Intracoronary levosimendan prevents myocardial ischemic damages and activates survival signaling through ATP-sensitive potassium channel and nitric oxide*§,§§

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Abstract

Objective: Levosimendan has been reported to exert cardioprotection. In this study, we have examined the cardiac effects of different doses of intracoronary levosimendan on ischemia/reperfusion injuries, and the involvement of KATP channels and nitric oxide (NO). Methods: The experiments were performed in a total of 56 anesthetized pigs. In 21 pigs, 1.5, 5 and 12 μg min⁻¹ levosimendan was infused over 15 min into the coronary artery at the onset of 1 h reperfusion following 2-h ischemia and the effects on cardiac function, infarcted area, and on apoptosis/autophagy were examined. In addition, the activation of Akt and extracellular receptor kinase (ERK) was analyzed. The findings were compared with those obtained in a further 14 pigs where the highest dose levosimendan was infused after glibenclamide and L-nitro-arginine methyl ester (L-NAME). Results: Intracoronary 1.5, 5 and 12 μg min⁻¹ levosimendan caused an increase of segmental shortening, dP/dtmax and cardiac output of 7.8%, 22.6%, and 31.6%; 7.6%, 16.9%, and 21.6%; 2.8%, 5.9%, and 6.2%, respectively, from values measured at the end of ischemia. The beneficial effects elicited by levosimendan were still evident at the end of reperfusion when the increase of segmental shortening, dP/dtmax and cardiac output caused by the three doses of levsimendan amounted to 3.7%, 13.3%, and 16.5%; 1.5%, 9.4%, and 11%; 1.4%, 2.7%, and 3.9%, respectively. When doses of 5 and 12 μg min⁻¹ levosimendan were used, a reduction of infarcted area to about 69% and 67% of area at risk was observed, and was significantly different from that of about 79% measured in control animals. In addition, after intracoronary levsimendan, the inhibition of apoptosis and activation of autophagy and a dose-related increase of the level of phosphorylation of ERK and Akt were observed. These responses were completely prevented by glibenclamide and significantly reduced by L-NAME. Conclusions: The results of this study show that intracoronary levsimendan prevents myocardial ischemic damages and activates survival signaling through KATP channel opening and NO. These findings support interesting implications for cardioprotection in interventional cardiology and cardiac surgery. © 2010 European Association for Cardio-Thoracic Surgery. Published by Elsevier B.V. All rights reserved.

Keywords: Akt; Apoptosis; ERK; Levosimendan; NO; Potassium channels

1. Introduction

Acute decompensated heart failure (ADHF) is a common and growing medical problem associated with major morbidity and mortality. The main cause of ADHF is ischemic heart disease in 60–70% of patients, with approximately 30% of patients being in cardiogenic shock at the time of hospital admission. Levosimendan, a member of the Ca²⁺-sensitizer drugs, meets most of the goals of treatment in ADHF and also promotes cardioprotection [1]. Levosimendan has been shown to exert a positive inotropic effect as a result of an increase in calcium sensitivity of the subunit C of troponin [2]. Further, the anti-ischemic properties of levosimendan have been related to the vasodilating and preconditioning effects related to both the interaction with KATP channels and to nitric oxide (NO) production [3]. Interestingly, recent in vitro experimental evidence suggests that levosimendan may protect cardiomyocytes from apoptotic cell death through...
the opening of $K_{ATP}$ channels, as well [4]. These in vitro observations are in keeping with some recent in vivo findings, according to which levosimendan-induced clinical improvement of patients with ADHF is related with the reduction of circulating soluble apoptosis signaling molecules [5].

Although systemic levosimendan administration has been reported to induce beneficial hemodynamic effects in the presence of stunned or global ischemic myocardium [6], in the case of acute regional myocardial ischemia, all beneficial effects of intravenously administered levosimendan were diminished by the accompanying hemodynamic changes [1]. We have previously shown in both an animal model [7] and in patients suffering from post-pericardiotomy heart failure [8] that such interactions can be avoided by giving intracoronary levosimendan in doses corresponding to those clinically given as a bolus [9,10]. It is also noteworthy that in experiments recently performed in the acute cardiac ischemic animal model, the intracoronary administration of low doses of levosimendan has been found to reduce apoptotic genes’ activation and to increase autophagy without influencing cardiac function [11]. As the basic functional unit of the myocardium, the cardiac myocytes are the ultimate target of both the pathogenesis and possible therapies in this paradigm. Indeed, maintaining adequate numbers of these terminally differentiated units in the myocardium is critical to the overall preservation of both structural integrity and function of the heart, and has been the focus of therapies used in ischemic syndromes, including reperfusion strategies. Programmed cell death, in the forms of apoptosis, necrosis, and autophagic cell death are the final arbiters of myocyte numbers following myocardial infarction. It is widely accepted that modulation of such forms of programmed cell death could have a significant impact in ventricular remodeling. However, the conditions that decrease the damage to cells in terms of apoptosis might also have a role in the support of heart function acutely. It is therefore possible that, in addition to acting as a calcium sensitizer, the antiapoptotic and proautophagic properties of levosimendan may be an important biologic mechanism that prevents further cytotoxic and hemodynamic consequences of abnormal oxidative response in ADHF, leading to cardioprotection and beneficially intervening in the progression of the syndrome. Hence, these properties could be of crucial importance for improving cardiac protection during interventional cardiology and/or cardiac surgery in end-stage cardiac patients [12].

Thus, as an extension of previous studies, the aim of this research was to examine the effects of different doses of intracoronary levosimendan on cell death and survival signaling activation in an ischemia/reperfusion animal model and to study the involvement of $K_{ATP}$ channels and NO.

2. Material and methods

The experiments were carried out in 56 domestic pigs, weighing 65–73 kg, supplied by an accredited dealer (Azienda Agricola Invernizzi, Olengo, Novara, Italy). The animals, fasted overnight, were anesthetized with intramuscular ketamine (20 mg kg$^{-1}$, Parke-Davis, Milan, Italy) followed after nearly 15 min by intravenous sodium pentobarbinate (15 mg kg$^{-1}$, Siegfried, Zofingen, Switzerland), and artificially ventilated with oxygen-enriched air using a respiratory pump (Harvard 613; Harvard Apparatus, South Natick, USA). Anesthesia was maintained throughout the experiments by the continuous intravenous infusion of sodium pentobarbinate (7 mg kg$^{-1}$ h$^{-1}$) and assessed, as previously reported [13], from responses of the animals to somatic stimuli. The experiments were carried out in accordance with national guidelines (DLGS 27/01/1992, license no. 116), with the European Communities Council Directive (86/609/EEC), and with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, revised 1996). Moreover, the study was approved by the institutional ethics committee.

Pressures in the ascending aorta and right atrium were recorded via catheters connected to pressure transducers (Statham P23 XL; Gould, Valley View, USA) inserted into the right femoral artery and the right external jugular vein, respectively. The chest was opened in the left fourth intercostal space, the pericardium was cut, and an ultrasound flowmeter probe (model 420; Transonic Systems Inc., Ithaca, USA) was positioned around the left anterior descending coronary artery (LAD) to record coronary blood flow. Left-ventricular pressure was measured by means of a catheter connected to a pressure transducer (Gould) inserted through the left atrium. The frequency response of the catheter—manometer system was found to be flat (±$5\%$) up to 40 Hz. To measure regional contractile function, expressed as a percentage of segmental shortening ($%SS$), pairs of 2-mm ultrasonic segment length microtransducer crystals (Sonometrics Corporation, London, Ontario, Canada) were implanted in the left anterior ventricular wall in the distribution area of the LAD. Two additional crystals were placed at opposite ends of the short axis of the left ventricle to measure changes in ventricular dimension throughout cardiac cycles. Each pair of crystals was implanted in the midmyocardial layer approximately 10-mm apart and parallel to the direction of the fibers, so that the segmental shortening was in line with the orientation of fibers. Arterial blood samples were used to measure pH, arterial partial pressures of $O_2$ ($pO_2$) and $CO_2$ ($pCO_2$; with a gas analyzer; Radiometer ABL505, Copenhagen, DK), and hematocrit. The acid–base status of the animals was kept within normal limits as previously reported [13].

Coagulation of blood was avoided by an intravenous injection of heparin (Parke-Davis; initial doses of 500 IU kg$^{-1}$, and subsequent doses of 50 IU kg$^{-1}$ every 30 min) to maintain an activated clotting time of greater than 400 s until the end of the experiment. The rectal temperature of the pigs was monitored and kept between 38 and 40 °C using an electric pad. The heart rate was obtained from the electrocardiogram with a ratemeter (electrocardiograph (ECG)/Biostach amplifi- er, model 13-4615-65 A; Gould). The QT interval corrected for heart rate (QTc) was calculated from ECG recordings by means of Bazett’s formula. The cardiovascular parameters were monitored and recorded, together with the maximum rate of change of left-ventricular systolic pressure ($dP/dt_{\text{max}}$), using a micro1401 A/D converter (Cambridge Electronic Design (CED), Cambridge, UK) displayed on a PC and processed by using Spike2 Software (CED). The maximum rate of change of $dP/dt_{\text{max}}$ was used to define the timing of
the cardiac cycle for segment length measurements with ultrasonic crystals. The end-diastolic length was measured at the onset of the rapid increase in \( \frac{dP}{dt_{\text{max}}} \) and end-systolic length was measured at peak negative \( \frac{dP}{dt_{\text{max}}} \). End-diastolic and end-systolic ventricular volumes were obtained from end-diastolic length and end-systolic length data using the specific software (Sonometrics Corporation). The percentage of segmental shortening (%SS) was calculated using the formula:

\[
%SS = \left( \frac{\text{End-diastolic length} - \text{End-systolic length}}{\text{End-diastolic length}} \right) \times 100
\]

The data from using the Sonomicrometer crystals were digitally processed by specific hardware and software (Sonometrics Corporation). Cardiac output was derived using the Sonosoft System from data recorded by piezoelectric crystals. At the end of the experiment, each animal was euthanized by an intravenous injection of 90 mg kg\(^{-1}\) sodium pentobarbital.

3. Experimental protocol

In the 56 pigs, 2-h myocardial ischemia was regionally induced by the occlusion of the distal portion of the LAD obtained through a tourniquet placed around the vessel. Coronary artery occlusion was verified by epicardial cyanosis and subsequent decrease in blood pressure, and by ST-segment elevation that was accompanied by inversion of the T waves of the ECG. At the end of the ischemic period, 1-h reperfusion was performed. The time course of 2-h ischemia/1-h reperfusion was similar to that previously used in pigs [14]. Moreover, the length of reperfusion was chosen based on the pharmacokinetics of levosimendan: an elimination half-life of nearly 1 h in pigs [14].

Thus, 1-h reperfusion represents the time course of the full effects of bolus levosimendan administration. In three groups of seven pigs each, levosimendan (SIMDAX; Orion-Pharma) dissolved in 15-ml solution of povidone, citric acid anhydrous, and ethanol anhydrous (vehicle; Sigma, Saint Louis, MO, USA), was infused over 15 min into the LAD at the beginning of reperfusion at three different doses of 1.5, 5, and 12 \( \mu \)g min\(^{-1}\) through an infusion pump (Model 22, Harvard Apparatus, Holliston, USA) working at constant rate of 1 ml min\(^{-1}\). These doses were similar to those of 6 and 12 \( \mu \)g min\(^{-1}\) levosimendan given in clinics as systemic bolus administration and have been calculated taking into consideration the cardiac output and the coronary blood flow of each animal [9]. Moreover, intracoronary doses of 3.75 and 12.5 \( \mu \)g min\(^{-1}\) levosimendan have been given as 15-min bolus in patients with left-ventricular dysfunction [10], and similar doses have been previously used in the same animal model as well as in dogs [11,16]. In other seven animals (taken as control), the vehicle only of the highest dose of levosimendan was administered through the coronary artery following the same time course. All the above pigs have been randomized to receive either levosimendan or vehicle.

In other two groups of seven pigs each, the intracoronary 12 \( \mu \)g min\(^{-1}\) levosimendan infusion was performed immediately after the intravenous administration of 1 mg kg\(^{-1}\) glibenclamide (Sigma), and after the intracoronary administration of l-nitro-arginine methyl ester (l-NNAME; Sigma) at a dose of 2 mg ml\(^{-1}\) of measured coronary blood flow, respectively. Both glibenclamide and l-NNAME were infused in approximately 1 min at the end of ischemia. In a further 14 pigs, glibenclamide (seven pigs) and l-NNAME (seven pigs) alone were infused.

After a full-time steady-state pre-ischemic period of 30 min, measurements of basal hemodynamic data were performed immediately before ischemia, before and after levosimendan, vehicle, glibenclamide, and l-NNAME administration (at 15 min from the beginning of reperfusion), and at the end of reperfusion. The same time course was followed to perform transmural myocardial tissue sampling using biopsy needles (HS, Hospital Programs, Milan, Italy) in the cyanotic area around the LAD. In each experiment, biopsy samples were stored in liquid nitrogen and then at \(-80^\circ\text{C}\) to perform Western blot analysis. A schematic representation of the experimental protocol is depicted in Fig. 1.

3.1. Western blot analysis

For Western blot, 50 mg of each tissue sample were lyzed in a buffer (1 M Tris base, 1 M NaCl, 0.5 M Ethylene diamine tetraacetic acid (EDTA), 10% NP40, and 10% Triton X-100; Sigma), containing 1:100 protease inhibitors (Sigma), 1:200 sodium orthovanadate (Sigma), 1:1000 phenylmethylsulfonyl fluoride (PMSF, Sigma), and 0.1 M sodium fluoride (NaF; Sigma). Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, USA). Thirty micrograms of each lysate were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Laboratories, Milan, Italy). After blotting through polyvinylidene fluoride membrane (PVDF; Amersham, Buckinghamshire, UK), nonspecific binding sites were blocked by incubations at room temperature in methanol for 5 min. The blots were then probed with Bax (1:500; Calbiochem, EMD Biosciences, San Diego, CA, USA), Beclin 1 (1:500; Santa Cruz, St. Louis, MO, USA), cytochrome c (1:10000; Sigma), p-p44/p42 mitogen-activated protein (MAP) kinase (Thr202/Tyr204 1:1000; Cell Signaling Technology, Danvers, USA), p-Akt (Ser473 1:1000; Cell Signaling Technology), Caspase 9 (1:300; Sigma) and Caspase 3 antibody (H-277, 1:500; Santa Cruz), and with \(\beta\)-actin (1:5000; Sigma) overnight at 4 °C in agitation. After being washed, they were incubated with specific secondary antibody (at 1:5000) for 45 min at room temperature.

![Fig. 1. Scheme of experimental protocol.](https://academic.oup.com/ejcts/article-abstract/39/4/e59/526603)
Proteins were detected by enhanced chemiluminescence according to the manufacturer’s instructions (Perkin Elmer, Waltham, USA).

3.2. Infarct-size quantifications

In the pigs, infarcted area and area at risk were delineated by a dual staining technique [11]. At the end of levosimendan or vehicle administration, 20 ml of Evans blue dye solution (0.1 g ml\(^{-1}\) in 50 mM phosphate-buffered saline (PBS), pH 7.4; Sigma) was injected into the jugular vein, to stain the non-ischemic area blue. The pigs were then sacrificed as described above, and the hearts were rapidly harvested and sectioned into five transverse slices (0.6-cm thick) from apex to base. Morphological changes in size, shape, and transmural distribution of myocardial infarction were measured using 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) staining. Tissue slices were rinsed with a cold, isotonic, saline solution and then incubated at 38 °C for 15–20 min in a phosphate-buffered solution of TTC (1% in 0.1 M, pH 7.4). This produced a brick-red coloration in the presence of dehydrogenase enzymes in intact myocardium, whereas infarcted regions remained unstained due to the collapse of enzyme activity. Infarcted and area at risk size were calculated by planimetry as a percentage of left-ventricular mass. The infarcted area was expressed as a percentage of area at risk. Tissue samples were taken from the infarcted area to perform Western blot analysis, as well.

3.3. Statistical analysis

All data were recorded using the institution’s database. Statistical analysis was performed using STATVIEW version 5.0.1 for Microsoft Windows (SAS Institute Inc., Cary, NC, USA). Data were checked for normality before statistical analysis. Group data are presented as mean \pm standard deviations (SDs; range). Student’s paired \(t\)-test was used to examine statistical significance within each animal before and after vehicle. Multiple groups were compared through one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls and Dunnet’s post hoc test. A value of \(p < 0.05\) was considered statistically significant.

4. Results

In all pigs, recordings commenced approximately 5 h after the induction of anesthesia. The mean pH, \(pO_2\), and \(pCO_2\) of arterial blood were 7.38 ± 0.02 (7.37–7.42), 118.2 ± 8.9 mmHg (103–139), and 40 ± 1.2 mmHg (38–42), and the hematocrit was 38.5 ± 1.7% (37–40).

4.1. Effects of intracoronary levosimendan administration on myocardial function, cell death, and survival signaling

In the seven pigs that received vehicle only, arterial blood pressure at the end of the reperfusion phase was 79.6 ± 7.2 mmHg (71–89) reducing from basal values of 92 ± 10.3 mmHg (80–112). The decrease of arterial blood pressure was statistically significant from basal values (\(p < 0.05\)), and was accompanied by an increase of heart rate (100.8 ± 9.2 beats/min from basal values of 86.6 ± 9.7 beats/min; \(p < 0.05\)), and of left-ventricular end-diastolic pressure (5.56 ± 0.5 mmHg from basal values of 5.2 ± 0.5 mmHg; \(p > 0.05\)), and by a deterioration of \(dP/\text{d}t_{\text{max}}\) (1812 ± 110 mmHg from basal values of 2510 ± 197.8 mmHg; \(p < 0.05\)) and %SS (8.56 ± 2.4% from basal values of 14.94 ± 2.4%; \(p < 0.05\)). Intracoronary 1.5, 5 and 12 \(\mu\)g min\(^{-1}\) levosimendan administration at the onset of reperfusion caused significant dose-related increases of %SS, \(dP/\text{d}t_{\text{max}}\), and cardiac output, which were already evident at the end of levosimendan infusion and were still present at the end of reperfusion (Fig. 2). In addition, the intracoronary...
administration of levosimendan was found to counteract the reduction of arterial blood pressure (which amounted to \(-3.7\%, -2.1\%, \text{ and } -1.3\%\) of basal values in the three groups of pigs treated with 1.5, 5, and 12 \(\mu\text{g min}^{-1}\) levosimendan, respectively) and the increase of left-ventricular end-diastolic pressure (which amounted to 3.1\%, 1.96\%, and 1.1\% of basal values in the three groups of pigs treated with 1.5, 5, and 12 \(\mu\text{g min}^{-1}\) levosimendan, respectively). Moreover, no significant changes in QTc or arrhythmias have been observed.

In the seven control pigs, which have received vehicle only, the infarcted area amounted to 79.5\% ± 8.3\% (64—90) of area at risk (Fig. 3). Moreover, a further worsening of apoptosis and a decrease of the survival signaling pathway was observed. Indeed, the increase of Bax, cytochrome c, Caspase 3, and Caspase 9 observed after 1-h reperfusion amounted to nearly 17\%, 16.9\%, 56\%, and 30\% in comparison with values measured after 2-h ischemia (\(p < 0.05\)); the decrease of Beclin 1, extracellular receptor kinase (ERK), and Akt amounted to approximately 55\%, 72\%, and 35\% in comparison with measurements performed after 2-h ischemia (\(p < 0.05\)). These findings were not different from those found in preliminary experiments performed in the same animal model with intracoronary saline infusion only, which excluded any effect of vehicle on cardiac function and on cell death.

In the pigs, which have received intracoronary 1.5, 5, and 12 \(\mu\text{g min}^{-1}\) levosimendan, the infarcted area amounted to 74.8\% ± 8.4\% (59—85), 69.4\% ± 7.1\% (55—78), and 67.4\% ± 6.7\% (54—76) of area at risk. Although a reduction of infarcted area has been observed after each levosimendan dose, only after 5 and 12 \(\mu\text{g min}^{-1}\) levosimendan was the infarcted area significantly lower than the one measured in control animals (\(p < 0.05\); Fig. 3).

As depicted in Fig. 4, levosimendan administration caused a dose-dependent reduction of Bax, Caspase 3, Caspase 9, and cytochrome c activation and an increase of Beclin 1, ERK, and Akt. The effects of levosimendan were present at the end of each dose and were still significant after 1 h of reperfusion.

4.2. Role of K\(_{\text{ATP}}\) channels and NO in the cardioprotection exerted by intracoronary levosimendan

The administration of glibenclamide and of L-NAME worsened cardiac function, as was evident at 15-min reperfusion when cardiac function was decreased in comparison with what was observed at the end of ischemia (Tables 1 and 2). Moreover, in the seven pigs treated with glibenclamide and L-NAME, the infarcted areas amounted to 83 ± 8.7\% (68—90) and 82.6 ± 9.5\% (66—90) of area at risk, respectively (Fig. 3).

As shown in Fig. 5, glibenclamide administration caused the worsening of programmed forms of cell death and survival signaling. In addition, the K\(_{\text{ATP}}\) blockade abolished all effects of 12 \(\mu\text{g min}^{-1}\) levosimendan on cardiac function, apoptotic/autophagic genes activation, and on the levels of phosphorylation of ERK and Akt (Figs. 2 and 5). Finally, in pigs treated with levosimendan after glibenclamide, infarcted area amounted to 81 ± 7.1\% (69—86), which was not different from that found in seven pigs treated with glibenclamide alone (\(p > 0.05\); Fig. 3).

It is noteworthy that, in pigs treated with L-NAME, apoptosis activation increased, whereas autophagy, ERK, and Akt decreased (Fig. 5). When 12 \(\mu\text{g min}^{-1}\) levosimendan was administered after L-NAME, the cardiac response was still present although reduced in comparison with that observed with levosimendan alone (Fig. 2(b)). In addition, infarcted...
Table 1. Changes in hemodynamic variables observed during ischemia and reperfusion in control pigs treated with glybenclamide and L-NAME at the onset of reperfusion.

<table>
<thead>
<tr>
<th>Glybenclamide (n=7)</th>
<th>L-NAME (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>max CO (%)</td>
<td>8.3 (49—65)</td>
</tr>
<tr>
<td>min CO (%)</td>
<td>8.5 (47—65)</td>
</tr>
<tr>
<td>max HR (bpm)</td>
<td>92 ± 7 (63—99)</td>
</tr>
<tr>
<td>min HR (bpm)</td>
<td>92 ± 7 (63—99)</td>
</tr>
<tr>
<td>max LVEF (%)</td>
<td>28 ± 2 (10—34)</td>
</tr>
<tr>
<td>min LVEF (%)</td>
<td>28 ± 2 (10—34)</td>
</tr>
<tr>
<td>max SBP (mmHg)</td>
<td>172 ± 8 (150—210)</td>
</tr>
<tr>
<td>min SBP (mmHg)</td>
<td>157 ± 8 (150—209)</td>
</tr>
</tbody>
</table>

The results of this study show for the first time, that a decrease in systolic blood pressure, LVEF, left ventricular end diastolic pressure, CO, mean coronary blood flow, do propranolol (mmHg), and ERK caused by 1.5, 3, 6 levosimendan alone.

Finally, in pigs treated with L-NAME, intracoronary administration of levosimendan alone (0.5, 1 mg/kg/min) with 12 mg/min do propranolol, had a less significant effect on coronary flow than that observed in seven animals treated with 12 mg/min of do propranolol alone which was significant.

<table>
<thead>
<tr>
<th>Data and measures</th>
<th>Control</th>
<th>Glybenclamide</th>
<th>L-NAME</th>
<th>P</th>
<th>p vs baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO (ml/min)</td>
<td>23 ± 2 (150—210)</td>
<td>28 ± 2 (10—34)</td>
<td>28 ± 2 (10—34)</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>28 ± 2 (10—34)</td>
<td>36.2 (64—86)</td>
<td>35.9 (82—112)</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>172 ± 8 (150—210)</td>
<td>173.6 ± 9.2 (150—210)</td>
<td>177.6 ± 13.4 (150—210)</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>92 ± 7 (63—99)</td>
<td>92 ± 7 (63—99)</td>
<td>92 ± 7 (63—99)</td>
<td>0.05</td>
<td>0.05</td>
</tr>
</tbody>
</table>

p < 0.05 versus baseline.
Levosimendan, a member of the Ca\(^{2+}\)-sensitizer drugs, has been developed for the treatment of ADHF. Its mechanism of action combines inotropic and vasodilatory effects. By selective binding to domains of subunit C of troponin, it causes a prolongation of this interaction without impairing diastolic relaxation [17] and without increasing intracellular \(\text{Ca}^{2+}\) concentration [17]. In the present study, the intracoronal levosimendan administration at the onset of 1-h reperfusion following 2-h ischemia caused a dose-related increase of cardiac function, and counteracted the changes in hemodynamic variables, which have been observed in animals treated with vehicle only.

These effects were accompanied by a reduction of infarcted area, which was significantly different from the one measured in control animals when 5 and 12 \(\mu\)g min\(^{-1}\) levosimendan were used. It is noteworthy that the beneficial effects elicited by intracoronary levosimendan were still significant at 1-h reperfusion when the coronary blood flow had almost returned to basal values, which would exclude any secondary effect related to vasodilation. Although the intracoronary administration of bolus of levosimendan would have resulted in selective cardiac effects, the possibility could be speculated that some systemic effects could have arisen from the distribution of levosimendan during its first pass through the myocardium. Such a possibility would entail that the intracoronary levosimendan administration could produce a higher intracardiac drug concentration and a faster effect on the myocardium compared with conventional intravenous administration. Similar results have been observed after intracoronary levosimendan bolus administration in animal models of stunned myocardium [16], and in patients suffering from heart failure [8,10]. In one report, the bolus administration of levosimendan in a patient suffering from post-pericardiotomy heart failure induced a significant increase of left-ventricular kinesis, cardiac output, and in graft blood flow without altering cardiac rate and reducing arterial blood pressure [8]. Moreover, in patients with left-ventricular dysfunction, intracoronary levosimendan at doses similar to those used in the present study (3.75 and 12.5 \(\mu\)g min\(^{-1}\) for 15 min) exerted positive inotropic and lusitropic effects [10]. Further, infarct-size reduction afforded by levosimendan was similar to that previously reported as the response of single dose of 24 \(\mu\)g kg\(^{-1}\) in anesthetized rats [18].

It is noteworthy that the dose-related cardiac effects of intracoronary levosimendan were accompanied by the modulation of apoptotic and autophagic genes activation and, interestingly, by activation of the intracellular survival pathway. These findings are in agreement with previous reports about inhibitory effects of levosimendan on apoptosis [4]. Hence, in advanced decompensated patients, systemic levosimendan administration caused the improvement of cardiac function and a significant reduction of soluble receptor Fas with Fas ligand, as well [5]. Moreover, similar findings have recently been obtained in preliminary experiments performed in the same animal model [11].

In the present study, and for the first time, a dose-related increase of both Akt and ERK was found in response to levosimendan in comparison with findings obtained in control animals where Akt and ERK were strongly reduced. These findings are in line with those reported by Hausenloy et al., who showed that both arms of the so-called reperfusion injury salvage kinase (RISK) pathway, PI3K-Akt, and ERK1/2,
are essential for infarct-size reduction in response to ischemic preconditioning [19]. Hence, this signaling pathway represents one of the major protein kinase programs, which leads to cardioprotection by inhibition of mitochondrial permeability transition pores (mPTP) opening. Regarding levosimendan, Honisch et al. have recently reported that its use in post-conditioning in the anesthetized rat has been able to trigger the PI3K-Akt cascade of the RISK pathway rather than ERK [18]. Different results have been obtained by du Toit et al., showing that pre-treatment of guinea pig hearts with levosimendan before ischemia increased activity of ERK on reperfusion [20]. The differences observed could be related to the different experimental protocol or animal model.

In the present study, the administration of glibenclamide before reperfusion caused the worsening of cardiac function and increased necrotic area. These effects have been accompanied by an increase of Bax, cytochrome c, Caspase 3, and Caspase 9 and by a reduction of Beclin 1, Akt, and ERK1/2. Indeed, the KATP channels have been shown to play a dominant role in affording cardioprotection in ischemic/reperfused hearts because of their energy-modulating properties resulting in more efficient cardiomyocyte bioenergetics [21]. In addition, in pigs that have received glibenclamide, all effects of intracoronary 12 μg min⁻¹ levosimendan were abolished. These findings are in agreement with previous reports providing evidence for a role of K<sub>ATP</sub> channels in the beneficial effects exerted by levosimendan against ischemia/reperfusion injuries [18, 20].

Moreover, the administration of the NOS blocker increased apoptotic genes’ activation, and caused the reduction of autophagy and the inhibition of the RISK pathway. These results are in agreement with reports from literature, which suggest a beneficial role of NO against ischemia/reperfusion injury and apoptosis through interference with the activation of the caspases cascade [22], and through the modulation of PI3K and Akt [23]. In particular, low levels (nM) of NO have been shown to reversibly inhibit mPTP opening, whereas at higher than physiological release rates (>2 μM s⁻¹), NO would accelerate mPTP opening and cytochrome c release [24].

Moreover, in pigs treated with L-NAME before reperfusion, not only the effects of levosimendan on cardiac contractility but also on apoptosis and on the RISK pathway have been significantly reduced. These results are in agreement with findings obtained in anesthetized rabbits where pharmacological preconditioning by levosimendan has been found to be mediated by nitric oxide synthase (NOS) [25].

It is noteworthy that, in coronary endothelial cells, levosimendan has been recently reported to induce the phosphorylation of ERK1/2, p38, and Akt and the activation of endothelial NOS (eNOS) either directly or through the opening of K<sub>ATP</sub> channels [3]. All together, these findings seem to support a relationship between NO and K<sub>ATP</sub> channels in the cardiovascular actions of levosimendan.

In conclusion, the results of the present study show, for the first time, that the intracoronary levosimendan administration at the onset of reperfusion would exert beneficial cardiac effects through the modulation of myocardial cell death and the activation of components of the RISK pathway, such as Akt and ERK1/2. These effects would be related to activation of the pathway mediated by both K<sub>ATP</sub> channels’ opening and NO production. It is noteworthy that these pathways are the common targets for cardioprotection shared by both ischemic pre- and post-conditioning, which are interventional strategies for protecting ischemic hearts. Thus, levosimendan could represent a pharmacological agent that is beneficial for patients undergoing myocardial ischemia/reperfusion injury, such as patients undergoing thrombolysis or percutaneous coronary intervention, or patients undergoing cardiac surgery.

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References


Appendix A. Conference discussion

Dr H. Vetter (Wuppertal, Germany): Having read your paper, I can state that this basic experimental work was conducted in a proper experimental setup of the study outline.

In clinical studies, like the LIDO or RUSSLAN, where levosimendan was compared to dobutamine, there was a significant survival benefit outcome for the new drug. In postcardiotomy and post-infarction patients, however, results were less convincing. I think that was your reason for performing further investigations. Levosimendan has not yet become a drug for routine use in the intensive care unit. I have some questions and some comments which arose from studying your paper.

Would it have been advantageous to analyze blood samples from the coronary sinus? And in clinical use, the most frequent adverse events described were ventricular tachycardia, hypertension and ventricular extrasystoles. Did you see these complications during your experiments? You have taken several myocardial biopsies; I think there were as many as seven samples taken from one myocardial area. Are you sure that this did not impair ventricular function in the pig’s heart by the end of the experiment?

Last question: in clinical practice, do you think that a single intracoronary infusion would be sufficient?

Dr Caimmi: About the arrhythmias, we used the Bazett’s formula. So we did not have arrhythmias.

In clinical practice, if the drug is properly used, it is not described as an arrhythmogenic drug. The problem is the trials. The trials are not sufficient at the moment, because there is a lot of bias. Also, they did not discriminate the ischemic patients. In our opinion, ischemic patients should not be treated with levosimendan before revascularization, only after revascularization. Because if you treat the ischemic patients with levosimendan, you have a bad impact on regional kinesis.

On the other hand, after revascularization, the effects will be very, very interesting. So for the cardioprotective effects, also the preoperative revascularization treatment will be good, but you cannot demonstrate this because you have a negative impact on the haemodynamics (systemic hypotension). After revascularization you will have both good effects, hemodynamic and cardioprotective.

Regarding the use in intracoronary administration, this approach was studied by our group especially to avoid the hemodynamic impact that we have when we administer levosimendan in the systemic circulation. So intracoronary administration probably is the safest because it doesn’t affect the pressure in so severe patients.

Dr D. Chambers (London, United Kingdom): That was a very interesting study, although you went through it very quickly and you showed a lot of data, so it was difficult to follow.

But it’s interesting, your data. I wondered why you used glibenclamide as a potassium channel blocker, because it’s a very nonselective drug. And there are other drugs that could target the KATP channels in the mitochondria or the sarcosome, and I wondered why you didn’t look at that, because some of the cardioprotective effects have been shown to be in the mitochondrial KATP channels.

Dr Caimmi: We chose this blocker because it is one of the most used in the literature in a similar model. So we chose it to compare with other studies.

Dr Chambers: But it doesn’t dissect out where your effect is occurring, that’s the problem.

Dr Caimmi: When we used glibenclamide, we observed a worsening of the damage. So it’s clear the target is right. Most reports use glibenclamide.

Dr D. Taggart (Oxford, United Kingdom): Why are you not doing these studies in man, if this is a drug that’s approved? If I’ve understood you and this drug is approved for use, why are you not trying these studies in the clinical setting?

Dr Caimmi: Our direction is to use this to protect the heart. But we have to validate the method experimentally. So with our data at the moment, we have just performed a study in a similar setting in humans, evaluating the hemodynamic results, and it was very good. Now we also have the support from the study I presented here, that shows the primary cellular effect of the drug. I think the direction would be this. The problem is when to use the levosimendan: not when the patient is not vascularized, is not reperfused, but after reperfusion. Because in ischemic patients, the first approach can be very, very dangerous. We have to reperfuse the patients: in this situation we have the best results with levosimendan in our experience.