The calcium sensitizer levosimendan improves human diaphragm function

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At a Glance Commentary

Scientific Knowledge on the Subject

Respiratory muscle weakness frequently occurs in chronic diseases and contributes to morbidity and mortality. There is no specific pharmacological treatment available to improve respiratory muscle function in patients with impeding respiratory muscle failure. Previous studies have demonstrated that calcium sensitizers improve *in vitro* function of the respiratory muscles.

What this Study Adds to the Field

We report that the calcium sensitizer levosimendan improves neuro-mechanical efficiency of the human diaphragm. In addition, levosimendan restored the loss of diaphragm contractility after an intervention of loaded breathing. These findings suggest that levosimendan treatment may be an effective strategy to improve muscle function in patients with respiratory muscle weakness.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

ABSTRACT

Rationale: Acquired diaphragm muscle weakness is a key feature in several chronic diseases including chronic obstructive pulmonary disease, congestive heart failure, and difficult weaning from mechanical ventilation. However, no drug is available to improve respiratory muscle function in these patients. Recently, we have shown that the calcium sensitizer levosimendan enhances force generating capacity of isolated diaphragm fibers.

Objectives: To investigate the effects of the calcium sensitizer levosimendan on *in vivo* human diaphragm function.

Methods: In a double-blind randomized crossover design, 30 healthy subjects performed two identical inspiratory loading tasks. After the first loading task, subjects received levosimendan (40 μg/kg bolus followed by 0.1/0.2 μg/kg/min continuous infusion) or placebo. Transdiaphragmatic pressure, diaphragm electrical activity and their relationship (neuro-mechanical efficiency) were measured during loading. Magnetic phrenic nerve stimulation was performed before the first loading task and after bolus administration to asses twitch contractility. Center frequency of diaphragm electrical activity was evaluated to study the effects of levosimendan on muscle fiber conduction velocity.

Measurements and Main results: The placebo group showed a 9% ($P=0.01$) loss of twitch contractility after loaded breathing, whereas no loss in contractility was observed in the levosimendan group. Neuro-mechanical efficiency of the diaphragm during loading improved by 21% ($P<0.05$) in the levosimendan group. Baseline center frequency of diaphragm electrical activity was reduced after levosimendan administration ($P<0.05$).

Conclusions: The calcium sensitizer levosimendan improves both neuro-mechanical efficiency and contractile function of the human diaphragm. Our findings suggest a new therapeutic approach to improve respiratory muscle function in patients with respiratory failure.

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Key words: diaphragm; muscle weakness; calcium sensitization; levosimendan

INTRODUCTION

Impaired force generation of the respiratory muscles has been recognized in a variety of diseases including chronic obstructive disease (COPD), congestive heart failure and in the critically ill.[1-7] The pathophysiological substrate of diaphragm dysfunction in these disorders is multifactorial and includes muscle fiber atrophy and contractile protein dysfunction.[3;8-11] We found that diaphragm muscle fibers of patients with COPD display reduced sensitivity of the contractile proteins to calcium.[9;12] In other words more calcium is needed to develop the same amount of force as in the non-COPD diaphragm, resulting in impaired contractile efficiency of the diaphragm muscle. Subsequent studies have shown that calcium sensitivity is also reduced in the diaphragm of animal models for congestive heart failure and prolonged mechanical ventilation.[2;13] Despite a better understanding of respiratory muscle dysfunction in chronic diseases, currently no drug is available to improve respiratory muscle function in humans.

Levosimendan is a clinically used calcium sensitizer, approved to enhance cardiac contractility in patients with acute heart failure. Clinical studies have shown that levosimendan improves cardiac function in these patients.[14] Levosimendan enhances the binding of calcium to troponin C, thereby improving the responsiveness of myofilaments to calcium. Accordingly, a greater amount of force is generated for the same level of cytosolic calcium, resulting in enhanced contractile efficiency. Besides calcium sensitizing, levosimendan has vasodilatory properties mediated by the opening of the ATP-sensitive potassium (K_{ATP}) channels [15].

Recently, we have shown that levosimendan also enhances calcium sensitivity of permeabilized muscle fibers obtained from the human diaphragm, including patients with COPD.[12] However, the effects of calcium sensitizing on the human diaphragm *in vivo* have not been studied. Based on our *in vitro* data [12] we hypothesize that levosimendan improves

contractile function of the human diaphragm *in vivo* through calcium sensitizing. In addition, levosimendan would decrease muscle fiber conduction velocity of the diaphragm, through its effects on K_{ATP} channels in diaphragm fibers. We tested this hypothesis in a double-blind placebo controlled crossover design in healthy subjects performing inspiratory loading tasks. Some of the results of this study have been previously reported in the form of an abstract.[16]

METHODS

We enrolled 30 healthy volunteers in this trial. The protocol was approved by the ethical committee of the Radboud University Nijmegen Medical Centre and registered at ClinicalTrials.gov (NCT01101620). All subjects gave their informed consent.

Esophageal catheter

Diaphragm EMG (EMGdi), esophageal (Pes) and gastric (Pga) pressure were obtained with a multi-electrode esophageal catheter with two balloons; see the *online supplement* for details. Pdi was calculated as Pga-Pes.

Magnetic stimulation and maximal inspiratory effort

Cervical magnetic stimulation of the phrenic nerves was performed to measure twitch Pdi (Pdi_{tw}) and compound muscle action potential of the diaphragm (CMAPdi). See the *online supplement* for stimulation protocol details.

Maximal voluntary Pdi (Pdi_{max}) was measured as mean Pdi in the first second during a maximal inspiratory effort against a closed valve at functional residual capacity.

Inspiratory loading task

Each subject performed two identical loading tasks, before and after administration of study medication. Sitting in upright position, with uncast abdomen subjects breathed through a mouthpiece wearing a nose clip. Subjects performed intermittent inspiratory maneuvers of 10 second against a closed valve (near-isometric contractions) followed by 7 seconds of unloaded breathing. Duty cycle was imposed by a sound signal and subjects were asked to target 40% of Pdi_{max}. Visual feedback of Pdi was provided. Total loading task duration was 10 minutes. During loading, EMGdi and Pdi were recorded continuously, as well as in unloaded

conditions approximately 5 minutes before and after loading. Respiratory effort sensation was scored with a Borg scale (range 6-20) at one, three, six, and nine minutes into loading.

Experimental protocol

The protocol is presented in Figure 1. Pdi_{tw} and Pdi_{max} were measured and followed by the first loading task and 30 minutes of unloaded breathing. After randomization subjects received study medication, either levosimendan bolus (40 μg/kg bodyweight, iv) or an equal volume of placebo in 10 minutes. Pdi_{tw} and Pdi_{max} measurements were repeated and followed by 30 minutes of continuous levosimendan (0.1 μg/kg bodyweight/min, iv) or placebo infusion. A second loading task was performed while infusing levosimendan (0.2 μg/kg/min, iv) or placebo.

Heart rate, end tidal carbon dioxide ($etCO_2$) and peripheral oxygen saturation (SpO_2) were monitored continuously. Blood pressure was measured non-invasively every 10 minutes and during bolus administration each minute. In six subjects cardiac output was determined by transthoracic echocardiography directly before and after bolus administration.

Data Analysis and Statistics

Measurement variables were analyzed offline in Matlab R2009b (The Mathworks, Natick, MA); see the *online supplement* for details. The ratio of mean inspiratory Pdi and EMGdi amplitude was calculated as a measure of the neuro-mechanical efficiency of the diaphragm. Changes in muscle fiber conduction velocity were evaluated using the power spectrum center frequency (CFdi) of the EMGdi; see the *online supplement* for details. Comparisons were made with the appropriate t-test. Changes over time during loaded breathing were analyzed using repeated measures ANOVA. Values are means ± SEM, and $P < 0.05$ was considered significant. Statistical analyses were performed with SPSS 16.0 (SPSS, Chicago, IL, USA).

RESULTS

Baseline characteristics of the subjects (male/female 23/7; age 22 ± 0.4 yrs; bodyweight 74 ± 1 kg; and body mass index 23 ± 0.4 kg/m^2) were not different between the placebo and levosimendan group. All subjects (n=30) received study medication as dictated by the protocol. One subject was excluded due to technical problems with the esophageal catheter. Two subjects were not able to maintain targeted Pdi (less than 30% of Pdimax) during the first loading task and were excluded from data analysis. The remaining subjects (placebo n=13 and levosimendan n=14) were able to keep their targeted Pdi during both loading tasks (44 ± 1% and 43 ± 2%), indicating compliance to the protocol. SpO_2 was above 96% in all subjects during the study, without supplemental oxygen. There were no differences in $etCO_2$ between the placebo and levosimendan group during loaded breathing.

Furthermore, there were no serious adverse events during the experimental protocol. One subject, receiving levosimendan, experienced a mild degree of nausea after completion of the study.

Diaphragm contractility

Cervical magnetic stimulation of the phrenic nerve resulted in reproducible twitch pressures in all subjects. Data from magnetic stimulation and maximal inspiratory maneuvers, as well as hemodynamic data, are given in Table 1. There was no significant difference in Pdi_{tw} and Pdi_{max} between the placebo and levosimendan group before study medication.

Figure 2 shows a decreased contractile response of the diaphragm after the first loading task in a subject receiving placebo. On average, loaded breathing in subjects receiving placebo resulted in significant reductions in Pdi_{tw} (-9 ± 3 %, P=0.01) and Pdi_{max} (-5% ± 3, P<0.05). The group receiving levosimendan after the first period of loaded breathing revealed no significant decrease in Pdi_{tw} and Pdi_{max} (Figure 3 and Table 1).

Neuro-mechanical efficiency of the diaphragm

In Figure 4, representative tracings of EMGdi and Pdi are presented for the first loading task (before study medication) and the second loaded task (with study medication) for both groups. Neuro-mechanical efficiency of the diaphragm (Pdi/EMGdi) in the first loading task was not different between the placebo and levosimendan group (both 1.3 ± 0.2 $cmH_2O/\mu V$) and remained stable throughout the loading task (Figure 5). Neuro-mechanical efficiency during unloaded breathing and during the second loading task was improved by 21% ($P<0.05$, compared to the first loading task) in the levosimendan group, whereas neuro-mechanical efficiency was not affected in the placebo group (Figure 5). The improved neuro-mechanical efficiency in subjects receiving levosimendan sustained throughout the entire loading task.

Center frequency of diaphragm electrical activity

During both loaded breathing protocols, CFdi decreased over time ($P<0.001$; Figure E1 *online supplement*) in both groups. Administration of levosimendan resulted in a downward shift in baseline CFdi during the second loaded breathing protocol ($P<0.05$; Figure E1, *online supplement*), whereas placebo did not affect CFdi.

Respiratory effort sensation

Respiratory effort sensation increased over time ($P<0.001$) during loaded breathing, until a score of 16. Neither levosimendan nor placebo affected respiratory effort sensation.

DISCUSSION

The present study is the first to evaluate the effect of the calcium sensitizer levosimendan on human diaphragm function *in vivo*. In this double-blinded and randomized study an intervention of loaded breathing resulted in significant loss of diaphragm contractility in the placebo group but not in the levosimendan group, suggesting that levosimendan restored diaphragm contractility. Loaded breathing following drug administration was associated with improved neuro-mechanical efficiency of the diaphragm in the levosimendan group. Furthermore, levosimendan reduced baseline center frequency of diaphragm electrical activity. Levosimendan was well tolerated with negligible side effects.

Calcium sensitivity and muscle fiber conduction velocity: effects of levosimendan

Skeletal muscle force develops as intracellular calcium rises and binds to troponin C, resulting in conformational changes in the troponin complex, allowing interaction between actin and myosin to form force generating cross-bridges. Reuptake of calcium from the cytoplasm into the sarcoplasmic reticulum is a high energy consuming process (up to 40% of total energy expenditure). Thus, reduced sensitivity of the troponin complex for calcium requires higher levels of cytoplasmic calcium to generate the same amount of force, requiring higher energy consumption and elevated CO_2 production.

Levosimendan belongs to a relatively new class of drugs, the calcium sensitizers and is approved in more than 40 countries worldwide to improve cardiac function in patients with acute heart failure.[14] Although the loading dose of levosimendan in this study is higher than recommended for the treatment of acute heart failure,[14] the total dose of levosimendan administered is lower than used in clinical practice.

Calcium sensitization by levosimendan occurs through stabilization of the interaction between calcium and the troponin complex during muscle activation.[17] Recently, we have shown that

levosimendan improves *in vitro* calcium sensitivity of human diaphragm (skeletal) muscle fibers as well.[12;18] Improvement in calcium sensitivity will affect contractile efficiency of the diaphragm.[19] In the current study, loaded breathing reduced Pdi_{tw} in placebo treated subjects (Table 1 and Figure 3), which is consistent with previous data by Laghi and colleagues.[20] However, levosimendan reversed the development of fatigue, as Pdi_{tw} after loading in these subjects was not different from baseline. Neuro-mechanical efficiency of the diaphragm during loaded breathing, expressed as Pdi/EMGdi ratio, was enhanced by 21% following administration of levosimendan (Figure 5). A 21% improvement in the ability to generate inspiratory pressure for a given neural respiratory effort should be considered a clinically relevant improvement in contractility. *In vitro* studies have shown that impaired calcium sensitivity of force generation develops during muscle loading and may contribute to contractile failure.[21] This is in line with the data from the current study which show that levosimendan reverses the effects of diaphragm force loss (Table 1 and Figure 3).

Administration of levosimendan resulted in a downward shift of the CFdi curve during loaded breathing (figure E1, *online supplement*). The decrease in CFdi over time indicates the development of reduced muscle fiber conduction velocity; see the *online supplement* for details. Reduced muscle fiber conduction velocity is an increased difficulty of muscle fiber action potentials to propagate along the sarcolemma and into T tubuli.[22] Therefore, the downward shift in baseline CFdi in subjects receiving levosimendan most likely reflects a baseline decrease in muscle fiber conduction velocity. These data are in line with the established effects of levosimendan on K_{ATP} channel activation;[15] and that extracellular $[K^+]$ accumulation contributes to reduced muscle fiber conduction velocity.[23;24] Since administration of levosimendan improves neuro-mechanical efficiency of the diaphragm with 21%, it is unlikely that extracellular $[K^+]$ accumulation results in functional impairment of the

diaphragm. Moreover, preserving a hyperpolarized sarcolemma by activating K_{ATP} channels might even play a myoprotective role under conditions of metabolic stress.[25]

All in all, enhancing calcium sensitivity with levosimendan appears a rational and effective approach to improve respiratory muscle function, in particular in patients with imminent respiratory failure.

Clinical implications

Reduced force generation of the respiratory muscles has been demonstrated in numerous disorders, including chronic obstructive disease (COPD), congestive heart failure, pulmonary hypertension and patients on mechanical ventilation.[1-7] Today, there are no pharmacological interventions available to improve respiratory muscle function.

Aubier and colleagues have previously shown that dopamine [26] and aminophylline [27] improve contractility of the human diaphragm, although the effects of aminophylline are controversial.[28] Neither dopamine, nor aminophylline have been used in clinical practice to optimize respiratory muscle function, probably due to the small therapeutic window, with risk of severe side effects (aminophylline, dopamine) and the very short half life time (dopamine). Instead, the active levosimendan metabolite OR-1896 has a half life time of 70-80 hours resulting in clinical effect up to one week after 24 hour infusion.[29] More importantly, both aminophylline and dopamine improve contractility by elevating intracellular calcium concentration, resulting in an increased ATP utilization. As discussed previously [12], inorganic phosphate accumulation contributes to the development of muscle fatigue and depresses calcium sensitivity.[30] In addition, elevated energy expenditure increases CO_2 production, requiring a higher level of ventilation. This limits the clinical utility of these drugs for the improvement of respiratory muscle function in patients with imminent ventilatory failure. Rather, calcium sensitizers improve muscle contractility without elevating energy

expenditure.[31] In addition, more efficient breathing may fasten liberation from mechanical ventilation.[32] This may be important for patients with respiratory failure such as acute exacerbation of COPD. Of note, our previous *in vitro* work showed that the effect of levosimendan is more profound in type-1 diaphragm fibers than in type-2 fibers.[12] Because a fiber type shift towards type-1 fibers is known to occur in the diaphragm of patients with COPD [8] and congestive heart failure [33], levosimendan treatment can potentially be more effective in these patients than in healthy subjects. Accordingly, the rationale for evaluating the effect of levosimendan on respiratory muscle function in patients with respiratory failure appears highly appropriate.

Experimental model

The contractile performance of the diaphragm was evaluated using magnetic stimulation and maximal inspiratory efforts. Magnetic stimulation has a clear benefit over voluntary maneuvers because it is effort independent and highly reproducible.[34] Values for Pdi_{tw} and Pdi_{max} reported in our study are similar as reported by other groups for healthy subjects.[20;35] Due to coincidence, baseline Pdi_{tw} as well as CMAPdi amplitude were lower (though insignificant) in the levosimendan group (Table 1), suggesting these subjects were more difficult to stimulate. Correcting for CMAPdi amplitude abolished the baseline difference between the groups (data not shown).

To further evaluate the function of the diaphragm we measured neuro-mechanical efficiency of the diaphragm (Pdi/EMGdi) during inspiratory loaded breathing. We found that diaphragm neuro-mechanical efficiency at 40% of Pdi_{max} was constant over time and that CFdi decreased over time during loaded breathing, which are in line with previous data from Sinderby.[36] We also found that, despite evidence of twitch force loss, the diaphragm neuro-mechanical efficiency was not reduced in the placebo group during the second loaded breathing task.

Thus, the reductions in maximal contractility were not reflected in the neuro-mechanical efficiency during submaximal contractions. Regardless of the reason for this inconsistency, the double-blinded randomized design of the study should ensure that the finding of improved neuro-mechanical efficiency - only in the levosimendan group - was not due to bias, carry over effects, or other subjective influences.

For an accurate physiological measurement of EMGdi and CFdi during voluntary breathing it is necessary to control for changes in muscle-to-electrode distance, electrode positioning with respect to the muscle fiber direction and location, influence of cross-talk from other muscles (including the heart and the esophagus) and electrode movement-induced artifacts. The technology used to measure and process the EMGdi and CFdi in our study minimizes all these influences.[37;38]

It could be postulated that improved contractile function of the diaphragm resulted from cardiac inotropic effects of levosimendan. However, subjects in the present study did not have a medical history of cardiac disease and the effect of levosimendan on cardiac output was modest. Development of Pdi_{tw} occurs through a sharp anaerobic maneuver of the diaphragm; therefore it is unlikely that an improved cardiac output (and oxygen delivery) would explain the restored diaphragm contractility with levosimendan. Moreover, levosimendan improved efficiency already during unloaded breathing and immediately at the start of the loading task, which is unlikely the result of improved oxygen delivery to the diaphragm

The dose of levosimendan used in the current study was derived from earlier studies in healthy subjects, demonstrating limited side effects.[39] This is in line with our study. Future studies should evaluate the effects of lower doses of levosimendan on respiratory muscle function in humans.

In conclusion, the present study demonstrates that the calcium sensitizer levosimendan improves both contractile function and neuro-mechanical efficiency of the human diaphragm. These findings suggest a new therapeutic approach for patients with acute respiratory muscle dysfunction.

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LEGENDS

Table 1: Diaphragm contractility and hemodynamic variables at baseline and after loading + bolus administration. Twitch transdiaphragmatic pressure (Pdi_{tw}), compound muscle action potential of the diaphragm (CMAPdi), maximum transdiaphragmatic pressure (Pdi_{max}), and maximum electromyographic activity ($EMGdi_{max}$) were measured before the first loading task and after bolus administration of study medication. Mean arterial pressure (MAP), heart rate (HR) and cardiac output (CO) were measured directly before and after bolus administration. Cardiac output has only been measured in n=2 (placebo group) and n=4 (levosimendan group). Data are presented as mean ± SEM. Abbreviations: * Significantly different from before study medication ($P < 0.05$).

Figure 1: Schematic description of the protocol. Abbreviations: Pdi_{tw} = twitch transdiaphragmatic pressure; Pdi_{max} = maximum transdiaphragmatic pressure.

Figure 2: (A) the compound muscle action potential of the diaphragm (CMAPdi) and (B) twitch transdiaphragmatic pressure (Pdi_{tw}) elicited by cervical magnetic stimulation before (black solid line) and after the first loading task (blue striped line) in a subject receiving placebo. In response to equal diaphragm activation (i.e. equal CMAPdi), there is decreased contractility after loaded breathing. Note the difference in scale of the x-axis between figure A and B.

Figure 3: Percentage change in transdiaphragmatic twitch pressure (Pdi_{tw}) from baseline after loading + bolus administration in the placebo (filled squares) and levosimendan (open circles)

group. Data are presented as mean ± SEM (placebo n=13, levosimendan n=14). *Significantly different from baseline (P=0.01) and levosimendan group after loading + bolus administration (P=0.01).

Figure 4: Diaphragm electromyography (EMGdi) and transdiaphragmatic pressure (Pdi) during the first (black) and second loading task (blue) in a subject receiving placebo (A and B) and levosimendan (C and D). Subjects were asked to target 40% of Pdi_{max}. In the subject receiving placebo there is no change in EMGdi (i.e. neural activation), whereas the subject receiving levosimendan shows decreased neural activation and thus increased neuromechanical efficiency of the diaphragm.

Figure 5: Neuro-mechanical efficiency of the diaphragm (Pdi/EMGdi) before, during (at one, three, six, and nine minutes) and after the first and second loading task in the placebo (A) and levosimendan (B) group. Administration of levosimendan resulted in an increase in diaphragm efficiency ($P<0.05$) during loaded and unloaded breathing, whereas placebo had no effect on diaphragm efficiency. Data are presented as mean ± SEM (placebo n=13, levosimendan n=14).

FIGURES AND TABLES

Table 1

	Placebo (n=13)		Levosimendan (n=14)	
	baseline	after loading + bolus administration	baseline	after loading + bolus administration
Pdi_{tw} (cmH_2O)	35 ± 2	32 ± 2 *	30 ± 2	31 ± 2
CMAPdi (mV)	1.3 ± 0.2	1.3 ± 0.2	1.0 ± 0.1	1.1 ± 0.1
Pdi_{max} (cmH_2O)	130 ± 8	122 ± 7 *	123 ± 11	120 ± 8
$EMGdi_{max}$ (µV)	76 ± 9	81 ± 10	77 ± 98	74 ± 10
MAP (mmHg)	92 ± 2	89 ± 4	90 ± 3	86 ± 4
HR (bpm)	71 ± 3	73 ± 3	68 ± 2	83 ± 3 *
CO (l/min)	4.5 ± 1.3	4.6 ± 0.4	6.2 ± 0.7	7.0 ± 0.7 *

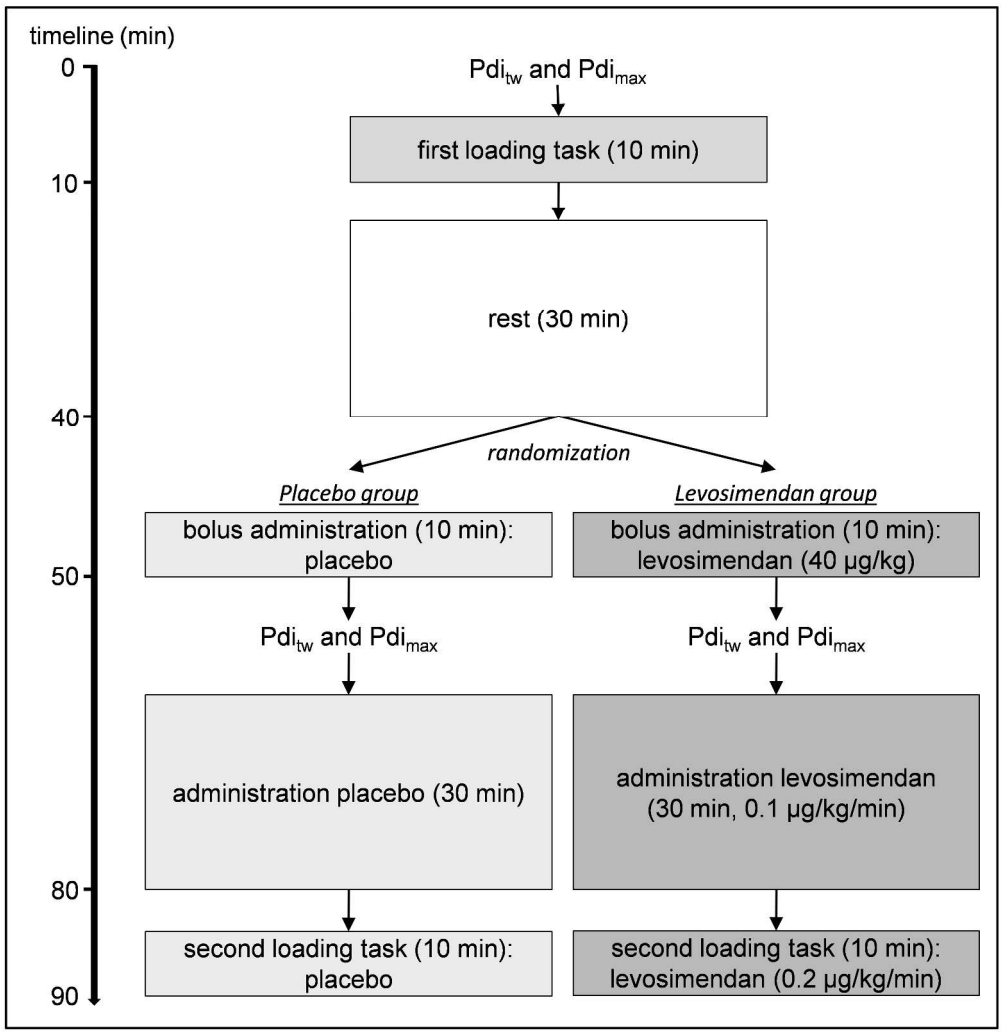

Figure 1
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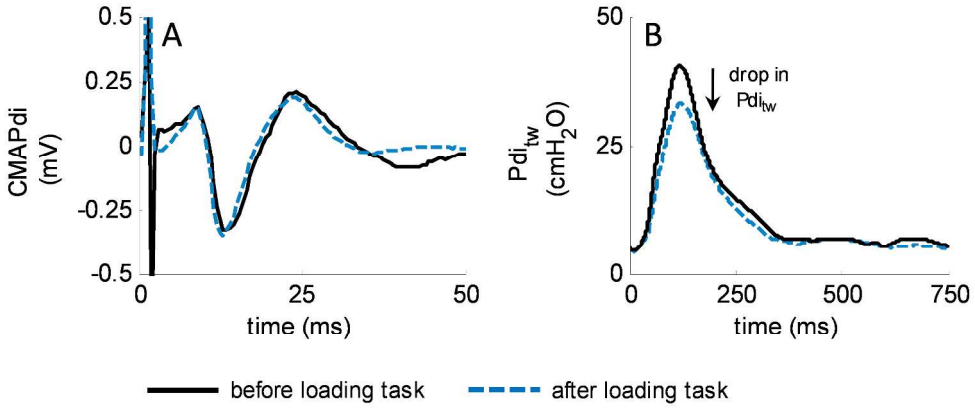

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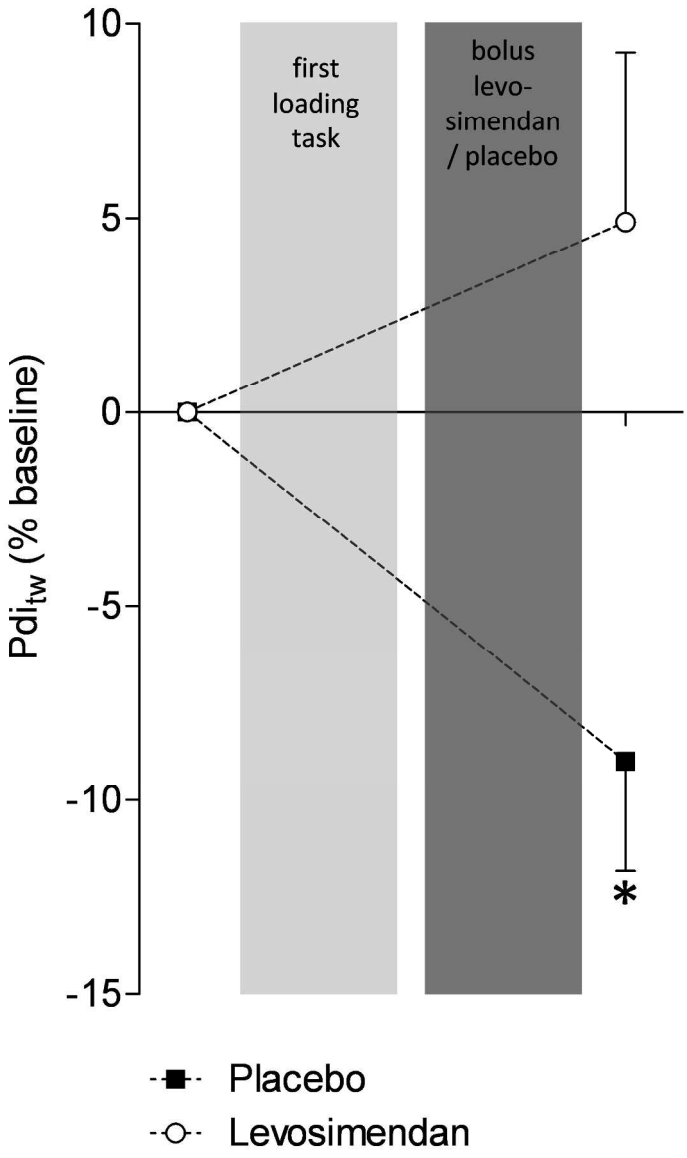

Figure 3
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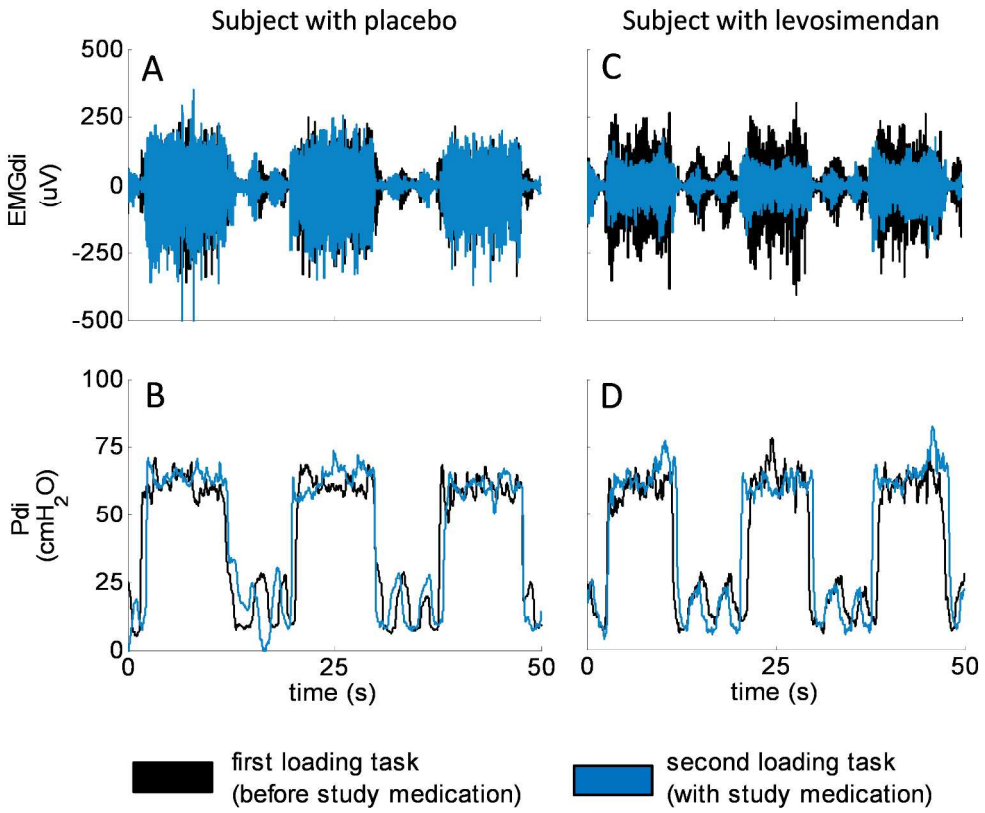

Figure 4
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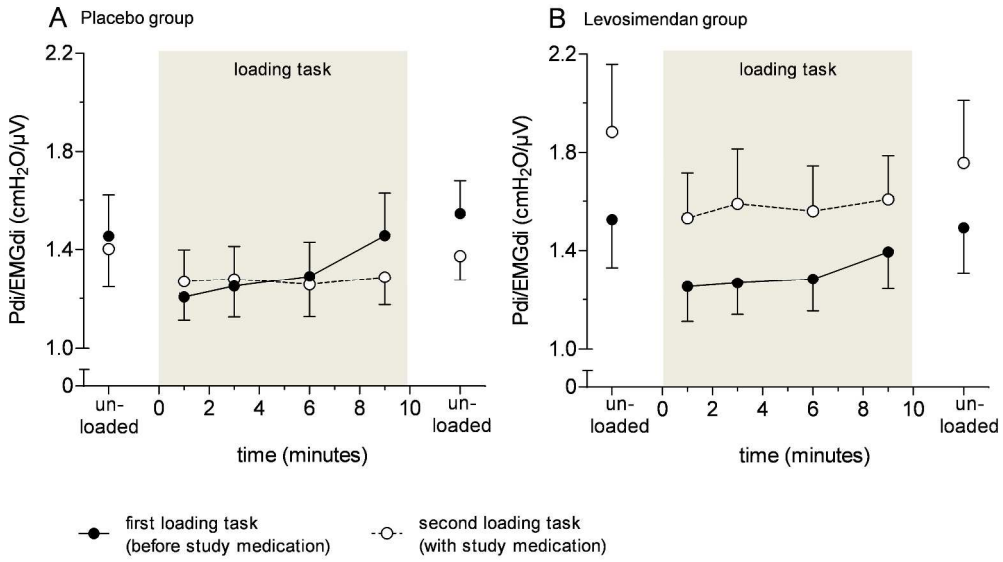

Figure 5
1131x646mm (96 x 96 DPI)

The calcium sensitizer levosimendan improves human diaphragm function

Jonne Doorduin, Christer A Sinderby, Jennifer Beck, Dick F Stegeman, Hieronymus WH van Hees, Johannes G van der Hoeven, and Leo MA Heunks

Online supplement

EXTENDED METHODS

Randomization and masking

Healthy subjects were randomly assigned to receive either levosimendan or placebo (ratio 1:1). The randomization code list, with a block size of six, was generated by an independent investigator of the Radboud University Nijmegen Medical Centre. The investigator and subject were masked to treatment. Both the active compound (levosimendan) and the placebo were similar in appearance (yellow solution) and could not be distinguished from one another. Levosimendan and placebo were labelled and stored by the department of Clinical Pharmacology of the Radboud University Nijmegen Medical Centre.

Esophageal catheter and its positioning

Diaphragm electromyogram (EMGdi) was measured via an esophageal catheter (NeuroVent Research Inc, Toronto, Canada) with nine stainless steel wire electrode rings placed 16 mm apart on 8 French polyurethane triple lumen tubing (2.5 mm diameter) with the tenth ring, the ground, placed 4 cm above the most proximal ring, creating an array of eight sequential electrode pairs. Two 4 cm long, 1.5 cm diameter polyurethane balloons were mounted 2.75 cm below the most distal ring and 1 cm above the ground ring for the measurement of gastric (Pga) and esophageal (Pes) pressures, respectively. The catheter was passed through the nose, swallowed, and the electrodes were positioned near the gastroesophageal junction.[E1;E2] The balloons were connected to two differential pressure transducers (range ± 50 kPa, Freescale, Tempe, AR) via two PVC tubes (0.75 mm diameter) inserted in each pressure lumen. The balloons were filled with 1.5 ml of air in order to distend the walls of the balloons evenly. Following this, air was withdrawn until the desired amount (0.3 – 0.4 ml) remained in the balloon.

Magnetic stimulation and maximal inspiratory effort

Cervical magnetic stimulation of the phrenic nerve was performed using a 90 mm circular coil (P/N 9784-00) powered by a Magstim 200^2 stimulator (Magstim Company Ltd, Whitland, UK). While seated with their neck flexed, a closed mouth and wearing a nose clip, magnetic stimulation was performed at functional residual capacity (FRC) with unbound abdomen. Stimulation position was determined and marked according to Similowski and colleagues. [E3] Subsequently, the increase in twitch Pdi (Pdi_{tw}) and compound muscle action potential of the diaphragm (CMAPdi) were measured at 100% Magstim output as the mean values of three twitches at least 30 seconds apart.

Pressure generating capacity of the diaphragm can be assessed by different techniques, including Pdi_{sniff} and maximal inspiratory effort against occluded airway. The latter technique was selected as this value was needed to calculate the load during the loading task (i.e, 40% maximal Pdi).

Data acquisition

EMGdi signals were amplified and digitized (Porti 16, 22 bits, 71.5 nV/least significant bit, TMSi;The Netherlands) at a sampling frequency of 2 kHz. Pressure signals were digitized (Porti 16, 22 bits, 1.4 μV/least significant bit, TMSi; The Netherlands) at a sampling frequency of 100 Hz. Data were stored and buffered on a hard disk for offline analysis in Matlab (R2009b, The Mathworks, Natick, MA) and to provide visual feedback.

Signal processing

EMGdi

The segments of EMGdi used in the analysis were automatically selected between the electrocardiogram R-R intervals to avoid contamination of the QRS complex. The relative

position of the centre of the electrical active region of the contracting diaphragm with respect to the electrode array was determined.[E1;E2] The "double subtraction technique" was then applied to enhance EMGdi signal quality.[E2] The double subtracted signal was converted from the time domain into the frequency domain by fast Fourier transform. Signal quality was evaluated from the power spectrum according to Sinderby and coworkers.[E4] For those signals deemed to be of acceptable quality, we calculated EMGdi (as the root mean square [RMS]) from the time domain and center frequency of the diaphragm (CFdi) from the EMGdi power spectrum. During loading, EMGdi and CFdi were calculated as means per 10 second isometric contraction.

CMAPdi

Compound muscle action potentials of the diaphragm (CMAPdi) were recorded from the electrode pair with the largest amplitude. Visual inspection for each individual CMAPdi was performed to avoid contamination of the QRS complex or motion artifacts. CMAPdi amplitude was calculated as the maximal displacement from negative to positive peak value. Mean values of three measurements were calculated.

Pressure tracings

Twitch transdiaphragmatic pressure (Pdi_{tw}) and maximum transdiaphragmatic pressure (Pdi_{max}) were calculated as the displacement from baseline to peak value. Mean values of three measurements were calculated. During loading, voluntary Pdi was calculated as means per 10 second isometric contraction.

Relation between center frequency and muscle fiber conduction velocity

As reported previously,[E5] there are strong reasons to assume that changes in CFdi, as measured in the present study, represent changes in muscle fiber conduction velocity. The muscle fiber conduction velocity depends on the cable properties of the fiber, which remain relatively stable during muscle contractions,[E6] and the membrane excitability.[E7,E8] The latter depends on ion gradients across the membrane generating the driving electric force and the properties of the proteins making up the gating ion channels. If the electrolyte imbalance across the sarcolemma leads up to a slight depolarization of the membrane, inactivation of the voltage-dependent sodium channels occurs, reducing membrane excitability and slowing propagation of the action potentials. [E7-E9] Proportionality between APCV and the power spectrum frequency shift has been theoretically described in detail,[E1,E10] and more recently described in a review as a method to estimate muscle fiber conduction velocity.[E11]

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LEGENDS SUPPLEMENT

Figure E1: Center frequency of the diaphragm electromyogram (CFdi) at one, three, six, and nine minutes into the first and second loading task in the placebo (A) and levosimendan (B) group. CFdi decreased over time ($P<0.001$) in both loading tasks in the placebo and levosimendan group. Administration of levosimendan resulted in a downward baseline shift in CFdi ($P<0.05$), whereas administration of placebo had no effect on CFdi. Data are presented as mean ± SEM (placebo n=13, levosimendan n=14).

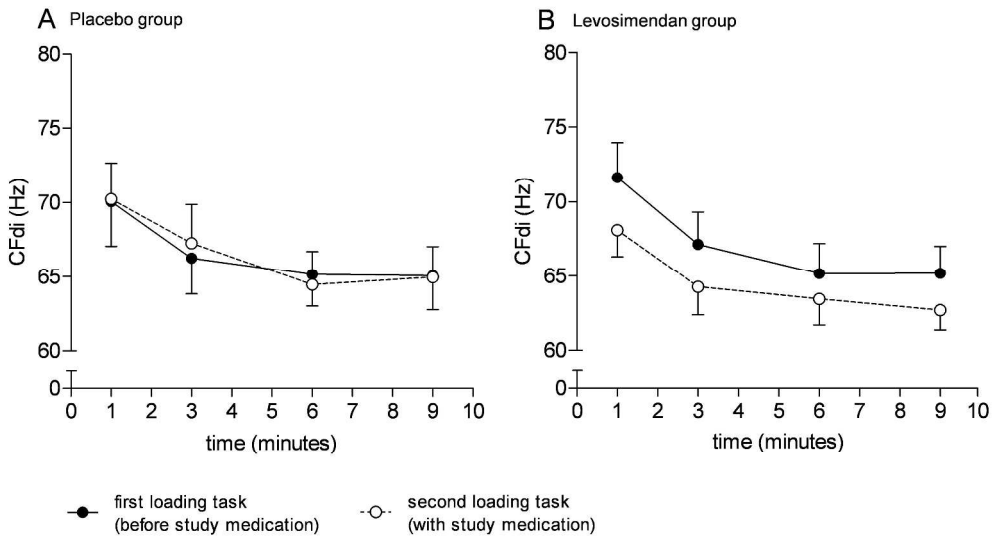

Figure E1
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