Levosimendan Protection against Kidney Ischemia/Reperfusion Injuries in Anesthetized Pigs

Elena Grossini, Claudio Molinari, Piero Pollesello, Giorgio Bellomo, Guido Valente, David Mary, Giovanni Vacca, and Philippe Caimmi

Laboratory of Physiology and Experimental Surgery, Department of Translational Medicine (E.G., C.M., D.M., G.V., P.C.) and Laboratory of Anatomical Pathology, Department of Health Sciences (G.V.), University East Piedmont, Novara, Italy; Orion Pharma, Espoo, Finland (P.P.); and Laboratory Analysis, Azienda Ospedaliero Universitaria Maggiore della Carità, Novara, Italy (G.B.)

Received March 2, 2012; accepted May 4, 2012

ABSTRACT

Ischemia/reperfusion (I/R) injury is an important cause of acute renal failure because of oxidative, inflammatory, and apoptotic mechanisms. The aim of the present study was to examine any possible protective effects of levsimendan in an in vivo pig model of renal I/R injury. In 40 anesthetized pigs (eight groups of five pigs each), I/R was induced by clamping-reopening the left renal artery. During ischemia, in three groups of pigs, levsimendan and the multiorgan preservation solution Custodiol, alone or in combination with levsimendan, were infused in the renal artery. In two other groups of animals, levsimendan in combination with Custodiol was administered after the intrarenal artery. In two other groups of pigs, levosimendan and the nitric-oxide (NO) synthase blocker L-NAME or the mitochondrial ATP-sensitive K⁺ channel (KATP channel) inhibitor 5-hydroxydecanoate (5-HD). In the other animals, saline, L-NAME, or 5-HD were administered alone. Throughout the experiments, urinary N-acetyl-β-glucosaminidase (NAG) release was measured, and renal function was assessed. Moreover, renal biopsy samples were taken for the detection of apoptosis and tissue peroxidation. In pigs treated with levsimendan or the combination of levsimendan and Custodiol, NAG, peroxidation, and apoptotic markers were lower than in animals treated with Custodiol alone. In addition, renal function was better preserved, and cell survival and antioxidant systems were more activated. All beneficial effects were prevented by L-NAME and 5-HD. In conclusion, levsimendan alone or in combination with Custodiol exerted better protection against renal I/R injuries than Custodiol alone through antioxidant, antiapoptotic, and prosurvival actions depending on mitochondrial KATP channels and NO-related mechanisms.

Introduction

In spite of significant advances in critical-care medicine, renal I/R injury remains a major cause of acute renal failure, and high morbidity and mortality rates have not significantly decreased over the last 50 years (Star, 1998; Lameire and Vanholder, 2001; Lien et al., 2003). Renal I/R also contributes to the considerable morbidity associated with cardiac surgery and anesthesia (McCombs and Roberts, 1979; Aronson and Blumenthal, 1998) and occurs during transplantation, caused by reperfusion injury in the early transplant period associated with late allograft failure (Boom et al., 2000). At the basis of such injuries there would not only be the activation of inflammatory processes within tissues (Sutton et al., 2002) but also the modulation of mitochondrial function, changes in intracellular calcium levels, and the increased production of free oxygen radicals (ROS), which could lead to cell death through necrosis and apoptosis (Devalaraja-Narashimha et al., 2005). Among various suggested pharmacological interventions, kidney preconditioning has been shown to afford the greatest protection (Bonventre, 2002). In addition, alleviation of the effects of I/R injury has been attempted, particularly in transplantation, with the development of complex organ preservation solutions such as Custodiol, Euro-Collins, HTK (histidine, tryptophan, α-ketoglutarate), UW (University of Wisconsin), and Marshall’s (hypertonic citrate) solution that contain a variety of buffers, glucose metabolites, and free radical scavengers. Nevertheless, the addition of the above agents to preservation solu-
tions has been accompanied only by limited success (Dhalla et al., 2000; Jones et al., 2003). Among the various suggested therapies against I/R injuries, the “calcium sensitizer” levosimendan could be of particular interest. For instance, in a clinical series of patients with cardiogenic shock who have been treated with levosimendan a significant reduction of polyuric syndrome after a stable hemodynamic recovery has been achieved (Caimmi et al., 2011a). Moreover, experimental evidence has shown tubular protection exerted by levosimendan against ischemic injuries (Yakut et al., 2008). Hence, levosimendan, in addition to improving myocardial contraction and exerting vasodilation, has been demonstrated to have the potential to protect against I/R damage through a preconditioning-like effect (Kersten et al., 2000; Soeding et al., 2011) and the reduction of inflammatory and oxidative stress profiles (Avgeropoulou et al., 2005). Moreover, levosimendan has been shown to protect cardiomyocytes from apoptotic cell death caused by I/R injuries (Grossini et al., 2010; Caimmi et al., 2011b; Uberti et al., 2009), and mitochondrial function (Kaheinen et al., 2001; Grossini et al., 2010). Mitochondrial KATP channels (Kaheinen et al., 2001; Grossini et al., 2010; Caimmi et al., 2011b; Uberti et al., 2011), the modulation of NO release (Grossini et al., 2005, 2009), and mitochondrial function (Kaheinen et al., 2001; Grossini et al., 2010; Caimmi et al., 2011b; Uberti et al., 2011). Taken together, these findings would suggest that renal protective effects caused by levosimendan occur not only through hemodynamic improvement but also by direct cellular action. Thus, the present work was planned in anesthetized pigs to examine any protective effects of levosimendan against kidney I/R injuries and the involvement of mitochondrial KATP channels and NO.

### Materials and Methods

The experiments were carried out in 40 pigs, weighing 69 to 71 kg, supplied by an accredited dealer (Azienda Invernizzi, Olengo, Novara, Italy). The animals were fasted overnight and then anesthetized with intramuscular ketamine (20 mg/kg; Parke-Davis, Milan, Italy) followed after approximately 15 min by intravenous sodium pentobarbitone (15 mg/kg; Siegfried, Zofingen, Switzerland), after which they were artificially ventilated with oxygen-enriched air by using a respiratory pump (Harvard 613; Harvard Apparatus Inc., Holliston, MA). Anesthesia was maintained throughout the experiments by continuous intravenous infusion of sodium pentobarbitone (7 mg/kg/h) and assessed as reported previously (Linden and Mary, 1983). Throughout the experiments arterial blood pressure was measured by means of a catheter connected to a pressure transducer (Statham P23 XL; Gould Instrument Systems Inc., Cleveland, OH) inserted into the right carotid artery. Heart rates were obtained from electrocardiograms, and renal blood flow of the left kidney was measured by an ultrasonic flowmeter probe (Transonic Systems Inc., Ithaca, NY) positioned around the renal artery. The jugular veins were cannulated for volume replacement by using warm 0.9% saline. Arterial blood samples were used to measure pH, pO₂, and pCO₂ and the hematocrit. Coagulation of the blood was avoided by the intravenous injection of heparin (initial doses of 500 IU/kg and subsequent doses of 50 IU/kg every 30 min) to maintain an activated clotting time of more than 400 s until the end of the experiment. The rectal temperature was monitored and kept between 38 and 40°C by using an electric pad. The experiments were carried out in accordance with local ethical committees, national guidelines (Decreto legislativo 27 gennaio 27/01/1992, license 116), Good Research Practice Guidelines in the European Community, and the Guide for the Care of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) as adopted and promulgated by the National Institutes of Health. In each animal, after a midline laparotomy, the left renal artery was clamped and cannulae were inserted into the artery distal to the clamp position to infusion the pleogic solution and into the renal vein to discharge renal pressure overloading and collect the infused solutions. In addition, the left ureter was cannulated to collect urine samples.

In five pigs (group GA), immediately after renal artery closure levosimendan (Simdax; Orion Pharma, Espoo, Finland) dissolved in 2500 ml of saline at 4°C was administered into the left renal artery beyond the clamp at 80 mm Hg, through a pressurized reservoir, over the ischemic period at a constant flow rate. In five other pigs (group GB), 2500 ml of the Custodiol HTK-Multiorgan Solution (1000 ml: 0.8766 g of sodium chloride, 0.6710 g of potassium chloride, 0.1842 g of potassium hydrogen 2-ketoglutarate, 0.8132 g of magnesium chloride, 3.7733 g of histidine HCl, 0.4085 g of tryptophan, 5.4651 g of mannitol, and 0.0022 g of calcium chloride in sterile H2O; Artech s.r.l., Cavezzo, Italy) was given following the same protocol of group GA. In five other animals (group GC), 2500 ml of the Custodiol solution containing 1 μM levosimendan was administered into the left renal artery over the ischemic period as described for groups GA and GB. In five more pigs (group GD, the same protocol as for group GC was followed but after the intrarenal administration of the mitochondrial KATP channel inhibitor 5-hydroxydecanoate (5-HD; 150 μg/kg; Sigma, Milan, Italy). In five other animals (group GE), the same protocol of group GC was followed but after the intrarenal administration of the NOS inhibitor N⁶-nitro-L-arginine methyl ester (L-NAME; 2 mg/min for each ml/min of measured renal blood flow; Sigma). Two more groups of five pigs each (GF and GG) were investigated following the same experimental protocol of groups GD and GE, respectively, but without levosimendan. Finally, in five more pigs (control group GH), 2500 ml of saline was infused for 90 min in the renal artery after the clamping.

The kidneys were maintained at 4°C by using an apposite refrigerating bag throughout ischemia that lasted 90 min. After the isch- emic period, renal vessels were declamped, and reperfusion was maintained for 120 min. In all pigs, blood samples were collected during the experiments according to the following time course: t₀ = control (before kidney clamping); t₁ = at the end of 90-min ischemia; and t₂ = at the end of 120-min reperfusion. Urinary volumes were measured, and urinary samples were taken at t₀ and t₂.

Throughout the experiments, plasma and urinary creatinine and urinary N-acetyl-β-glucosaminidase (NAG) (Bioquant, San Diego, CA) (a widely used and specific marker of renal tubular injury) release were examined at t₀ and t₂. In addition, plasma creatinine (Siemens Healthcare Diagnostics, Deerfield, IL) was measured at t₁. Proteinuria, α-1-microalbuminuria (Siemens Healthcare Diagnostics), and the urinary α-1-microalbumin-to-creatinine ratio (a non-specific marker of glomerular and proximal tubular injury) were determined at t₀ and t₂. An ADVIA 2400 Chemistry Analyzer (Siemens Healthcare Diagnostics) was used for creatinine, albumin, and protein determination. The analytical variabilities for creatinine, albuminuria, and proteinuria measurements were 1.47, 3.62, and 6.52%, respectively.

Creatinine clearance was calculated from urine and serum samples by the standard formula: creatinine clearance (ml/min) = [urine creatinine concentration (μmol/ml) × urine volume (ml/min)]/plasma creatinine concentration (μmol/ml).

Biopsy samples were taken from kidneys at t₀, t₁, and t₂. Moreover, sections from kidneys removed at the end of experiments were taken for histological analyses. All biopsy samples were cut in two portions of which one was fixed in formalin and embedded in paraffin for hematoxylin-eosin staining and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay (Millipore Corp., Milan, Italy). The other one was snap-frozen by liquid nitrogen and stored at −80°C for Western blot analysis.
Frozen biopsy kidney samples and kidney sections were processed for the quantification of glutathione (GSH) (Sigma) content and the determination of caspase-3 activity (Tema Ricerca, Bologna, Italy).

**Western Blot.** The renal biopsies, conserved at −80°C, were further cut into five parts, and 20 mg of each tissue sample was lysed in a buffer (1 M Tris base, 1 M NaCl, 0.5 M EDTA, 10% nonyl phenoxypolyethoxylethanol, and 10% Triton X-100; Sigma) containing 1:100 protease inhibitors (Sigma), 1:200 sodium orthovanadate (Sigma), and 1:1000 phenylmethylsulfonyl fluoride (Sigma). Protein concentrations were determined by using the bicinchoninic acid (Sigma) assay. Forty micrograms of each lysate was subjected to electrophoresis on SDS-polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Hercules, CA) and transferred to polyvinylidene difluoride (Thermo Fisher Scientific, Waltham, MA) membranes and finally probed with p-Bax (Thr167; 1:500; Assay Biotechnology Company, Sunnyvale, CA), Bax (1:500; Sigma), cytochrome c (1:500; Sigma), p-p44/42 mitogen-activated protein kinase (1:1000; Cell Signaling Technology, Danvers, MA), p44/42 mitogen-activated protein kinase (1:1000; Cell Signaling Technology), p-Akt (1:1000; Cell Signaling Technology), Akt (1:1000; Cell Signaling Technology), and actin (1:5000; Sigma) detection.

**Caspase-3 Activity.** The tissue renal lysates were also used for the caspase-3 assay according to the manufacturer's instructions (Tema Ricerca). In brief, 50 μl of each sample was added into 75 μl of caspase-3 colorimetric substrate in a 96-well ready to use. The samples were vortexed and left in ice for 10 min and then centrifuged at 10,000g for 10 min at 4°C. The supernatants were diluted 5-fold to stay in the detection range. The results of the sample were read at 405 nm by a spectrometer (BS1000 Spectra Count) and compared with those obtained with the standard curve generated with various GSH dilutions. The results were expressed as GSH nM.

**TUNEL Assay.** For DNA fragmentation analysis caused by renal ischemia/reperfusion, the TUNEL technique was performed (Millipore Corporation). Working Strength mixture (110 μl) containing terminal deoxynucleotidyl transferase was added for 60 min in the dark in a humidified camera to the renal sections previously deparaffinized, rehydrated, and treated with protein-digesting enzyme (20 μg/ml proteinase K). Nuclear counterstaining was obtained by the incubation in 0.5% (w/v) methyl green for 10 min at room temperature. The reaction was analyzed with a microscope, and positive cells were counted in 10 different expositions of at least five fields for each experimental protocol.

**Statistical Analysis.** All data were recorded by using the University East Piedmont, Department of Translational Medicine’s database. Statistical analysis was performed by using Statview version 5.0.1 for Microsoft Windows (SAS Institute, Cary, NC). Data were checked for normality before statistical analysis. The Student’s paired t test was used to examine statistical significance within each animal. One-way analysis of variance followed by Bonferroni post hoc test was used to examine changes among the different groups. Data are expressed as mean ± S.D. (minimum and maximum values). A value of p < 0.05 was considered statistically significant.

**Results**

**Renal Protection against I/R Injuries by Levosimendan.** As shown in Table 1, arterial blood pressure and heart rate measured at the end of ischemia, at 15-min reperfusion, and at the end of reperfusion did not change significantly among the various groups of animals (p > 0.05), thus excluding confounding factors on observed renal effects elicited by the various agents used.

As shown in Fig. 1, 90-min ischemia/120-min reperfusion caused slight histological changes represented mainly by ballooning of tubular cells. To examine renal damages caused by I/R, molecular markers of renal damages such as TBARS and NAG and the level of antioxidant GSH were measured throughout the experiments and compared with those obtained before ischemia (t0). In particular, TBARS and GSH were examined in renal tissue samples after both ischemia (t1) and reperfusion (t2), whereas NAG was measured in urinary samples only after reperfusion (t2). As shown in Fig. 2A, after 90-min ischemia TBARS was significantly increased in saline-treated animals (p < 0.05). After 120-min reperfusion TBARS was worsened and urinary NAG was increased (Fig. 2: p < 0.05), whereas GSH levels were markedly reduced (Fig. 3A; p < 0.05). The administration of Custodiol only significantly reduced changes in the above mark-
TABLE 1
Changes in hemodynamic variables throughout I/R in 30 pigs
Reported values refer to means ± S.D. of five different experiments.

<table>
<thead>
<tr>
<th>Arterial Blood Pressure</th>
<th>Heart Rate</th>
<th>Renal Blood Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>mm Hg</td>
<td>bpm</td>
<td>ml/min</td>
</tr>
<tr>
<td>Saline, group GH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>85.2 ± 8</td>
<td>77.5 ± 1.7</td>
</tr>
<tr>
<td>I</td>
<td>86 ± 7.7</td>
<td>77.2 ± 0.9</td>
</tr>
<tr>
<td>R 15 min</td>
<td>85.7 ± 7.9</td>
<td>77.7 ± 0.5</td>
</tr>
<tr>
<td>R 120 min</td>
<td>86 ± 8</td>
<td>77.7 ± 1.7</td>
</tr>
<tr>
<td>Levosimendan, group GA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>80 ± 3.6</td>
<td>79.8 ± 3.7</td>
</tr>
<tr>
<td>I</td>
<td>80.4 ± 3.5</td>
<td>79.6 ± 3.9</td>
</tr>
<tr>
<td>R 15 min</td>
<td>80.2 ± 3.7</td>
<td>79.8 ± 4.1</td>
</tr>
<tr>
<td>R 120 min</td>
<td>79.2 ± 3.4</td>
<td>79.8 ± 3.7</td>
</tr>
<tr>
<td>Custodiol, group GB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>78.6 ± 2.7</td>
<td>75.2 ± 6</td>
</tr>
<tr>
<td>I</td>
<td>79 ± 2.4</td>
<td>74.5 ± 6.3</td>
</tr>
<tr>
<td>R 15 min</td>
<td>79 ± 2.7</td>
<td>75 ± 6.7</td>
</tr>
<tr>
<td>R 120 min</td>
<td>78.5 ± 2.5</td>
<td>75.7 ± 5.9</td>
</tr>
<tr>
<td>Levosimendan + Custodiol, group GC</td>
<td>79.7 ± 3.3</td>
<td>73.7 ± 7</td>
</tr>
<tr>
<td>C</td>
<td>83.5 ± 5.2</td>
<td>76 ± 7.8</td>
</tr>
<tr>
<td>I</td>
<td>84.7 ± 5.1</td>
<td>74.7 ± 7.6</td>
</tr>
<tr>
<td>R 15 min</td>
<td>82.7 ± 6</td>
<td>75.5 ± 7.1</td>
</tr>
<tr>
<td>R 120 min</td>
<td>81.2 ± 5.5</td>
<td>76.7 ± 8.4</td>
</tr>
</tbody>
</table>

C, control values; I, after 90-min ischemia; R 15 min, at 15-min reperfusion; R 120 min, at 120-min reperfusion.

*p < 0.05 vs. C. **p < 0.05 vs. R 15 min.

Fig. 1. A, biopsy taken from one pig kidney before the onset of ischemia/reperfusion, with no evidence of histological changes in renal tubules. B, histological changes are detectable, represented mainly by the ballooning of tubular cells. Hematoxylin/eosin staining is shown at 430X.

Fig. 2. Effects of saline, levosimendan (Levo), Custodiol, and the combination of levosimendan and Custodiol on TBARS and NAG in presence of renal I/R. A, TBARS. Columns c, e, g, i, and m, p < 0.05 versus column a. Columns d, f, h, l, and n, p < 0.05 versus column b. Column h, p < 0.05 versus column I and n. Columns i and m, p < 0.05 versus column g. Column m, p < 0.05 versus column i. Column I, p < 0.05 versus column n. B, urinary NAG. Columns b to f, p < 0.05 versus column a. Columns e and f, p < 0.05 versus column d. C, control values; I, after 90-min ischemia; R, after 120-min reperfusion. Reported values are mean ± S.D. of five experiments.

ers of I/R injuries (p < 0.05). However, the protection against ischemic damages was the highest in pigs treated with levosimendan alone or, at a higher extent, in combination with Custodiol (Figs. 2 and 3). Indeed, TBARS was lower than that
found in pigs treated with saline and Custodiol alone, after both ischemia and reperfusion \((p < 0.05)\). Moreover, urinary NAG was lower than that measured in saline- and Custodiol-treated animals \((p < 0.05)\). Hence, in pigs treated with levo-
simendan in combination with Custodiol both TBARS and
NAG were not different from control values. Finally, GSH
levels were higher in pigs treated with levo-
simendan and
particularly in combination with Custodiol after both isch-
emia and reperfusion \((p < 0.05)\).

Hence, in pigs treated with levo-
simendan in combination with Custodiol GSH levels at t1 and t2 were higher by approx-
imately 6 and 10% than those observed before ischemia
\((p < 0.05)\), whereas in Custodiol-treated animals GSH levels were higher by approximately 1.2 and 3.6% than control
values, at t1 and t2, respectively \((p < 0.05)\).

**Antiapoptotic and Prosurvival Effects Elicited by Levosimendan.** Caspase-3 activity examination and Western blot analysis performed on renal tissue samples taken at
t1 (after ischemia) and t2 (after reperfusion) showed evidence of an increase of apoptosis and a reduction of eNOS and survival signaling in response to I/R injuries \((p < 0.05)\). As shown in Figs. 3B, 4, and 5A, caspase-3 activity, Bax, caspase-9, and cytochrome c were increased at both t1 and t2
\((p < 0.05)\). The administration of levo-
simendan alone or in combination with Custodiol counteracted the up-regulation of the above apoptotic markers. Hence, in animals treated with levo-
simendan alone and, above all, in pigs treated with levo-
simendan in combination with Custodiol, the reduction of caspase-3 activity was more effective than what was ob-
served in animals treated with Custodiol alone \((p < 0.05)\). In addition, Western blot analysis confirmed the protective ef-
fects elicited by levo-
simendan against apoptosis in pig kid-
ney. As shown in Figs. 4 and 5A, in animals treated with
either levo-
simendan or levo-
simendan in combination with Custodiol Bax, caspase-9, and cytochrome c activation after ischemia and reperfusion was lower than what was found in both saline- and Custodiol-treated animals \((p < 0.05)\). In particular, in pigs treated with levo-
simendan in combination with Custodiol cytochrome c at t1 was almost 57% lower than
that measured in animals treated only with Custodiol \((p < 0.05)\) and almost returned to control values at t2 (Fig. 5A).

Moreover, these findings were accompanied by higher eNOS (Fig. 5B; \(p < 0.05\)) and extracellular signal-regulated kinase 1/2 (ERK1/2; Fig. 6A; \(p < 0.05\)) and Akt (Fig. 6B; \(p < 0.05\)). It is noteworthy that SOD3 (Fig. 6C), which is the SOD isoform highly expressed in renal tissue and is involved in the preservation of renal function against oxidative stress (Schneider et al., 2010; Kawakami et al., 2012), was also higher in pigs treated with levo-
simendan in combination with Custodiol than in animals treated only with Custodiol, particularly after reperfusion \((p < 0.05)\). As shown in Figs. 5B and 6, A and C, in animals treated with levo-
simendan in combination with Custodiol eNOS and ERK1/2 activation at
t2 was almost 4-fold higher than that observed in Custodiol-
treated animals \((p < 0.05)\), whereas SOD3 activation was
3-fold higher \((p < 0.05)\).

The antiapoptotic effects elicited particularly by levo-
simendan in combination with Custodiol were also found by TUNEL assay. TUNEL-positive bodies were absent or rarely detected in this group of animals, whereas in saline- and Custodiol-treated pigs a mean number of 15 and 6 positive bodies/high-power field was counted, respectively (Fig. 7).

**Preservation of Renal Function against I/R by Levosimendan.** Renal function was examined throughout the experiments by plasma creatinine determination after isch-
emia (t1) and reperfusion (t2) and by urinary microalbumin-
uria, protein/creatinine ratio, and albumin/creatinine ratio
measured after reperfusion and compared with those found before ischemia (t0). Creatinine clearance evaluated after
reperfusion was also compared with that measured before ischemia. The findings on renal function showed higher protection against renal I/R injuries in pigs treated with levosimendan, particularly in combination with Custodiol. As depicted in Fig. 8A, plasma creatinine in animals treated with levosimendan and Custodiol was the lowest after both ischemia and reperfusion, whereas creatinine clearance was highly improved in animals treated with levosimendan and Custodiol (Fig. 8B). Hence, plasma creatinine at t2 in pigs treated with levosimendan in combination with Custodiol and in animals treated with Custodiol was lower than control values by approximately 20 and 43%, respectively.

Hence, at reperfusion the animals treated with levosimendan alone were better protected than those treated with Custodiol alone. Moreover, in animals treated with levosimendan and, particularly, in pigs treated with levosimendan in combination with Custodiol, urinary protein/creatinine and albumin/creatinine ratios and urinary microalbumin measured at 2 h reperfusion were lower than those measured in saline- and Custodiol-treated animals (Fig. 9). Urinary microalbumin, protein/creatinin ratio, and microalbumin/creatinine ratio were lower by approximately 74, 50, and
63%, respectively, in pigs treated with levosimendan in combination with Custodiol compared with what was found in animals treated with Custodiol alone, which provided evidence for higher protection exerted by levosimendan in combination with Custodiol against I/R injuries.

Role of Mitochondrial K\textsubscript{ATP} Channels and NO in the Renal Protective Effects Elicited by Levosimendan against I/R Injuries. As shown in Figs. 2 to 6, 8, and 9, in pigs treated with 5-HD and L-NAME all markers of apoptosis, cell survival signaling, and renal function were worsened, after both ischemia and reperfusion, in comparison with what was observed in animals treated with levosimendan and Custodiol ($p < 0.05$). These effects were not accompanied by any changes in arterial blood pressure, heart rate, and renal blood flow (Table 1; $p > 0.05$). Moreover, all protective effects elicited by levosimendan were prevented.

Discussion

The present study has shown for the first time the protective effects elicited in anesthetized pigs by levosimendan against renal I/R injuries through the modulation of apoptotic and oxidative cell death and activation of prosurvival cell signaling.

The pathophysiology of ischemic renal failure involves a complex interplay between renal hemodynamic variables,
tubular injury, and inflammation. Ischemic injury to the vascular endothelial cell results in the loss of its capacity to regulate inflammatory cells migration into the tissue, vascular tone, permeability, and coagulation (Molitoris et al., 2002; Devalaraja-Narashimha et al., 2005). In addition, damage occurs predominantly in the proximal tubule, leading to tubular cell dysfunction, swelling, desquamation, obstruction, back leakage, and consequently the reduction of the glomerular filtration rate (Bonventre, 1993; Devalaraja-Narashimha et al., 2005). Moreover, in humans and experimental models of renal ischemia, tubular cells in various nephron segments undergo necrotic and/or apoptotic cell death (Devalaraja-Narashimha et al., 2005). The return of blood flow to ischemic tissue can not only result in the recovery of normal cellular function, but paradoxically can increase the tissue injury as a consequence of the process of reperfusion. A key feature of I/R injury is the generation of ROS, such as superoxide radical, hydroxyl radical, or singlet oxygen, resulting from the return of oxygen to ischemic tissue (Zweier et al., 1994; Zweier and Talukder, 2006). Such highly reactive free radicals together could form a potent cytotoxic cocktail capable of inactivating cellular enzymes, damaging essential cellular constituents and modulating cell viability (Przyklenk and Kloner, 1986). Indeed, it has been reported that ROS are able to induce DNA strand breaks in ischemic kidneys as early as 1 h after reperfusion. The increased ROS
production at reperfusion has been reported to induce apoptosis through mitochondrial damages and release of proautophagic factors in many organs such as brain, heart, liver, and kidney (Javadov and Karmazyn, 2007).

In the present study 120-min reperfusion was able to worsen the renal deleterious effects elicited by 90-min ischemia. The activity of specific markers of renal tubular injuries and lipid peroxidation, NAG and TBARS (Meagher and FitzGerald, 2000; Snoeijis et al., 2011), and apoptotic markers, such as Bax, caspase-9, cytochrome c, and caspase-3, was significantly increased after reperfusion in comparison with what was observed after ischemia. These effects were in addition accompanied by a reduction of antioxidant system and prosurvival factors and a deterioration of renal function. The infusion of levosimendan into the renal artery during the ischemic phase at a dose previously used and considered as therapeutically relevant (Haikala et al., 1997; Grossini et al., 2009; Uberti et al., 2011) was more effective than Custodiol alone in protection against I/R injuries. Moreover, an increase of the protective effects was observed when levosimendan was administered in combination with Custodiol. Hence, Custodiol has widely been reported to be highly effective for organ preservation and could be used safely to lower the costs in solid organ preservation for transplantation (Klaus et al., 2007). Its composition stabilizes the cell membrane potential and prevents any increase of intracellular calcium concentration, thus preventing ROS generation and apoptosis (Hrabalová et al., 2003). Hence, in the presence of the multiorgan storing solution given alone, markers of peroxidation, tubular injuries, and cytochrome c were lower than those found in saline-treated animals after both ischemia and reperfusion. In addition, plasma creatinine, microalbuminuria, albumin/creatinine, and protein/creatinine ratio were significantly reduced, whereas creatinine clearance, ERK1/2, Akt, and antioxidant systems were increased, particularly after 120-min reperfusion. However, in the presence of levosimendan, a further improvement of all of the above parameters was observed. As reported previously, the
optimization of organ preservation solutions by the addition of buffers, glucose metabolites, and free radical scavengers is not so unusual (Dhalla et al., 2000; Jones et al., 2003). Levosimendan in combination with Custodiol infusion was able to significantly reduce oxidative and apoptotic renal damages caused by 90-min ischemia in comparison with Custodiol alone and preserve GSH, ERK1/2, Akt, and SOD3 levels and renal function. It is noteworthy that the renal protection against renal I/R injuries could be attributed to a direct action of levsimendan on kidneys beyond its hemodynamic effects, because almost all of the infused solutions were collected through the renal venous cannulae or ureters, and no significant changes in hemodynamic variables were observed during the experiments. In addition, renal blood flow at the end of reperfusion almost returned to control values without differences compared with what was observed in other groups of animals. Thus, the present findings confirmed previous observations in heart and cardiomyocytes (Grossini et al., 2010; Caimmi et al., 2011b; Uberti et al., 2011) regarding the effects of levsimendan as an antioxidant, antiapoptotic, and prosurvival agent.

These results are also in agreement with previous findings obtained in patients with heart failure or cardiogenic shock (Yilmaz et al., 2007; Caimmi et al., 2011a) where levsimendan administration was more effective than dobutamine in preserving kidney function or reducing polyuric syndrome. In those studies, however, the beneficial effects of levsimendan have been related to the improved renal blood flow secondary to the positive inotropic effects exerted by the calcium sensitizer. Actually, those effects on renal blood flow and function have been speculated to be offset by the drop in blood pressure caused by intravenous levsimendan administration (Damman and Voors, 2007), which did not happen in anesthetized pigs.

In the present study the blocking of mitochondrial KATP channels and NO release worsened the renal I/R injuries and abolished the protective effects elicited by levsimendan. In particular, the administration of 5-HD was effective in worsening the renal damage. These results highlight the role of mitochondrial KATP channel activity, in particular, and NO production in modulation of renal ischemic injuries and are in agreement with previous findings about this issue. The presence of mitochondrial KATP channels in the kidney has been demonstrated (Cancherini et al., 2003). Moreover, KATP channel opening prevented cytotoxicity and caspase-3 activation by the activation of ERK and inhibition of c-Jun NH2-terminal kinase signaling pathways (Nilakantan et al., 2010). Likewise, in a cellular model of renal I/R glibenclamide and 5-HD reduced the protection induced by propofol (Assad et al., 2009), and the blocking tubular ROS production by a mitochondrial KATP channel opener, diazoxide, was associated with the amelioration of all of the parameters of renal function (Sun et al., 2008). In addition, these data add further information about the role of mitochondrial KATP channels in antioxidant effects elicited by levsimendan (Uberti et al., 2011).

The results obtained about the involvement of NO in protection against renal I/R injuries in anesthetized pigs are in agreement with previous in vivo studies. NO is synthesized in the kidney and plays an important role in regulating renal hemodynamic variables and function (Ruilope et al., 1994). A great deal of evidence has suggested that NO is generated not only in the renal vascular endothelium but also in other renal cells such as the mesangium (Shultz et al., 1991), macula densa (Mundel et al., 1992), or tubular cells (Terada et al., 1992), thereby suggesting that endogenous NO plays an important role in the regulation of renal hemodynamic variables and functions.
Indeed, NOS inhibition through NG-monomethyl-L-arginine significantly caused the deterioration of renal function of the postischemic kidney in anesthetized rats, whereas pretreatment with L-arginine or NO donors abolished the NOS inhibitor-induced renal dysfunction (Chintala et al., 1993) and markedly attenuated ischemic acute renal failure in rats (Matsumura et al., 1998). In the present study, the role of NO in protection against I/R injuries has been shown by experiments performed with L-NAME, which was able to worsen apoptosis and peroxidation and reduce antioxidant system and cell survival pathways. Moreover, Western blot analysis revealed decreased eNOS phosphorylation in ischemic kidneys, which was prevented by levosimendan administration. Again, these results are in agreement with previous findings showing that eNOS-derived NO is responsible for maintaining physiological renal hemodynamic performance and function regarding the involvement of NO in the effects elicited by levosimendan.

Fig. 9. Effects of saline, levosimendan, Custodiol, and the combination of levosimendan and Custodiol on microalbuminuria (A), urinary protein/creatinine ratio (B), and urinary microalbumin/creatinine ratio (C) in the presence of renal I/R. Columns b to f, p < 0.05 versus column a. Abbreviations are as in Fig. 2. Reported values are mean ± S.D. of five experiments.
Thus, in the present study the administration of Custodiol solution in combination with levosimendan was found to be more effective than Custodiol alone in protection against renal IR injuries. These results are not surprising considering that levosimendan is a calcium sensitizer that acts without causing changes in intracellular calcium levels and by stabilizing energy balance (García González and Domínguez Rodrigo, 2006) in a similar manner to Custodiol. It is also noteworthy that those findings were obtained in the renal cold-ischemia model and could, thus, have important consequences in the clinical results of renal transplantation therapy. Moreover, the improved outcome of transplanted patients could have economic implications determining a reduction of the costs related to transplantation therapy.

Acknowledgments
We thank staff at Azienda Ospedaliero Universitaria Maggiore della Carità, Novara, for help.

Authorship Contributions
**Participated in research design:** Grossini, Pollesello, and Caimmi.
**Conducted experiments:** Grossini and Molinari.
**Contributed new reagents or analytic tools:** Bellomo and Valente.
**Performed data analysis:** Mary.
**Wrote or contributed to the writing of the manuscript:** Grossini, Pollesello, Vacca, and Caimmi.

References


**Address correspondence to:** Dott. E. Grossini, Department of Translational Medicine, University East Piedmont, via Solaroli 17, I-28100 Novara, Italy. E-mail: grossini@med.unipmn.it