British Journal of Pharmacology (2008) 154, 41–50

0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

A role for the RISK pathway and K_{ATP} channels in pre- and post-conditioning induced by levosimendan in the isolated guinea pig heart

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Background and purpose: Myocardial reperfusion injury prevents optimal salvage of the ischaemic myocardium, and adjunct therapy that would significantly reduce reperfusion injury is still lacking. We investigated whether (1) the heart could be pre- and/or post-conditioned using levosimendan (levosimendan pre-conditioning (LPC) and levosimendan post-conditioning (LPostC)) and (2) the prosurvival kinases and/or the sarcolemmal or mitochondrial K_{ATP} channels are involved.
Experimental approach: Isolated guinea pig hearts were treated with two 5 min cycles of levosimendan (0.1 μM) interspersed with vehicle perfusion, or two 5 min cycles of ischaemia/reperfusion, before coronary artery ligation (CAL) for 40 min at 36.5 °C. Hearts were treated with mitochondrial or sarcolemmal K_{ATP} channel blockers before LPC or LPostC. For post-conditioning, hearts received three 30 s cycles of ischaemia/reperfusion or levosimendan/vehicle. Hearts were pretreated with levosimendan immediately before CAL (without washout). Cardiac function, infarct size and reperfusion injury salvage kinase activity was assessed.
Key results: LPC and LPostC halved the infarct size compared with controls ($P<0.05$). Treatment with K_{ATP} channel blockers before LPC or LPostC reversed this decrease. Pretreating hearts with levosimendan increased activity of extracellular signal-regulated kinase (ERK) 42/44 on reperfusion and had the most marked infarct-lowering effect ($P<0.05$).
Conclusions and implications: (1) Hearts could be pharmacologically pre- and post-conditioned with levosimendan; (2) levosimendan pretreatment is the most effective way to reduce infarct size, possibly by increasing ERK 42/44 activity; (3) benefits of LPC and LPostC were abolished by both K_{ATP} channel blockers and (4) LPC may be useful before elective cardiac surgery, whereas LPostC may be used after acute coronary artery events.
British Journal of Pharmacology (2008) **154**, 41–50; doi:10.1038/bjp.2008.52; published online 25 February 2008

Keywords: levosimendan; myocardial infarct; myocardial function; RISK pathway; K_{ATP} channel; reperfusion injury

Abbreviations: ASP, aortic systolic pressure; CAL, coronary artery ligation; IPC, ischaemic pre-conditioning; IPostC, ischaemic post-conditioning; LPC, levosimendan pre-conditioning; LPostC, levosimendan post-conditioning; LPT, levosimendan pretreatment; Qa, aortic output; Qe, coronary flow; RISK, reperfusion injury salvage kinase

Introduction

Acute myocardial infarction (AMI) represents a major cause of death and heart failure in industrialized countries (McGovern *et al*., 1996). Although early reperfusion therapy for AMI has reduced mortality, reperfusion-induced injury in the form of apoptosis (Fliss and Gattinger, 1996; Haunstetter and Izumo, 1998) prevents the optimal salvage of the ischaemic myocardium (Kloner and Rezkalla, 2004). This problem may partially explain why only one-third of the life years lost by MI were regained by reperfusion (Van Domberg *et al*., 2005). Adjunct therapy that would, when given together with rapid reperfusion, significantly reduce reperfusion injury and further limit infarct size has been elusive. The search for pharmacological cardioprotective agents that target the reperfusion phase continues, and although pre-conditioning is only feasible during elective cardiac procedures, knowledge about the underlying mechanisms has been used to devise new cardioprotective therapies. Post-conditioning is, however, feasible after AMI and was recently used in patients undergoing coronary angioplasty (Staat *et al*., 2005). This group showed that post-conditioning reduced creatine kinase release, which was used as an indirect measure of the severity of the myocardial infarction.

Patients with AMI often develop heart failure and recent clinical trials demonstrate that the calcium sensitizer,

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Received 5 November 2007; revised 3 December 2007; accepted 31 January 2008; published online 25 February 2008

levosimendan, is effective in the treatment of heart failure (Follath *et al*., 2002; Cleland *et al*., 2004; Duygu *et al*., 2007; Givertz *et al*., 2007; Pollesello and Papp, 2007; Sargento *et al*., 2007). Levosimendan is also a K_{ATP} channel opener (Yokoshiki *et al*., 1997; Kopustinskiene *et al*., 2001) and therefore has the potential to protect the heart against ischaemic/reperfusion injury (Cammarata *et al*., 2006).

Recent studies have demonstrated that pharmacological pre-conditioning leads to activation of PKB/Akt and extracellular signal-regulated kinase (ERK) 42/44 during reperfusion (Hausenloy *et al*., 2003; Hausenloy and Yellon, 2004; Yang *et al*., 2004a, b), the so-called reperfusion injury salvage kinase (RISK) pathway (Hausenloy and Yellon, 2004). In a more recent review, it was proposed that the RISK pathway could potentially be a viable target for both pre- and post-conditioning (Hausenloy *et al*., 2005). To this end, we investigated the effects of levosimendan pre-conditioning (LPC), pretreatment and post-conditioning on the activity of PKB/Akt and ERK 42/44 during reperfusion.

We hypothesized that levosimendan with its K_{ATP} channel-opening properties has the potential to (1) protect the heart when used to pretreat the heart; (2) act as a trigger for protection when given briefly before the onset of ischaemia (pre-conditioning) or immediately after reperfusion (post-conditioning) and (3) has its effect by stimulating the RISK pathway and/or opening the K_{ATP} channels in the heart. We found that the guinea pig heart could be protected by levosimendan pretreatment or pre- or post-conditioning and that ERK 42/44 activation and the K_{ATP} channel may be implicated in these protective effects.

Methods

Animal model

This study was conducted in accordance with the Principles of Laboratory Animal Care of the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (NIH publication no. 80-23, revised 1985). Guinea pigs weighing 250–500 g were anaesthetized with pentobarbital (30 mg kg^{-1} i.p.), hearts were then removed and transferred to the working heart perfusion apparatus. They were retrogradely perfused at a perfusion pressure of 75 cm H_2O for 5 min with Krebs–Henseleit buffer (containing (in mmol l^{-1}) NaCl 121.5, KCl 3.8, $MgCl_2 \cdot 6H_2O$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 15.5, KH_2PO_4 1.2, Na-pyruvate 2.0, glucose 11.0 and mannitol 16.0) equilibrated with 95% O_2 and 5% CO_2 at 37 °C. For the evaluation of mechanical function, hearts were perfused in the working heart mode (preload 15 cm H_2O and afterload 75 cm H_2O) for 5 min before being pre-conditioned with ischaemia or levosimendan. Aortic output (Qa), coronary flow (Qe), heart rate (HR) and aortic diastolic pressure (ADP) and aortic systolic pressure (ASP) were measured before and directly after the pre-conditioning intervention had been applied. Data were collected and analysed using a PhysiTutor data acquisition system.

Pretreatment and pre- and post-conditioning protocols

For reperfusion function and infarct size measurements, hearts were randomly allocated to one of the study groups and mechanical function documented after 5 min of working-heart perfusion. Hearts that were pretreated with levosimendan (0.1 μM) were perfused with the drug for 10 min before coronary artery ligation (CAL) without washout. The hearts that were pre-conditioned were subjected to 2×5 min cycles of levosimendan (0.1 μM) interspersed with vehicle perfusion, or 2×5 min cycles of global ischaemia/reperfusion, before CAL. In all procedures, CAL was instituted for 40 min and the heart temperature was maintained at 36.5 °C before the suture was released to induce reperfusion. For post-conditioning, hearts were subjected to 3×30 s cycles of levosimendan (0.1 μM) interspersed with vehicle perfusion, or 3×30 s cycles of ischaemia/reperfusion.

For treatment with K_{ATP} channel blockers before LPC, hearts were perfused with the respective blockers for 5 min before the intermittent levosimendan perfusions were commenced. The blocker was present throughout the perfusion period until CAL was performed. For hearts pretreated with levosimendan for 10 min before sustained ischaemia, the same approach was followed. Hearts were treated with the blocker for 5 min before co-perfusion with both the blocker and levosimendan for a further 10 min. The control hearts for these experiments were continuously perfused with the respective blockers for the appropriate time (25 min for pre-conditioned hearts and 15 min for pretreated hearts) before induction of sustained ischaemia. Hearts treated with the K_{ATP} channel blocker during post-conditioning were reperfused with the blockers for the first 3 min of reperfusion.

To determine whether LPC or pretreatment merely altered cardiac function and therefore the metabolic demand on hearts, we performed an additional series of experiments in which hearts were pre-conditioned or pretreated with levosimendan or subjected to ischaemic pre-conditioning before being made to work for an additional 5 min. During this working heart perfusion phase, cardiac function was assessed and compared with the values obtained before the interventions (ischaemic pre-conditioning, LPC or levosimendan pretreatment) were applied. Aortic output and coronary flow were not altered by the pre-conditioning or pretreatment protocols employed before the CAL was performed.

Infarct size determinations

Myocardial infarct size was determined as previously described (du Toit *et al*., 2005). Briefly, after 30 min reperfusion of the regionally ischaemic heart, the coronary artery was re-occluded at the end of the reperfusion period and a solution of 2.5% Evans blue injected into the coronary arteries to delineate the area at risk. We chose to use 30 min reperfusion for the infarct determinations because our group has previously shown that infarct size does not change significantly during reperfusion for 30–120 min (Marais *et al*., 2005). Hearts were then frozen and cut into 5–7 slices, which were incubated in sodium phosphate buffer containing 1% (w/v) triphenyltetrazolium chloride for 15 min to visualize the unstained infarcted region. Infarct and risk zone areas were determined with planimetry and infarct area was expressed as a percentage of the area at risk. The area at risk was determined for all experimental groups and was found

to be similar in all groups. The average value for all the groups was 36.7 ± 1.1% of the volume of the left ventricle.

Western blot analysis

In additional series of experiments, hearts were (1) pretreated or pre-conditioned with levosimendan and then subjected to ischaemia and reperfusion as described above; or (2) they were post-conditioned with ischaemia or levosimendan or (3) they were co-perfused with one of the K_{ATP} channel blockers while being post-conditioned with ischaemia or levosimendan. After 5 or 10 min reperfusion, ischaemic and nonischaemic zones of the hearts were separated and freeze-clamped with Wollenberger tongs. Samples were stored at −80 °C until western blot analysis was performed.

Cardiac ERK 42/44 and PKB/Akt were extracted with a lysis buffer containing Tris 20 mM, *p*-nitrophenylphosphate 20 mM, EGTA 1 mM, NaF 50 mM, sodium orthovanadate 0.1 mM, phenylmethylsulphonyl fluoride 1 mM, dithiothreitol 1 mM, aprotinin 10 μg ml^{-1} and leupeptin 10 μg ml^{-1}. The tissue lysates were diluted in Laemmli sample buffer, boiled for 5 min and 10 μg protein was separated by 10% SDS-polyacrylamide gel electrophoresis. The lysate protein content was determined using the Bradford technique (Bradford, 1976). The separated proteins were transferred to a PVDF membrane (Immobilon P; Millipore, Bangalore, India). These membranes were routinely stained with Ponceau Red for visualization of proteins. Nonspecific binding sites on the membranes were blocked with 5% fat-free milk in TBST (Tris-buffered saline–0.1% Tween 20) and then incubated with the primary antibodies that recognize phospho-specific ERK p42/p44 (Thr^{202}/Tyr^{204}) or PKB (Ser^{473} and Thr^{308}). Membranes were subsequently washed with large volumes of TBST (5 × 5 min) and the immobilized antibody was conjugated with a diluted horseradish peroxidase-labelled secondary antibody (Amersham Life Science, Buckinghamshire, UK). After thorough washing with TBST, membranes were covered with ECL (enhanced chemiluminescence) detection reagents and quickly exposed to an autoradiography film (Hyperfilm ECL, RPN 2103) to detect light emission through a nonradioactive method (ECL western blotting). Films were densitometrically analysed (UN-SCAN-IT, Silk Scientific Inc., Orem, Utah, USA) and phosphorylated protein values were corrected for minor differences in protein loading, if required. Experiments were performed (data not shown) to ensure that all signals were within the linear range of detection on the autoradiographs under our assay and gel-loading conditions.

Statistical methods

We performed experiments with a minimum of six hearts per group. For multiple comparisons, the two-way ANOVA followed by the Bonferroni correction was applied. A value of $P<0.05$ was considered significant. Infarct size was expressed as a percentage of the area at risk, and for functional recovery data, reperfusion aortic output was expressed as a percentage of the pre-ischaemic value.

Drugs

Levosimendan was prepared with 50 μl of 1 M NaOH in 10 ml phosphate buffer (2.32% Na_2HPO_4 in distilled water) and from this 1 mM stock solution, 100 μl was added to 1000 ml of perfusion buffer. 5-Hydroxydecanoic acid (5HD) was used at a concentration of 200 μM and glibenclamide at 5 μM. 5HD is a mitochondrial K_{ATP} channel blocker, whereas glibenclamide blocks both the mitochondrial and sarcolemmal K_{ATP} channels. For selected experiments, these blockers were applied during the levosimendan pretreatment or pre-conditioning protocols.

Results

The effect of ischaemic pre-conditioning, LPC and levosimendan pretreatment on myocardial infarct size

Ischaemic pre-conditioning reduced infarct size in the guinea pig heart to about half of that in control hearts ($P<0.05$; Figure 1). Pre-conditioning hearts with two cycles of levosimendan perfusion interspersed with vehicle perfusion (LPC) reduced infarct size similarly and combining ischaemic and levosimendan pre-conditioning did not further reduce infarct size ($P<0.05$ vs control; Figure 1).

Pretreating hearts with levosimendan for 10 min (without washout before ischaemia) decreased infarct size to about 10% of that in control hearts ($P<0.05$; Figure 1).

Effect of K_{ATP} channel blockers on the infarct size in levosimendan-pre-conditioned and -pretreated hearts

Co-perfusing hearts with levosimendan and glibenclamide or 5HD during the trigger phase of LPC precluded the hearts from being protected by pre-conditioning, and there was no difference in myocardial infarct size between control hearts and those that were pre-conditioned with levosimendan while being perfused with K_{ATP} channel blockers (Figure 2).

Co-perfusing hearts with the blockers during levosimendan pretreatment also prevented the effects of levosimendan on infarct size (48.9 ± 2.1% for glibenclamide vs 48.5 ± 2.9% for levosimendan + glibenclamide and 39.1 ± 2.8% for 5HD vs 38.6 ± 2.4% for levosimendan + 5HD).

Effect of ischaemic or levosimendan post-conditioning on infarct size

Post-conditioning hearts with either ischaemia or levosimendan decreased myocardial infarct size ($P<0.05$; Figure 3). Ischaemic and levosimendan post-conditioning were not additive and did not further reduce the infarct size when compared with one of their individual interventions (data not shown).

Effect of K_{ATP} channel blockers on the infarct size in levosimendan- and ischaemic-post-conditioned hearts

Co-perfusing hearts with levosimendan and glibenclamide or 5HD during the trigger phase of post-conditioning prevented the hearts from being protected by levosimendan. Myocardial infarct size was similar for control hearts and hearts treated with K_{ATP} channel blockers alone or in combination with post-conditioning with levosimendan or ischaemia (Figure 3).

Aortic output recovery for the respective experimental groups

Reperfusion aortic output recovery was improved with LPC or pretreatment with levosimendan ($P<0.05$; Figure 4a).

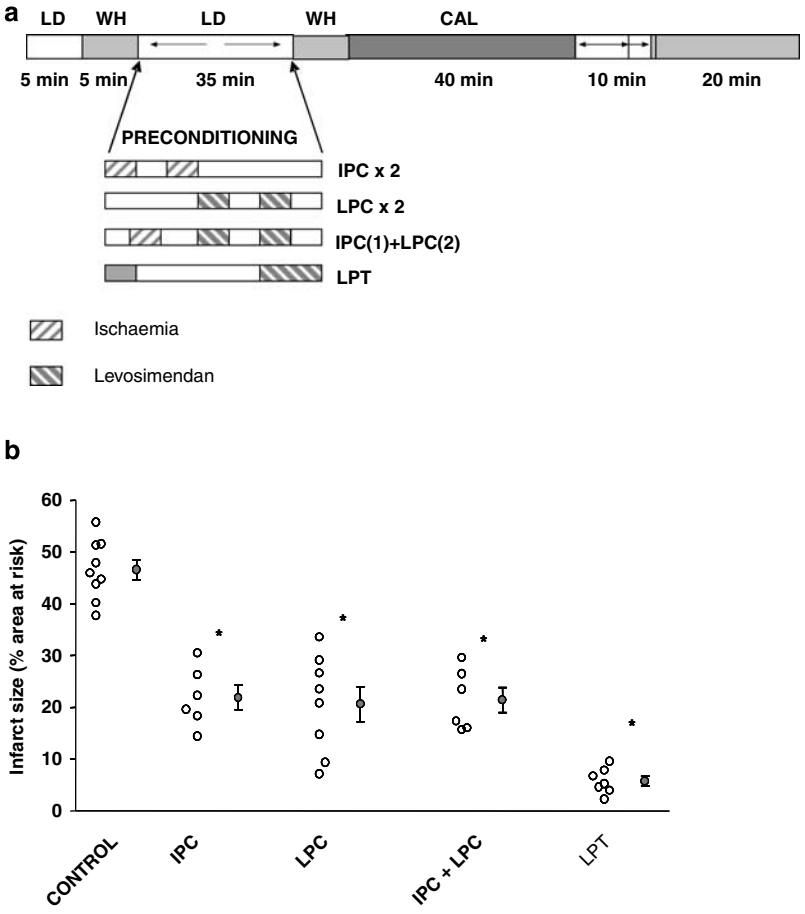

Figure 1 Experimental perfusion protocol used to induce ischaemic and levosimendan pre-conditioning and to pretreat the hearts with levosimendan (**a**). Myocardial infarct size in control, untreated hearts; hearts pre-conditioned using ischaemic pre-conditioning (IPC), or levosimendan pre-conditioning (LPC) or the combination of both; or hearts pretreated with levosimendan (LPT) (**b**). LD, Langendorff perfusion; WH, working heart perfusion; CAL, coronary artery ligation; $n = 6–9$; $^*P < 0.05$ vs control.

However, post-conditioning, with either ischaemia or levosimendan, did not improve aortic output recovery on reperfusion (Figure 4b).

Effect of LPC or levosimendan pretreatment on myocardial PKB/Akt and ERK 42/44 activity during reperfusion

We measured PKB/Akt and ERK 42/44 activity on reperfusion in both ischaemic and nonischaemic tissue of the CAL hearts. The PKB/Akt and ERK 42/44 activities after 5 and 10 min reperfusion were comparable in the nonischaemic and ischaemic tissue of the control and levosimendan-pretreated or -pre-conditioned hearts (data not shown). Neither levosimendan pre-conditioning nor pretreatment had any effects on post-ischaemic myocardial PKB/Akt activity when compared with control, untreated hearts. Both total and phosphorylated PKB/Akt levels were similar in control and levosimendan-pre-conditioned or -pretreated groups at 5 and 10 min of reperfusion (data not shown).

Total ERK levels were similar in all groups (control, levosimendan-pretreated and -pre-conditioned) investigated. ERK 44 activity was increased above basal levels in the levosimendan-pretreated group after 5 min reperfusion. Levosimendan pretreatment also increased ERK 44 activity above the levels of control, nontreated hearts (Figure 5a). ERK 42 activity was increased above basal (pre-ischaemic) levels after 5 min reperfusion in the levosimendan-pre-conditioned or -pretreated hearts (Figure 5b). ERK 42 activity was also elevated in the levosimendan-pretreated heart after 5 min reperfusion when compared with the control, non-treated, reperfused hearts (Figure 5b). At 10 min reperfusion, only the levosimendan-pretreated hearts had elevated ERK 42 activity when compared with basal levels.

Effect of LPostC or ischaemic post-conditioning and co-perfusion with K_{ATP} channel blockers on myocardial PKB/Akt and ERK 42/44 activity during reperfusion

Post-conditioning with levosimendan or ischaemia did not increase myocardial PKB/Akt and ERK 42/44 expression or activity. We were also unable to demonstrate any effects of the co-perfusion with K_{ATP} channel blockers and levosimendan or ischaemic post-conditioning on myocardial PKB/Akt and ERK 42/44 activity during reperfusion.

Discussion and conclusions

These data demonstrate that levosimendan reduces myocardial infarct size in the guinea pig heart when used either to

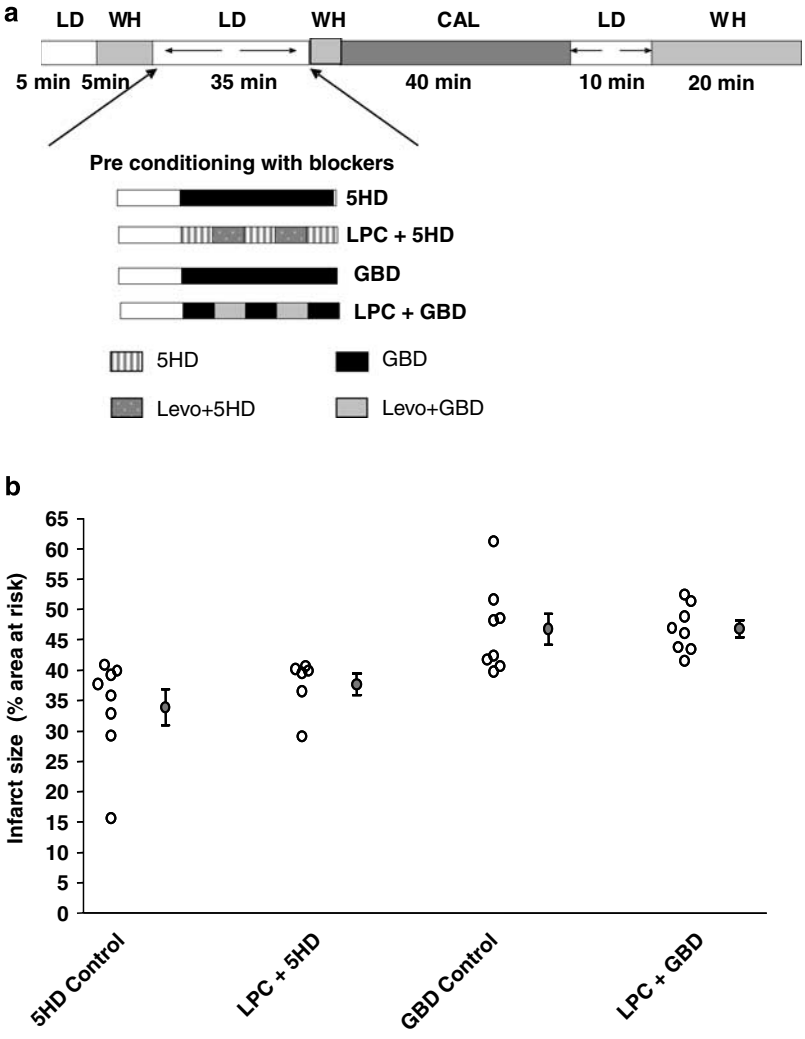

Figure 2 Experimental perfusion protocol used to induce levosimendan pre-conditioning in the absence or presence of K_{ATP} channel blockers (**a**). Effects of K_{ATP} channel blockers (5HD or GBD) on the myocardial infarct size in hearts pre-conditioned with levosimendan (**b**). $n = 6$–8; LD, Langendorff perfusion; WH, working heart perfusion; CAL, coronary artery ligation; Levo, levosimendan; 5HD, 5-hydroxydecanoic acid; GBD, glibenclamide; 5HD Control, 5HD without intermittent levosimendan pre-conditioning; LPC + 5HD, 5HD with intermittent levosimendan pre-conditioning; GBD Control, GBD without intermittent levosimendan pre-conditioning; LPC + GBD, GBD with intermittent levosimendan pre-conditioning.

pretreat the heart or as a pre-conditioning or post-conditioning procedure. The most effective cardioprotection was observed with pretreatment with levosimendan, with lesser but still significant protection by levosimendan-induced pre- or post-conditioning. Both levosimendan pre-conditioning and pretreatment also improved the mechanical function of the heart on reperfusion. The cardioprotective effect of levosimendan pre-conditioning or pretreatment may relate to its ability to increase ERK 1/2 activity during reperfusion. These cardioprotective effects in both pre- and post-conditioning were abolished by blocking the mitochondrial K_{ATP} channels, an effect that implicates these channels in the resultant protection observed by us.

AMI and the importance of optimizing reperfusion therapy

Myocardial infarction is set to become the leading cause of death by 2020 (Murray and Lopez, 1997). Despite efforts to develop reperfusion strategies that could salvage all viable myocardium after a myocardial infarction, a recent study has highlighted the fact that only one-third of the life years lost by MI were regained with reperfusion therapy (van Domberg *et al*., 2005). These observations suggest that there is still significant damage to the myocardium that may be attributed to reperfusion injury (Becker and Ambrosio, 1987; Forman *et al*., 1990; Opie, 1991) and reperfusion-induced apoptosis (Fliss and Gattinger, 1996; Haunstetter and Izumo, 1998). With this in mind, several research groups have set out to determine how ischaemic and reperfusion injury can be minimized.

Although pre-conditioning is only clinically applicable before elective cardiac surgery, several researchers have endeavoured to understand the mechanisms responsible for the cardioprotective effects of pre- and post-conditioning. This has been done in the hope that this knowledge could be used to formulate pharmacological cardioprotective agents that would be clinically applicable. Ischaemic post-conditioning has however been effective in protecting the

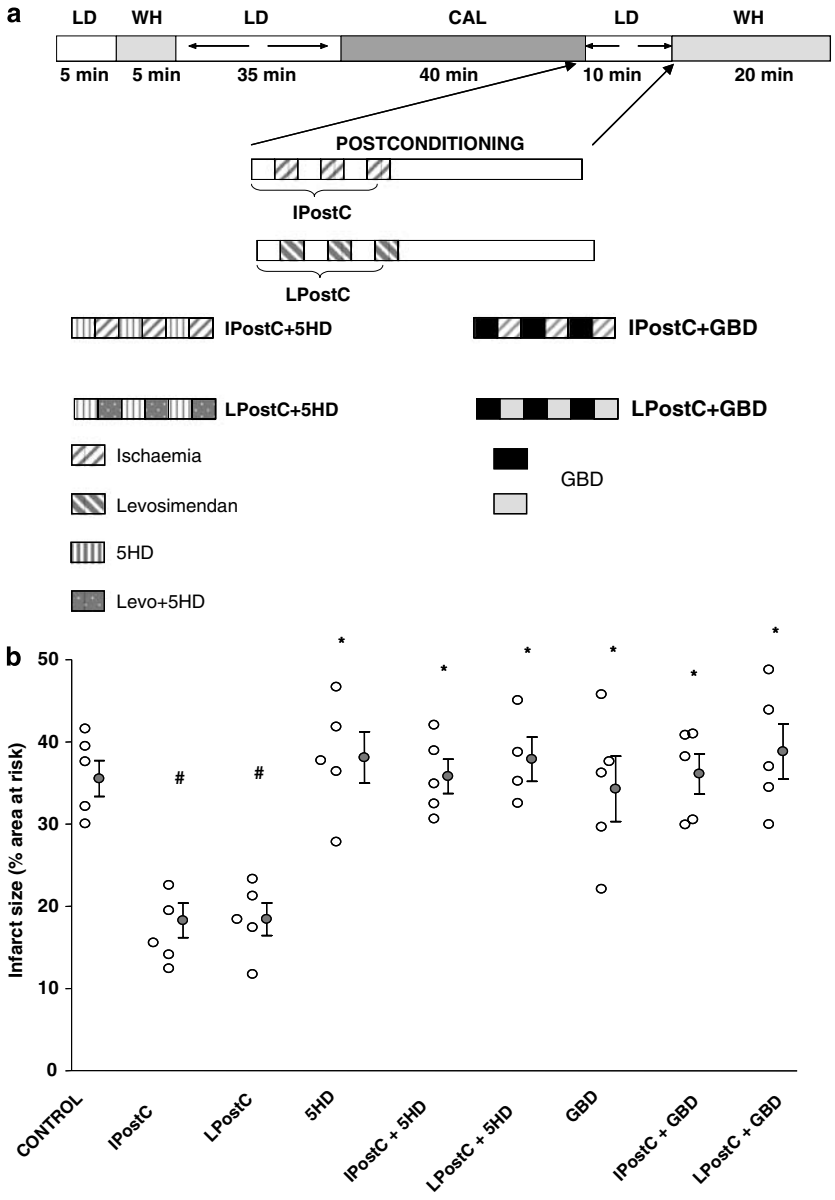

Figure 3 Experimental perfusion protocol used to induce ischaemic (IPostC) or levosimendan post-conditioning (LPostC) in the presence and absence of K_{ATP} channels blockers (**a**). Myocardial infarct size in control hearts, hearts post-conditioned with ischaemia or hearts post-conditioned with levosimendan (**b**). $n=4$–5; $^*P<0.05$ vs control; LD, Langendorff perfusion; WH, working heart perfusion; CAL, coronary artery ligation; Levo, levosimendan; 5HD, 5-hydroxydecanoic acid; GBD, glibenclamide.

heart in the laboratory (Zao *et al*., 2003; Halkos *et al*., 2004; Kin *et al*., 2004) and clinical (Staat *et al*., 2005) setting. These studies illustrate that, notwithstanding the deleterious effects of ischaemia, salvage of the ischaemic myocardium can be greatly enhanced by interventions confined to reperfusion.

Levosimendan as a cardioprotective agent

Levosimendan decreases infarct size in a dog (Kersten *et al*., 2000), improves reperfusion function in a guinea pig (du Toit *et al*., 1999) and improves both these variables in an isolated perfused rabbit heart model (Lepran *et al*., 2006). It has also recently been shown to improve cardiopulmonary resuscitation and 48 h survival rate in rats with experimentally induced ventricular fibrillation (cardiac arrest) (Cammarata *et al*., 2006). These beneficial effects of levosimendan could be negated by treating the animals with glibenclamide before the induction of arrhythmias in rat (Cammarata *et al*., 2006) or coronary artery occlusion in dog (Kersten *et al*., 2000), suggesting a role for the K_{ATP} channel-opening properties of levosimendan.

Levosimendan as a cardioprotective inotropic agent after AMI

A recent study performed in a porcine model of ischaemia and reperfusion suggests that levosimendan treatment before and after a myocardial infarction improves pre-ischaemic and reperfusion mechanical function, but not infarct size (Busk *et al*., 2006).

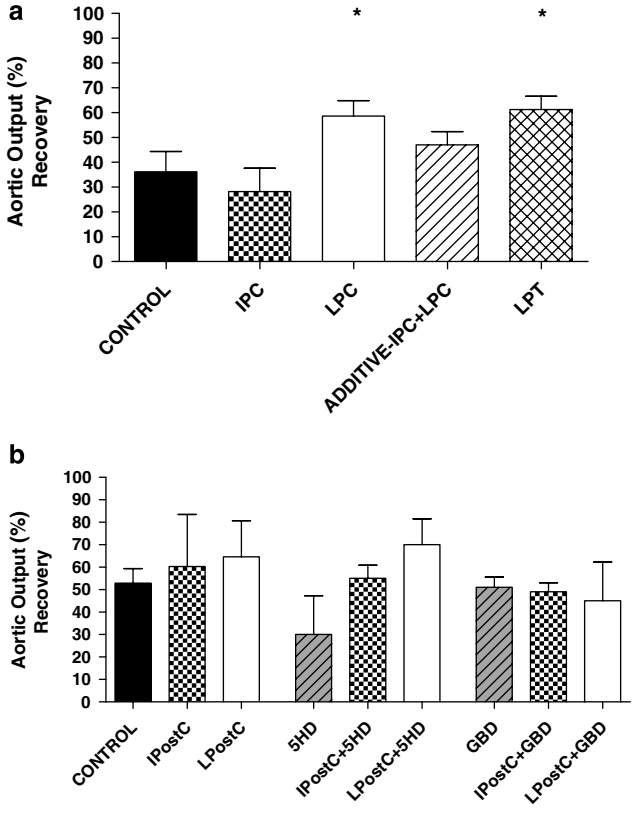

Figure 4 Aortic output recovery (%) for control hearts and hearts pretreated with levosimendan (LPT), or pre-conditioned using ischaemia (IPC) or levosimendan (LPC) (**a**). Aortic output recovery (%) for control hearts and hearts post-conditioned with ischaemia (IPostC), or levosimendan (LPostC) or in combination with K_{ATP} channel blockers (**b**). $n=6$–9; *$P<0.05$ vs control.

Patients with AMI often develop heart failure. In this context, several recent clinical trials have been performed to assess the efficacy of levosimendan as an inotrope in patients with decompensated heart failure (Nieminen *et al*., 2000; Follath *et al*., 2002; Cleland *et al*., 2004; Michaels *et al*., 2005; Duygu *et al*., 2007; Givertz *et al*., 2007; Pollesello and Papp, 2007; Sargento *et al*., 2007). The results from these studies have been encouraging and suggest that levosimendan has favourable haemodynamic effects and improved cardiac function and efficiency.

Levosimendan as a pre- and post-conditioning treatment

Downstream signalling pathways that have been implicated in the triggering of pre-conditioning varies, but the K_{ATP} channels have been implicated as a possible end-effector in calcium (Hiayawaki *et al*., 1996; Meldrum *et al*., 1996; Kouchi *et al*., 1998) and ischaemic pre-conditioning procedures (Auchampach *et al*., 1992; Gross and Auchampach, 1992; Garlid *et al*., 2003). These studies suggested that K_{ATP} channels play a central role in classic pre-conditioning and that opening of these channels during sustained ischaemia possibly protects the heart against the negative consequences of ischaemia. Recently, opening of these channels has also been suggested as a trigger of protection (see Yellon and Downey, 2003).

Based on the knowledge that levosimendan has K_{ATP} channel–opening properties (Yokoshiki *et al*., 1997; Kopustinskiene *et al*., 2001) and that these channels play a central role in pre-conditioning, we set out to determine whether the drug could act as a pre- and post-conditioning treatment. This is one of the first studies to demonstrate that levosimendan can act as a 'trigger' for pre- and post-conditioning in an isolated guinea pig heart. We found that LPC not only decreased myocardial infarct size, but also improved reperfusion mechanical function in the working heart model. We also demonstrated that co-perfusion with levosimendan and either the mitochondrial K_{ATP} channel blocker (5HD) or the nonspecific K_{ATP} channel blocker (glibenclamide), during the 'trigger' phase of LPC, attenuated the cardioprotective effects of pre-conditioning. These data support the concept that levosimendan protected the ischaemic heart by opening these K^+ channels and thus conferred protection on the ischaemic heart.

As it has been demonstrated that the mitochondrial K_{ATP} channels are also involved in the cardioprotection induced by post-conditioning (Yang *et al*., 2004a, b), we also tested the effects of 5HD and glibenclamide on post-conditioning. The infarct-reducing effect of post-conditioning could be abolished by treating rabbits with glibenclamide before the induction of coronary artery occlusion and post-conditioning during the reperfusion phase (Yang *et al*., 2004a, b). Our data also suggest that the effects of levosimendan on these channels may be implicated in its post-conditioning effects in this study.

Despite glibenclamide being considered a nonspecific K_{ATP} channel blocker, we chose this compound, as the efficacy of HMR 1098 (a putative selective sarcolemmal K_{ATP} channel blocker) under conditions of metabolic stress has been questioned by Rainbow *et al*. (2005). In electrophysiological studies, this group found that HMR 1098 became an ineffective sarcolemmal K_{ATP} channel blocker under ischaemic conditions.

We found that pretreatment with levosimendan without washout before index ischaemia was the most effective intervention to protect the heart and improved both infarct size and reperfusion aortic output recovery. These cardioprotective effects were lost when hearts were co-perfused with levosimendan and either of the mitochondrial K_{ATP} channels blockers. These data again suggest that the cardioprotective effect of pretreatment with levosimendan was due to K_{ATP} channel-opening before the sustained ischaemic episode.

The fact that levosimendan pretreatment conferred greater cardioprotection against ischaemia and reperfusion injury than LPC may indicate that the drug is more effective as an anti-ischaemic compound when present in the heart during ischaemia. It may open the K_{ATP} channels directly without activating the conventional pre-conditioning signalling pathways when used as a pretreatment drug. During LPC procedures, it is possible that the compound is washed out of the heart during the 5 min before sustained ischaemia and therefore has less effect on the ischaemic myocardium. This possibility will have to be investigated in the future.

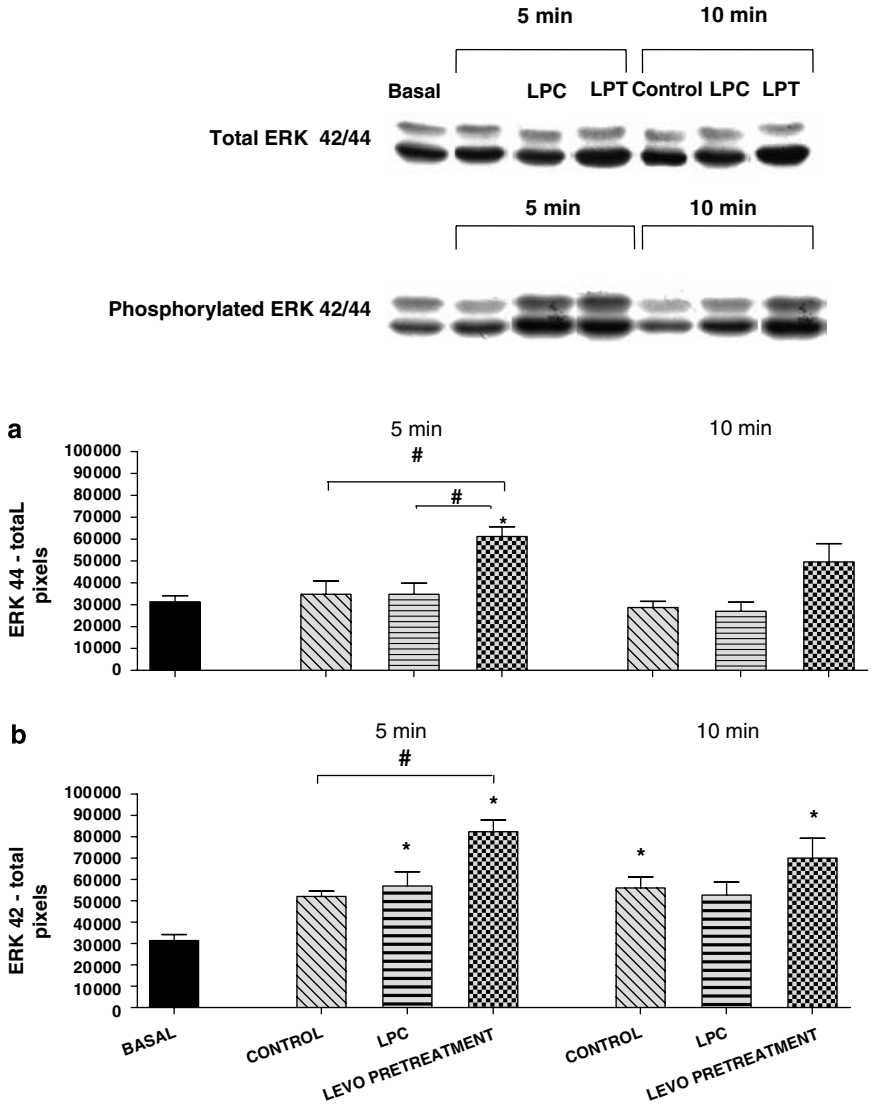

Figure 5 Representative blots for total ERK and phospho-ERK 1/2 and densitometry showing the effect of levosimendan pre-conditioning (LPC) or levosimendan pretreatment (LPT) on ERK 44 (**a**) and ERK 42 (**b**) phosphorylation of the ischaemic tissue at 5 and 10 min reperfusion. $n=6$; $^{*}P<0.05$ vs basal; $^{\#}P<0.05$.

The effect of levosimendan pretreatment or LPC on the RISK pathway activity

Recent studies have demonstrated that pharmacological activation of the RISK pathway during reperfusion reduces both necrotic and apoptotic cell death and thus infarct size (Yellon and Baxter, 1999; Hausenloy and Yellon, 2004). The exact downstream effects of RISK pathway activation have not been established but probably involve closing the mitochondrial permeability transition pores during reperfusion (Hausenloy *et al.*, 2005). The reperfusion-induced opening of these pores is thought to be secondary to mitochondrial calcium overload, oxidative stress and ATP depletion after an ischaemic event (Hausenloy and Yellon, 2003). Closing of these pores possibly occurs through ERK-induced phosphorylation and activation of eNOS, phosphorylation and inactivation of glycogen synthase kinase-3β or phosphorylation and mitochondrial translocation of PKCε (Hausenloy *et al.*, 2005). We monitored the activity of both PKB/Akt and ERK 42/44 during the first 10 min of reperfusion in levosimendan-pretreated and -pre-conditioned hearts. We found no differences in the activity of these kinases on reperfusion, when comparing control hearts with levosimendan-pretreated or -pre-conditioned hearts. We did however find that ERK 42 and ERK 44 activities were increased within the first 5 min of reperfusion in the levosimendan-pretreated hearts. This is the group that was best protected against ischaemia/reperfusion injury as reflected by the reduced infarct size and improved reperfusion function. These data implicate the ERK 42/44 in the cardioprotection afforded by levosimendan pretreatment of the guinea pig heart. Although a protective role for RISK pathway activation has been demonstrated in pre- and post-conditioning (Hausenloy *et al.*, 2005; Schwartz and Lagranha, 2006), our data suggest that the RISK pathway, and more specifically ERK 42/44, can also be activated by pharmacological pretreatment with levosimendan. We believe that this

Table 1 Aortic output and coronary flow rates for groups after the pre-conditioning or pretreatment procedure has been performed but before coronary artery ligation was performed (A) and pre-ischaemic aortic output and coronary flow rates for groups of hearts that were post-conditioned (B)

	Control	*IPC*	*LPC*	*IPC + LPC (IPC and LPC combined)*	*LPC + 5HD*	*LPC + GBD*	*Pretreatment*	*Pretreatment + 5HD*	*Pretreatment + GBD*
(A)									
Aortic output ($ml\,min^{-1}$)	54.78 ± 3.76	54.50 ± 6.20	60.50 ± 3.57	50.17 ± 3.20	62.50 ± 3.97	54.75 ± 3.16	62.00 ± 3.94	63.00 ± 4.18	52.88 ± 3.12
Coronary flow ($ml\,min^{-1}$)	19.89 ± 1.41	20.00 ± 2.62	21.13 ± 1.19	19.67 ± 1.12	19.83 ± 1.54	21.38 ± 2.97	23.43 ± 0.80	22.50 ± 1.37	20.63 ± 1.54

(B)	*Control*	*IPostC*	*IPostC + 5HD*	*IPostC + GBD*	*LPostC*	*LPostC + 5HD*	*LPostC + GBD*
Aortic output ($ml\,min^{-1}$)	61.50 ± 4.70	53.63 ± 6.28	64.30 ± 1.95	62.88 ± 2.83	53.86 ± 5.31	61.80 ± 2.41	62.14 ± 3.47
Coronary flow ($ml\,min^{-1}$)	24.75 ± 2.17	22.00 ± 1.77	22.20 ± 0.80	22.25 ± 1.66	20.29 ± 1.14	22.80 ± 1.82	25.29 ± 1.90

Abbreviations: IPC, ischaemic pre-conditioning; LPC, levosimendan pre-conditioning; 5HD, 5-hydroxydecanoate; GBD, glibenclamide; Ipost C, ischaemic post-conditioning; LpostC, levosimendan post-conditioning.

is the first study that has demonstrated a strong association between the cardioprotective effects of pharmacological pretreatment of the heart and activation of the ERKs. Future work would include investigating the possible link between levosimendan pretreatment, ERK activation and susceptibility to the opening of the mitochondrial permeability transition pores.

The effect of levosimendan or ischaemic post-conditioning on the RISK pathway activity

Several studies have reported increased ERK 42/44 activity during reperfusion after post-conditioning (Darling *et al*., 2005). We were unable to demonstrate ERK or PKB activation in the post-conditioned hearts studied by us 5 min after reperfusion. The exact reason for this is unclear but may relate to the fact that we only measured the activity and expression of these signalling kinases at 5 min reperfusion after post-conditioning, which may not have allowed enough time for activation of these pathways to occur.

We conclude that levosimendan can be used to either precondition or post-condition the heart. Also, levosimendan pretreatment was the most effective way to protect the heart against ischaemic/reperfusion injury. Pre-conditioning with levosimendan was abolished by either a nonspecific, or a mitochondrial K_{ATP} channel blocker suggesting a role for the mitochondrial K_{ATP} channel in levosimendan-induced cardioprotection. Finally, levosimendan pretreatment or pre-conditioning may protect the heart by activating components of the RISK pathway.

Regarding future clinical application, levosimendan-induced pre-conditioning may be useful before elective cardiac surgery, whereas LPostC could be applicable immediately after coronary reperfusion. The major experimental effect of levosimendan pretreatment also warrants clinical trials, especially in those with large AMIs when this agent can be expected to protect from both left ventricular failure and subsequent reperfusion injury (Table 1).

Acknowledgements

This study was partially funded by Orion Pharma, Espoo, Finland. We also wish to thank the Medical Research Council for their support and Dr Erna Marais for her expert advice and technical support.

Conflict of interest

Dr Piero Pollesello from Orion Pharma, Finland, provided the levosimendan and limited financial support. Other authors state no conflict of interest.

References

Auchampach JA, Grover GJ, Gross GJ (1992). Blockade of ischaemic pre-conditioning in dogs by the novel ATP dependent potassium channel antagonist sodium 5-hydroxydecanoate. *Cardiovasc Res* **26**: 1054–1062.

Becker LC, Ambrosio G (1987). Myocardial consequences of reperfusion. *Prog Cardiovasc Dis* **30**: 23–44.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254.

Busk M, Meang M, Kistensen J, Berg JS, Mortensen UK, Nielsen TT *et al*. (2006). Effects of levosimendan on myocardial infarct size and hemodynamics in a closed chest porcine ischaemia–reperfusion model. *Cardiovasc Drugs Ther* **20**: 335–342.

Cammarata GA, Weil MH, Sun S, Huang L, Fang X, Tang W (2006). Levosimendan improves cardiopulmonary resuscitation and survival by K(ATP) channel activation. *J Am Coll Cardiol* **47**: 1083–1085.

Cleland JG, Nikitin N, McGowan J (2004). Levosimendan: first in a new class of inodilator for acute and chronic severe heart failure. *Expert Rev Cardiovasc Ther* **2**: 9–19.

Darling CE, Jiang R, Maynard M, Whittaker P, Przyklenk K (2005). Post-conditioning via stuttering reperfusion limits myocardial infarct size in rabbit hearts: role of ERK1/2. *Am J Physiol Heart Circ Physiol* **289**: H1618–H1626.

du Toit EF, Muller CA, McCarthy J, Opie LH (1999). Levosimendan: effects of a calcium sensitizer on function and arrhythmias and cyclic nucleotide levels during ischaemia/reperfusion in the

Langendorff-perfused guinea pig heart. *J Pharmacol Exp Ther* **290**: 505–514.
du Toit EF, Rossouw E, Salie R, Opie LH, Lochner A (2005). Effect of sildenafil on reperfusion function, infarct size, and cyclic nucleotide levels in the isolated rat heart model. *Cardiovasc Drugs Ther* **19**: 23–31.
Duygu H, Ozerkan F, Nalbantgil S, Yildiz A, Akilli A, Akin M *et al*. (2007). Effect of levosimendan on right systolic and diastolic functions in patients with ischaemic heart failure. *Int J Clin Pract* **62**: 228–233.
Fliss H, Gattinger D (1996). Apoptosis in ischemic and reperfused rat myocardium. *Circ Res* **79**: 949–956.
Follath F, Cleland JG, Just H, Papp JG, Scholz H, Peuhkurinen K *et al*. (2002). Efficacy and safety of intravenous levosimendan compared with dobutamine in severe low-output heart failure (the LIDO study): a randomised double-blind trial. *Lancet* **360**: 196–202.
Forman MB, Virmani R, Puett DW (1990). Mechanisms of therapy of myocardial reperfusion injury. *Circulation* **81**: IV69–IV78.
Garlid KD, Dos Santos P, Xie ZJ, Costa ADT, Paucek P (2003). Mitochondrial potassium transport: the role of the mitochondrial ATP-sensitive K+ channel in cardiac function and cardioprotection. *Biochim Biophys Acta* **1606**: 1–21.
Givertz MM, Andreou C, Conrad CH, Colucci WS (2007). Direct myocardial effects of levosimendan in humans with left ventricular dysfunction: alterations of force–frequency and relaxation–frequency relationships. *Circulation* **115**: 1218–1224.
Gross GJ, Auchampach JA (1992). Blockade of ATP-sensitive potassium channels prevents myocardial pre-conditioning in dogs. *Circ Res* **70**: 223–227.
Halkos ME, Kerendi F, Corvera JS, Wang NP, Kin H, Payne CS *et al*. (2004). Myocardial protection with post-conditioning is not enhanced by ischaemic pre-conditioning. *Ann Thor Surg* **78**: 961–969.
Haunstetter A, Izumo S (1998). Apoptosis: basic mechanisms and implications for cardiovascular disease. *Circ Res* **82**: 1111–1129.
Hausenloy DJ, Yellon DM (2003). The mitochondrial permeability transition pore: its fundamental role in mediating cell death during ischaemia and reperfusion. *J Mol Cell Cardiol* **35**: 339–341.
Hausenloy DJ, Yellon DM (2004). New directions for protecting the heart against ischaemia/reperfusion injury: targeting the reperfusion injury salvage kinase (RISK) pathway. *Cardiovasc Res* **61**: 448–460.
Hausenloy DJ, Tsang A, Yellon DM (2005). The reperfusion injury salvage kinase pathway: a common target for both ischaemic pre-conditioning and post-conditioning. *Trends Cardiovasc Med* **15**: 69–75.
Hausenloy DJ, Mocanu MM, Yellon DM (2003). Activation of pro-survival kinase cascades (PI3Kinase-Akt-p70S6K kinase and Erk 1/2 -p70S6K kinase) at reperfusion is essential for pre-conditioning induced protection. *Circulation* **108**: I-288.
Hiayawaki H, Zhou X, Ashraf M (1996). Calcium pre-conditioning elicits strong protection against ischaemic injury via protein kinase C signaling pathway. *Circ Res* **79**: 137–146.
Kersten JR, Montgomery MW, Pagel PS, Waltier DC (2000). Levosimendan, a new positive inotropic drug, decreases myocardial infarct size via activation of K(ATP) channels. *Anesth Analg* **90**: 5–11.
Kin H, Zhao ZQ, Sun HY, Wang NP, Corvera JS, Halkos ME *et al*. (2004). Post-conditioning attenuates myocardial ischaemia/reperfusion injury by inhibiting events in the early minutes of reperfusion. *Cardiovasc Res* **62**: 74–85.
Kloner RA, Rezkalla SH (2004). Cardiac protection during acute myocardial infarction: where do we stand in 2004? *J Am Coll Cardiol* **44**: 276–286.
Kopustinskiene DM, Pollesello P, Saris NE (2001). Levosimendan is a mitochondrial K(ATP) channel opener. *Eur J Pharmacol* **428**: 311–314.
Kouchi I, Murakami T, Nawada M, Akao M, Sasayama S (1998). K_{ATP} channels are common mediators of ischaemic and calcium pre-conditioning in rabbits. *Am J Physiol* **274**: H1106–H1112.
Lepran I, Pollesello P, Vajda S, Varro A, Papp JG (2006). Pre-conditioning effects of levosimendan in a rabbit cardiac ischaemia–reperfusion model. *J Cardiovasc Pharmacol* **48**: 148–152.
Marais E, Genade S, salie R, Huisamen B, Lochner A (2005). The temporal relatonship between p38 MAPK and HSP27 activation in ischemic and pharmacological pre-conditioning. *Basic Res Cardiol* **100**: 35–47.
McGovern PG, Pankow JS, Shahar E, Doliszny KM, Folsom AR, Blackburn H *et al*. (1996). Recent trends in acute coronary heart disease—mortality, morbidity, medical care, and risk factors. *N Eng J Med* **334**: 884–890.
Meldrum DR, Cleveland JCJ, Sheridan BC, Rowland RT, Banerjee A, Harken AH (1996). Cardiac pre-conditioning with calcium; clinically accessible myocardial protection. *J Thorac Cardiovasc Surg* **112**: 778–786.
Michaels AD, McKeown B, Kostal M, Vakharia KT, Jordan MV, Gerber IL *et al*. (2005). Effects of intravenous levosimendan on human coronary vasomotor regulation, left ventricular wall stress, and myocardial oxygen uptake. *Circulation* **111**: 1504–1509.
Murray CJ, Lopez AD (1997). Alternative projections of mortality and disability by cause 1990–2020: Global Burden of Disease Study. *Lancet* **349**: 1498–1504.
Nieminen MS, Akkila J, Hasenfuss J, Kleber FX, Lehtonen LA, Mitrovic V *et al*. (2000). Hemodynamic and neurohumoral effects of continuous infusion of levosimendan in patients with congestive heart failure. *J Am Coll Cardiol* **36**: 1903–1912.
Opie LH (1991). Role of calcium and other ions in reperfusion injury. *Cardiovasc Drugs Ther* **75**: 237–247.
Pollesello P, Papp Z (2007). The cardioprotective effects of levosimendan: preclinical and clinical evidence. *J Cardiovasc Pharmacol* **50**: 257–263.
Rainbow RD, Norman RI, Davies NW, Standen NB (2005). Reduced effectiveness of HMR 1098 in blocking cardiac sarcolemmal K_{ATP} channel during metabolic stress. *J Mol Cel Cardiol* **39**: 637–646.
Sargento L, Brito D, Matias JS, Madeira H (2007). Evaluation of the clinical, hemodynamic and neurohormonal response to levosimendan administration in decompensated heart failure patients. One month follow-up. *Rev Port Cardiol* **26**: 717–726.
Schwartz LM, Lagranha CJ (2006). Ischaemic post-conditioning during reperfusion activates Akt and ERK without protecting against lethal myocardial ischaemia–reperfusion injury in pigs. *Am J Physiol Heart Circ Physiol* **290**: H1011–H1018.
Staat P, Rioufol G, Piot C, Cottin Y, Cung TT, L'Huillier I *et al*. (2005). Post-conditioning the human heart. *Circulation* **112**: 2143–2148.
Van Domberg RT, Sonnenschein K, Nieuwlaat R, Kamp O, Storm CJ, Bax JJ *et al*. (2005). Sustained benefit 20 years after reperfusion therapy in acute myocardial infarction. *J Am Coll Cardiol* **46**: 15–20.
Yang XM, Krieg T, Cui L, Downey JM, Cohen MV (2004a). NECA and bradikinin at reperfusion reduce infarction in rabbit hearts by signaling through PI3K, ERK and NO. *J Mol Cell Cardiol* **36**: 411–421.
Yang XM, Proctor JB, Cui L, Krieg T, Downey JM, Cohen MV (2004b). Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. *J Am Coll Cardiol* **44**: 1103–1110.
Yellon DM, Baxter GF (1999). Reperfusion injury revisited: is there a role for growth factors signalling in limiting lethal reperfusion injury? *Trends Cardiovasc Med* **9**: 245–249.
Yellon DM, Downey JM (2003). Pre-conditioning the myocardium: from cellular physiology to clinical cardiology. *Physiol Rev* **83**: 1113–1151.
Yokoshiki H, Katsube Y, Sunagawa M, Sperelakis N (1997). The novel calcium sensitizer levosimendan activates the ATP-sensitive K^+ channel in rat ventricular cells. *J Pharmacol Exp Therap* **283**: 375–383.
Zao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA (2003). Inhibition of myocardial injury by ischaemic post-conditioning during reperfusion: comparison with ischaemic pre-conditioning. *Am J Physiol* **258**: 579–588.