ARTICLE

Preferential vasodilator effects of levosimendan in resistance pulmonary arteries in a rodent pulmonary embolism model

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Abstract

Background: We compared the vasoactive effects of levosimendan on isolated conduit pulmonary arteries (CPA) and resistance pulmonary arteries (RPA) *versus* mesenteric arteries, and we assessed the pulmonary artery (PA) vascular function and the PA vasoactive effects of levosimendan in a rodent pulmonary embolism (PE) model.
Methods: One group of male Wistar rats (200-300 g) was killed by decapitation to obtain pulmonary and mesenteric rings. Another group was assigned to a massive PE or saline solution infusion. After euthanasia, mesenteric arteries and CPA (1-2 mm) and RPA (≤0.5 mm) were dissected and cut into 2-3 mm wide rings, recording contractile tension. We obtained the concentration-response curves of cumulative doses of levosimendan on pre-contracted arterial rings from decapitated and sham/embolized animals. A set of RPA rings was exposed to acute hypoxia. The effect of PE on the pulmonary vasoactive function was assessed by dose-response curves of acetylcholine (ACh) and endothelin-1 (ET-1) of PA rings from sham/embolized animals.
Results: Levosimendan relaxant potency of RPA was similar to that of mesenteric arteries and higher than CPA, while mesenteric rings showed the maximal relaxant effect, followed by RPA and CPA, respectively. PE did not affect the vasoactive response of PA rings either to ACh or to ET-1, and the relaxant effects of CPA and RPA to levosimendan were also preserved. Acute hypoxia reduced ($p<0.05$) but did not avoid the RPA relaxant effect of levosimendan.
Conclusions: Levosimendan is a more specific vasodilator of RPA with a similar relaxant potency as mesenteric arteries, which is preserved after PE but significantly reduced during hypoxia.

Introduction

The pulmonary vasculature consists of large, elastic, extra-parenchymal conduit pulmonary arteries (CPA, order 1 to 2) that arise from the sixth aortic arch and small muscular resistance intrapulmonary arteries (RPA, ≥4th order), that originate from the mesenchymal lung bud by capillary plexus expansion.[1] This subdivision is associated with different responses to several stimuli. While CPA dilates or fails to constrict to hypoxia, RPA is responsible for hypoxic pulmonary vasoconstriction, control the regional distribution of blood flow and largely determines pulmonary vascular resistance. This functional difference mainly depends on the distribution of electrophysiologically distinct myocytes in CPAs and RPAs arteries.[1,2]

Levosimendan is a positive inotropic agent (by increasing the sensitivity of troponin C to calcium) with vasodilating properties (by lowering intracellular free Ca^{++}, the opening of different potassium channels and the inhibition of phosphodiesterase type III), also termed inodilator.[3,4]

There are several animals studies in different acute pulmonary hypertension (PH) models secondary to thromboxane A_2 infusion,[5] endotoxemia,[6] acute pulmonary embolism (PE),[7,8] and hypoxia,[9] and some clinical studies that demonstrated the

vasodilator effect of levosimendan on the pulmonary circulation, restoring right ventricular-arterial coupling as it increased right ventricular contractility concomitantly.[10,11] Acute PE-induced PH results from two main mechanisms: the mechanical obstruction of pulmonary vessels (passive mechanism) and the arterial vasoconstriction secondary to pulmonary neurohumoral activation (*e.g.*, thromboxane A2, serotonin), neurogenic reflex, increased oxidative stress and hypoxemia (active mechanism).[12] There is experimental evidence that secondary pulmonary vasoconstriction is the major contributor to the potentially fatal increase in pulmonary dynamic afterload in PE. Therefore the treatment should not only focus on removing the obstructing blood clot but also on reducing this vasoconstrictor response.[13] We have previously reported a significant decrease of the dynamic afterload despite the persistence of mechanical obstruction by clots during levosimendan infusion in a PE-induced PH ovine model.[14] The concomitant decrease in total pulmonary vascular resistance with the preservation of the pulmonary arterial characteristic impedance suggests a predominant vasodilator effect on distal arterial vessels.[14]

We hypothesized that levosimendan would have a different vasodilator potency between CPA and RPA, with a predominant distal vasodilatation effect. The aims of the present work were: to compare the vasoactive effects of levosimendan on isolated CPA and RPA *versus* mesenteric arteries of rats; to assess the pulmonary arterial vasoconstriction and vasodilatation response and the pulmonary arteries vasoactive effects of levosimendan in a rodent PE model and to assess the effects of hypoxia on the RPA relaxant responses to levosimendan.

Materials and methods

This study was approved by the Institutional Animal Care and Use Committee (CHEA, Facultad de Medicina, Universidad de la República. N° 070153-000611-13. http://www.chea.ude-lar.edu.uy/). We rigidly performed all institutional protocols to handle animals under experimentation according to the Guide for Care and Use of Laboratory Animals (NIH Publication N° 85-23, revised 1996), prepared by the National Academy of Sciences' Institute for Laboratory Animal Research.

Male Wistar rats (200-300 g) were used in this study. Rats fasted overnight with free access to water. One group of animals was killed by decapitation, and the lungs and mesenteric beds were rapidly immersed in Krebs solution to obtain pulmonary and mesenteric rings. Another group of animals was anesthetized with pentobarbital [40 mg/kg, intraperitoneal (i.p.)] and fentanyl (50 mg/kg, i.p.). Both were maintained with pentobarbital (10 mg/kg/h), and fentanyl (1-2 mg/kg/h) administered intravenously throughout an infusion pump (syringe pump, Graseby 3400™, Smiths-Medical, Minneapolis, MN, USA). Normothermia was kept using a heating pad. The animals were tracheotomized and mechanically ventilated (ServoVentilator™ model 300, Siemens AG, Munich, Germany). The tidal volume and the fraction of inspired oxygen were set at 8 mL/Kg and 60%, respectively. The respiratory rate was adjusted to maintain a baseline physiologic arterial oxygen and carbon dioxide tension. Blood samples were taken regularly (every 30 min) to analyze arterial oxygen and carbon dioxide tension (blood gas analyzer, Radiometer ABL520™, Copenhagen, Denmark).

We placed two 22G fluid-filled catheters into both external jugular veins for blood withdrawal and drug infusion, respectively. Another fluid-filled catheter was positioned into the common carotid artery to monitor systemic arterial pressure. We performed a sternotomy and placed a 22G fluid-filled catheter into the PA through a minimal stab in the right ventricular outflow. The distal tip of the catheter was positioned in the main PA before its bifurcation. All pressure transducers (P23Db Gould Statham, Hato Rey, PR, USA) were zeroed to atmospheric pressure at the mid-axillary level.

Once we completed the instrumentation, the animals were allowed to stabilize for 15 minutes. Baseline hemodynamic data were obtained.

Animals were divided into two groups:

I. Sham group (saline solution, n=8): mL of saline infusion. Animals were euthanized once they reached 60 minutes.
II. Embolized group (PE, n=8): 1 mL of blood was collected and allowed to clot at room temperature for five minutes.

Clots were mechanically disaggregated, achieving a diameter ~0.5 mm and venous embolization was carried out progressively through the right jugular vein every 15 min over 60 min until systemic hypotension was reached, ensuring to produce a massive PE. Once hypotension developed, the animal was euthanized with an overdose of pentobarbital. After euthanasia, heart and lungs were removed, and lungs were rapidly placed in Krebs solution.

Tissue preparation and contractile tension recording

Mesenteric arteries (i.d. 1-2 mm), CPA (i.d. 1-2 mm, second order) and RPA (i.d. ≤0.5 mm, fifth order) were carefully dissected from surrounding tissue and cut into 2-3 mm wide rings for studies on intact preparations.[15] During manipulation of the arteries, care was taken not to touch their intimal surface to preserve the endothelium layer.

The rings were suspended between two wire hooks in 5 mL organ baths for contractile tension recording with an isometric transducer (myograph model 610M, Danish Myo Technology, Aarhus, Denmark; for mesenteric arteries and RPA rings and KG-20 force transducers, World Precision Instruments, Sarasota, FL, USA for CPA rings).[16] The organ baths contained Krebs solution maintained at 37°C and continuously bubbled with a 95% O_2 − 5% CO_2 mixture as previously described. Tissues were stretched to a predetermined optimal resting tension of 0.5 g for pulmonary rings and 2.0 g for mesenteric rings.[15] The presence of functional endothelium was tested by the assessment of the relaxant response to acetylcholine (ACh; 10^{-6} M) in rings pre-contracted with 5HT (10^{-5} M). The ability of ACh to induce relaxation was taken as an indicator of the presence of functional endothelium. We discarded those rings with a relaxant response ≤20% of maximal tension. Total time of the experiments never exceeded

120 min. Based on previous experiments there was no spontaneous relaxation in pre-contracted rings during this period.

Experimental protocol

Six to eight mesenteric and PA rings were obtained from each animal. The rings were first stimulated by raising the K^+ concentration of the buffer to 80 mM and then washing three times, allowing to recover the resting tension.

Comparative analysis of the effects of levosimendan on mesenteric arteries, RPA, and CPA rings: after reaching a stable tension, the rings were contracted with a mixture of 3×10^{-9} M ET-1, 3×10^{-8} M thromboxane A_2 mimetic U46619 and 3×10^{-6} M 5HT. This mixture allowed us to avoid possible vasoactive response differences among arteries depending on the vasoconstrictor used,[15] and mimics several forms of PH including PE, achieving a high vascular tone that presumably reflects what happens in massive PE.[17] Once equilibration (about 25 min), a concentration-response curve to levosimendan (10^{-9} to 3×10^{-5} M) was carried out by cumulative addition of drug after a steady-state relaxant response was reached after each increment. The range of levosimendan doses and the interval between doses (7 min) are based on previous experiments.

Assessment of the PA vascular vasoconstrictor and vasodilation properties: the analysis of the endothelium function was performed through concentration-response curves of cumulative doses of acetylcholine (ACh, 10^{-9} to 10^{-5} M) in rings previously contracted by serotonin (5HT, 10^{-5} M). The vascular smooth muscle function was assessed by concentration-response curves of cumulative doses of endothelin-1 (ET-1, 10^{-11} to 10^{-8} M).

Assessment of the effects of hypoxia on levosimendan RPA relaxant responses: a set of RPA rings extracted from euthanized rats by decapitation was exposed to acute hypoxia. Hypoxia was induced by aerating the organ bath with 95% N_2-5% CO_2 (PO_2 24±1 mmHg)[18] and then we obtained the dose-response curve to levosimendan.

Drugs and reagents

Acetylcholine chloride, serotonin hydrochloride, endothelin-1 and thromboxane A2 mimetic U46619 and dimethyl sulphoxide (DMSO) were obtained from Sigma-Aldrich, SPAIN. Levosimendan was obtained from Sigma Chemical Co, St. Louis, MS, USA. They were dissolved in distilled deionized water except for levosimendan which was dissolved in DMSO. The final concentration of DMSO in the organ bath was less than 0.1% and had no effect on the vessel reactivity. The concentration of drugs was expressed as a final molar concentration in the tissue chamber.

Data analysis

The maximal vasoactive effect (Emax, expressed as a percentage of the initial contractile response), which is an index of the efficacy of the vasoactive drug, and the drug concentration exhibiting 50% of the Emax (EC_{50}, expressed as negative logarithmic concentration, -log EC_{50}: pD_2), which is an index of the potency of the vasoactive drug, were calculated from the fitted concentration-response curves for each ring. Data were averaged for each animal in all experiments. Relaxation responses to levosimendan are expressed as percentages of tension developed with KCl 80 mM.

Statistical analysis

Results are expressed as the mean±standard error of the mean (SEM), with n equal to the number of animals. Individual cumulative concentration-response curves were fitted assuming the sigmoid dose-response curves (Levenberg-Marquardt algorithm) by using Origin Pro Software (version 9.1, San Diego, CA, USA). For multiple comparisons (*e.g.* the vasoactive effects of levosimendan on the mesenteric arteries, CPA and RPA), statistical analysis was performed using a one-way ANOVA followed by a Bonferroni *post hoc* test, otherwise (*e.g.* rings from sham *versus* PE rats, CPA *versus* RPA rings and normoxic *versus* hypoxic RPA rings) we used a two-tailed unpaired Student's *t*-test. Differences were considered statistically significant when $p < 0.05$.

Results

Vasodilator effects of levosimendan

Levosimendan induced a concentration-dependent relaxation but was unable to fully relax 3×10^{-9} M ET-1, 3×10^{-8} M thromboxane A_2 mimetic U46619 and 3×10^{-6} M 5HT-induced contractions in mesenteric as pulmonary rings. The highest concentration tested (3×10^{-4}) produced a maximal relaxation of 85±6% of the mesenteric arteries. RPA and mesenteric rings showed the maximal relaxant potency, while the highest maximal relaxant effect was obtained in mesenteric arteries followed by RPA and CPA, respectively ($p < 0.05$) (Table 1, Figure 1).

Effects of pulmonary embolism on pulmonary artery vasodilatation and vasoconstriction response

Table 2 shows systemic and pulmonary arterial pressures of sham and embolized animals. Neither the endothelium function (Sham: pD_2 6.9±0.07 and Emax 20±7% *vs*. PE: pD_2 7.1±0.09 and Emax 21±17%) nor the vascular smooth muscle function (Sham: pD_2 8.4±0.2 and Emax 94±11% *vs*. PE: pD_2 8.6±0.2 and Emax 90±17%) of the PA rings from embolized rats was changed in comparison of PA rings from sham animals (Figure 2).

Effects of levosimendan on conduit and resistance pulmonary arteries from embolized rats

The relaxant effect of levosimendan was not significantly affected by PE. Like in the sham group, rings from embolized rats showed a significantly higher levosimendan potency in RPA rings in comparison to CPA rings (Table 3, Figure 3).

Effects of hypoxia on the resistance pulmonary arteries relaxant responses to levosimendan

Figure 4 shows the effects of hypoxia on the RPA relaxant responses to levosimendan. Exposure to hypoxia induced a significant decrease in the relaxant potency of levosimendan in RPA rings with similar efficacy (Emax) (normoxia: pD_2 7.11±0.06 and Emax 65±5% *vs.* hypoxia: pD_2 6.03±0.09 and Emax 65±7%).

Table 1. Parameters (pD_2 and Emax) of the concentration-response curves of mesenteric arteries, conduit, and resistance pulmonary arteries to levosimendan calculated from Figure 1.

	Conduit PA	Resistance PA	Mesenteric artery
Emax %	55±5	70±6*	85±6*°
pD_2	5.71±0.22	7.00±0.21*	6.57±0.13*
No.	8	8	3

PA, pulmonary arteries.
pD_2 is the negative logarithm of concentration which relaxed 50% and Emax is the maximal relaxant effect achieved with the highest concentration of levosimendan tested. *p<0.05 *vs.* conduit PA; °p<0.05 *vs.* resistance PA.

Table 2. Hemodynamic data of sham and pulmonary embolized rats.

	Sham	Pulmonary embolism
mPAP, mmHg	13±2	26±3*
mPAo, mmHg	96±12	43±11*
No.	8	8

mPAP, mean pulmonary arterial pressure; mPAo, mean aortic pressure.
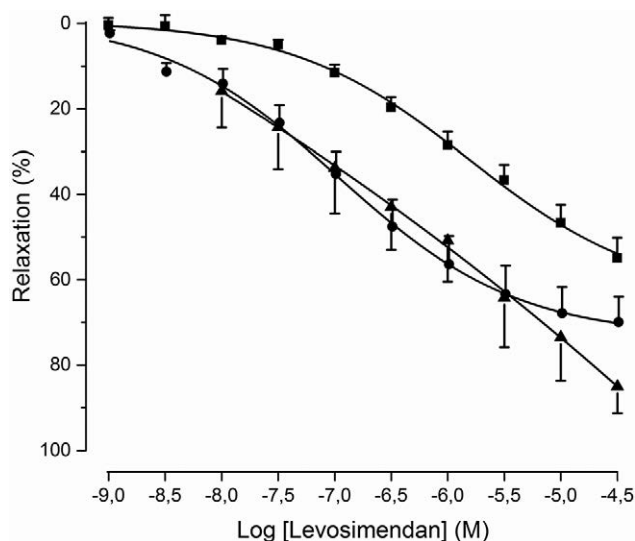*p<0.05 *vs.* sham.

Figure 1. Relaxant effects of cumulative doses of levosimendan on mesenteric arteries (triangles), conduit (squares), and resistance (circles) pulmonary arteries pre-contracted with a mixture of $3×10^{-9}$ M endothelin-1, $3×10^{-8}$ M thromboxane A2 mimetic U46619 and $3×10^{-6}$ M serotonin (n=3 for mesenteric rings, and n=8 for pulmonary arteries).

Discussion

In the present study, we compared the effect of levosimendan, a new class of inodilator drugs in isolated mesenteric and conduit and resistance pulmonary arteries of adult rats. We evaluated the effect of PE on the vascular smooth muscle vasoconstriction and endothelial vasodilatation response through the concentration-response curves to accumulating doses of ET-1 and ACh, respectively in PA rings from pulmonary embolized *versus* sham rats. Finally, we analyzed the relaxant effects of levosimendan on the proximal conduit PA *versus* distal resistance PA rings from sham and embolized rats, assessing the effect of hypoxia on the RPA relaxant effect of levosimendan, concomitantly. Our results indicate that: i) levosimendan produced different relaxant effects depending on the artery. Relaxant potency was higher in RPA and mesenteric arteries than CPA, while mesenteric rings showed the maximal relaxant effect (efficacy), followed by RPA and CPA, respectively; ii) PE did not affect the in vitro response of PA rings either to ACh (endothelial function) or ET-1 (vascular smooth muscle function), and the relaxant effects of CPA and RPA to levosimendan were also preserved; iii) hypoxia reduced but did not avoid the RPA relaxant effect of levosimendan.

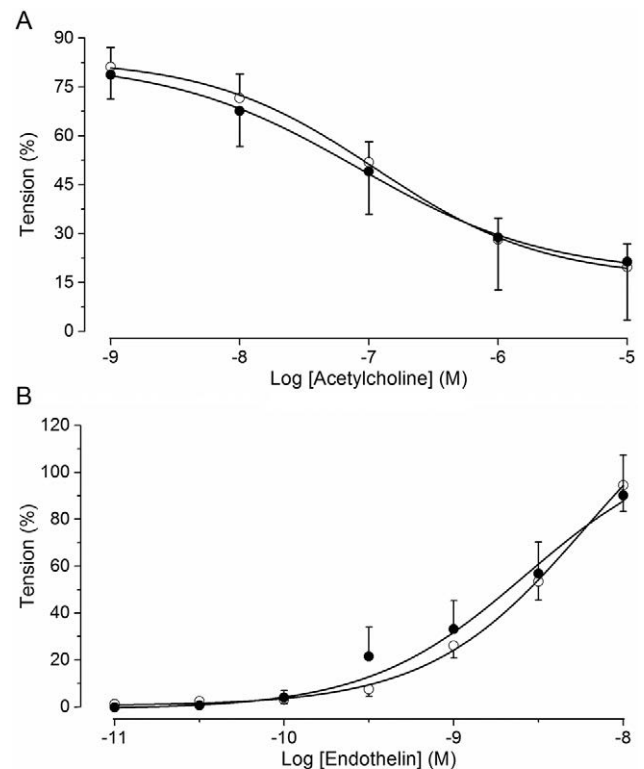The higher vasodilatation potency of levosimendan on RPA

Figure 2. Effects of acetylcholine (endothelium function) (A) and endothelin-1 (vascular smooth muscle function) (B) on pulmonary arterial rings of sham (open circles) and pulmonary embolized (filled circles) rats. The acetylcholine concentration-response curves correspond to pre-contracted rings by 10^{-5} M serotonin (% of tension developed with KCl 80 mM) (n=8).

versus CPA is consistent with our previous results in an ovine model of blood clot PE-induced PH. Briefly, we have demonstrated that levosimendan reduced the dynamic afterload increase in PE-induced PH with a decrease in total pulmonary vascular resistance (input impedance) and relative preservation of main PA impedance (characteristic impedance), suggesting a predominant vasodilator effect over distal resistive pulmonary vessels.[14]

The fact that PA rings without functional endothelium (≤20% of tension decrease during ACh exposition) showed similar dose-response curves of an accumulative addition of levosimendan, would make the levosimendan relaxant effects endothelium-independent, like in other vascular beds.[19,20] Mesenteric rings relaxation to levosimendan showed a similar dose-response parameters of other systemic vascular beds as the human internal mammary arteries (pD$_2$ 6.8±0.1 and Emax 75±5%).[20]

PE is associated with the release of vasoconstrictors and secondary pulmonary vasoconstriction, which adds to the mechanical obstruction. This vasoconstriction is at least partially reversed by different vasodilator therapy that attenuates PE-induced PH.[21] De Witt *et al*.[5] showed that levosimendan induces vasodilatation in the pulmonary vascular bed of the cat in thromboxane A$_2$-induced PH. Furthermore, levosimendan has been shown to prevent endotoxin-induced PH.[6] Wiklund *et al*.[9] showed that levosimendan attenuated hypoxic pulmonary vasoconstriction in a porcine model, suggesting that the pulmonary vasodilatation effect of the drug is apparent when PH is present. To the best of our knowledge, this is the first study on the comparative effects of levosimendan in isolated CPA and RPA rings in a rodent PE model.

The predominant distal vasodilator effect – RPA – of levosimendan with mild vasodilatory effect on CPA in the vessels from the PE animals could preserve the ventricular-arterial coupling and the proximal-distal PA coupling during the treatment of PE-induced PH through avoiding the increase of proximal arterial stiffness.[7,22] We have previously shown that proximal PA vasoconstriction induced by vascular smooth muscle activation improves both the buffering and conduit functions of the PA during acute PH is mainly due to the increase in wall viscosity, preventing increased wall stiffness secondary to the recruitment of collagen fibers.[23]

This different response to levosimendan could be linked to the differential distribution of electrophysiologically distinct smooth muscle cells in CPAs and RPAs arteries.[1] It is well known the role of smooth muscle contractile phenotypic diversity in the vascular system in determining the unique

Table 3. Parameters (pD$_2$ and Emax) of the concentration-response curves of conduit and resistance pulmonary arteries (PA) from sham and embolized animals to levosimendan calculated from Figure 3.

	Sham		Pulmonary embolism	
	Conduit PA	Resistance PA	Conduit PA	Resistance PA
Emax %	50±4	66±5	52±7	69±9
pD$_2$	5.83±0.2	6.85±0.19*	6.02±0.13	6.82±0.12*
No.	8	8	8	8

PA, pulmonary arteries.
pD$_2$ is the negative logarithm of concentration which relaxed 50% and Emax is the maximal relaxant effect achieved with the highest concentration of levosimendan tested. *$p<0.05$ *vs.* conduit PA.
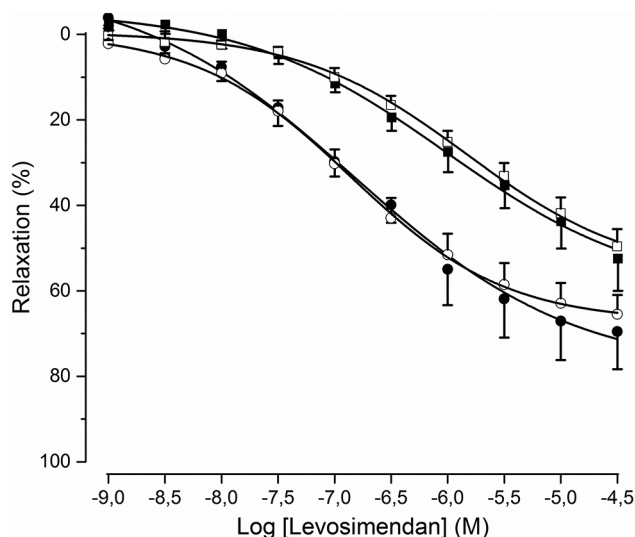

Figure 3. Relaxant effects of cumulative addition of levosimendan on conduit (squares) and resistance (circles) pulmonary rings pre-contracted with a mixture of 3×10^{-9} M endothelin-1, 3×10^{-8} M thromboxane A2 mimetic U46619 and 3×10^{-6} M serotonin from sham (open symbols) and embolized (filled symbols) animals (n=8).
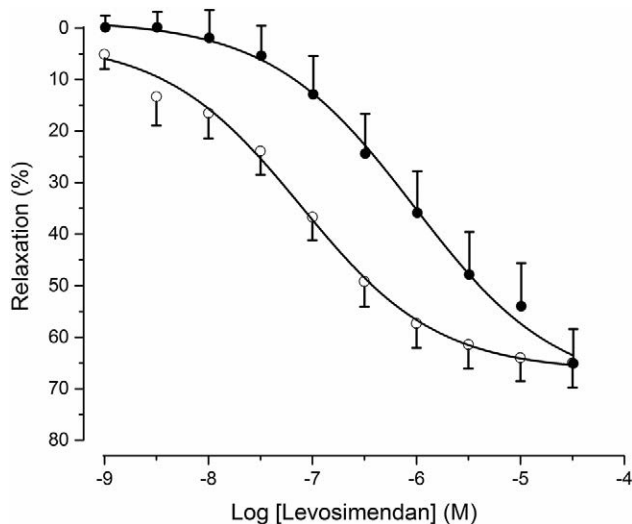

Figure 4. Relaxant effects of cumulative doses of levosimendan on resistance pulmonary arteries in normoxia (open circles) and hypoxia (filled circles) pre-contracted with a mixture of 3×10^{-9} M endothelin-1, 3×10^{-8} M thromboxane A2 mimetic U46619 and 3×10^{-6} M serotonin (n=6).

properties of selected regional circulations and their potential influence on drug targeting in disease.[24]

It is noteworthy that neither endothelium nor vascular smooth muscle dysfunction was observed in PA rings from rats embolized since both the increased oxidative stress and alterations in the availability of nitric oxide are implicated in the pathogenesis of PH associated with PE.[25,26] The lack of vascular dysfunction observed would be attributable to at least two considerations. One explanation would be that vascular dysfunction is observed *in vivo*, and once PA rings are isolated, their response *in vitro* to the diverse stimulus was similar to vessels from sham animals. Another explanation would be related with the lower time spent on the PE-induced PH on other PE models (from 180 minutes to several hours).[27,28] Anyway, if the activation of the inflammatory response with the release of vasoactive mediators and increase of oxidative stress play some role in the pathogenesis of PH associated with PE,[21,29] levosimendan would also have the advantage of attenuating the PE-induced PH through mechanisms involving antioxidant effects.[30,31]

The fact that exposure to hypoxia reduces the relaxant potency without entirely avoiding the RPA relaxant effect of levosimendan, would be an in vivo advantage since it would allow decreasing RV afterload (high relaxant potency in normoxic alveolar-capillaries units) without completely preventing the hypoxic vasoconstriction (main mechanism for distributing the pulmonary arterial flow to ventilated alveoli and preserving an adequate ventilation/perfusion ratio).

Lowering pulmonary arterial pressure while maintaining systemic vascular resistance and adequate cardiac output is crucial for several clinical scenarios like submassive and massive PE. Although levosimendan showed similar relaxant potency of both mesenteric and resistance pulmonary arteries which could be disadvantageous in clinical settings, there are different strategies to ameliorate the appearance of hypotension as to not use the loading dose and use in combination with other vasopressor agents.[14,32]

Limitations

Some limitations of our study should be taken into consideration. We used the same concentrations of the mixture of vasoconstrictors in pulmonary and mesenteric arteries and therefore, the vasodilator response of levosimendan was not evaluated under equi-effective concentrations of endothelin-1, U46619, and serotonin for a given artery. The effect of levosimendan on the whole systemic arterial tree is the sum of the effects in all vascular beds and, thus, extrapolation of mesenteric arteries to the whole systemic circulation has to be done with caution. We have not measured any oxidative stress parameters like plasma nitrite/nitrate concentrations and plasma lipid peroxide concentrations to demonstrate the presence of an increase in oxidative stress after lung embolization in PE group. Although we employed a relatively short period to embolize the lungs, a significant increase in plasma nitrite/nitrate concentrations were observed after 60 minutes

of lung embolization in a canine PE model.[29] We were careful to analyze several PA rings from different lobs of both lungs of each animal to obtain a representative sample beyond the final distribution of blood clots.

Conclusions

The results of the present study suggest that levosimendan is a more specific vasodilator of resistance PA with a similar relaxant potency as mesenteric arteries, which are preserved after PE but significantly reduced during hypoxia. Thus, levosimendan could reduce pulmonary dynamic afterload with lesser effects on conduit PA and the hypoxic vasoconstriction, improving the right ventricular-arterial coupling and proximal-distal vascular coupling, and preserve adequate ventilation/perfusion ratio, respectively, during PH treatment. Further studies are required to demonstrate the mechanisms of action of levosimendan in conduit and resistance pulmonary arteries.

Contributions

The authors contributed equally.

Conflict of interest

The authors declare no conflict of interest.

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Conference presentation

This study was was partly presented at the XVIII World Congress of Cardiology, Melbourne, Australia, May 2014 (Global Heart 2014;9:e182).

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