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Levosimendan and its metabolite OR-1896 elicit K_{ATP} channel-dependent dilation in resistance arteries *in vivo*

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Abstract

Background—Levosimendan and its long-lived metabolite OR-1896 produce vasodilation in different types of vessels by activating ATP-sensitive (K_{ATP}) and other potassium channels.

Methods—In the present study we applied intravital videomicroscopy to investigate the *in situ* effects of levosimendan and OR-1896 on the diameters of real resistance arterioles (rat cremaster muscle arterioles with diameters of ~ 20 μ m).

Results—Levosimendan and OR-1896 induced concentration-dependent (1 nM – 100 μ M) dilations to similar extents in these arterioles (maximal dilation from 23 ± 2 to 33 ± 2 μ m and from 22 ± 1 to 32 ± 1 μ m, respectively). The arteriolar dilations induced by the selective K_{ATP} channel opener pinacidil (1 nM – 10 μ M) (maximal dilation from 22 ± 4 μ m to 35 ± 3 μ m) were diminished in the presence of the selective K_{ATP} channel blocker – glibenclamide (5 μ M) (maximal diameter attained: 22 ± 1 μ m). Glibenclamide also counteracted the maximal dilations in response to levosimendan or OR-1896 (to 23 ± 3 μ m or 22 ± 5 μ m, respectively).

Conclusions—In conclusion, this is the first demonstration that levosimendan and OR-1896 elicit arteriolar dilation *in vivo*, via activation of K_{ATP} channels in real resistance vessels in the rat.

Keywords

levosimendan; OR-1896; cremaster muscle; vasodilation; K_{ATP} channels; intravital microscopy

Introduction

Levosimendan was developed as a Ca^{2+} -sensitizer drug. Its positive inotropic effect in the heart develops in combination with the vasodilation of peripheral and coronary arteries and

of veins [15]. We earlier demonstrated that OR-1896, a long-lived metabolite of levosimendan, is a strong vasodilator in *ex vivo*, cannulated skeletal muscle and coronary arteries of the rat, and identified OR-1896 as a probable mediator of the long-term cardiovascular effects of levosimendan [6]. The vasodilations induced by levosimendan or OR-1896 have been found to be mediated by ATP-sensitive potassium (K_{ATP}) channels and possibly other potassium channels (e.g., large-conductance Ca^{2+} -activated potassium (BK_{Ca}) channels and/or voltage-sensitive potassium channels) [6–8, 18, 19]. The relative contributions of the various potassium channels to the drug-induced vasodilation in the above *ex vivo* tested large-diameter preparations did not seem to be uniform, but was rather dependent on vessel type, vessel size and/or species. For example, in isolated porcine epicardial coronary artery, the vasorelaxing mechanism of levosimendan probably involved the activation of voltage-sensitive and BK_{Ca} channels to a significant degree [19]. Similarly, the activation of BK_{Ca} channels appeared essential for OR-1896-induced coronary arterial dilation in the rat [6]. In contrast, the activation of K_{ATP} channels predominated as concerns levosimendan-induced vasodilation in the coronary system of the guinea pig [8], human portal veins [18], human saphenous veins [7], the pulmonary system of the cat [4], the OR-1896 responses in rat skeletal muscle arteries [6]. The studies by Banfor et al. [3] and Segreti et al. [22] indicated that the infusion of rats with levosimendan or OR-1896 resulted in significant reductions in systemic peripheral vascular resistance, suggesting that these drugs exert their vasodilator effects predominantly on resistance-sized vessels. However, the vasomotor effects of levosimendan and OR-1896 on real resistance arteries *in vivo* have not yet been investigated.

Our present aim was therefore, to characterize the microvascular effects of levosimendan and OR-1896 *in vivo*, and hence, to provide evidence as to their vasodilator roles in the microcirculation. The changes in the diameters of third order-cremaster muscle arterioles (diameter: ~ 20 μ m) in the presence of increasing concentrations of levosimendan or OR-1896 were monitored by means of intravital videomicroscopy. This allowed a comparative assessment of the vasodilator effects induced by levosimendan and OR-1896 in the microcirculation *in vivo*. Since levosimendan- and OR-1896-induced vasodilations have been shown to be associated with various degrees of K_{ATP} channel activation [7, 19, 28], K_{ATP} channel function modulators were also employed.

Materials and Methods

Animals and chemicals

All experimental procedures were approved by the Institutional Animal Care and Use Committee, in compliance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes. Experiments were carried out on male Wistar rats ($n = 15$, 8–10 weeks old, weighing ~ 200 g). The animals were housed under a 12-h light-dark cycle in the animal care facility of the University of Debrecen, and were fed with standard rat chow and drank tap water *ad libitum*. At the end of the experiments the animals were euthanized by an injection of sodium pentobarbital (150 mg/kg).

Levosimendan and OR-1896 were from Orion Pharma, Espoo, Finland. All other salts and chemicals were from Sigma-Aldrich Co. (St. Louis, MO, USA). Levosimendan, OR-1896, pinacidil and glibenclamide were prepared by dissolution in dimethyl sulfoxide (100 mM stock solutions), which were subsequently diluted with ethanol. Solutions were prepared fresh on the day of the experiment.

Intravital microscopy of the rat cremaster muscle

After overnight fasting rats were anesthetized with a subcutaneous injection of sodium pentobarbital (50 mg/kg). A constant level of anesthesia was maintained throughout the experiments by the subcutaneous injection of supplemental doses (20% of the original dose) of the anesthetic agent every 30–45 min. The trachea was cannulated to facilitate respiration. The left cremaster muscle was exposed through a midline scrotal incision, as described previously [7, 14]. An anesthetized rat was placed on a platform, and the cremaster muscle, with nerves and vessels intact, was spread over a heated, transparent pedestal. The whole preparation was then placed on the x–y stage of a microscope (Nikon Eclipse, FN1), at 35°C Krebs buffer was superfused continuously over the muscle at a constant flow rate (2 ml/min). After the surgical procedure, the preparation was allowed to equilibrate for at least 30 min before the start of the experimental protocol. We studied third- and fourth-order cremaster muscle arterioles with internal diameters of ~ 20 µm. Images were collected with a CCD camera and were recorded on a VHS recorder. The internal arteriolar diameters were measured offline with the aid of videocalipers (Colorado Instruments, USA), by two blinded, independent investigators.

Experimental protocols

During an incubation period of 30 min, a spontaneous tone developed in the cremaster muscle arterioles. The arteriolar responses to acetylcholine (1 µM) and adenosine (10 µM) were then recorded to assess the viability of the preparation.

In the first series of experiments, cumulative concentrations of levosimendan (1 nM – 100 µM) or OR-1896 (1 nM – 100 µM) were administered (100 µl in a bolus) topically to the surface of the cremaster muscle and the changes in arteriolar diameter were recorded.

In a separate set of experiments, the potential role of K_{ATP} channels in levosimendan or OR-1896-induced vasodilation was specifically tested. First, the arteriolar responses to cumulative concentrations of the K_{ATP} channel opener pinacidil (1 nM – 10 µM) were recorded in the absence or presence of glibenclamide (5 µM, in the superfusion solution for 30 min), a selective inhibitor of K_{ATP} channels [6]. This was followed by observation of vascular diameter changes in response to levosimendan (1 nM – 100 µM) or OR-1896 (1 nM – 100 µM) in the absence or presence of glibenclamide.

Data analysis

Drug-induced arteriolar diameter changes are presented in absolute values. Vascular diameter changes were recorded continuously and peak arteriolar responses were selected to illustrate the drug-induced effects. Data were analyzed with GraphPad Prism Software (version 5.00 for Macintosh, San Diego, California, USA). Data are expressed as the means

± SEM. Statistical analyses were performed with two-way repeated-measures ANOVA, followed by Bonferroni's *post-hoc* test. A probability level $p < 0.05$ was regarded as statistically significant.

Results

Effects of OR-1896 and levosimendan on arteriolar diameter in vivo

During the 30-min incubation period a spontaneous tone developed in the cremaster muscle arterioles (active diameter $25 \pm 6 \mu\text{m}$). The endothelium-dependent vasodilator acetylcholine ($1 \mu\text{M}$) and the endothelium-independent agonist adenosine ($10 \mu\text{M}$) elicited substantial dilations in the cremaster muscle arterioles (dilations from 25 ± 2 to $33 \pm 3 \mu\text{m}$ and from 24 ± 4 to $35 \pm 2 \mu\text{m}$, respectively), indicating the viability of the endothelium and smooth muscle cells in the preparations.

The responses of the cremaster muscle arterioles to increasing concentrations of levosimendan or of OR-1896 were then recorded. The topical administration of levosimendan induced concentration-dependent ($1 \text{ nM} - 100 \mu\text{M}$) arteriolar dilation (maximal dilation from 23 ± 2 to $33 \pm 2 \mu\text{m}$) (Fig. 1). The application of OR-1896 ($1 \text{ nM} - 10 \mu\text{M}$) likewise induced dilation in the cremaster muscle arterioles (maximal dilation from 22 ± 1 to $32 \pm 1 \mu\text{m}$). The magnitudes of the dilations induced by levosimendan or by OR-1896 were similar at all investigated concentrations. The maximum concentration of the solvent vehicle, DMSO was 0.015% in the superfusion solution, the concentration that had no significant effect on arteriolar diameter (Fig. 1).

Role of K_{ATP} channels in mediating levosimendan and OR-1896-induced arteriolar dilations

The changes in cremaster muscle arteriolar diameter in response to the K_{ATP} channel opener pinacidil ($1 \text{ nM} - 10 \mu\text{M}$) were first recorded in the absence or presence of glibenclamide ($5 \mu\text{M}$), a selective blocker of K_{ATP} channels. Incubation with glibenclamide alone had no effect on the basal arteriolar tone (the diameters were 23 ± 2 and $23 \pm 2 \mu\text{m}$ before and after the incubation with glibenclamide). However, in the presence of glibenclamide, the dilations in the cremaster arteriole in response to pinacidil were found to be completely abolished (Fig. 2).

Next, the potential involvement of K_{ATP} channels in levosimendan- or OR-1896-induced dilation was investigated. Similarly to the findings with pinacidil, the dilation in response to levosimendan was diminished by glibenclamide (maximal dilation to $23 \pm 3 \mu\text{m}$; Fig. 3A). The OR-1896-induced dilation was also opposed by glibenclamide (maximal dilation to $22 \pm 5 \mu\text{m}$; Fig. 3B). The vascular diameters at the beginning of the experiments (initial diameters) and those at maximal levosimendan or OR-1896 concentration did not differ significantly in the presence of $5 \mu\text{M}$ glibenclamide.

Discussion

The present study has revealed pronounced vasodilator potentials for both levosimendan and its long-lived positive inotropic metabolite OR-1896 in the rat skeletal muscle microcirculation, through K_{ATP} channel activation *in vivo*.

The long-term hemodynamic responses [10] following the administration of short-lasting levosimendan [13, 30] relate to the combination of differences in elimination half-life [11, 20], and plasma protein binding [12, 20] of levosimendan and OR-1896 [17, 24–27]. In fact, in addition to a calcium-sensitizing effect similar to that of levosimendan, OR-1896 exerts a potent vasodilatory effect on isolated coronary and skeletal muscle arteries in the rat [6]. Both levosimendan and OR-1896 have been demonstrated to act as vasodilators *ex vivo* in arteries and veins with diameters larger than 100 μm [2, 6, 7, 12, 18, 19]. Moreover, indirect microvascular effects of levosimendan and OR-1896 in whole animals have been investigated through measurement of the changes in systemic vascular resistance following systemic drug infusion [3, 22], whereas no experimental evidence has been provided for their direct effects on the microcirculation and resistance-sized arterioles *in vivo*.

In the present study, we monitored the effects of levosimendan and OR-1896 on the arteriolar responses directly by means of intravital microscopy on the rat cremaster muscle. The results demonstrated that both levosimendan and OR-1896 may decrease the vascular resistance in vessels which are not antagonized by cardiovascular reflex regulation. The maximal vasodilations achieved in response to levosimendan or OR-1896 were similar at all concentrations investigated. It should be noted that levosimendan and OR-1896 also induced significant vasodilations at the therapeutically relevant concentration of 10 nM. These *in vivo* features of levosimendan and OR-1896 were reminiscent of those observed *in vitro* in a previous study [6].

The activation of K_{ATP} channels by levosimendan was first demonstrated under voltage- and current-clamp conditions in isolated rat vascular smooth muscle cells [28] and isolated ventricular cardiomyocytes [29]. Since the levosimendan-induced opening of potassium channels was opposed by glibenclamide, it was proposed that levosimendan hyperpolarizes the smooth muscle cells of the vascular bed *via* glibenclamide-sensitive K_{ATP} channel activation. Experiments with K_{ATP} channel blockers on a number of vascular preparations furnished further evidence supporting the link between levosimendan and K_{ATP} channel activation [4, 6–8, 18]. Overall, the present results confirm earlier observations on the cardiovascular effects of levosimendan and OR-1896, and extend our understanding of the mechanisms of levosimendan and OR-1896-induced vasodilation in the peripheral microcirculation.

Previous studies suggested that levosimendan-induced vasodilation was only partially dependent on K_{ATP} channel activation in certain, relatively large-diameter vascular preparations (obtained from regions other than skeletal muscles); blockers of BK_{Ca} channels and/or voltage-dependent potassium channels effectively inhibited glibenclamide-insensitive components of levosimendan or OR-1896-induced vasorelaxation [6, 7, 18, 19]. Iberitoxin, a selective blocker of BK_{Ca} channels provoked a reduction in levosimendan-induced

vasodilation in porcine coronary arteries, suggesting the involvement of BK_{Ca} channel activation in levosimendan-induced vascular relaxation [19]. Similar combined mechanisms for smooth muscle relaxation have also been revealed in guinea pig tracheal preparations [5]. Moreover, our earlier results indicated that BK_{Ca} channel activation was essential for OR-1896-induced coronary dilation, whereas these channels made only a limited contribution in the skeletal muscle arteries [6]. Collectively, these *in vitro* data suggested that levosimendan and OR-1896-induced vasorelaxation in the coronary artery involves primarily the BK_{Ca} channel activation, whereas dilation in the skeletal muscle can be ascribed solely to K_{ATP} channels. We currently attempted to elucidate the relative contribution of K_{ATP} channel activation in levosimendan- and OR-1896-induced vasodilation by using K_{ATP} channel function modulators in the cremaster arteriole of the rat. Both levosimendan- and OR-1896-induced arteriolar dilations proved to be effectively diminished by the selective K_{ATP} channel blocker glibenclamide, similarly as when the K_{ATP} channel opener pinacidil was combined with glibenclamide. Glibenclamide practically abolished levosimendan or OR-1896-induced vasodilation, implicating K_{ATP} channels as the most significant mediators of these induced microcirculatory responses. Nevertheless, we would like to point out that the above regulatory effect was demonstrated in male animals in this study, and hence, the involvement of K_{ATP} channels in vasodilation might be affected by gender to some degree [1].

At the moment, we do not have a clear-cut explanation for the complexity of levosimendan-induced vascular responses in various vessels and species, and we therefore speculate that the expressions and/or regulations of K_{ATP} and BK_{Ca} channels are not uniform in the investigated vascular preparations [9, 16, 23]. The present results do not support the hypothesis of additional vasodilator mechanisms [21, 26] involving partial mediation of the action of levosimendan and OR-1896 by cAMP. In fact, these two molecules were equipotent vasodilators *in vitro* and *in vivo*; both could be antagonized by glibenclamide and, as indicated earlier, displayed profound differences as phosphodiesterase inhibitors [24].

The data reported here suggest that levosimendan and its metabolite OR-1896 act on the same molecular target in resistance arterioles of the systemic circulation by activating K_{ATP} channels and leading to substantial vasodilation of the rat cremaster muscle arteriole *in vivo*. It can be assumed that levosimendan and OR-1896 mediate effects on microvascular resistance through this molecular interaction in skeletal muscle, thereby contributing to both the acute and long-term hemodynamic effects subsequent to levosimendan administration.

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Fig. 1.

Changes in diameter of rat cremaster muscle arterioles in response to cumulative concentrations of levosimendan (1 nM– 100 μM, n = 9) or OR-1896 (1 nM – 100 μM, n = 7). Data are the means ± SEM. The asterisk indicates a significant difference from the initial diameter (id) or from the solvent vehicle (p < 0.05)

Fig. 2.

Changes in diameter of rat cremaster muscle arterioles in response to cumulative concentrations of pinacidil (1 nM – 10 μM), before and after the incubation with glibenclamide (5 μM; n = 5). Data are the means ± SEM. The asterisk indicates a significant difference (p < 0.05); id – initial diameter

Fig. 3.

Changes in diameter of rat cremaster muscle arterioles in response to cumulative concentrations of levosimendan (1 nM– 100 μM , $n = 4$, panel **A**) or OR-1896 (1 nM – 100 μM , $n = 4$, panel **B**), before and after incubation with glibenclamide (5 μM ; $n = 4$). Data are the means $\pm$ SEM. The asterisk in each panel indicates a significant difference ($p < 0.05$); id – initial diameter