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Effects of Levosimendan on Myocardial Contractility and Oxygen Consumption[1]

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ABSTRACT

Levosimendan is hypothesized to be primarily a calcium sensitizer *in vitro*. Therefore, its inotropic action may be similar in both the normal and the congestive heart failure (CHF) state, and it may be associated with a decreased energetic cost of inotropism *in vivo*. To test these hypotheses, we gave levosimendan to cross-circulated isolated hearts from normal (n = 11) and CHF (n = 7, 4-week rapid pacing) dogs. Peak isovolumic left ventricular pressure at an end-diastolic pressure of 5 mm Hg ($P_{max,5}$) measured by an intraventricular balloon was 120 ± 15 mm Hg in normal dogs, and it was increased by ~40% in response to ~0.63 μM levosimendan. In CHF dogs, base-line $P_{max,5}$ was only 60 ± 12 mm Hg ($P < .01$ compared to normals), and ~8.4 μM levosimendan ($P < .05$) was required to increase $P_{max,5}$ by ~40%. The inotropic actions were associated with increases in unloaded myocardial oxygen consumption by comparable amounts in normal and failing hearts. The blunted inotropic response in CHF and the energetic cost of inotropism were also comparable to those obtained with isoproterenol. In other studies, there was no significant inotropic action of levosimendan in Langendorff-perfused rat hearts (n = 5), and intracellular calcium concentration, estimated by macroinjected aequorin, in ferret hearts (n = 2) increased dose-dependently. These findings suggest that inotropic actions of levosimendan *in vivo* may be mediated in part by factors other than calcium sensitization.

Levosimendan, the levo-isomer of racemic simendan, is a pyridazinone dinitrile with inotropic actions that is being developed for the treatment of heart failure. It has been proposed that the primary inotropic action of levosimendan is through a calcium sensitization effect on the myofilaments: levosimendan stabilizes the conformational change of troponin after calcium binding, which results in increased contractile force without increased intracellular calcium (Haikala *et al.*, 1993). Levosimendan was originally identified on the basis of its ability to bind to porcine troponin and later to human recombinant troponin C (Haikala *et al.*, 1995). Furthermore, the troponin C binding affinity of levosimendan is calcium-dependent (Pollesello *et al.*, 1994; Ovaska *et al.*, 1992), so the calcium-sensitizing effect is, in principle, maximal during systole and minimal during diastole. Thus, in contrast to some other calcium sensitizers (Lee and Allen, 1993), levosimendan does not impair relaxation (Haikala *et al.*, 1992b; Haikala *et al.*, 1992a; Pagel *et al.*, 1994). However, levosimendan is also known to have PDE-III inhibitory activity (Edes *et al.*, 1995). It has been shown in a recent study of isolated guinea pig hearts and papillary muscles that the calcium-sensitizing action is relatively specific at concentrations up to 0.03 μM, at which point contractile force is increased by about 25%. At higher concentrations, PDE-III inhibitory actions are evident (Edes *et al.*, 1995). Studies in humans and in chronically instrumented conscious dogs have shown that in addition to significant inotropic actions, levosimendan has vasodilatory actions that reduce both ventricular preload and afterload pressures (Pagel *et al.*, 1994; Sundberg *et al.*, 1995; Lilleberg *et al.*, 1994).

In contrast to traditional inotropic agents whose actions are mediated by increasing cAMP concentration that ultimately leads to an increase in systolic intracellular calcium, the theoretical advantages of a calcium sensitizer include 1) that the increased contractile state could be achieved without

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ABBREVIATIONS: PDE, phosphodiesterase; CHF, congestive heart failure; A-VO_2, difference between arterial and venous oxygen content; mV_{O_2}, myocardial oxygen consumption; LV, the left ventricle; EDP, end-diastolic pressure; ESPVR, end-systolic pressure-volume relationship; EDPVR, end-diastolic pressure-volume relationship; $t_{1/2}$, pressure half-time; PVA, pressure-volume area; CBF, coronary blood flow; CPP, coronary perfusion pressure; P_{max}, peak isovolumic pressure.

added energy costs for calcium cycling and 2) that the inotropic action may be similar in both the normal and the heart failure state, because inotropic actions are independent of cAMP-generating pathways that, in CHF, are down-regulated (Feldman *et al.*, 1987; Perreault *et al.*, 1992).

Accordingly, the primary purpose of this study was to test, in normal and failing hearts, the dose-dependent actions of levosimendan on ventricular contractile state, myocardial oxygen demand and the dynamics of relaxation. In order to obviate potential confounding effects of autonomic reflexes and changes in ventricular afterload and preload, these studies were performed in isolated blood-perfused canine hearts. To further characterize the actions of this agent, we tested the effects of levosimendan in different species (rats and ferrets) on contractile state and on changes in intracellular calcium. For the latter, intracellular calcium transients were assessed by measuring regional luminescence of macroinjected aequorin in perfused ferret hearts.

Materials and Methods

Isolated heart preparation. In order to test the impact of levosimendan on the interrelationship among contractile state, the dynamics of relaxation and energy consumption, we studied hearts of 11 normal dogs (21.3 ± 1.5 kg) and 7 dogs with tachycardia-induced heart failure (27.9 ± 4.1 kg, methods described below) using a standard isolated heart preparation. Details of this preparation have been provided previously (Burkhoff *et al.*, 1991). Briefly, the heart from the dog of interest was excised and metabolically supported by blood provided from a second support dog. The femoral arteries of the support dog were cannulated and connected to a perfusion circuit consisting of two peristaltic pumps, a heater, a blood filter and an air trap. The pumps were arranged so that the first pump maintained constant total blood flow from the support dog (to provide a stable metabolic demand), and blood flow to the isolated heart could be controlled by regulating the flow through the second pump, which shunted blood back to the support dog. The pressure in the aortic root of the isolated heart, which is the perfusion pressure for coronary flow, was measured and used as the feedback signal for a servo system that regulated the speed of the second pump and maintained perfusion pressure at ~80 mm Hg, except in experiments that were designated *constant blood flow protocols* as described later. Blood traveled through the coronary vasculature of the isolated heart and returned to the support dog by gravity. Coronary flow was collected through a wide bore cannula placed in the right atrium and right ventricle and was measured by an in-line ultrasonic flowmeter (Transonic Systems model T108, Ithaca, NY). The difference between arterial and venous oxygen content (A-VO_2) was measured on-line by a commercially available spectrophotometer (AVOX Systems, San Antonio, TX). Oxygen consumption of the whole heart (mV_{O_2}) was determined by multiplying coronary flow by A-VO_2.

A water-filled balloon was placed within the LV *via* the mitral valve. The volume of the balloon, and therefore of the LV, was controlled by a piston pump servo system. A micromanometer (Millar Instruments Model SPC-360, Houston, TX) placed within the balloon was used to measure LV pressure. The heart was paced from the LV apex at a constant rate (143 ± 17/min) and was constrained to contract isovolumically. Blood temperature was kept at ~37°C by a heat exchanger.

Tachycardia-induced heart failure. Seven mongrel dogs were anesthetized (1%–2% inhaled isoflurane) and underwent sterile surgery for chronic instrumentation *via* a left thoracotomy. Fluid-filled catheters were inserted to the left ventricle, left atrium and aorta. Pacing wires were fixed to the LV free wall. After 2 to 3 weeks of recovery, base-line hemodynamics were recorded while the dogs were awake. Pacing was then initiated at 210 beats/min for 3 weeks followed by 240 beats/min during the fourth week. Upon completion of 4 weeks of pacing, hemodynamic measurements were repeated about 2 hr after the pacer was turned off and in the conscious state with dogs lying quietly on their sides. The results, summarized in table 1, show that there was a marked decrease in dP/dt_{max}, an elevation in LV EDP and an increase in resting HR ($P < .05$, paired t test) after the 4-week pacing regimen, which indicated that the animals were in significant heart failure. The hearts of these animals were studied in isolation as described above and made up the CHF group.

Isolated heart protocols and data analysis. Inotropic, lusitropic and metabolic effects of levosimendan were assessed in normal and failing hearts. Hemodynamic recordings (LV pressure, coronary blood flow, A-VO_2) were made at base line, during low-dose levosimendan infusion (titrated to increase contractile state by ~25%) and during high-dose levosimendan infusion (titrated to increase contractile state by a total of ~50%). Levosimendan was dissolved fresh each day in phosphate buffer (pH 8.4) at concentrations ranging between 11 and 110 μg/ml (depending on heart sensitivity to levosimendan) and was infused directly into the arterial perfusion line approximately 1.5 m from the heart, which allowed ample mixing volume. The same measurements were made at four different volumes (spanning EDPs between 0 and 15 mm Hg) under each of these conditions. The results obtained with levosimendan were compared with those obtained with isoproterenol titrated to create similar degrees of inotropism. Because of the relatively long half-life of levosimendan, the order of drug infusion could not be randomized. Therefore, some hearts received only levosimendan, some hearts received isoproterenol followed by levosimendan and some hearts received only isoproterenol. Results from the former two groups were analyzed separately to test whether isoproterenol pretreatment modified the effects of levosimendan. In all, the levosimendan group consisted of eight normal hearts (two of which were pretreated with isoproterenol) and six CHF hearts (four of which were pretreated with isoproterenol), and the isoproterenol group consisted of five normal and five CHF dogs.

Ventricular contractile state was assessed by the linear ESPVR, which was characterized by a slope (E_{es}) and volume axis intercept (V_0) with end-systolic pressures defined as the peak isovolumic pressures (P_{es}) measured at the different volumes (LVV): $P_{es} = E_{es}(LVV - V_0)$. Contractile state was indexed by a single value, $P_{max,5}$, which is the interpolated maximum systolic pressure at a volume that provides an end-diastolic pressure of 5 mm Hg (V_5): $P_{max,5} = E_{es}(V_5 - V_0)$. $P_{max,5}$ is thus a *contractility index* that simultaneously accounts for changes in slope and volume axis intercept of the ESPVR and therefore simplifies pooling and statistical comparison of data. End-diastolic properties and the rate of relaxation were assessed by the EDPVR and by $t_{1/2}$, respectively; $t_{1/2}$ was defined as the time for LV pressure to fall to 50% of its value at the point of maximal negative dP/dt.

To assess the metabolic cost of inotropism, we determined the relationship between mV_{O_2} and total mechanical work, indexed by the PVA: $mV_{O_2} = A \cdot PVA + B$. PVA was defined in the usual manner as the area on the pressure-volume diagram contained within the triangular region bounded by the ESPVR, the EDPVR and the vertical line corresponding to the volume at which the

TABLE 1

Hemodynamic measurements obtained from conscious dogs after 4 weeks of rapid pacing

	Base line	After 4 Weeks of Pacing
HR (beats/min)	95 ± 15	141 ± 20**
AoP (mm Hg)	101 ± 4	95 ± 8
LVEDP (mm Hg)	2.2 ± 1.3	15.5 ± 4.0**
dP/dt_{max} (mm Hg/sec)	3300 ± 520	1790 ± 320**

Postpacing values obtained 2 hr after pacer was turned off. AoP, mean aortic pressure; LVEDP, left ventricular end-diastolic pressure; dP/dt_{max}, peak rate of rise of LV pressure. ** $P < .01$ by paired t test.

isovolumic contraction occurred (Suga, 1990). The mV_{O_2} intercept of this relationship (*i.e.*, unloaded mV_{O_2}, *B*) has been shown to vary directly with contractile state, whereas the slope, *A*, is relatively independent of contractile state (Suga, 1990). Because changes in contractile state are generally brought about by changes in intracellular calcium, changes in *B* have been hypothesized to reflect altered energy demands for calcium cycling (Suga, 1990).

Inotropic, lusitropic and metabolic effects were related to plasma concentrations of levosimendan, which were estimated from the rate of drug infusion, the measured CBF and a calibration curve. The calibration curve was derived from 12 freshly frozen samples spanning a wide range of concentrations (0–350 ng/ml) in which levosimendan plasma concentrations were determined by an HPLC technique employing UV spectrometry. Samples were transported overnight on dry ice from our laboratory to Orion-Farmos, where the assay was performed. The formula determined from this procedure was

$$[\text{levosimendan}]_{\text{plasma}}\ (\text{ng/ml}) = \frac{\text{Infusion rate (ng/min)}}{\text{CBF (ml/min)}} \times 2.5$$

where 2.5 is the empirically determined scaling factor.

CPP was maintained at ~80 mm Hg in the protocol described above; because levosimendan is a coronary vasodilator, CBF increased in a dose-dependent manner. An increase in CBF may cause an increase in mV_{O_2} independent of any change in contractile state [the Gregg effect (Gregg, 1963; Feigl, 1983)]. In order to test whether mV_{O_2} increases during levosimendan infusion independently of an increase in CBF, constant-CBF studies were performed in three of the normal hearts, and the results obtained were compared with those obtained with a vasodilator devoid of significant inotropic actions (adenosine). For these studies, LV volume was set to provide an EDP of ~5 mm Hg, and CBF was set at the beginning of the experiment to provide a CPP of ~100 mm Hg. Adenosine was then infused at a rate of 1 mg/min; measurements of P_{max} (index of contractile strength), CPP and mV_{O_2} were made once steady-state conditions were reached. We then repeated these measurements after increasing the adenosine infusion rate to 2 mg/min. After a 45-min washout period, the same constant-CBF procedure was repeated for multiple doses of levosimendan in the same hearts. Coronary resistance under any given condition was defined as the ratio between CPP and CBF. Changes in inotropic state and in oxygen consumption were then related to changes in coronary vascular resistance, and data from the adenosine and levosimendan runs were compared.

Isolated rat and ferret heart studies. Rat hearts (n = 5) and ferret hearts (n = 2) were excised from heparinized (1000 U i.p.), anesthetized (ketamine 100 mg/kg i.p. and xylazine 1 mg i.p.) animals and perfused with oxygenated, warmed (37°C) crystalloid solution (modified Tyrode composed of, in mmol/l: glucose, 15; NaCl, 140; KCl, 5; $MgCl_2$, 0.9; $CaCl_2$, 2.0; HEPES, 6). A water-filled balloon was placed in the LV, and LV pressure was measured by a transducer connected to the balloon. LV volume was set to provide an EDP between 5 and 10 mm Hg and was kept constant. Levosimendan, which was prepared fresh daily, was dissolved in phosphate buffer (pH 8.3) and was infused into the perfusion line so that final concentrations reaching the heart spanned the range from 10 to 640 ng/ml (0.04 to 2.29 μM). Systolic function was assessed by LV P_{max}.

In order to test the effects of levosimendan on intracellular calcium, the calcium-sensitive photoprotein aequorin was macroinjected into the LV apex of the two ferret hearts studied. Techniques for measuring calcium transients from the epicardial surface of crystalloid perfused hearts were similar to those described previously (Bentivegna *et al.*, 1991; Kihara *et al.*, 1989). We injected 3 to 5 μl of an aequorin solution (aequorin 1 mg/ml, NaCl 154 mmol/l, KCl 5.4 mmol/l, $MgCl_2$ 1 mmol/l, HEPES 12 mmol/l, glucose 11 mmol/l and EDTA 0.1 mmol/l, adjusted to pH 7.40) just under the epimysium in the infero-apical region, using a low-resistance glass micropipette with an inner diameter of about 30 μm. To record the aequorin luminescence, the heart and a portion of the perfusion apparatus were placed inside a light-tight box (Blinks, 1982). Luminescence emitted from the heart was directed to the surface of a photo multiplier tube (9235QA, Thorn EMI, Fairfield, NJ). Initial filtering was performed on-line using an analog filter with a corner frequency of 100 Hz.

The method of calibrating the light signal into an absolute concentration of intracellular calcium $[Ca^{++}]_i$ was the same as used previously (Kihara *et al.*, 1989). In brief, the heart was perfused with a 50 mmol/l calcium–5% Triton X-100 solution that lysed the cells and exposed the remaining aequorin to high amounts of calcium at the end of the experiment (Bentivegna *et al.*, 1991). Luminescence signals to be converted to calcium signals, *L*, were normalized by the total light emission, L_{max}, which was estimated as the integral of the aequorin signal collected during the lysis procedure multiplied by the rate constant for aequorin consumption (2.11/sec) (Kihara *et al.*, 1989). The instantaneous L/L_{max} was then converted to $[Ca^{++}]_i$ (t) according to the following equation:

$$L/L_{max} = (\{1 + K_r[Ca^{++}]_i\}/\{1 + K_{tr} + K_r[Ca^{++}]_i\})^3$$

where $K_r = 4.5 \times 10^6$/M, and $K_{tr} = 130$. Values of K_r and K_{tr} were determined previously (Kihara *et al.*, 1989).

Statistical analysis. Two-way analysis of variance (ANOVA) was used for simultaneous comparison of base-line parameters from the normal and CHF groups and from isoproterenol and Levosimendan groups. Multiple linear regression was used to test for statistical significance of difference in trend, such as effect of drug concentration on contractile function. $P < .05$ was regarded as significant.

Results

Effects of levosimendan on systolic function in isolated canine hearts. Pressure-volume relationships measured under base-line conditions and after administration of levosimendan from a representative normal isolated canine

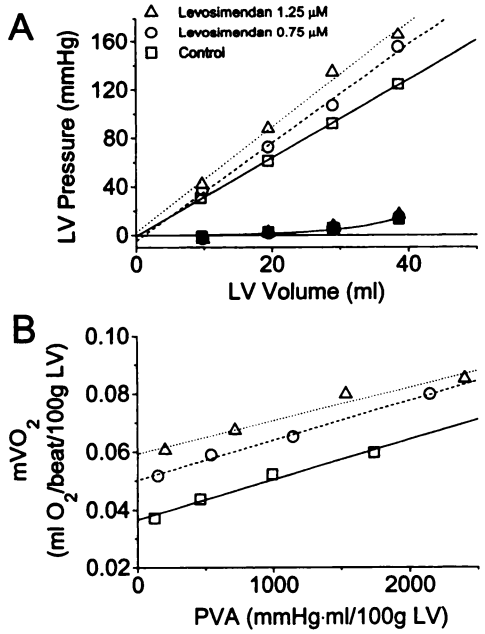

Fig. 1. A) Left ventricular pressure-volume relationships from a representative normal heart at base line and with levosimendan infusion at two concentrations. The slope of the ESPVR increased dose-dependently without appreciable changes in either V_0 or the end-diastolic relation. B) PVA *vs.* mV_{O_2} relationships from the same heart. The relationship was shifted upward as contractility was enhanced by levosimendan infusion.

heart are shown in figure 1A. There was a levosimendan dose-dependent increase in the slope of the ESPVR with little change in the volume axis intercept. Furthermore, levosimendan did not influence the EDPVR, which indicates that there was no effect on end-diastolic properties.

Results from both normal and CHF animals are summarized in figure 2A (all values are mean + S.D.). The average base-line $P_{max,5}$ value from failing hearts was significantly lower than that from normal hearts ($P < .01$), consistent with a heart failure state. In normal hearts, levosimendan increased $P_{max,5}$ by approximately 40% at an estimated concentration of 0.63 μM. There was also a significant inotropic action of levosimendan in the CHF hearts, but the concentration needed to achieve a ~40% increase in $P_{max,5}$ was about 8.5 μM, approximately 13 times greater than in the normal hearts. The changes in levosimendan responsiveness in heart failure were similar to those in that of isoproterenol (fig. 2B). Isoproterenol at an estimated concentration of 0.76 nM increased contractile strength by ~30% in normal dogs. In the heart failure state, however, an estimated concentration of 4.1 nM was required to increase contractile strength by the same amount (an approximately 5 fold increase in concentration). Statistical analysis (multiple linear regression analysis applied to plots of normalized $P_{max,5}$ *vs.* drug concentration) revealed that the changes in inotropic responsiveness in CHF hearts compared to normal hearts were statistically significant ($P < .05$) for both levosimendan and isoproterenol.

Effects of levosimendan on mV_{O_2} in isolated canine hearts. Typical mV_{O_2}-PVA relationships measured concurrently with the pressure-volume relationships of figure 1A are shown in figure 1B; they reveal that there was a dose-dependent, nearly parallel upward shift of this relationship (*i.e.*, an increase in the mV_{O_2} axis intercept with little influence on the slope). The slope of the mV_{O_2}-PVA relationship averaged $1.61 \pm 0.46 \times 10^{-5}$ ml $O_2 \cdot beat^{-1} \cdot mm\ Hg^{-1} \cdot ml^{-1}$ in normal animals and $1.62 \pm 0.37 \times 10^{-5}$ ml $O_2 \cdot beat^{-1} \cdot mm\ Hg^{-1} \cdot ml^{-1}$ in failing hearts (not significant), and furthermore, the value of the slope did not vary significantly with either levosimendan infusion or with isoproterenol infusion. The mV_{O_2} axis intercept (which corresponds to mV_{O_2} measured with the heart in a mechanically unloaded state) was lower in CHF hearts than in normal hearts ($P < .01$). Changes in unloaded mV_{O_2} (observed with both levosimendan and with isoproterenol), plotted as functions of changes in inotropic state indexed by normalized $P_{max,5}$, are summarized in figure 3, A and B. There was a contractility-dependent increase in unloaded mV_{O_2} for both isoproterenol and levosimendan in both normal and CHF hearts. Statistical analysis (multiple linear regression analysis) revealed that the slopes of these curves were the same (not significant), which suggests that the metabolic costs of inotropism were similar in normal and failing hearts and were similar for both levosimendan and isoproterenol.

Effects of levosimendan on relaxation in isolated canine hearts. The effect of levosimendan on the rate of relaxation, indexed by $t_{1/2}$, is summarized in figure 4A for both normal and failing hearts. In this graph, changes in $t_{1/2}$ are related to the change in inotropic state (indexed by $P_{max,5}$) achieved by drug infusion. Base-line values of $t_{1/2}$ were similar in normal and failing hearts, and there was an inotropic-dependent decrease in $t_{1/2}$ that was also similar in normal and failing hearts. Finally, the relationship between $t_{1/2}$ and $P_{max,5}$ obtained when inotropy was achieved with levosimendan (fig. 4A) was the same as that obtained when inotropy was achieved with isoproterenol (fig. 4B) (not significant, by multiple linear regression analysis).

Pretreatment with β-agonist does not diminish inotropic effectiveness of levosimendan. As noted previously, the half-life of levosimendan is relatively long, so isoproterenol had to be infused before levosimendan in all experiments in which both drugs were used, and no study could be done in which levosimendan preceded isoproterenol administration. In order to test whether isoproterenol pretreatment modified the results, we administered isoproterenol to six of the hearts (two normal and four failing) at the beginning of the experiment and allowed it to wash out for 30 to 45 min. Then the hearts were exposed to levosimendan. These results were compared to those obtained with hearts that received only levosimendan. The results of this analysis,

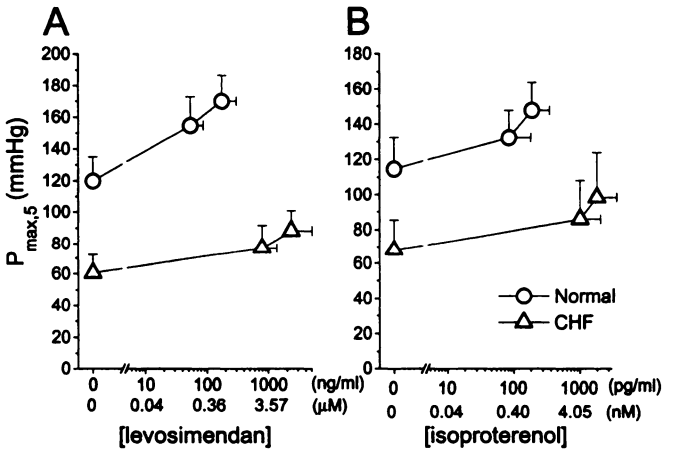

Fig. 2. Impact of either levosimendan (A) or isoproterenol (B) on contractile state (indexed by $P_{max,5}$) in normal (○) and failing (△) hearts. Base-line $P_{max,5}$ was markedly reduced in failing hearts compared with normal hearts. The inotropic responses of failing hearts to drug infusion were also blunted for both drugs ($P < .05$ by multiple linear regression). Points show mean (+S.D.) data obtained from all hearts studied.

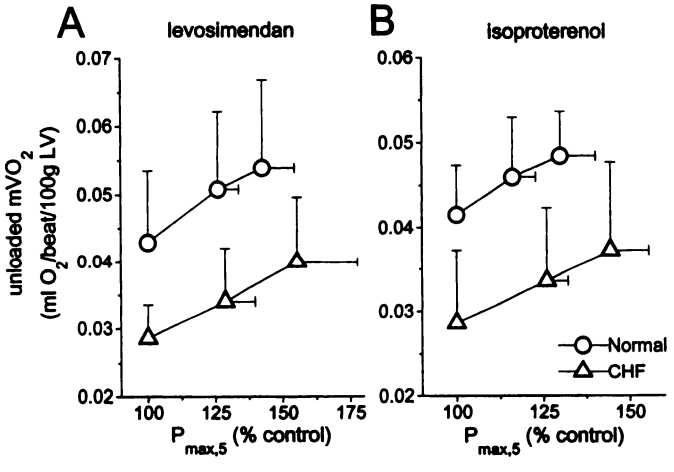

Fig. 3. Average unloaded mV_{O_2} plotted as a function of the simultaneously measured contractile state (indexed by $P_{max,5}$) for levosimendan (A) or isoproterenol (B). In both normal and failing hearts, unloaded mV_{O_2} increased in proportion to $P_{max,5}$ with either levosimendan or isoproterenol ($P < .01$ by multiple linear regression). There were no differences among the slopes of these relationships.

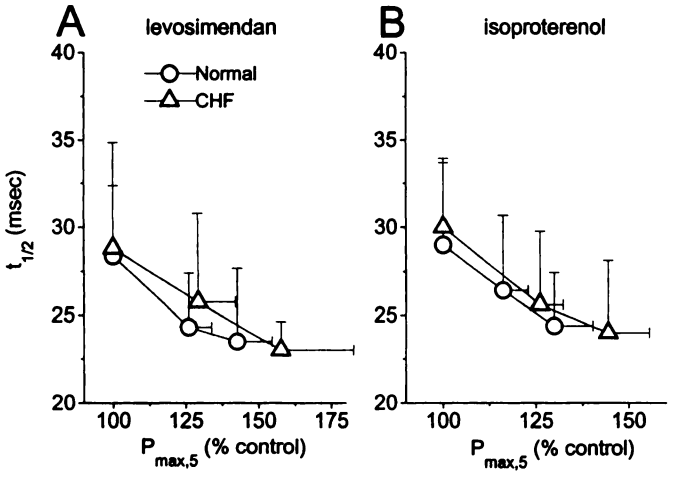

Fig. 4. Average changes in the rate of relaxation (indexed by $t_{1/2}$) plotted as a function of contractile state for levosimendan (A) and for isoproterenol (B). In both normal and failing hearts, $t_{1/2}$ decreased as $P_{max,5}$ was increased by either drug ($P < .01$ by multiple linear regression). There were no differences among the slopes of these relationships.

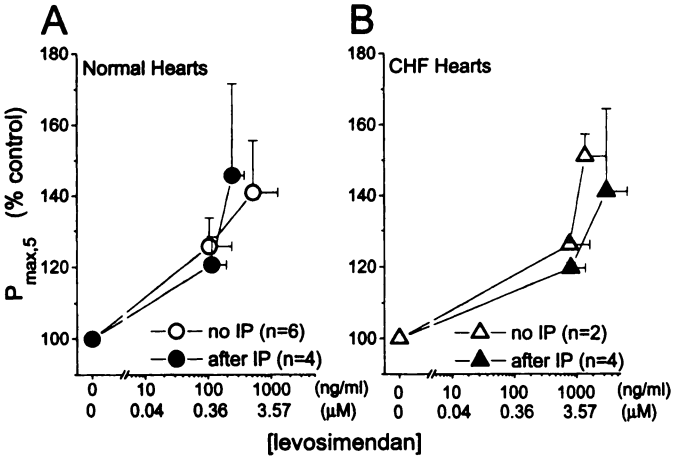

Fig. 5. Effect of isoproterenol pretreatment (IP) on isolated canine heart responsiveness to levosimendan in both normal (A) and failing (B) hearts. IP did not influence the dose-response curve of contractile state to levosimendan in either normal or failing hearts. Points are mean (+S.D.); numbers of animals contributing to the sets are specified in the insets.

summarized in figure 5, indicated that inotropic responses to levosimendan in these pretreated hearts (● or ▲) were indistinguishable from those in hearts that were not pretreated with isoproterenol (○ or △). Thus isoproterenol pretreatment did not modify the inotropic effectiveness of levosimendan.

Impact of coronary vasodilating properties of levosimendan on contractile performance and energetics. Levosimendan was administered under conditions of constant coronary blood flow in three normal hearts, and the results were compared to those obtained during adenosine infusion. The results, summarized in figure 6, show that reductions in coronary resistance due to levosimendan infusion were associated with increases in ventricular contractile state (as indexed by P_{max}) and increases in mV_{O_2}. Adenosine infusion was associated with comparable decreases in coronary resistance, but neither ventricular contractile state nor mV_{O_2} was influenced. These data suggest that in this preparation, changes in mV_{O_2} observed with levosimendan are not dependent on changes in CBF or coronary vascular resistance but rather are linked to changes in inotropic state.

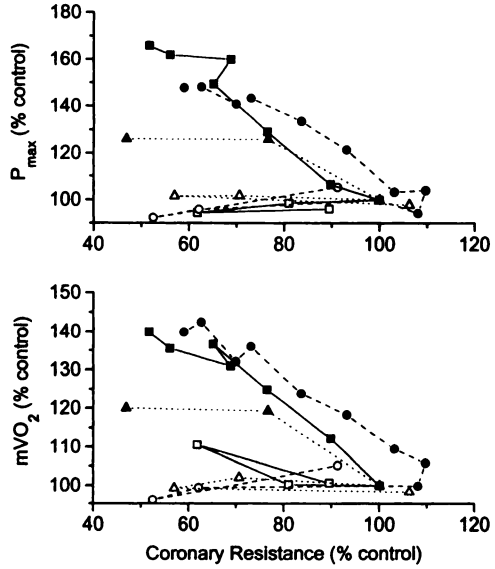

Fig. 6. Effects of adenosine (open symbols) on contractility (P_{max}) and on myocardial oxygen consumption (mV_{O_2}) compared with the effects of levosimendan (closed symbols) assessed in three hearts with constant coronary blood flow. Although adenosine dilated coronary artery about as much as levosimendan (as indexed by coronary vascular resistance), adenosine induced no positive inotropic effect and no increase in mV_{O_2}. Thus the inotropic and metabolic effects of levosimendan are independent of its coronary vasodilatory effects.

Effects of levosimendan on contractile state and calcium transients in crystalloid perfused rat and ferret hearts. The dose-dependent influence of levosimendan on contractile performance (P_{max}) in isolated, Langendorff perfused rat hearts is summarized in figure 7; note that EDP (data not shown) increased by about 10 mm Hg at the largest dose of levosimendan tested in these hearts. This series of experiments revealed that there was no positive inotropic

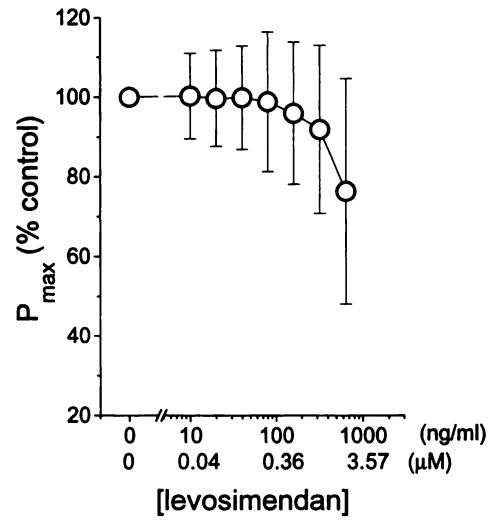

Fig. 7. Average effect of levosimendan on contractile performance (indexed by peak isovolumic pressure, P_{max}) in isolated Langendorff perfused rat hearts. There was no positive inotropic effect; rather, as observed previously, a negative inotropic effect was recorded at high doses.

effect of levosimendan in this preparation and that the contractile state decreased at the higher doses.

There was also a small dose-dependent increase in diastolic pressure, but, as in the rat hearts, no significant increase in developed pressure was observed with levosimendan concentrations up to 1.0 μM in the ferret hearts. Intracellular calcium transients estimated from measured aequorin luminescence tracings obtained from these hearts, shown in figure 8, reveal that at concentrations above 0.03 μM there was an increase in peak calcium despite there being no change in developed pressure.

Discussion

Levosimendan is a calcium sensitizer *in vitro* that also has PDE-III inhibitory actions at high concentrations. Although the relative contributions of calcium-sensitizing and PDE-III inhibitory actions are relatively easy to define *in vitro*, this is more difficult under *in vivo* conditions. The isolated blood-perfused canine heart preparation, the primary experimental model employed in the present study, allowed for relatively precise characterization of the direct myocardial effects of levosimendan in the absence of the confounding influences of autonomic reflexes or changing preload and afterload conditions. Using this model, we sought indirect evidence that levosimendan acts as a calcium sensitizer.

It was first demonstrated that levosimendan caused an upward shift of the ESPVR and an increase in $P_{max,5}$ (a contractile index derived from the ESPVR) in both normal and failing isolated dog hearts. In accordance with previous studies (Pagel *et al.*, 1994; Udvary *et al.*, 1995), these findings indicate that levosimendan is a positive inotropic agent in canine myocardium. The inotropic actions of levosimendan were blunted in failing hearts. Concentrations about 10-fold higher were required to achieve increases in contractile state comparable to those in normal hearts; this paralleled the increases in isoproterenol concentrations required for inotropism in the failing hearts. Furthermore, the estimated concentrations at which inotropic actions were observed even in the normal isolated canine hearts were within the range wherein levosimendan exerts PDE-III inhibitory actions in isolated guinea pig hearts (Edes *et al.*, 1995). These findings contrast with the estimated therapeutic dose in humans, which is about 50 ng/ml (with free levosimendan concentrations of as little as 1 to 2 ng/ml) (Lehtonen *et al.*, 1995). However, the degree to which hemodynamic actions of levosimendan are mediated by inotropic or peripheral vascular effects in the human has not yet been established.

Levosimendan did not affect the EDPVR. Furthermore, as in previous studies (Pagel *et al.*, 1994), levosimendan increased the rate of relaxation (as indexed by $t_{1/2}$) and thus acted as a positive lusitropic agent like other positive inotropic agents.

The impact of levosimendan on mV_{O_2} was examined within a previously proposed framework of myocardial energetics that proposes to break total mV_{O_2} down into contributions due to 1) basal metabolism, 2) total mechanical work (indexed by PVA and dependent on cross-bridge interactions) and 3) "contractility." The contractility dependent component affects the mV_{O_2}-PVA intercept (B) without causing a change in the slope (A) (Suga, 1990). In the absence of changes in basal metabolism, B is presumed to be tightly linked to changes in energy demands for calcium cycling (Suga, 1990). Accordingly, these concepts have led to the expectation that calcium sensitizers (which would not affect the amount of calcium cycling) should increase the contractile state with relatively little increase in B (Suga, 1990). However, the inotropic actions of levosimendan were accompanied by increases in B by amounts that were similar to those observed with isoproterenol for similar increases in contractile performance. Although this is contrary to expectations for a calcium sensitizer, it is noteworthy that the same finding has been obtained with other reported calcium sensitizers (Suga, 1990; Futaki *et al.*, 1992; de Tombe *et al.*, 1992), and it has recently been questioned whether changes in B solely reflect changes in the energy demands for calcium cycling (Higashiyama *et al.*, 1994). Aside from this evolving controversy, the

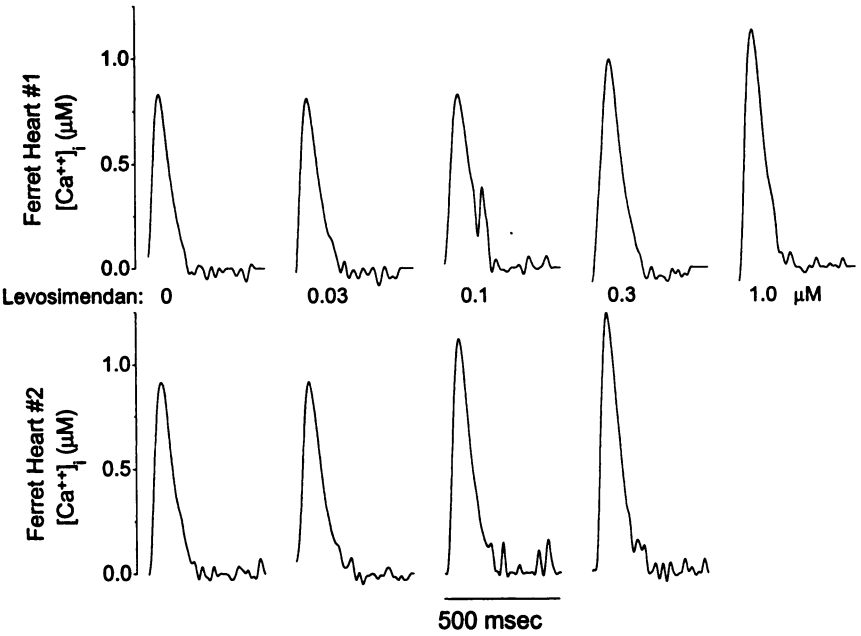

Fig. 8. The effect of levosimendan on calcium transients, estimated from macroinjected aequorin luminescence, in two ferret hearts. Levosimendan concentrations up to 0.03 or 0.1 μM did not increase aequorin signal; beyond this concentration, however, peak intracellular calcium increased.

present results show on a phenomenologic level that as an inotropic agent, levosimendan is energetically equivalent to a β-agonist in normal and failing isolated canine hearts.

The coronary vasodilatory actions of levosimendan potentially complicate the interpretation of changes in mV_{O_2}, because under some conditions, an increase in coronary flow can cause an increase in oxygen consumption independently of changes in contractile performance [Gregg's phenomenon (Gregg, 1963; Feigl, 1983)]. However, this possibility was excluded as the sole cause of the contractility-related increase in mV_{O_2} with levosimendan in studies performed under conditions of constant coronary blood flow. Inotropic state and oxygen consumption both increased under this condition, whereas these parameters were unaffected when a *pure* vasodilator (adenosine) was used. Thus the increases in oxygen consumption were related to the inotropic effects of levosimendan, not to its vasodilatory actions.

Levosimendan was not inotropic in crystalloid perfused rat hearts; the reason for this was not determined in the present study. In addition, a negative inotropic action was evident at high concentrations (>0.6 μM), a finding that has been observed previously in rabbit hearts (Rump *et al.*, 1994), though the mechanism is also unclear. If the inotropic action of levosimendan is dominated by PDE-III inhibitory activity, then the lack of inotropic actions in rats may be related to compartmentalization of PDE-III that renders this species insensitive to PDE-III inhibitors (Weishaar *et al.*, 1987). However, other PDE-III inhibitors are inotropic in ferret hearts (Gwathmey and Morgan, 1985), so this cannot be the only explanation. Thus it may be more plausible that the lack of inotropic action in these studies reflects a species difference in the troponin C binding affinity of levosimendan. The intracellular calcium transient (assessed from macroinjected aequorin luminescence in ferret hearts) was unaffected at low concentrations but increased significantly at concentrations greater than 0.03 μM. It was at concentrations above this level at which positive inotropic actions were noted in the dog hearts.

One potential limitation of the present study is related to uncertainty about the status of myofilament phosphorylation in isolated canine hearts. Studies in guinea pig myocardium *in vitro* have shown that myofilament phosphorylation decreases the calcium-sensitizing effects of levosimendan (Dr. Heimo Haikala, personal communication). Because β-agonists lead to myofilament phosphorylation, and because basal β-agonist (epinephrine and norepinephrine) blood concentrations may be high in the isolated heart preparation as a result of elevated sympathetic tone of the support dog, it is possible that inotropic potency mediated by calcium sensitization may be reduced in this preparation. However, in view of the fact that significant catecholamine concentrations are present in all patients and that these are markedly elevated in patients with heart failure, studies in the isolated heart model may adequately reflect conditions in which this agent will be used.

It is difficult to prove, in an *in vivo* model, that an inotropic agent works *via* myofilament calcium sensitization. Therefore, we sought indirect evidence to test this hypothesis for levosimendan. Our findings of altered inotropy in failing *vs.* normal hearts, equivalent energetic costs for inotropism and for a β-agonist and blood concentrations required for inotropism above the range shown *in vitro* to provide "pure" calcium-sensitizing action do not support a calcium-sensitizing mechanism of action. Lack of inotropy in crystalloid perfused hearts, despite increases in aequorin luminescence at high concentrations, is unexplained. Species differences in the troponin C binding affinity of levosimendan and differences in experimental conditions (background catecholamine concentrations, myofilament phosphorylation status, *etc.*) may be contributing factors. Studies in human subjects showing that levosimendan decreases LV EDP and arterial resistance while increasing stroke volume and ejection fraction indicate that it has potent hemodynamic actions. The degree to which these actions are related to peripheral vascular *vs.* direct myocardial effects is as yet undetermined, as is the degree to which any myocardial effects may be related to calcium sensitization. However, elucidation of the mechanisms of action will help define the clinical uses of this agent. The results of the present study, obtained in multiple experimental settings, suggest that mechanisms other than calcium sensitization (perhaps PDE-III inhibition) contribute to the hemodynamic profile of this compound *in vivo*. However, because all measurements we made address this fundamental question indirectly, the results of the present study in no way exclude the possibility that calcium sensitization may be an important contributing factor.

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