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Author

Du Toit, EF, Genis, A, Opie, LH, Pollesello, P, Lochner, A

Published

2008

Journal Title

British Journal of Pharmacology

DOI

[10.1038/bjp.2008.52](https://doi.org/10.1038/bjp.2008.52)

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**A ROLE FOR THE RISK PATHWAY AND K_{ATP} CHANNELS IN
LEVOSIMENDAN INDUCED PRE- AND POST-CONDITIONING IN THE
ISOLATED GUINEA PIG HEART.**

Eugene F du Toit, Amanda Genis, ¹Lionel H Opie, ²Piero Pollesello, Amanda Lochner.

Department of Biomedical Sciences, Faculty of Health Sciences, University of Stellenbosch. ¹The Hatter Heart Research Institute, Faculty of Health Sciences, University of Cape Town. ²Orion Pharma, Espoo, Finland.

Address for correspondence:

Dr. EF du Toit

Department of Biomedical Sciences

Faculty of Health Sciences

University of Stellenbosch

PO Box 19063

Tygerberg

7505

South Africa

Tel: 27 21 938 9613

Fax: 27 21 938 9476

e-mail: efdt@sun.ac.za

Running title: Levosimendan pre- and postconditioning

Summary

Background and purpose. Myocardial reperfusion injury prevents optimal salvage of the ischaemic myocardium. The search for adjunct therapy that would significantly reduce reperfusion injury is ongoing. We investigated whether: 1) the heart could be pre- and/or postconditioned using levosimendan (levo) (LPC & LPostC) and, 2) the pro-survival kinases and/or the sarcolemmal or mitochondrial K_{ATP} channels are involved.

Experimental approach. Isolated guinea pig hearts were subjected to 2X5min cycles of levo (0.1 μ M) interspersed with vehicle perfusion or 2X5min cycles of ischaemia/reperfusion before coronary artery ligation (CAL-40min@36.5°C). Hearts were treated with mitochondrial or sarcolemmal K_{ATP} channel blockers before LPC or LPostC. For postconditioning, hearts were subjected to 3X30sec cycles of ischaemia/reperfusion or levo/vehicle. Hearts were pretreated with levo immediately before CAL (without washout). Cardiac function, infarct size and reperfusion injury salvage kinase activity was assessed.

Results. LPC and LPostC decreased infarct size compared with controls (20.6 \pm 3.1% and 20.5 \pm 1.8% vs. 46.4 \pm 1.8% for controls, p <0.05). Treatment with K_{ATP} channel blockers before LPC or LPostC abolished these infarct lowering effects. Pretreating hearts with levo increased reperfusion ERK 42/44 activity and had the most marked infarct lowering effect (5.7 \pm 0.9% (p <0.05)).

Conclusions and implications. 1) Hearts could be pharmacologically pre- and post-conditioned with levo, 2) levo pretreatment is the most effective way to reduce infarct size, possibly acting by increasing ERK 42/44 activity, 3)

LPC and LPostC was abolished by both K_{ATP} channel blockers, 4) LPC may be useful before elective cardiac surgery while LPostC may be used after acute coronary artery events.

Key words: Levosimendan; myocardial infarct; myocardial function, RISK pathway; K_{ATP} channel; reperfusion injury.

List of abbreviations

ADP, aortic diastolic pressure

ASP, aortic systolic pressure

CAL, coronary artery ligation

GBD, glibenclamide

IPC, ischaemic preconditioning

IPostC, ischaemic postconditioning

Levo, levosimendan

LPC, levosimendan preconditioning

LPT – levosimendan pre-treatment

LPostC, levosimendan postconditioning

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 & Gattinger, 1996; Haunstetter & Izumo, 1998) prevents the optimal salvage of the ischaemic myocardium (Kloner & Rezkalla, 2004). This problem may partially explain why only a 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 preconditioning is only feasible during elective cardiac procedures, knowledge about the underlying mechanisms has been used to devise new cardioprotective therapies. Postconditioning is, however, feasible after AMI and was recently used in patients undergoing coronary angioplasty (Staat *et al.*, 2005). This group showed that postconditioning 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, levosimendan, is effective for the treatment of heart failure (Follath *et al.*, 2002; Cleland *et al.*, 2004; Givertz *et al.*, 2007; Duygu *et al.*, 2007; Sargento *et al.*, 2007; Pollesello and Papp, 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 preconditioning leads to activation of PKB/Akt and ERK 42/44 during reperfusion (Hausenloy *et al.*, 2003; Hausenloy & Yellon, 2004; Yang *et al.*, 2004), the so-called reperfusion injury salvage kinase (RISK) pathway (Hausenloy & Yellon, 2004). In a more recent review, it was proposed that the RISK pathway could potentially be a viable target for both pre- and postconditioning (Hausenloy *et al.*, 2005). To this end, we investigated the effects of levosimendan preconditioning, pretreatment, and postconditioning 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 pre-treat the heart, 2) act as a trigger for protection when given briefly before the onset of ischemia (preconditioning) or immediately after reperfusion (postconditioning) 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 levosimendan pre- or postconditioning and that ERK 42/44 activation and the K_{ATP} channel may be implicated in these protective effects.

Methods

Animals model used

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 grams were anaesthetized with pentobarbital (30mg 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 cmH₂O for 5 min with a Krebs-Henseleit buffer equilibrated with 95% O₂ and 5% CO₂ at 37°C (in mmol L⁻¹ - NaCl 121.5, KCl 3.8, MgCl₂.6H₂O 1.2, CaCl₂ 2.5, NaHCO₃ 15.5, KH₂PO₄ 1.2, Na-pyruvate 2.0, glucose 11.0, mannitol 16.0). For evaluation of mechanical function, hearts were perfused in the working heart mode (preload 15 cmH₂O and afterload 75 cmH₂O) for 5 minutes before being preconditioned with ischaemia or levosimendan. Aortic output (Qa), coronary flow (Qe), heart rate (HR) and aortic diastolic (ADP) and aortic systolic pressure (ASP) were measured before and directly after the preconditioning intervention had been applied. Data were collected and analyzed using a PhysiTutor® data acquisition system.

Pretreatment and pre- and postconditioning protocols employed

For reperfusion function and infarct size measurements, hearts were randomly allocated to one of the study groups and mechanical function documented after 5 min working heart perfusion. Hearts that were pre-treated with levosimendan (0.1µM) were perfused with the drug for 10 min before coronary artery ligation (CAL) without washout. The hearts that were preconditioned were subjected to 2X5min cycles of levosimendan (0.1µM) interspersed with vehicle perfusion or 2X5min cycles of global ischaemia/reperfusion before CAL. Coronary artery ligation was instituted for 40 min and heart temperature was maintained at 36.5°C before the suture was released to induce reperfusion. For post-conditioning, hearts were

subjected to 3X30sec cycles of levosimendan (0.1 μ M) interspersed with vehicle perfusion or 3X30sec cycles of ischaemia/reperfusion.

For the K_{ATP} channel blocker treatment before levosimendan preconditioning, 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 coronary artery ligation was performed. For hearts pre-treated 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 preconditioned 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 levosimendan preconditioning or pretreatment merely altered cardiac function and therefore the metabolic demand on the hearts, we performed an additional series of experiments in which hearts were preconditioned or pretreated with levosimendan or subjected to ischaemic preconditioning before being made to work for an additional 5 minutes. During this working heart perfusion phase, cardiac function was assessed and compared with the values obtained before the IPC, LPC and levosimendan pretreatment interventions were applied. Aortic output and coronary flow was not altered by the preconditioning or pre-treatment protocols employed before the coronary artery ligation 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 ischemic 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 whether we reperfused for 30 min or 120 min (Marais *et al.*, 2005). Hearts were then frozen and cut into 5-7 slices, incubated in sodium phosphate buffer containing 1% w/v triphenyltetrazolium chloride (TTC) 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 $36.7 \pm 1.1\%$ of the volume of the left ventricle.

Western blot analysis

In additional series of experiments hearts were: 1) pretreated or preconditioned with levosimendan and then subjected to ischaemia and reperfusion as described above or, 2) they were postconditioned by ischaemia or levosimendan or, 3) they were co-perfused with one of the K_{ATP} channel blockers while being postconditioned with ischaemia or levosimendan. After 5 or 10 min reperfusion ischaemic and non-ischaemic 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 (in mM): Tris 20, *p*-nitrophenylphosphate 20, EGTA 1, NaF 50, sodium orthovanadate 0.1, phenylmethyl sulfonyl fluoride (PMSF) 1, dithiothreitol (DTT) 1, aprotinin 10 µg ml⁻¹, 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% PAGE-SDS 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). These membranes were routinely stained with Ponceau Red for visualization of proteins. Non-specific binding sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline — 0.1% Tween 20 (TBST) and then incubated with the primary antibodies that recognize phospho-specific ERK p42/p44 (Thr²⁰²/Tyr²⁰⁴) or PKB (Ser⁴⁷³ and Thr³⁰⁸). Membranes were subsequently washed with large volumes of TBST (5×5 min) and the immobilized antibody conjugated with a diluted horseradish peroxidase-labeled secondary antibody (Amersham LIFE SCIENCE). After thorough washing with TBST, membranes were covered with ECL 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 analyzed (UN-SCAN-IT, Silkscience) 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.

Drugs used

Levosimendan was prepared with 50 μ l 1 M NaOH in 10ml phosphate buffer (2.32% Na₂HPO₄ in distilled water) and from this 1mM stock solution 100 μ l was added to 1000ml of perfusion buffer. 5-Hydroxy decanoic acid (5HD) was used at a concentration of 200 μ M and glibenclamide (GBD) at 5 μ M. 5HD is a mitochondrial K_{ATP} channels blocker while GBD blocks both the mitochondrial and sarcolemmal K_{ATP} channels. For selected experiments these blockers were applied during the levosimendan pretreatment or preconditioning protocols.

Statistical methods

We performed a minimum of 6 hearts/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-ischemic value.

Results

The effect of ischaemic preconditioning, levosimendan preconditioning and levosimendan pretreatment on myocardial infarct size

IPC reduced infarct size in the guinea pig heart from 46.6 \pm 1.9% for control hearts to 21.9 \pm 2.8% for the preconditioned hearts ($p < 0.05$) Preconditioning hearts with two cycles of levosimendan perfusion interspersed with vehicle perfusion (LPC) reduced infarct size to 20.6 \pm 3.1%

($p < 0.05$ vs. control). Combining IPC and LPC did not further reduce infarct size (IPC + LPC)(Figure 1).

Pre-treating hearts with levosimendan for 10 min (without washout prior to ischaemia) decreased infarct size to $5.7 \pm 0.9\%$ ($p < 0.05$) (Figure 1).

Effect of K_{ATP} channel blockers on the infarct size in levosimendan preconditioned and pretreated hearts.

Co-perfusing hearts with levosimendan and glibenclamide/5HD during the trigger phase of LPC precluded the hearts from being protected by LPC. Myocardial infarct size was similar in control hearts to hearts that were preconditioned with levosimendan while being perfused with K_{ATP} channel blockers ($46.8 \pm 1.3\%$ for LPC + GBD vs. $46.8 \pm 2.4\%$ for GBD and $37.7 \pm 1.6\%$ LPC + 5HD vs. $33.9 \pm 2.8\%$ for 5HD (Figure 2)).

Co-perfusing hearts with the blockers during levosimendan pretreatment abrogated the infarct reducing effects of levosimendan. Infarct size was $48.9 \pm 2.1\%$ for GBD vs. $48.5 \pm 2.9\%$ LPT+GBD and $39.1 \pm 2.8\%$ (5HD) for 5HD vs. $38.6 \pm 2.4\%$ LPT+5HD.

Effect of ischaemic- or levosimendan postconditioning on infarct size

Post-conditioning hearts with either ischaemia (IPostC) or levosimendan (LPostC) decreased myocardial infarct size ($37.8 \pm 1.7\%$ control vs. $16.8 \pm 1.7\%$ and $18.4 \pm 1.8\%$ respectively, $p < 0.05$) (Figure 3). Ischaemic- and levosimendan postconditioning were not additive and did not further reduce infarct size when compared with one of the interventions on their own (data not presented).

Effect of K_{ATP} channel blockers on the infarct size in levosimendan and ischaemic postconditioned hearts.

Co-perfusing hearts with levosimendan and glibenclamide/5-HD during the trigger phase of postconditioning prevents the hearts from being protected by LPostC. Myocardial infarct size was similar for control hearts and hearts treated with K_{ATP} channel blockers alone or in combination with levosimendan or ischaemic postconditioning ($36.1 \pm 2.1\%$ for IPostC+5HD, $37.8 \pm 2.2\%$ for LPostC+5HD, $36.1 \pm 2.2\%$ for IPostC+GBD, and $38.8 \pm 3.1\%$ for LPostC+GBD (Figure 3)).

Aortic output recovery for the respective experimental groups

Reperfusion aortic output recovery was improved with LPC (control: $36.2 \pm 7.7\%$ vs LPC: $58.6 \pm 5.8\%$ ($p < 0.05$)) and pretreatment with levosimendan (control: $36.3 \pm 7.4\%$ vs. $61.3 \pm 4.9\%$ for pretreatment ($p < 0.05$))(Figure 4 A).

Postconditioning did not improve reperfusion aortic output recovery. IPostC resulted in reperfusion aortic output recoveries of $60.0 \pm 9.2\%$ vs. $50.2 \pm 4.0\%$ for controls and LPostC aortic output recoveries were $64.0 \pm 13.1\%$ (Figure 4 B).

Effect of LPC or levosimendan pre-treatment on myocardial PKB/Akt and ERK 42/44 activity during reperfusion.

We documented reperfusion PKB/Akt and ERK 42/44 activity in both the ischaemic and non-ischaemic tissue of the CAL hearts. The PKB/Akt and ERK 42/44 activity after 5 and 10 min reperfusion was comparable in the non-ischaemic and ischaemic tissue of the control and levosimendan pretreated and LPC hearts (data not presented). Neither LPC nor levosimendan 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, LPC and levosimendan pre-treated groups at 5 and 10 min reperfusion (data not shown).

Total ERK levels were similar in all groups (control, levosimendan pretreatment and LPC) 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 non-treated hearts (Figure 5A). ERK 42 activity was increased above basal (pre-ischaemic) levels after 5 min reperfusion in the LPC and levosimendan pre-treated 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 pre-treated hearts had elevated ERK 42 activity when compared to basal levels.

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

Postconditioning 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 coperfusion with K_{ATP} channel blockers and LPostC or IPostC 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 pretreat the heart or as a preconditioning or postconditioning-mimetic. The most effective cardioprotection was observed with pretreatment with levosimendan, with lesser but still significant protection by levosimendan-induced pre- or postconditioning. Both levosimendan preconditioning and pretreatment also improved reperfusion mechanical function of the heart. The cardioprotective effect of LPC and levosimendan pretreatment may relate to its ability to increase ERK 1/2 activity during reperfusion. These cardioprotective effects in both pre- and postconditioning can be abrogated by blocking the mitochondrial K_{ATP} channels, an effect which implicates these channels in the resultant protection we observed.

Acute myocardial infarction and the importance of optimizing reperfusion therapy

Myocardial infarction is set to become the leading cause of death by 2020 (Murray & 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 a third of the life years lost by the 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 & Ambrosio, 1987; Forman *et al.*, 1990; Opie, 1991) and reperfusion induced apoptosis (Fliss & Gattinger, 1996; Haunstetter & Izumo, 1998). With this in mind several research groups have set out to determine how ischaemic and reperfusion injury can be minimized.

Although preconditioning is only clinically applicable before elective cardiac surgery, several researchers have endeavoured to understand the mechanisms responsible for the cardioprotective effects of pre- and postconditioning. This has been done in the hope that this knowledge could be used to formulate pharmacological cardioprotective agents that would be clinically applicable. Ischaemic postconditioning has however been effective in protecting the 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 the guinea pig (Du Toit *et al.*, 1999) and improves both these parameters in the isolated perfused rabbit heart model (Lepran *et al.*, 2006). It has also recently been shown to improve

cardiopulmonary resuscitation and 48 hour 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 the arrhythmias in the rat (Cammarata *et al.*, 2006) or coronary artery occlusion in the dog (Kersten *et al.*, 2000) suggesting a role for the K_{ATP} channel opening properties of the drug.

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).

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 (Follath *et al.*, 2002; Cleland *et al.*, 2004; Niemenen *et al.*, 2000; Michaels *et al.*, 2005; Duygu *et al.*, 2007; Givertz *et al.*, 2007; Sargento *et al.*, 2007; Pollesello and Papp, 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 postconditioning mimetic

Downstream signalling pathways that have been implicated in the triggering of preconditioning varies, but the K_{ATP} channels have been

implicated as a possible end-effector in calcium preconditioning (Hiyawaki *et al.*, 1996; Meldrum *et al.*, 1996; Kouchi *et al.*, 1998) and IPC (Gross & Auchampach, 1992; Auchampach *et al.*, 1992; Garlid *et al.*, 2003). These studies suggested that K_{ATP} channels play a central role in classic preconditioning 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 (for review 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 preconditioning, we set out to determine whether the drug could act as a pre- and postconditioning mimetic. This is one of the first studies to demonstrate that levosimendan can act as a “trigger” for pre- and postconditioning in the 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 non-specific K_{ATP} channel blocker (glibenclamide) during the “trigger” phase of LPC attenuates the cardioprotective effects of LPC. These data support the concept that levosimendan protects the ischaemic heart by opening these channels and thus conferring protection on the ischaemic heart.

As it has been demonstrated that the mitochondrial K_{ATP} channels are also involved in the cardioprotection induced by postconditioning (Yang *et al.*, 2004), we also tested the effects of 5HD and glibenclamide on

postconditioning. The infarct reducing effect of postconditioning could be abrogated by treating rabbits with glibenclamide prior to the induction of coronary artery occlusion and postconditioning during the reperfusion phase (Yang *et al.*, 2004). Our data also suggests that the effects of levosimendan on these channels may be implicated in its postconditioning mimetic effects in this study.

Despite glibenclamide being considered a non-specific K_{ATP} channel blocker, we chose this compound as the efficacy of HMR 1098 (a putative sarcolemmal K_{ATP} channel blocker) in conditions of metabolic stress have been questioned by Rainbow and co-workers (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 prior to 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 were due to K_{ATP} channels opening prior to the sustained ischaemic episode.

The fact that levosimendan pretreatment confers greater cardioprotection against ischaemia and reperfusion injury than does 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 PC signalling pathways when used as a

pretreatment drug. During LPC procedures, the possibility exists that the compound is washed out of the heart during the 5 min prior to sustained ischaemia and therefore has less effect on the ischaemic myocardium. This possibility will have to be investigated in the future.

The effect of levosimendan pretreatment or levosimendan preconditioning 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 & Baxter, 1999; Hausenloy & Yellon, 2004). The exact downstream effects of RISK pathway activation have not been established but probably involve closing the mPTP 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 & Yellon, 2003). Closing of these pores possibly occurs through ERK induced phosphorylation and activation of eNOS, phosphorylation and inactivation of GSK3 β or phosphorylation and mitochondrial translocation of PKC ϵ (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 LPC hearts. We found no differences in reperfusion PKB/Akt activity when comparing control hearts with levosimendan pretreated or LPC hearts. We did however find that reperfusion ERK 42 and ERK 44 activity was 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 postconditioning (Hausenloy *et al.*, 2005, our data suggests that the RISK pathway, and more specifically ERK 42/44, can also be activated by pharmacological pretreatment with levosimendan. We believe that this 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 mPTP opening.

The effect of levosimendan or ischaemic postconditioning on the RISK pathway activity.

Several studies have reported increased ERK 42/44 activity during reperfusion after postconditioning (Darling *et al.*, 2005; Schwartz and Lagranha, 2006). We were unable to demonstrate ERK or PKB activation in our postconditioned hearts 5 min after reperfusion. The exact reason for this is unclear but may relate to the fact that we only measure the activity and expression of these signalling peptides at 5 min reperfusion after postconditioning which may have not allowed enough time for activation of these pathways to occur.

We conclude that: 1) Levosimendan can be used to either precondition or postcondition the heart. 2) Levosimendan pretreatment is the most effective way to protect the heart against ischaemic/reperfusion injury. 3) LPC was abolished by either a non-specific, or a mitochondrial K_{ATP} channel blocker suggesting a role for the mitochondrial K_{ATP} channel in levosimendan induced cardioprotection. 4) Levosimendan pretreatment and LPC may protect the heart by activating components of the RISK pathway.

Regarding future clinical application, levosimendan-induced preconditioning may be useful before elective cardiac surgery while levosimendan postconditioning could be applicable immediately after coronary reperfusion. The major experimental effect of levosimendan given as pretreatment also warrants clinical trials, especially in those with large AMIs when this agent can be expected to protect from both LV failure and subsequent reperfusion injury.

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.

Statement of conflict of interest

None. Dr Piero Pollesello from Orion Pharma, Finland provided the levosimendan and limited financial support. Dr Pollesello was involved during the planning stages of the study and the final preparation of the manuscript. All experimental work and data analysis was performed in the laboratory at the University of Stellenbosch, Cape Town, South Africa.

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A	CONTROL	IPC	LPC	IPC+LPC (IPC and LPC combined)	LPC + 5HD	LPC + GBD	PRETREAT- MENT	PRETREAT- MENT+5HD	PRETREAT- MENT+GBD
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		

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

Legends

Fig. 1. Experimental perfusion protocol used to induce ischaemic and levosimendan preconditioning and to pre-treat the hearts with levosimendan (A). Myocardial infarct size in control untreated hearts, hearts preconditioned using ischaemic preconditioning, or levosimendan preconditioning, or the combination of both, or hearts pretreated with levosimendan (B).

LD = Langendorff perfusion

WH = working heart perfusion

CAL = coronary artery ligation

LPT = levosimendan pretreatment

IPC = ischaemic preconditioning

LPC = levosimendan preconditioning

n=6-9

* $p < 0.05$ vs. control

Fig 2. Experimental perfusion protocol used to induce levosimendan preconditioning 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 preconditioned with levosimendan (B).

n=6-8

LD = Langendorff perfusion

WH = working heart perfusion

CAL = coronary artery ligation

5HD Control = 5HD without intermittent levosimendan preconditioning

LPC + 5HD = 5HD with intermittent levosimendan preconditioning

GBD Control = glibenclamide without intermittent levosimendan preconditioning

LPC + GBD = GBD with intermittent levosimendan preconditioning

Fig 3. Experimental perfusion protocol used to induce ischaemic and levosimendan postconditioning in the absence and presence of K_{ATP} channels blockers (A). Myocardial infarct size in control hearts, hearts postconditioned with ischaemia, or hearts postconditioned with levosimendan (B).

n=4-5

* $p < 0.05$ vs. control

LD = Langendorff perfusion

WH = working heart perfusion

CAL = coronary artery ligation

IPostC = ischaemic postconditioning

LPostC = levosimendan postconditioning

Fig 4. Aortic output recovery (%) for control hearts and hearts pretreated with levosimendan or, pre-conditioned using ischaemia, or levosimendan (A). Aortic output recovery (%) for control hearts and hearts postconditioned with ischaemia, or levosimendan or in combination with K_{ATP} channels blockers (B). $n=6-9$

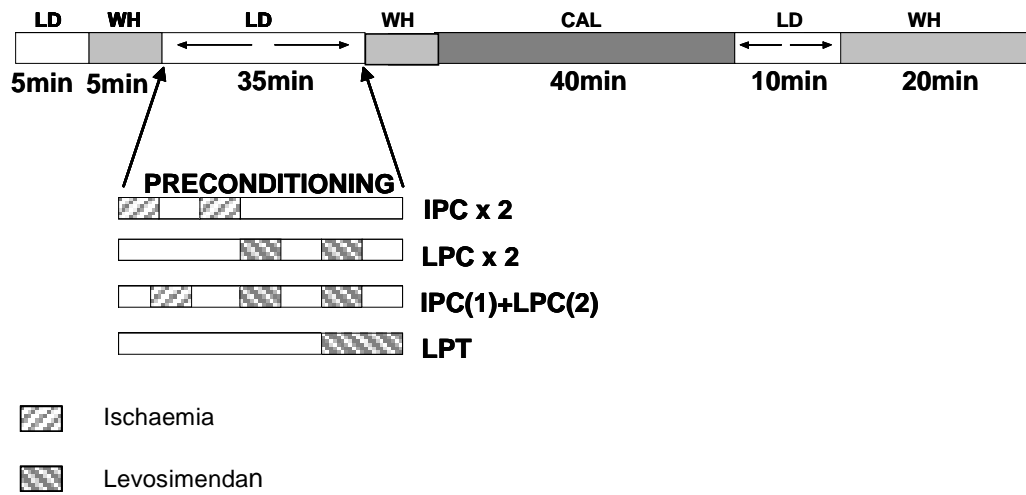
* $p < 0.05$ vs. control

Fig 5. Representative blots for total ERK and phospho ERK 1/2 and densitometry showing the effect of LPC and LPT on ERK 44 (A) and ERK 42 (B) phosphorylation of the ischaemic tissue at 5 min and 10 min reperfusion.

$n=6$

* $p < 0.05$ vs. basal, # $p < 0.05$

A



B

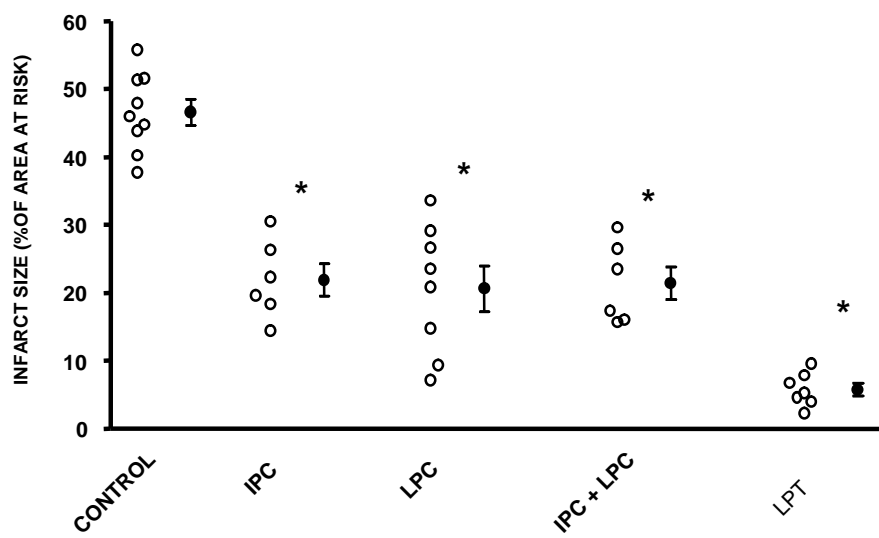


Fig. 1.

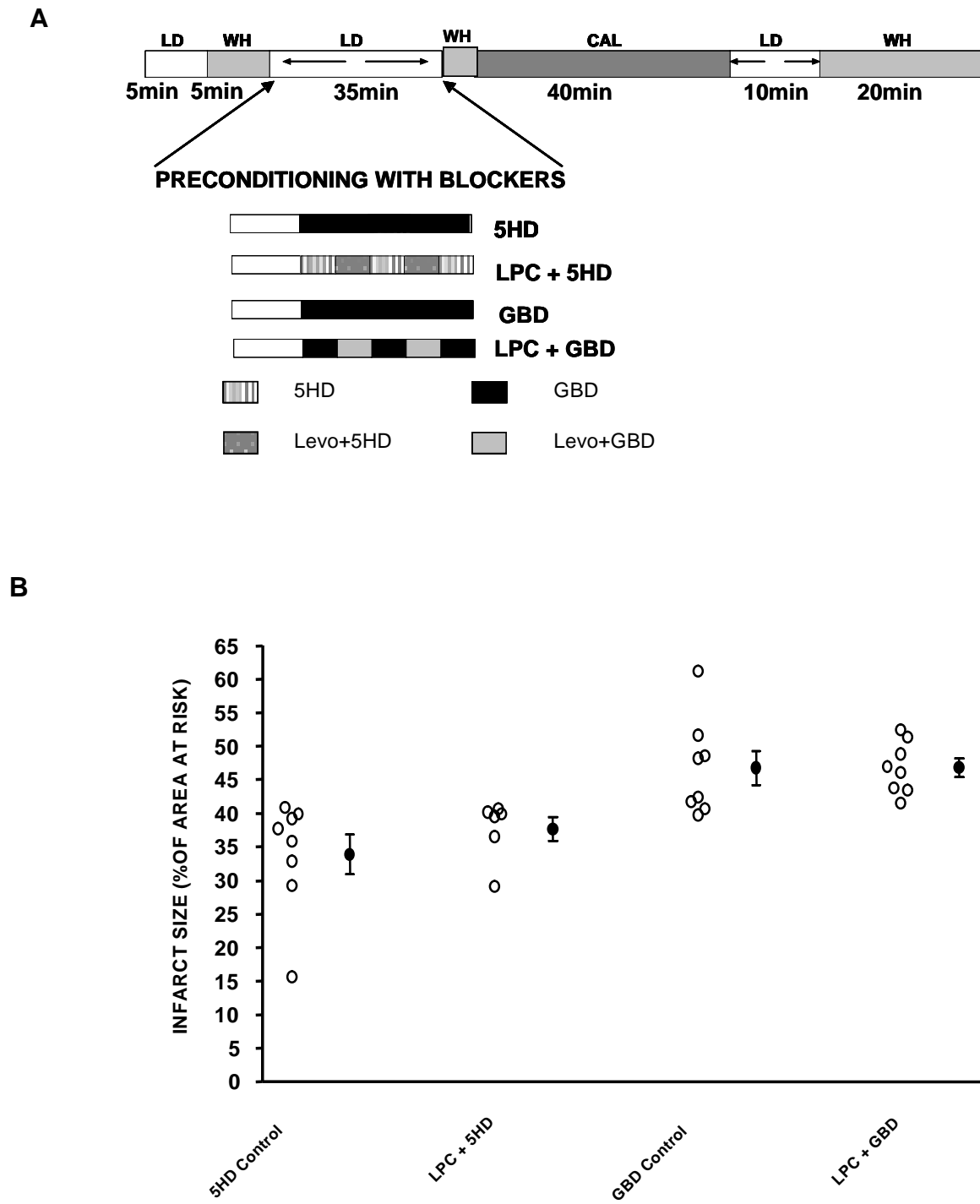
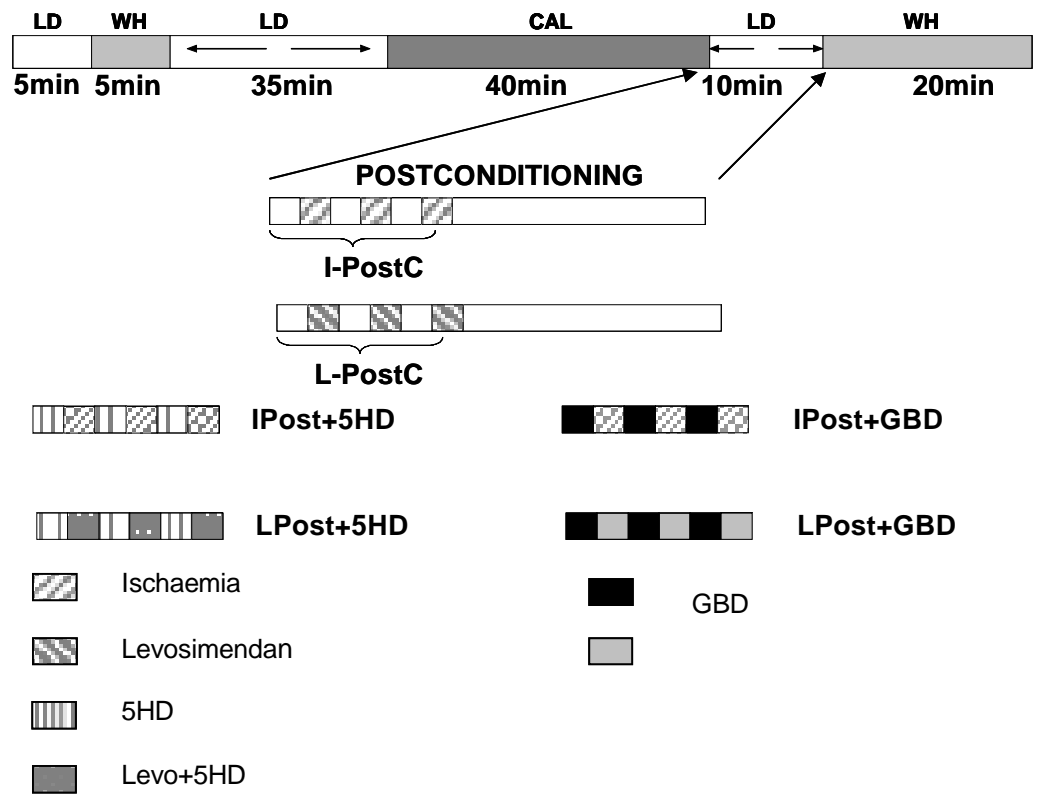


Fig. 2.

A



B

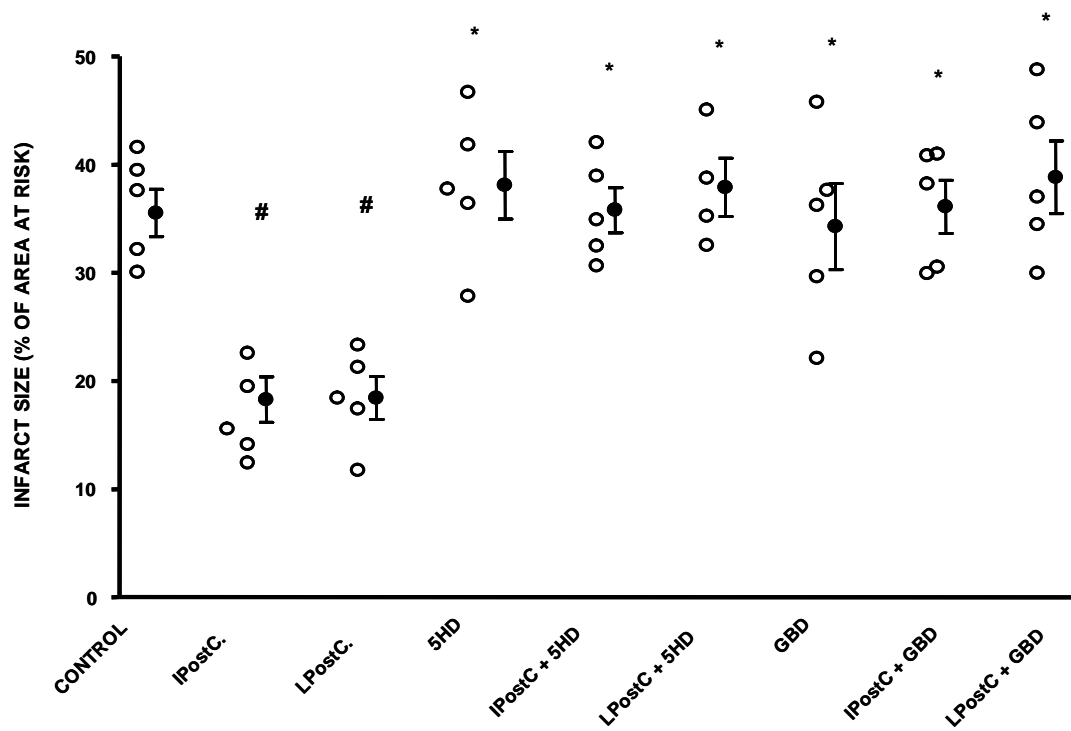
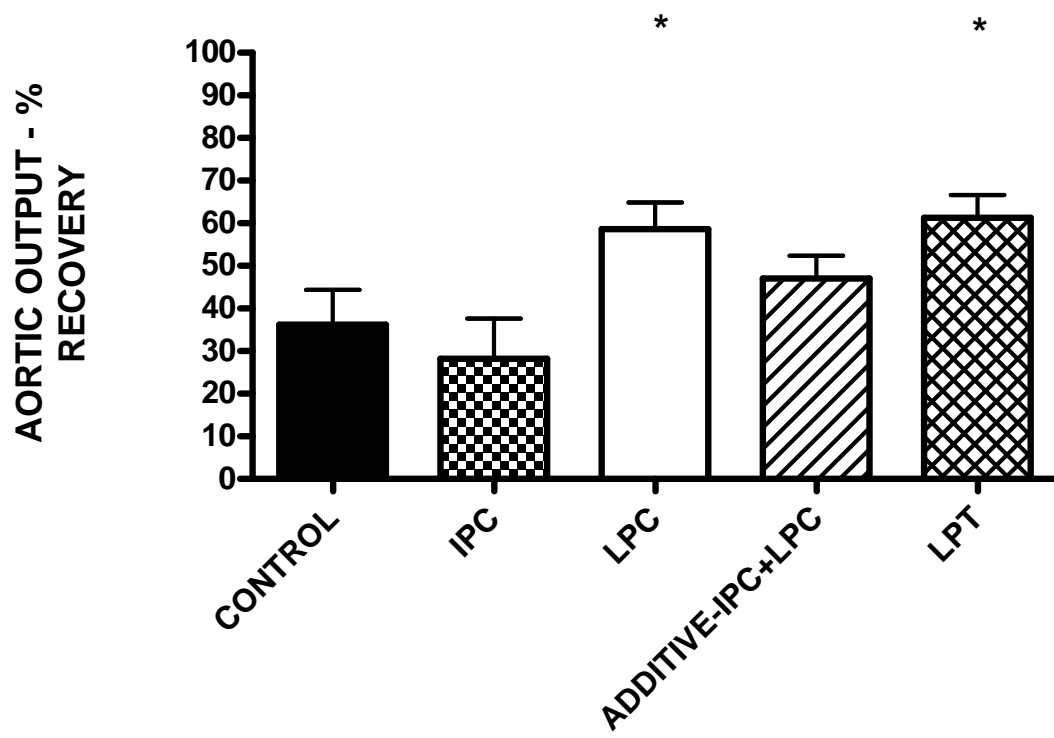
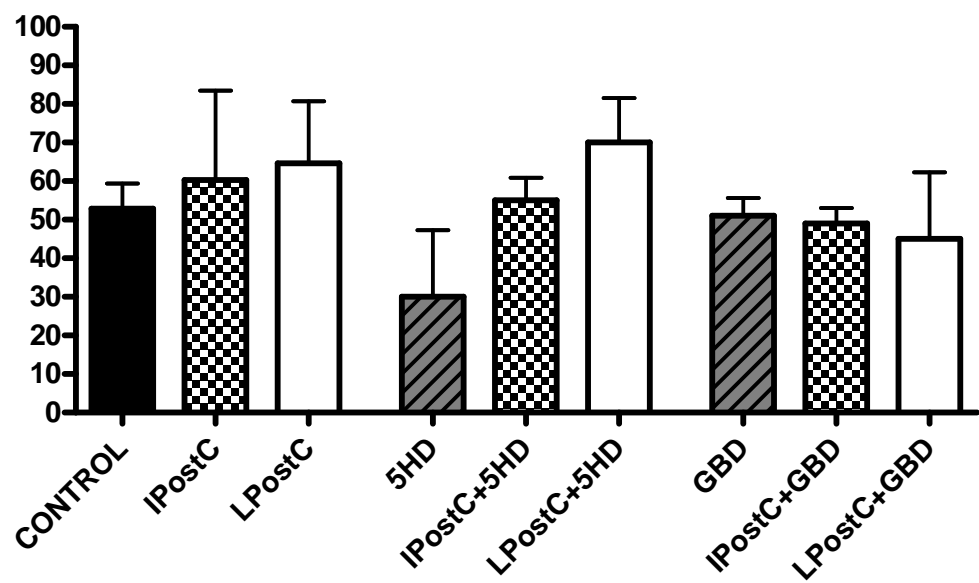


Fig. 3.

A



B

**Fig. 4.**

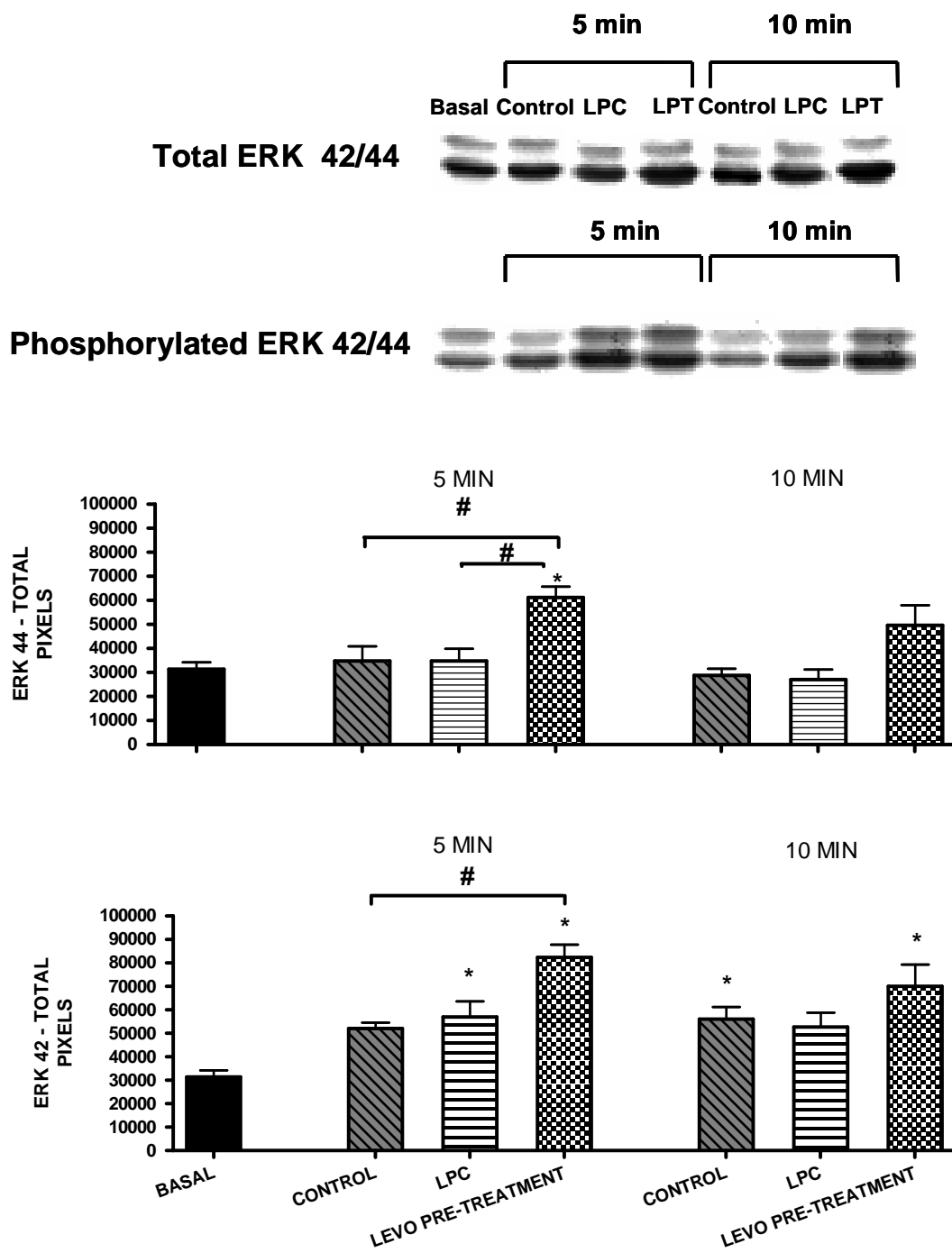


Fig. 5.