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Transient limb ischaemia remotely preconditions through a humoral mechanism acting directly on the myocardium: evidence suggesting cross-species protection

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ABSTRACT

rIPC (remote ischaemic preconditioning) is a phenomenon whereby short periods of ischaemia and reperfusion of a tissue or organ (e.g. mesentery, kidney) can protect a distant tissue or organ (e.g. heart) against subsequent, potentially lethal, ischaemia. We, and others, have shown that transient limb ischaemia can provide potent myocardial protection experimentally and clinically during cardiac surgery. Nonetheless, our understanding of the signal transduction from remote stimulus to local effect remains incomplete. The aim of the present study was to define the humoral nature of rIPC effector(s) from limb ischaemia and to study their local effects in isolated heart and cardiomyocyte models. Using a Langendorff preparation, we show that infarct size after coronary artery ligation and reperfusion was substantially reduced by rIPC *in vivo*, this stimulus up-regulating the MAPKs (mitogen-activating protein kinases) p42/p44, and inducing PKCε (protein kinase Cε) subcellular redistribution. Pre-treatment with the plasma and dialysate of plasma (obtained using 15 kDa cut-off dialysis membrane) from donor rabbits subjected to rIPC similarly protected against infarction. The effectiveness of the rIPC dialysate was abrogated by passage through a C_{18} hydrophobic column, but eluate from this column provided the same level of protection. The dialysate of rIPC plasma from rabbits and humans was also tested in an isolated fresh cardiomyocyte model of simulated ischaemia and reperfusion. Necrosis in cardiomyocytes treated with rIPC dialysate was substantially reduced compared with control, and was similar to cells pre-treated by 'classical' preconditioning. This effect, by rabbit rIPC dialysate, was blocked by pre-treatment with the opiate receptor blocker naloxone. In conclusion, *in vivo* transient limb ischaemia releases a low-molecular-mass (<15 kDa) hydrophobic circulating factor(s) which induce(s) a potent protection against myocardial ischaemia/reperfusion injury in Langendorff-perfused hearts and isolated cardiomyocytes in the same species. This cardioprotection is transferable across species, independent of local neurogenic activity, and requires opioid receptor activation.

Key words: limb ischaemia, myocardium, mitogen-activated protein kinase (MAPK), opioid, protein kinase C (PKC), remote ischaemic preconditioning.

Abbreviations: AAR, area at risk; BP, blood pressure; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; I/R, ischaemia/reperfusion; IPC, ischaemic preconditioning; LAD, left anterior descending coronary artery; LV, left ventricle; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; rIPC, remote IPC; SI, simulated ischaemia; SR, simulated reperfusion.

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INTRODUCTION

The phenomenon of IPC (ischaemic preconditioning), induced by short periods of ischaemia and reperfusion, is well recognized as one of the most potent innate protective mechanisms against I/R (ischaemia/reperfusion) injury [1]. However, despite profound protective effects demonstrable in experimental models, the practical difficulties in delivering the stimulus has limited the clinical application of IPC generally, and to the heart in particular [2,3].

rIPC (remote IPC) was first described by Przyklenk et al. [4] in 1993. They showed the transfer of protection between vascular territories within the heart, and went on to demonstrate that another heart could be preconditioned by transfer of 'preconditioned' coronary effluent, strongly suggesting a humoral mechanism for this remote protection [5]. It has become clear that remote transient ischaemia of a variety of tissues, including the kidney, gut, liver and skeletal muscle, can protect the myocardium [6,7] and also other tissues such as remotely located skeletal muscle [8]. From the perspective of a clinical application of rIPC, repeated short periods of limb ischaemia using a BP (blood pressure) cuff or tourniquet is a practical approach, and we have demonstrated at our institution by randomized clinical trial that this form of rIPC is effective in protecting children undergoing cardiac surgery from multi-organ injury [9]. This 'first in man' demonstration of clinical protection has been confirmed in subsequent studies showing reduced myocardial damage and post-operative renal failure in patients undergoing abdominal aortic aneurysm surgery [10], and reduced troponin T release in adults undergoing surgical coronary revascularization [11].

In an excellent recent review by Hausenloy and Yellon [12], the current state of knowledge and gaps in our understanding of the mechanistic pathways implicated in the myocardial protection afforded by rIPC are discussed. They point out that the exact nature of the signalling pathways involved remain to be clarified not only because of the widely variable experimental methods employed in prior studies, but also because of a demonstrable variability in response depending on species and the mode of induction of the rIPC stimulus.

In the present study, we address some of these gaps in understanding by focusing solely on the protective substance(s) entering the bloodstream after repetitive limb ischaemia, the most practical means of achieving rIPC clinically. We used a rabbit hindlimb ischaemia stimulus for the induction of rIPC that allowed testing of the mechanisms of protection in an isolated Langendorff-perfused rabbit heart model of regional myocardial ischaemia, and an isolated rabbit cardiomyocyte model of SI (simulated ischaemia) and SR (simulated reperfusion).

MATERIALS AND METHODS

Ethics

All animal protocols were approved by the Animal Care and Use Committee of the Hospital for Sick Children in Toronto and conformed with the Guide for the Care and Use of Laboratory Animals published by NIH (NIH publication N0. 85-23, revised 1996). The experiments involving human volunteers were approved by the Research Ethics board of the Hospital for Sick Children, Toronto, and written informed consent was obtained.

rIPC induced by intermittent skeletal muscle ischaemia

New Zealand White rabbits were anaesthetized with akmezine (0.25 mg/kg of body weight) and pentobarbital (30 mg/kg of body weight), intubated and ventilated, and anticoagulated with heparin (100 units/kg of body weight) given via a marginal ear vein. Animals were remotely preconditioned (rIPC; $n=6$) by subjecting them to four-cycle hindlimb ischaemia (5 min) and reperfusion (5 min) (Figure 1A), as described previously [13,14]. Control rabbits (non-rIPC; $n=6$) were anaesthetized and ventilated for the duration of the rIPC protocol (Figure 1A). Next, each rabbit heart was excised and immediately perfused on a Langendorff apparatus using Krebs–Henseleit buffer (118 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l $MgSO_4$, 1.8 mmol/l $CaCl_2$, 1.2 mmol/l KH_2PO_4, 25 mmol/l $NaHCO_3$ and 10 mmol/l glucose) for 20 min (stabilization period). Each heart was then subjected to I/R {30 min of regional myocardial ischaemia [LAD (left anterior descending coronary artery) occlusion] and 120 min of reperfusion} (Figure 1C). Infarct size was used as the end point to determine protection by rIPC in this model.

In a separate series of experiments, hearts from control (non-rIPC; $n=5$) and rIPC rabbits ($n=7$) were excised either at the end of the four cycles of intermittent hindlimb ischaemia and reperfusion (rIPC hearts) or following a sham operation of the same duration as rIPC (control hearts) (Figure 1C). All hearts were immediately frozen in liquid nitrogen after excision for subsequent subcellular fractionation and Western blot analysis as described below.

Studies on rIPC protection induced by transfer of humoral effector(s) in plasma, plasma dialysate or hydrophobic eluate

Separate groups of Langendorff-perfused hearts were treated (as described above for 35 min prior to 30 min of LAD occlusion, followed by 120 min of reperfusion) with 50 ml of plasma ($n=6$), 1 litre of plasma dialysate ($n=6$), 1 litre of C_{18} column flow-through ($n=6$) or 1 litre of C_{18} column eluate ($n=6$) derived from either control or rIPC rabbit blood (Figure 1C). Infarct size was measured as the end point at the end of reperfusion as described below.

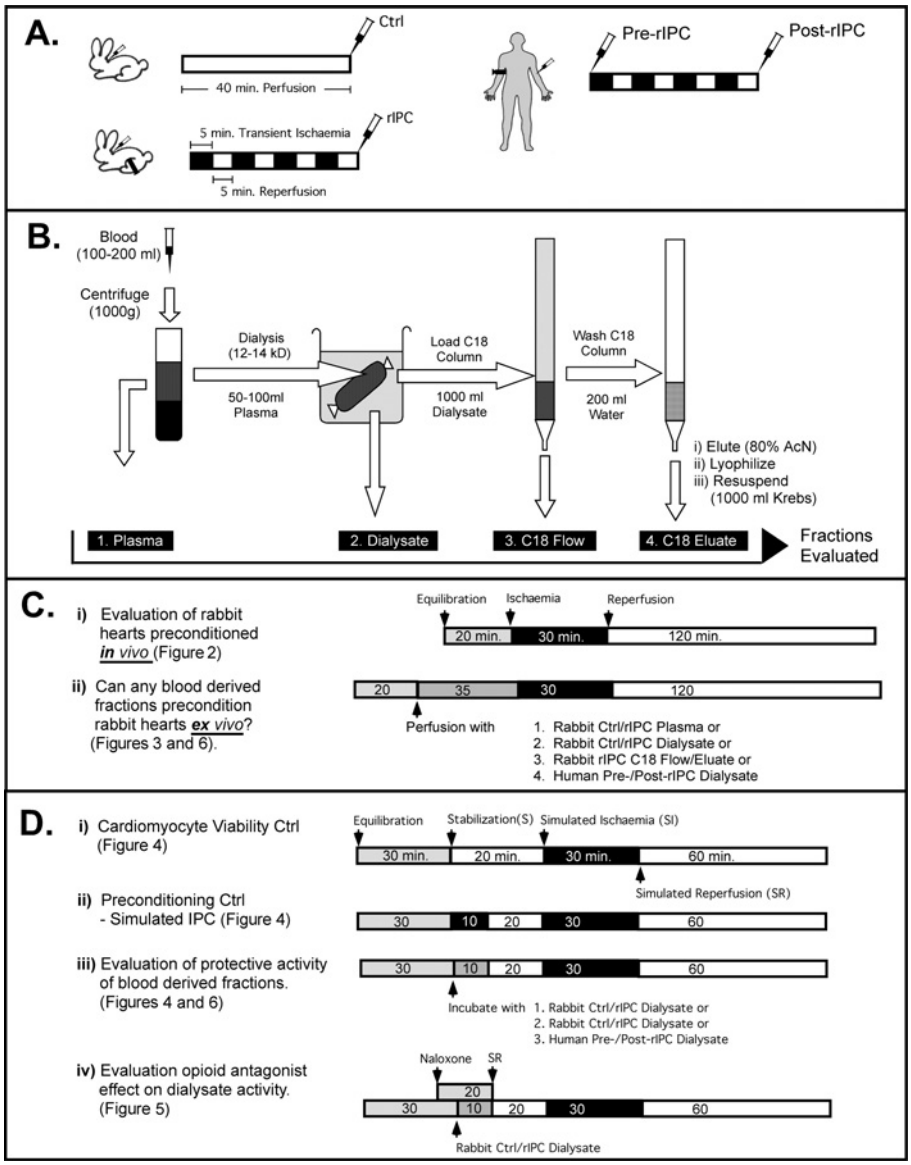

Figure 1 Langendorff-perfused heart and isolated cardiomyocyte experimental protocols

(A) Blood collection. In rabbits, blood was drawn (denoted by the needle symbol) from sham (Ctrl) or remotely preconditioned (rIPC) rabbits with four cycles of 5 min hindlimb ischaemia (filled box) and reperfusion (open box). In humans, blood was obtained prior to (Pre-rIPC) and following (Post-rIPC) a similar preconditioning protocol. (B) Generation of blood-derived fractions. Following centrifugation of rabbit blood (1), plasma was dialysed against water/Krebs buffer (2). Rabbit dialysate (lightly stippled box) was loaded on to a Sep-Pak C_{18} column and the flow-through (C18 Flow) was collected (3). Bound material (C18 Eluate) was eluted using acetonitrile (AcN), dried and resuspended in Krebs buffer (4). Human blood was only processed to obtain dialysate. (C) Langendorff model of I/R injury. Whole hearts were used to evaluate: (i) the cardioprotected status of donor hearts from control or rIPC-treated rabbits following local ischaemia (filled box) and reperfusion (open box); and (ii) the efficacy of plasma, dialysate, C18 Flow or C18 Eluate following perfusion (hatched box) of donor hearts from untreated animals. (D) Isolated fresh cardiomyocyte model of I/R injury. Prior to a long period of SI (filled box) and SR (open box), isolated cardiomyocytes were subjected to: (i) sham treatment (Ctrl); (ii) ischaemic preconditioning stimulus (IPC); (iii) treated with rIPC/Ctrl rabbit dialysate, rIPC/Ctrl rabbit C_{18} column eluate or human Ctrl/rIPC dialysate; or (iv) pre-treated with naloxone (lightly hatched box), followed by incubation with rabbit rIPC dialysate (heavy hatched box).

Preparation of plasma dialysate and fraction via reverse-phase chromatography

Control and rIPC rabbits were also cannulated in the left carotid artery with a 5 French catheter to draw blood. The protocol for preparation of plasma dialysate and fraction via reverse-phase chromatography is shown in Figure 1(B). Between 100 and 150 ml of blood was obtained from each animal at the end of the control or rIPC protocol. Bleeding was limited to less than 2 min to avoid secondary haemodynamic effects. Blood gases and

electrolyte measurements were taken at the end of the bleeding to confirm that each rabbit was well oxygenated and neither acidosis nor hyperkalaemia had been present during each experiment. Plasma was obtained by centrifugation (3000 *g* for 20 min) of the whole blood. To prepare dialysate, 50 or 100 ml of plasma (from control or rIPC rabbits/humans) was placed in dialysis tubing with a 12–14 kDa cut-off membrane (Spectrapor), dialysed against a 10–20-fold volume of water or Krebs–Henseleit buffer (500–1000 ml). When dialysis was performed against water, the osmotic strength of this dialysate in preparation for use in the Langendorff apparatus was adjusted to physiological levels using a 10-fold concentrate of Krebs–Henseleit buffer. In all cases the electrolyte levels in the dialysate were confirmed. Prior to perfusion of the donor hearts, D-glucose and $NaHCO_3$ were added to a final concentration of 11 and 10 mmol/l respectively, to the dialysate. No differences were observed in infarct size when comparing Krebs- or water-based dialysate. To fractionate the protective factor(s) in the dialysate further, reverse-phase chromatography based on a C_{18} matrix (Sep-pak; Waters) was utilized. Briefly, the C_{18} matrix in the column was first washed with 10 column volumes of 100% methanol/acetonitrile, followed by 10 column volumes of distilled water, and then equilibrated with Krebs–Henseleit solution. The dialysate was loaded on to the C_{18} column and allowed to flow through by gravity. This flow-through material was collected and treated, as described above, prior to evaluation for cardioprotective activity. The column was washed further with 10 column volumes of water prior to elution of the bound material with 5 column volumes of 80% acetonitrile. The C_{18} column eluate was diluted 2-fold with water, frozen and freeze-dried. Following freeze drying, the dried powder was dissolved in 20 ml of unbuffered Krebs buffer, filtered through a 0.25 μm filter and diluted to 1 litre with buffered and oxygenated Krebs buffer before use.

Cardioprotection of rabbit hearts by protective factor(s) present in blood obtained from remotely preconditioned humans

In these series of experiments, human volunteers ($n = 4$) were remotely preconditioned by application of four cycles of intermittent right arm ischaemia by inflation of a BP cuff (Figures 1A and 1B). Before rIPC (pre-rIPC) and at the end of the rIPC protocol (post-rIPC), 100 ml of blood was withdrawn from each volunteer's arm vein and used to obtain control and rIPC dialysate respectively, as described above for rabbit blood (Figure 1B). Human dialysate, prepared as described above, was given to buffer-perfused rabbit hearts for 35 min prior to subjecting them to the long index I/R episode. Infarct size was measured in each heart at the end of each experiment as described below.

Infarct size measurement

At the end of reperfusion, the LAD was re-occluded and 4 ml of 1% Evans Blue dye solution was infused into the coronary arteries to define the area at risk (not stained area). Then each heart ventricle was cross-sectioned to produce 4–5 3-mm-thick heart slices that were then stained with 1% TTC (2,3,5-triphenyltetrazolium chloride), maintained at 37 °C and pH 7.4 to distinguish dead tissue areas (white or tan colour) from viable tissue area (brick-red-stained areas). Individual slices were weighed and traced on to acetate sheets. Computer planimetry of the different areas [total LV (left ventricle) area, AAR (area at risk) and area not at risk] was performed on the tracings, and the infarct size area/AAR and AAR/LV area ratios were calculated, as described previously [13], and are expressed as percentages.

Subcellular fractionation and Western blot analysis

In brief, frozen heart samples were homogenized in ice-cold lysis buffer containing 150 mmol/l NaCl, 50 mmol/l Tris/HCl, 5 mmol/l EDTA, 2.5 mmol/l sodium orthophosphate, 1 mmol/l sodium vanadate, 1 mmol/l β-glycerolphosphate and a cocktail of proteases inhibitors (Roche). Each homogenate was initially centrifuged at 30000 *g* for 30 min. The resulting supernatant was then centrifuged at 100000 *g* for 60 min to obtain the cytosolic fraction (supernatant). The resulting pellet was solubilized in lysis buffer supplemented with Triton X-100 and centrifuged at 40000 *g* for 30 min to obtain the membrane fraction (supernatant). Total PKCε (protein kinase Cε) and p42/p44 MAPK (mitogen-activated protein kinase) Thr^{202}/Tyr^{204} expression were measured by Western immunoblotting. Proteins (30 μg) from each sample were resolved by SDS/PAGE [10% (w/v) acrylamide)] and transferred on to nitrocellulose membranes (Bio-Rad Laboratories). These membranes were incubated with primary monoclonal anti-PKCε (mouse) antibodies (Transduction Laboratories) and mouse anti-(phospho-p44/42 MAPK Thr^{202}/Tyr^{204}) antibodies (Cell Signaling Technology). Detection of protein bands was performed using a chemiluminescence system (Amersham Biosciences). Immunoblots were scanned using storm 840 (Molecular Dynamics), and protein band densities were measured using ImageQuant 5.0 software (Molecular Dynamics). Normalized density ratios (to total proteins observed on Coomassie Blue staining) of total PKCε (phosphorylated and non-phosphorylated) for each subcellular fraction (cytosol and membranes) were calculated and compared to determine the translocation of total PKCε from the cytosolic fraction to the membrane fraction. Normalized density ratios [p42+p44/GAPDH (glyceraldehyde-3-phosphate dehydrogenase)] were calculated to determine whether rIPC increased the expression of phosphorylated p42/p44 MAPK. In

addition, the p42/p44 MAPK densities were normalized to the total p42 MAPK protein detected by anti-(p42 MAPK) antibodies and the effect of rIPC on this ratio was measured.

Direct cardioprotection of isolated cardiomyocytes by protective factor(s) present in blood obtained from remotely preconditioned rabbits and humans

To confirm the cardioprotective properties of the rIPC factor(s) present in plasma dialysate and C_{18} column eluate, we tested whether pre-treatment of freshly isolated cardiomyocytes with dialysate or C_{18} column eluate rendered cardiomyocytes resistant to injury caused by a prolonged episode of SI, followed by SR in the absence of neural pathway influences (Figure 1D). We then tested whether human rIPC dialysate also protected freshly isolated cardiomyocytes against I/R injury under the same experimental conditions. The percentage of dead cardiomyocytes was assessed as the end point, as described below.

Isolated cardiomyocyte experimental protocol

Cardiomyocytes were isolated from New Zealand White rabbit (3.0–3.5 kg) hearts by enzymatic dissociation using a method described previously [15]. The cardiomyocyte isolate (3–4 ml) was initially divided into seven Eppendorff tubes (0.5 ml/tube) and then each isolate was transferred into a 12-well culture plate, which was incubated at 37 °C in a 100 % oxygen atmosphere for 30 min (stabilization period) (Figure 1D). Then each isolate was randomly assigned to an experimental group (oxygenated baseline, non-IPC control, classical IPC, rIPC and non-rIPC; $n = 5$/group) and was subjected to 45 min of SI (at 37 °C), followed by 60 min of SR (Figure 1D). Ischaemia and reperfusion were simulated as reported previously [15]. Oxygenated baseline cardiomyocytes were incubated in oxygenated buffer for the duration of the experimental protocols. Control (non-IPC) cardiomyocytes were not preconditioned, but were subjected to the same centrifugation protocol as IPC. Classical IPC cardiomyocytes were also subjected to 10 min of SI, followed by 20 min of SR before the long SI/SR protocol. Cardiomyocytes were remotely preconditioned by incubating them in plasma dialysate obtained from blood withdrawn previously from remotely preconditioned rabbits (rIPC; $n = 5$) as described above, and supplemented with 20 mmol/l Hepes and 10 mmol/l D-glucose (pH adjusted to 7.4) for a period of 10 min, followed by a 20 min washout period in reactive buffer before the long SI/SR (Figure 1D). Non-rIPC cardiomyocytes were incubated in plasma dialysate obtained from sham-operated rabbits for 10 min followed by the same washout period with reactive buffer as for rIPC cardiomyocytes. The reactive buffer consists 130 mmol/l NaCl, 4.7 mmol/l KCl, 0.5 mmol/l $MgSO_4$, 1.2 mmol/l KH_2PO_4, 10 mmol/l $NaHCO_3$, 20 mmol/l Hepes and 10 mmol/l D-glucose (pH 7.4). These experiments were performed in duplicate. Separate groups of experiments were also performed with human control and rIPC dialysate (two volunteers were used to obtain the pre-rIPC and post-rIPC plasma dialysates that were tested in five different rabbit cardiomyocyte isolates in each group). Cardiomyocyte mortality was assessed at different time points (end of stabilization, before SI and after the SR period) using hypotonic (85 mosm) Trypan Blue exclusion, as reported previously [15]. Dead cardiomyocytes were stained dark blue, whereas viable cardiomyocytes were not stained. At least 300 live cardiomyocytes were counted (magnification, ×20). The percentage of dead cardiomyocytes was then calculated (number of dead cardiomyocytes/number of live cardiomyocytes).

Role of opioid receptors on isolated cardiomyocytes in protection by humoral factor(s) present in plasma dialysate

To assess the participation of opioid receptor activation in the cardioprotection induced by plasma dialysate in freshly isolated cardiomyocytes, a separate series of experiments were performed with controls ($n = 5$/group) and rIPC dialysates ($n = 5$/group), where we assessed whether the cardioprotection induced by rIPC dialysate in cardiomyocytes was abolished by co-administration of naloxone (100 μmol/l), a specific opioid receptor blocker, together with the rabbit rIPC dialysate. In these studies, naloxone was present for 10 min prior to and during control or rIPC dialysate pre-treatment (10 min) (Figure 1D). Naloxone was washed out by a subsequent 20 min incubation period in buffer without drug before SI/SR (Figure 1D). The percentage of dead cardiomyocytes was measured before SI and after SR in each group. The percentage of dead cardiomyocytes was also measured in control baseline cardiomyocytes, with and without naloxone, at the end of stabilization and at the end of the experimental protocol.

Statistical analysis

All values are expressed as means ± S.E.M. All data were first tested for normality and homogeneity of variance. If these tests were passed, ANOVA followed by Scheffe's post-hoc test was performed to determine whether there were significant differences ($P < 0.05$) among groups and between two groups respectively. Where the criteria for parametric analysis were not met, non-parametric analysis (Kruskal–Wallis test followed by a Mann–Whitney U test) was used to determine whether or not

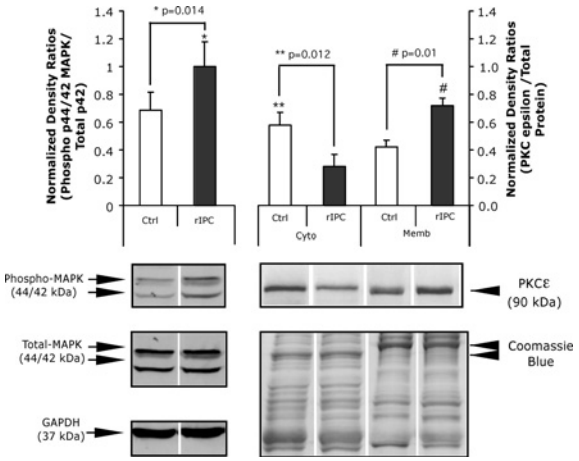

Figure 2 Comparison of PKCε and MAPK levels in control and rIPC rabbit hearts

Lower panels, Western blots of total or membrane/cytosolic fractions of lysates from control (Ctrl) or remotely preconditioned (rIPC) rabbit hearts probed with either anti-phospho-/total p42/p44 MAPK or anti-PKCε antibodies respectively. Arrows indicate the positions of relevant bands. Upper panels, normalized density ratios for bands corresponding to phospho-/total p42/p44 MAPK ($n = 4$ for control; $n = 5$ for rIPC) and total PKCε protein ($n = 3$ for control; $n = 3$ for rIPC) in cytosolic (Cyto) and membrane (Memb) fractions. Values are means ± S.E.M.

a significant difference ($P < 0.05$) existed between two groups.

RESULTS

In vivo remotely preconditioned rabbit heart studies

We used two criteria to demonstrate that our *in vivo* rabbit model of rIPC is cardioprotective: (i) that *in vivo* preconditioned hearts have reduced infarct size using a Langendorff-based model of local I/R injury; and (ii) that the signalling pathway associated with preconditioning is operative. Thus hearts derived from rabbits preconditioned by four cycles of transient limb ischaemia relative to sham operation had reduced infarct size when evaluated in a Langendorff-based model of local I/R injury (15.0 ± 2.7 compared with 49.2 ± 6.2 % in rIPC and controls respectively; $P = 0.0001$). In relation to signalling pathways induced in our *in vivo* rabbit model, rIPC significantly increased the phospho-p42/p44 MAPK/total p42/p44 MAPK ratio ($P = 0.014$; Figure 2) and the total p42/p44 MAPK/GAPDH ratio ($P = 0.0209$), indicating that rIPC not only produced a substantial increase in the expression of phosphorylated p44/p42 MAPK, but it also caused a statistically significant, but small, increase in total p42/p44 MAPK in heart (ventricular) tissue. In addition, rIPC produced a redistribution of PKCε in subcellular compartments, with significant translocation of PKCε from the cytosolic fraction to the membrane fraction (Figure 2).

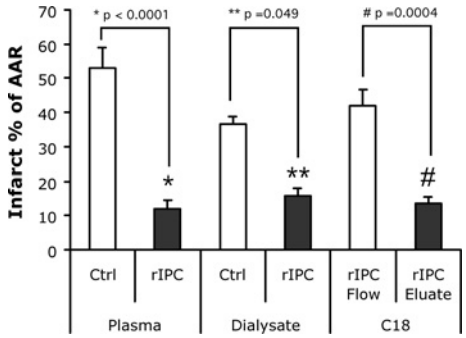

Figure 3 Effect of rabbit rIPC plasma, plasma dialysate and C_{18} column eluate on infarct size in Langendorff-perfused rabbit hearts subjected to 30 min of LAD occlusion, followed by 120 min of reperfusion

Values are means ± S.E.M. ($n = 6$ for all groups). Ctrl, control.

Langendorff-perfused rabbit heart studies: presence of a transferable cardioprotective factor(s) in plasma derived from remotely preconditioned rabbits

To demonstrate the presence of a transferable cardioprotective factor, plasma derived from rabbits that had undergone four cycles of transient limb ischaemia (rIPC plasma) or sham operation (control plasma) was used to perfuse naive donor hearts mounted on a Langendorff apparatus (Figure 3). rIPC-plasma-perfused hearts had significantly ($P < 0.0001$) reduced infarct size compared with control-plasma-perfused hearts. To characterize further the cardioprotective factor in terms of size (molecular mass), plasma was dialysed against a 10-fold volume of buffer using a membrane with a 12–14 kDa pore size. Dialysate (i.e. the liquid outside the dialysis tubing) derived from control and rIPC plasma was used to perfuse naive rabbit donor hearts. Perfusion with the rIPC dialysate resulted in significantly ($P = 0.049$) reduced infarct size compared with control dialysate. Reverse-phase chromatography of the rIPC dialysate was performed to provide further clues as to the size and hydrophobicity of the cardioprotective factor. Pre-treatment of hearts, before the long index I/R, with the material that flowed through the column following loading of the rIPC dialysate into the C_{18} column, did not reduce infarct size compared with control dialysate ($P < 0.05$). In contrast, pre-treatment of hearts with the material subsequently eluted from the column with 80 % acetonitrile and reconstituted in Krebs–Henseleit buffer resulted in significantly ($P = 0.004$) reduced infarct size. In all groups examined in these series of experiments, the AAR did not differ among the groups ($P = 0.4316$). Similarly, no correlation was found between AAR and infarct size ($R = -0.043$, $P = 0.78$).

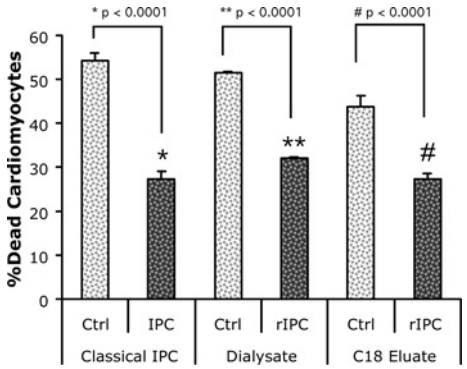

Figure 4 Effect of rabbit control and rIPC plasma dialysate and C_{18} column eluate on cardiomyocyte mortality following 45 min of SI and 60 min of SR

As positive and negative controls for preconditioning cardioprotection, cardiomyocytes were either left untreated (Ctrl) or underwent simulated 'local' IPC respectively. Values are means ± S.E.M. ($n = 5$/group).

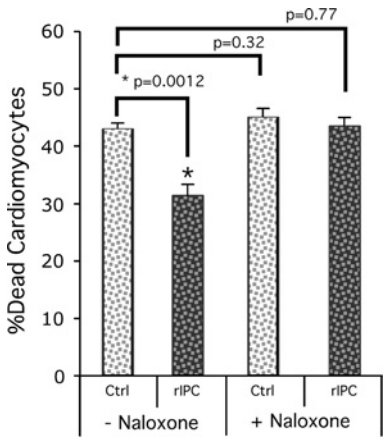

Figure 5 Effect of opioid receptor blockade

The percentage of dead rabbit cardiomyocytes with (+) or without (−) 100 μmol/l naloxone treatment prior to and during incubation with control (Ctrl) and rIPC rabbit dialysate and after SI/SR. Values are means ± S.E.M. ($n = 5$/group).

Isolated cardiomyocyte studies

To determine whether the rIPC dialysate and C_{18} column eluate were able to induce cardioprotection in the absence of neurogenic pathways, we assessed the protective effect of the rIPC dialysate and C_{18} column eluate in freshly isolated cardiomyocytes (Figure 4). Pre-treatment of cardiomyocytes for 10 min prior to SI/SR with either plasma dialysate or C_{18} column eluate obtained from rIPC rabbit blood significantly (both $P < 0.0001$) reduced the percentage of dead cardiomyocytes at the end of SI/SR compared with control cardiomyocytes pre-treated with respective products obtained from non-rIPC rabbit blood. This cardioprotection mirrored the significantly ($P < 0.0001$) protective effect obtained with classical IPC in cardiomyocytes.

Participation of opioid receptors in cardioprotection induced by humoral factor(s) present in plasma dialysate in isolated cardiomyocytes

To determine the participation of opioid receptors in rIPC-dialysate-induced cardioprotection, we assessed whether the cardioprotection induced by rIPC dialysate in cardiomyocytes was abolished by co-administration of naloxone, a specific opioid receptor blocker, together with the rabbit rIPC dialysate (Figure 5). Pre-treatment of cardiomyocytes with rIPC dialysate significantly ($P = 0.0012$) reduced the percentage of dead cardiomyocytes after a long SI/SR episode compared with cardiomyocytes pre-treated with control dialysate. Blockade of opioid receptors with naloxone (100 μmol/l), given for 10 min prior to and during pre-treatment with rIPC dialysate, completely abolished its protective effect ($P = 0.53$). Naloxone had no effect on cell viability in cardiomyocytes pre-treated with control dialysate ($P = 0.32$).

Transfer of cardioprotection from human to rabbit myocardium

To test whether the protective factor(s) present in rIPC dialysate (post-rIPC dialysate) is (are) transferable between species, we assessed whether the humoral protective effector(s) in human rIPC dialysate is (are) preserved and biologically active when applied to either rabbit Langendorff-perfused whole hearts or freshly isolated rabbit cardiomyocytes. Human rIPC dialysate significantly ($P = 0.02$) reduced infarct size in Langendorff-perfused rabbit hearts when compared with hearts treated with control (pre-rIPC) human dialysate (Figure 6). Similarly, human rIPC dialysate given for 10 min and washed out for 20 min prior to the long SI/SR episode significantly ($P = 0.0002$) reduced cardiomyocyte mortality, as compared with cardiomyocytes treated with human control dialysate (Figure 6). Pre-treatment of cardiomyocytes for 10 min prior to SI/SR with plasma dialysate obtained from rIPC human blood (post-rIPC) significantly ($P = 0.0002$) reduced the percentage of dead cardiomyocytes at the end of SI/SR compared with cardiomyocytes pre-treated with control human dialysate (pre-rIPC) obtained from the same human volunteers before rIPC.

DISCUSSION

The present study provides several novel observations that increase our understanding of the mechanisms of signal transduction for the cardioprotection afforded by repeated transient limb ischaemia and reperfusion. Although our finding that this stimulus results in the release

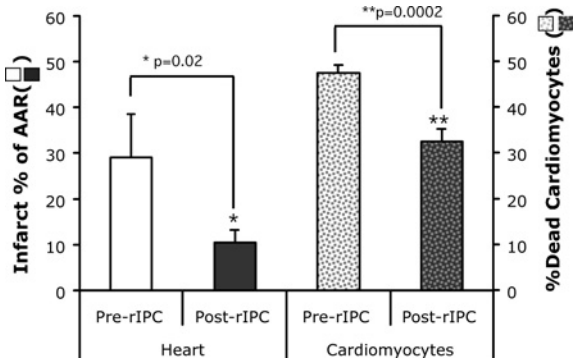

Figure 6 Evidence suggesting cross-species protective effects of human rIPC plasma dialysate

Left-hand bars, infarct size in rabbit hearts following perfusion with pre-rIPC (control) and post-rIPC human dialysates ($n = 4$ volunteers) subsequent to index ischaemia and reperfusion. Right-hand bars, percentage of dead rabbit cardiomyocytes following treatment of cells with pre-rIPC (control) and post-rIPC human dialysates ($n = 5$/group) from human volunteers ($n = 2$). Values are means $\pm$ S.E.M.

of one or more rIPC effector substances which circulate in the bloodstream and provide potent protection against myocardial I/R injury is perhaps not surprising, given our previous observations using this stimulus [13,16] and the findings of other groups examining other stimuli of rIPC [12], we show for the first time that transient limb ischaemia liberates one or more protective dialysable factor(s) with molecular masses below 15 kDa, resistant to freezing and thawing, the effect of which is abrogated completely by processing the dialysate through a C_{18} column. The fact that the effector(s) retained cardioprotective activity subsequent to elution from the C_{18} column with 80 % acetonitrile, a known denaturant, suggests it (they) is (are) both hydrophobic and not easily denatured.

Our approach of isolating the protective humoral factor(s) in a dialysate and demonstrating protection in both an isolated whole heart and, moreover, in cardiomyocytes allows further refinement of our understanding of signal transduction in rIPC. Previous studies of various modes of induction of rIPC, including transient mesenteric artery [17–19], renal artery [20–22] and limb ischaemia in rats [23,24], and limb ischaemia in humans [25], have each shown that intact neural pathways are vital to the protection afforded by rIPC. However, our previous observation [13], in a porcine cardiac transplant model, that cardioprotection was demonstrable in the denervated heart transplanted into a previously preconditioned recipient suggested that, although neural pathways may be a key component in the local release of the circulating effector(s) during transient ischaemia of the limb, intact afferent connections to the target organ may not be necessary for the distant cardioprotective effect of rIPC. Our present findings, showing protection of isolated cardiomyocytes by dialysate, reinforces this conclusion and suggests that neuronal involvement in the signal transduction of rIPC induced by transient limb ischaemia is minimal in the heart, or at least cardioprotection can be achieved without it.

We emphasized earlier that not only must potential interspecies differences be taken into account, but also that the mode of stimulating rIPC may result in significant differences when assessing the mechanisms of rIPC reported previously. In contrast with results from the rat mesenteric artery occlusion model [17–19], Weinbrenner et al. [26] could not block cardioprotection achieved by a 15 min occlusion of the infrarenal rat aorta by hexamethonium, although they demonstrated, like Wolfrum et al. [19], that the PKC inhibitor chelerythrine could block the cardioprotection. They concluded that a humoral factor must only be responsible for the remote protection. Subsequent experiments by Wolfrum et al. [27] have emphasized further the variability in signalling pathways that may exist depending on the model chosen and the nature of the stimulus.

Having discussed these interspecies differences in signalling pathways, our hitherto undescribed observation that a circulating cardioprotective factor(s) released after transient human forelimb ischaemia protects isolated rabbit heart and cardiomyocytes suggests some preservation in the response to this particular stimulus across species. However, given the relatively small number of human subjects studied, it would be premature to imply any degree of consistency of this phenomenon, either between humans and other species or in terms of the response in individual human subjects. Indeed, a review of the infarct sizes as a percentage of the AAR in the isolated rabbit hearts treated with the pre- and post-rIPC samples from each of the four humans in that element of our present study (#1: pre, 33 % and post, 9 %; #2: pre, 33 % and post, 8.6 %; #3: pre, 34.6 % and post, 14.3 %, #4: pre, 14.9 % and post, 9.5 %) suggest that one of the volunteers was capable of transferring substantial protection prior to being subjected to the repetitive limb ischaemia. Although further experiments will be required fully to characterize this phenomenon, we believe it does, however, represent a potential avenue for the study of the mechanisms and effector(s) of 'human rIPC' using *in vitro* models.

The exact nature of the circulating effector(s) of rIPC, whatever the mode of stimulus, remain(s) to be determined. Nonetheless, limited characterization of humoral protective factors in heart-to-heart rIPC using the effluent collected from hearts in Langendorff preparations undergoing local preconditioning has been reported. Similar to the dialysate obtained after transient limb ischaemia in the present study, Dickson et al. [28] demonstrated that when coronary effluent from isolated rabbit hearts preconditioned by repetitive ischaemia was flowed through a C_{18} column the eluate from that column transferred protection to another acceptor isolated rabbit heart. Furthermore, naloxone inhibited this transferred

protection while having no effect on infarct size in control isolated hearts. Serejo et al. [29] recently reported that a transfer of fresh preconditioned effluent from isolated rat hearts subjected to repetitive ischaemia protected other isolated rat hearts. Furthermore, lyophilized concentrate of the effluent subsequently dialysed to retain molecules above a molecular mass of 3500 Da also protected, as did hydrophobic compounds present in the eluate from C_{18} column cartridges after exposure to the same dialysis product. On the basis of these results, Serejo et al. [29] suggested that the protective substance(s) was in the 3.5–8 kDa range, the upper limit based on the proteomic analysis (two-dimensional gel electrophoresis combined with MS and LC) of rat *in vivo* cardioprotection by renal artery occlusion/reperfusion by Lang et al. [30], who reported that no differentially abundant proteins from rIPC with a known signalling function could be found over 8 kDa, the lower molecular mass limit of their search. Serejo et al. [29] concluded that their finding "excludes the participation of adenosine (267.24 Da), opioids (500–800 Da), bradykinin (1060.22 Da), and other substances with molecular weights below the dialysis cutoff (3.5 kDa) as putative mediators of preconditioning". We interpret their results more cautiously.

Dickson et al. [28] interpreted their C_{18} column eluate results in rabbits, which parallel ours, as indicating that "the transferred cardioprotection factor(s) include one or more hydrophobic compounds that mediate protection through, or in cooperation with, opioid receptor activation". They suggested that multiple triggers of preconditioning, in addition to opioids, may have been present in their C_{18} column eluate and, by blocking the opioid component of this 'cocktail', the cumulative effects of the transferred triggers would have been reduced to below the threshold for protection. We agree with this assessment and emphasize the importance of the 'threshold for protection' concept in the interpretation of preconditioning experiments. It may be that larger molecular-mass substances augment opioids in stimulating protection in cardiomyocytes and other opioid-independent substances may be involved.

Finally, although multiple circulating effectors and multiple cell-surface receptors appear to be implicated in different forms of IPC, phosphorylation of MAPK [31] and PKCε translocation from the cystolic fraction to the membrane fraction [32] have been shown to be a fundamental requirement for local IPC and rIPC protection in several experimental models and species. Our present results show, for the first time in rIPC induced by transient limb ischaemia, translocation of PKCε from the cytosol to membranes in myocardium subjected to rIPC *in vivo* (Figure 2B). We have also shown increased p42/p44 MAPK phosphorylation, confirming the findings of Heidbreder et al. [33], who recently demonstrated a similar involvement in myocardial rIPC induced by mesenteric artery ischaemia. It appears reasonable to suggest therefore that rIPC employs the same intracellular signalling machinery as classical IPC, although we cannot exclude the possibility that only a subset of the classical pathways is involved. Furthermore, both for rIPC and classical IPC the intracellular end effectors remain to be firmly established and definitive studies of the signalling taxonomy of these phenomena remain to be reported.

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