation of inorganic phosphate resulting from the excessive breakdown of phosphocreatine during periods of high energy demand (36). Elevated inorganic phosphate levels depress calcium sensitivity (37), which evidently impairs contractile muscle function. Hence, levosimendan seems highly appropriate to counteract fatigue-induced muscle weakness because it enhances the sensitivity for calcium without increasing energy demand. Very recently, Levine and colleagues showed that controlled mechanical ventilation induces rapid atrophy of human diaphragm muscle fibers (38). Although not studied, it is likely that atrophy will result in impaired diaphragm contractility in these patients. Therefore, levosimendan may appear beneficial in difficult mechanical ventilation weaning of patients without COPD as well. Our study demonstrated that levosimendan increased calcium sensitivity of diaphragm fibers from subjects without COPD.

Levosimendan is generally well tolerated by patients and hypotension is one of the most observed side effects but is not common when the treatment remains within the recommended dose. If necessary, hypotension can be counteracted by low-dose norepinephrine (39, 40).

In conclusion, the present study demonstrates that levosimendan enhances the force-generating capacity of diaphragm fibers from patients with and without COPD by increasing calcium sensitivity of force generation. The results of our study provide a strong rationale for testing the effect of calcium sensitizers on respiratory muscle contractility in patients with COPD during elevated loading, such as weaning from mechanical ventilation.

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