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Research Paper

Myocardial triggers involved in activation of remote ischaemic preconditioning

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New Findings

- **What is the central question of this study?**
  Ischaemia–reperfusion of peripheral tissues protects the heart from subsequent myocardial ischaemia–reperfusion injury, a phenomenon referred to as remote ischaemic preconditioning (rIPC). This study evaluated the possible myocardial triggers of rIPC.
- **What is the main finding and its importance?**
  Remote ischaemic preconditioning reduces infarct size through a vagal pathway and a mechanism involving phosphorylation of Akt and endothelial nitric oxide synthase, opening of mitochondrial ATP-dependent K^+ channels and an increase in mitochondrial H_2O_2 production. All these phenomena occur before the myocardial ischaemia; hence, they could act as 'triggers' of rIPC.

It has been proposed that remote ischaemic preconditioning (rIPC) activates a parasympathetic neural pathway. However, the myocardial intracellular mechanism of rIPC remains unclear. Here, we characterized some of the intracellular signals participating as rIPC triggers. Isolated rat hearts were subjected to 30 min of global ischaemia and 120 min of reperfusion (Non-rIPC group). In a second group, before the isolation of the heart, an rIPC protocol (three cycles of hindlimb ischaemia–reperfusion) was performed. The infarct size was measured with tetrazolium staining. Expression/phosphorylation of Akt and endothelial nitric oxide synthase (eNOS) and mitochondrial H_2O_2 production were evaluated at the end of the rIPC protocol, before myocardial ischaemia–reperfusion. The rIPC significantly decreased the infarct size and induced Akt and eNOS phosphorylation. The protective effect on infarct size was abolished by cervical vagal section, L-NAME (an NO synthesis inhibitor) and 5-hydroxydecanoate (a mitochondrial ATP-dependent K^+ channel blocker). Mitochondrial production of H_2O_2 was increased by rIPC, whereas it was abolished by cervical vagal section, L-NAME and 5-hydroxydecanoate. We conclude that rIPC activates a parasympathetic vagal pathway and a mechanism involving the phosphorylation of Akt and eNOS, the opening of mitochondrial

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ATP-dependent K^+ channels and the release of H_2O_2 by the mitochondria. All these phenomena occur before myocardial ischaemia and could act as triggers of rIPC.

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Introduction

Cardiac remote ischaemic preconditioning (rIPC) is a treatment strategy in which alternate cycles of preconditioning ischaemia and reperfusion performed in a remote organ (other than the heart) protect the heart against a subsequent index ischaemia- (sustained ischaemia) and reperfusion-induced injury. Different authors have described that the cardioprotective signal transference from the peripheral organ to the heart is a result of the action of humoral factors (Dickson *et al.* 1999), neural pathways (Mastitskaya *et al.* 2012) or neurohumoral interaction (Hausenloy *et al.* 2008). These hypotheses are not mutually exclusive and are probably part of the same mechanism. The neural hypothesis, in particular, was studied by Basalay *et al.* (2012), who demonstrated that rIPC activates a neural pathway, and the signal reaches the heart through the vagus nerves. In accordance with the pioneer findings of Gourine's group, we showed that rIPC activates a neural afferent pathway (femoral and sciatic nerves and spinal cord) and that the cardioprotective signal reaches the heart through the vagus nerve (efferent pathway) and acetylcholine activates the classic ischaemic preconditioning (IPC) phenomenon when acting on the muscarinic receptors (Donato *et al.* 2013). However, the remotely activated signal transductions participating in the intracellular mechanism of rIPC remain unclear.

Some authors (Hausenloy *et al.* 2008; Lim *et al.* 2012) have suggested that rIPC activates a signalling mechanism similar to that described for IPC, whereas others have shown that the cardioprotection conferred by rIPC follows a different pattern (Heinen *et al.* 2011). Recently, Heusch (2015) carefully reviewed the signal transduction pathways involved in the different ischaemic conditioning phenomena and noted that there are still some unsolved problems when studying myocardial protection; in particular, the absence of a temporal description of the cardioprotective signals involved.

A few years ago, Downey and co-workers proposed a classification of the signals participating in IPC that follows a logical, causal sequence of events and meets the temporal sequence of the preconditioning protocol (Yellon *et al.* 2003; Downey *et al.* 2008). They defined a trigger as a factor released during the preconditioning ischaemic periods that activates the cardioprotective phenomenon and defined mediators as factors that transmit the cardioprotective signal during the prolonged myocardial ischaemia to one or more end-effectors, which are responsible for attenuating the irreversible injury during the lethal ischaemic insult and/or during the subsequent reperfusion period.

From this point of view, several studies have evaluated the intracellular signalling pathway involved in the cardioprotection afforded by rIPC (Wolfrum *et al.* 2002; Li *et al.* 2010; Breivik *et al.* 2011). However, those authors have not considered the timing sequence of signalling activation. The aim of our study was to determine some of the intracellular signals that trigger the rIPC myocardial protection.

We hypothesize that pre-ischaemic activation of muscarinic receptors induces phosphorylation of the Akt enzyme and, through this pathway, the phosphorylation of endothelial nitric oxide synthase (eNOS). As a consequence of increased production of nitric oxide (NO), mitochondrial ATP-dependent K^+ (mK_{ATP}) channels open and mitochondrial production of hydrogen peroxide (H_2O_2) increases.

Methods

Ethical approval

The experiments were performed on male Wistar rats, 60–70 days old (200–250 g) supplied by the animal facility of the Faculty of Pharmacy and Biochemistry, University of Buenos Aires. The procedures used in this study were approved by the Animal Care and Research Committee of the University of Buenos Aires (protocol number 2948/10) and were in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health in 2011. The investigators understand the ethical principles under which the journal operates (Grundy, 2015).

Surgical procedure (*in vivo*)

Rats were anaesthetized with sodium pentobarbital (65 mg kg^{-1}, I.P.) and intubated for mechanical ventilation with a mixture of room air and oxygen. The level of anaesthesia was assessed by loss of the pedal reflex (toe pinch). Next, the left femoral artery was dissected and exposed, and the animals were randomized into different experimental groups.

Ischaemia–reperfusion (*in vitro*)

After completion of the *in vivo* protocols described below (see '*Experimental groups*'), the animals were killed with sodium pentobarbital (150 mg kg^{-1}, I.P.) and each heart was rapidly excised and mounted on a Langendorff apparatus by the aortic root in <1 min. Each heart was perfused with Krebs–Henseleit buffer [containing (mM): NaCl, 118.5; KCl, 4.7; $NaHCO_3$, 24.8; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.5; and glucose, 10] and bubbled with a 95% O_2–5% CO_2 gas mixture at 37°C, with a final pH of 7.2–7.4. Two electrodes were secured to the epicardial surface and connected to a pacemaker with a constant heart rate of 275 beats min^{-1}.

A saline-filled latex balloon connected by a catheter to a pressure transducer (Deltram II; Utah Medical System, Miavale, UT, USA) was inserted into the left ventricle. The volume of the balloon was adjusted to achieve a left ventricular end-diastolic pressure of 8–10 mmHg. Coronary perfusion pressure was also recorded through a pressure transducer connected to the perfusion line, and the coronary flow was adjusted to obtain a coronary perfusion pressure of ∼70 mmHg during the initial stabilization period. This flow was kept constant throughout the experiment.

Experimental groups (Fig. 1)

Non-remote ischaemic preconditioning ($n = 8$). Rats were anaesthetized and the left femoral artery was dissected and exposed as described above [see '*Surgical procedure (in vivo)*']. After 30 min of monitoring, the hearts were excised and perfused according to the Langendorff technique. After 15 min of stabilization, myocardial infarction was induced by 30 min of global no-flow ischaemia followed by 120 min of reperfusion. Global no-flow ischaemia was induced by abruptly decreasing the total coronary flow provided by the perfusion pump.

Remote ischaemic preconditioning ($n = 10$). After the rats had been anaesthetized and the left femoral artery dissected and exposed, the animals were remotely preconditioned by a three-cycle hindlimb ischaemia (5 min) and reperfusion (5 min) protocol by occlusion of the femoral artery with a vascular clamp. Subsequently, the hearts were subjected to the same protocol used in non-rIPC group (30 min of global no-flow ischaemia followed by 120 min of reperfusion) to induce myocardial infarction.

Remote ischaemic preconditioning with cervical vagal section (CVS; $n = 7$). The same protocol used in rIPC group was performed, but the left and right vagus nerves were sectioned at the middle cervical level before starting the rIPC protocol. Myocardial infarction was induced using the above-mentioned protocol.

Remote ischaemic preconditioning with subdiaphragmatic vagal section (SVS; $n = 7$). The same protocol used in rIPC group was performed, but the vagus nerves were sectioned at the subdiaphragmatic level before starting the rIPC protocol. Myocardial infarction was induced using the above-mentioned protocol.

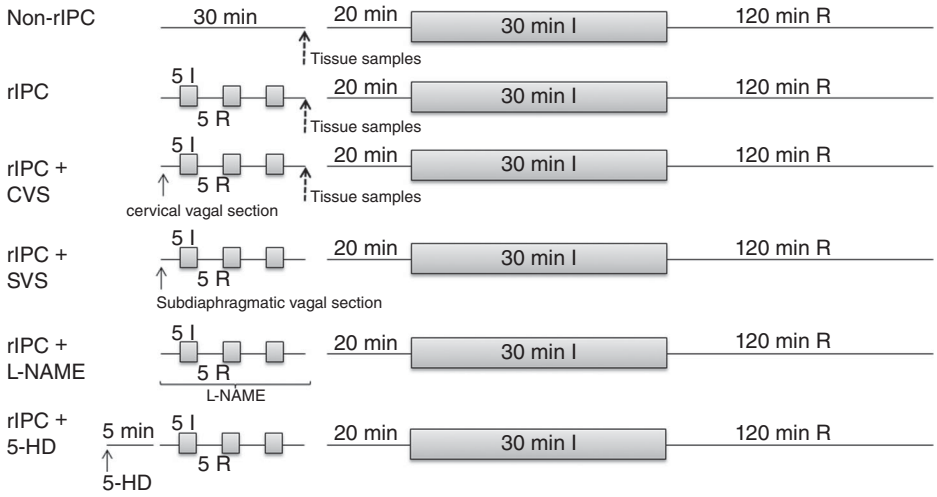

Figure 1. Representative scheme of the experimental protocols
Dashed arrows indicate the time when tissue samples were collected in a subset of experiments without ischaemia–reperfusion to study triggers that could be involved only in the rIPC mechanism. Abbreviations: CVS, cervical vagal section; I, ischaemia; R, reperfusion; rIPC, remote ischaemic preconditioning; and SVS, subdiaphragmatic vagal section. Non-rIPC, $n = 8$; rIPC, $n = 10$; rIPC + CVS, $n = 7$; rIPC +SVS, $n = 7$; rIPC + L-NAME, $n = 7$; and rIPC + 5HD, $n = 5$.

Remote ischaemic preconditioning with L-NAME (n = 7). The same *in vivo* protocol used in rIPC group was performed, but N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM kg^{-1} I.V.), an inhibitor of nitric oxide synthase (NOS), was administered during the rIPC protocol. Myocardial infarction was induced using the above-mentioned protocol.

Remote ischaemic preconditioning with 5-HD (n = 5). The same protocol used in rIPC group was performed, but 5-hydroxydecanoate (5-HD; 100 μM I.P.), a selective mK_{ATP} channel antagonist, was administered 5 min before the rIPC protocol. Myocardial infarction was induced using the above-mentioned protocol.

It is important to mention that the mean time between the cardioprotective stimulus and the onset of the period of global ischaemia was no longer than 25 min.

Measurement of infarct size

After 2 h of reperfusion in the Langendorff apparatus, the hearts were frozen and cut into 2-mm-thick transverse slices from apex to base. Sections were incubated for 20 min in 1% triphenyltetrazolium chloride (pH 7.4, 37°C) and then immersed in 10% formalin. Applying this technique, viable regions are stained red, whereas infarcted areas remain unstained. Finally, sections were scanned and the viable and infarcted areas measured (Image Pro Plus, Media Cybernetics, version 4.5). The infarct size was expressed as a percentage of the left ventricular area.

Western blot

In order to study only triggers that could drive the protection afforded by the rIPC, tissue samples of the entire left ventricle were taken from a different subset of experiments at the end of the rIPC protocol, without myocardial ischaemia–reperfusion.

Samples from left ventricles of animals subjected to Non-rIPC, rIPC and rIPC with cervical vagal section but not subjected to ischaemia–reperfusion (n = 8 per group) were homogenized in 3 vol (w/v) of ice-cold homogenization buffer [NaCl, 150 mM; Trizma–HCl, 50 mM; sodium deoxycholate, 1% (v/v); EGTA, 1 mM; NaF, 1 mM; phenylmethanesulfonylfluoride, 1 mM; and sodium pervanadate, 1 mM; All reagents were from Sigma Aldrich (St Louis, MO, USA)] and a protease inhibitor cocktail 4% (Roche, Welwyn Garden City, UK), pH 8.0, and centrifuged at 10,507g for 10 min at 4°C. The protein in supernatant was quantified by the Lowry method using bovine serum albumin (BSA) as standard, resuspended in 2× solution of SDS-sample buffer [Tris–HCl buffer, 62.5 mM, pH 6.8, containing: SDS, 2% (w/v); glycerol, 25% (w/v); β-mercaptoethanol, 5% (v/v); and Bromophenol Blue, 0.01% (w/v)] and heated at 95°C for 5 min. Equal amounts of protein (80 μg) were loaded onto 8% SDS-PAGE and transferred to nitrocellulose membranes. After blocking for 1 h in 3% (w/v) BSA in PBS, membranes were incubated overnight at 4°C with the corresponding primary antibodies, namely rabbit anti-phospho-Akt antibody (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA) and rabbit anti-phospho-eNOS (1:750 dilution; Cell Signaling Technology). The blots were hybridized with a secondary antibody coupled to horseradish peroxidase (1:5000 dilution; Santa Cruz Biotech, Inc., Dallas, TX, USA). Complexes were visualized by chemiluminescence detection (Pierce ECL Western blotting substrate, Buenos Aires, Argentina). The membranes were stripped and reprobed with anti-Akt (1:1000 dilution; Cell Signaling Technology) and anti-eNOS antibody (1:750 dilution; Cell Signaling Technology). Densitometric analysis of the bands was performed using ImageJ (National Institutes of Health, Bethesda, MD, USA). Protein band densities were normalized to the Akt and eNOS content.

Mitochondrial isolation and mitochondrial H_2O_2 production

Cardiac mitochondria were isolated from tissue homogenates of Non-rIPC, rIPC, rIPC + L-NAME and rIPC + 5HD left ventricles not subjected to ischaemia–reperfusion (n = 5–8 per group) by differential centrifugation in a Sorvall RC5C centrifuge (Sorvall, Buckinkhanshire, UK). Heart samples were washed and minced in STE buffer containing 250 mM sucrose, 5 mM Tris–HCl and 2 mM EGTA (pH 7.4). A brief digestion was performed in STE supplemented with 0.5% (w/v) fatty acid-free BSA, 5 mM $MgCl_2$, 1 mM ATP and 2.5 UI ml^{-1} type XXIV bacterial proteinase. After 4 min at 4°C, samples were homogenized in 1:10 STE with a Potter Elvejhem glass homogenizer and centrifuged at 8000g for 10 min. The obtained pellet was resuspended in STE and centrifuged at 700g for 10 min. The sediment was discarded, and mitochondria were pelleted from the supernatant by two centrifugation steps at 8000g for 10 min each. Finally, the pellet was washed, rinsed, and resuspended in 500 μl of STE buffer. The whole procedure was carried out at 0–4°C. The purity of isolated mitochondria was assessed by determining lactate dehydrogenase activity; only mitochondria with <5% impurity were used (Cadenas *et al.* 1980). The protein concentration was measured by the Lowry assay using BSA as the standard.

The mitochondrial H_2O_2 production rate was evaluated by the Amplex Red–horseradish peroxidase (HRP) method as described by Chen *et al.* (2003). The reaction buffer consisted of 125 mM sucrose, 65 mM KCl, 10 mM Hepes, 2 mM KH_2PO_4, 2 mM $MgCl_2$, and 0.01% (w/v) BSA (pH 7.2). Amplex Red (25 μM) oxidation was followed in

the presence of HRP (0.5 U ml^{-1}), with malate (2 mM) and glutamate (5 mM) as mitochondrial respiratory substrates. After an initial stabilization period, freshly isolated heart mitochondria (0.25 mg protein ml^{-1}) were added to the reaction mixture. Resorfurin formation, resulting from Amplex Red oxidation by HRP bound to H_2O_2, was measured in a Perkin Elmer LS 55 Fluorescence Spectrometer (Perkin Elmer, Waltham, MA, USA) at 563 nm (excitation) and 587 nm (emission). Fluorescence increments were suppressed in the presence of catalase, indicating that changes in fluorescence were attributable to H_2O_2 formation. Controls in the absence of isolated mitochondria or HRP indicated that non-specific probe oxidation was minimal (<1%). A calibration curve was performed using H_2O_2 solutions as the standard. The mitochondrial H_2O_2 production rate was expressed as nanomoles per minute per milligram of protein.

Statistical analysis

Data are expressed as means ± SEM. Intergroup comparisons were carried out using one way-ANOVA followed by unpaired Student's *t* tests, with the *P* value adjusted for multiple comparisons using the Bonferroni test. The data comparisons were not significant unless the corresponding P value was less than 0.05/*k*, where *k* represents the number of comparisons. The intragroup comparisons were analysed by two-factor repeated-measures ANOVA.

Results

Figure 2 shows the infarct size induced by 30 min of global ischaemia followed by 120 min of reperfusion, expressed as a percentage of the left ventricular area. In the Non-rIPC group, the infarct size was 52.31 ± 3.3%, and rIPC decreased the infarct size to 31.2 ± 2.6% ($P < 0.05$ *versus* Non-rIPC group), thus showing the well-established cardioprotection by rIPC. The CVS completely abolished the beneficial effect of rIPC, leading to an infarct size of 53.2 ± 4.2% ($P < 0.05$ *versus* rIPC group). The SVS did not modify the protective effect of rIPC, thus demonstrating that denervation of organs other than the heart does not contribute to the loss of rIPC.

Given that activation of muscarinic receptors can increase NO synthesis, we studied a possible involvement of NO in the observed reduction of infarct size afforded by the rIPC. In this regard, administration of L-NAME, during the rIPC protocol, completely abolished the protective effect of rIPC, leading to an infarct size of 49.1 ± 1.8%, indicating a central role of NO in the cardioprotection afforded by rIPC. Given that NO could induce the mK_{ATP} channels to open, we administered 5-HD before the rIPC protocol. The mK_{ATP} channel blocker completely abolished the effect of rIPC (infarct size 53.1 ± 4.2%), thus providing evidence of an involvement of mK_{ATP} channels in rIPC.

Activation of muscarinic receptors induces phosphorylation of the Akt enzyme (Krieg *et al.* 2003). To address this issue in rIPC, the phosphrylation of Akt in cardiac tissue was determined, and results are shown in Fig. 3*A*. Cardiac expression of phosphorylated Akt (Ser 473) can be observed in the Non-rIPC, rIPC and the rIPC + CVS groups at the end of the hindlimb preconditioning protocol. Most importantly, rIPC induced a significant increase of the cardiac Akt phosphorylation, which was abolished by the CVS, in hearts that were not yet subjected to ischaemia–reperfusion.

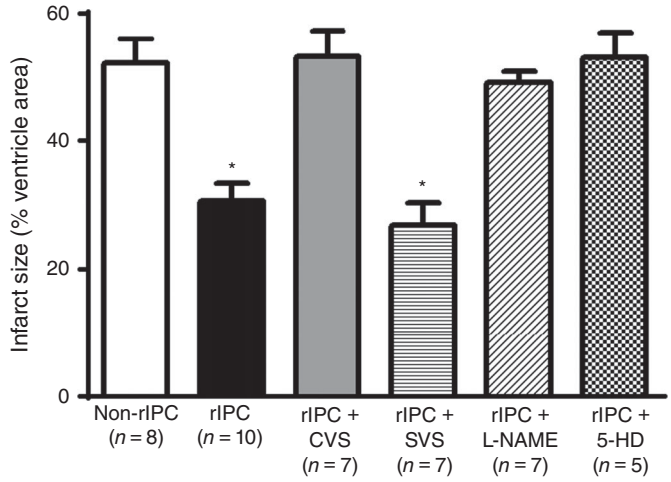

Figure 2. Infarct size, expressed as a percentage of the left ventricular area
The rIPC significantly reduced the infarct size, and this effect was abolished by the different treatments (CVS, L-NAME or 5-HD). Non-rIPC, $n = 8$; rIPC, $n = 10$; rIPC + CVS, $n = 7$; rIPC +SVS, $n = 7$; rIPC + L-NAME, $n = 7$; and rIPC + 5HD, $n = 5$. $^*P < 0.05$ *versus* each other group. Abbreviations are as in Fig. 1.

In order to evaluate phosphorylation of eNOS (Ser-1177), which is located downstream Akt (Krieg *et al.* 2003), the phosphorylated eNOS expression was studied. The results are shown in Fig. 3*B*, where it can be seen that rIPC induced a significant increase in the phosphorylation of this enzyme, which was abolished by CVS, before the myocardial ischaemia. Taken together, these results clearly indicate involvement of the Akt–eNOS pathway in the heart as triggers of the rIPC mechanism before the cardiac insult of ischaemia–reperfusion.

It has been suggested that mitochondrial reactive oxygen species (ROS) production might play a relevant role in IPC (Kalogeris *et al.* 2014), but this issue has not been addressed in rIPC. Therefore, the H_2O_2 production rate was evaluated in freshly isolated cardiac mitochondria. Figure 4*A* shows a representative trace during an initial stabilization period of the reaction mixture and after the addition of isolated mitochondria from the following groups: Non-rIPC and rIPC. It can be seen that there is an increased H_2O_2 release in the rIPC

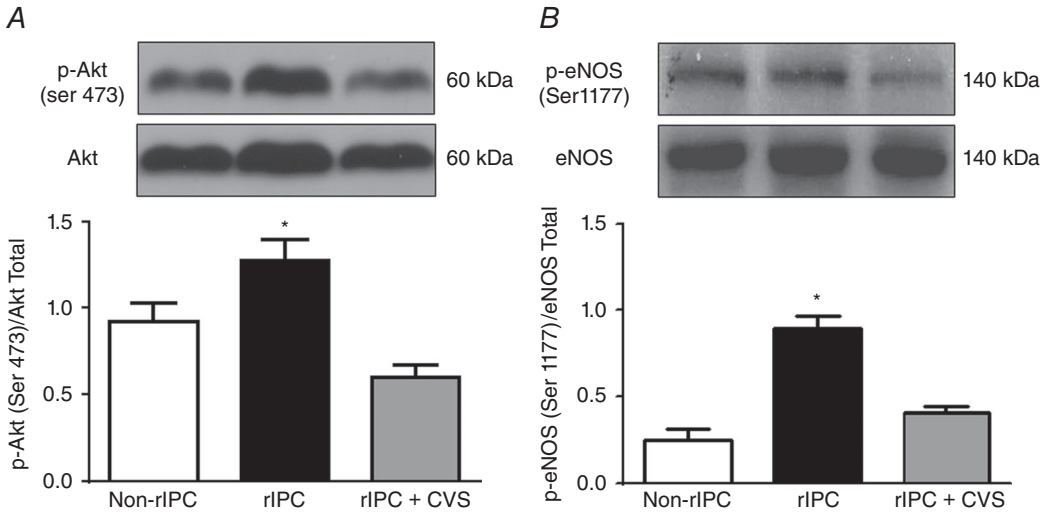

Figure 3. Expression of cardiac p-Akt and p-eNOS
In *A*, cardiac expression of phosphorylated Akt (Ser 473) can be observed in the Non-rIPC, rIPC and rIPC + CVS groups ($n = 8$ per group), immediately after hindlimb rIPC. The rIPC induced a significant increase of the Akt phosphorylation, which was abolished by CVS. In *B*, expression of phosphorylated eNOS (Ser 1177) can be observed. The rIPC induced a significant increase of the phosphorylation of this enzyme, which was abolished by CVS. $^*P < 0.05$. Abbreviations are as in Fig. 1.

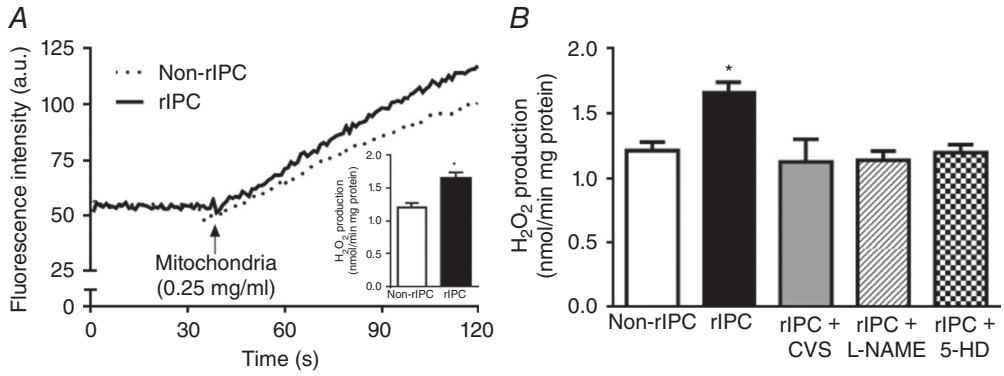

Figure 4. Cardiac mitochondrial H_2O_2 production
A shows a representative trace during an initial stabilization period and after the addition of isolated mitochondria from Non-rIPC or rIPC rats. The inset bar graph shows that the mitochondrial H_2O_2 production rate at the end of the experiments was significantly increased in rIPC hearts compared with the Non-rIPC group. *B* shows the mean mitochondrial H_2O_2 production rate in the different study groups. The CVS, L-NAME or 5-HD abolished the increase in mitochondrial H_2O_2 production rate. $^*P < 0.05$. Abbreviations are as in Fig. 1.

group. Indeed, as shown in the bar graph in the inset of the same panel, the mitochondrial H_2O_2 production rate was significantly increased, by 36%, in remotely preconditioned hearts in comparison with the Non-rIPC group at the end of the experiment ($P < 0.05$). Figure 4*B* shows that CVS, L-NAME and the mK_{ATP} channel blockade with 5-HD attenuated the H_2O_2 release.

Discussion

The present study was designed to evaluate cardiac mechanisms involved in rIPC. In order to exclude any factor other than the remote preconditioning stimulus, we have evaluated intracellular mechanisms activated by rIPC in tissue samples from hearts that received the preconditioning signal from the remote organ without being subjected to ischaemia–reperfusion injury. Our major findings were that rIPC could induce activation of the Akt enzyme and eNOS phosphorylation, mK_{ATP} channel opening and mitochondrial H_2O_2 production in the heart before the index myocardial ischaemia. Therefore, they could be considered as rIPC triggers. In addition, the protective effect of rIPC was abolished by CVS but not by SVS, reinforcing the hypothesis of a parasympathetic vagal pathway.

Different authors have evaluated the possible intracellular mechanisms involved in the rIPC pathway. However, most of these studies have investigated mechanisms at time points that are different from the ones used in the present study [during early reperfusion (Hausenloy *et al.* 2012) or in late reperfusion (Dow *et al.* 2012)] and also using different animal species (Konstantinov *et al.* 2005; Donato *et al.* 2013; Brandenburger *et al.* 2014), making difficult to establish a comparison. In agreement with our findings, Li *et al.* (2010) showed that rIPC induces activation of Phosphatidylinositol 3-kinase and inhibition of Glycogen synthase kinase before myocardial ischaemia. However, the authors proposed that rIPC would induce release of a factor to the bloodstream, which activates the PI3K–Akt–GSK3β pathway at the cardiac level, suggesting a myocardial mechanism similar to IPC. The hypothesis of a humoral pathway for rIPC has been proposed by different authors in animals (Skyschally *et al.* 2015) and in patients (Rassaf *et al.* 2014). At least in some experimental models, the reduction in size of the infarct induced by rIPC involves release of one or more humoral factors into the bloodstream. The proposed factors include a small hydrophobic molecule (3.5–15 kDa; still unknown), which is released during a brief episode of ischaemia–reperfusion (Serejo *et al.* 2007). However, there is also evidence suggesting that neural communication is stimulated by the release of adenosine, bradykinin etc. In some studies, it has been shown that communication of the protective stimulus does not seem to be explained by a neuronal pathway (i.e. protection was achieved by transfer of coronary effluent between isolated buffer-perfused hearts), whereas in other studies transmission appears to be multifactorial and requires humoral, neuronal and/or systemic mechanisms.

The results of our study support the existence of a vagal efferent pathway, and they extend current knowledge by showing that rIPC could activate the Akt pathway. Furthermore, the denervation of organs other than the heart does not contribute to the loss of rIPC, because SVS did not abolish the cardioprotection afforded by rIPC.

Phosphorylation of Akt would lead to activation of eNOS and, subsequently, to mK_{ATP} channel opening and mitochondrial changes. The role of NO in the mechanism of rIPC is difficult to evaluate, because it could participate in the tissue or organ where the preconditioning stimulus originates and/or at the cardiac level as part of the cardioprotection signalling. In the latter case, it is well known that infusion of a drug able to increase NO

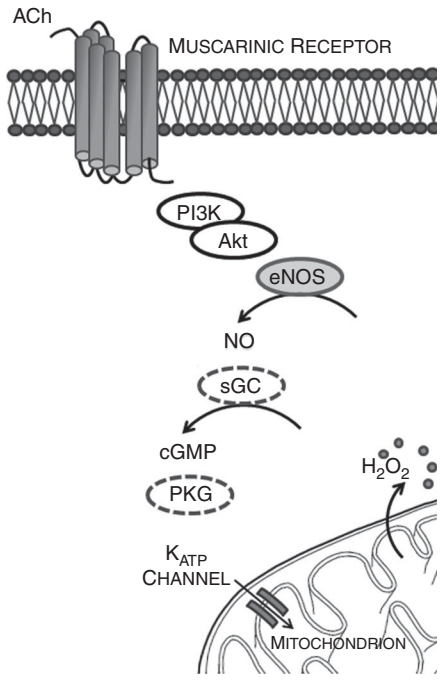

Figure 5. Schematic illustration of the intracellular pathways activated by remote ischaemic preconditioning before myocardial ischaemia
Acetylcholine, released from cardiac vagal nerve endings, activates muscarinic receptors located in the cardiomyocyte plasma membrane, inducing the phosphorylation of Akt and eNOS enzymes. Subsequently, activation of soluble guanylate cyclase and protein kinase G could lead to mK_{ATP} channel opening and an increase in mitochondrial production of H_2O_2. In this way, H_2O_2 could act as a second messenger of the rIPC protective signal. Abbreviations: eNOS, endothelial nitric oxide synthase; mK_{ATP}, mitochondrial K_{ATP} channels; NO, nitric oxide; P13K, Phosphatidylinositol 3-kinase; PKG, protein kinase G; and sGC, soluble guanylate cyclase.

bioavailability puts the heart into a preconditioned state (Sun *et al.* 2013). Our results demonstrate that the rIPC activation involves eNOS, because by administration of L-NAME we abolished the protective effect, but more importantly, they demonstrate that there is cardiac eNOS phosphorylation immediately after the rIPC protocol, before myocardial ischaemia. In addition, an increase in NO production is capable of acting directly on the mitochondria, inhibiting the respiratory chain and favouring production of ROS (Antunes *et al.* 2004), or activating the cGMP–protein kinase G pathway, which is involved in the cardioprotective mechanism of IPC.

Given that several authors showed that either IPC or the administration of diazoxide produces mK_{ATP} channel opening (Yao *et al.* 1993), we administered 5-HD to investigate a possible involvement of mK_{ATP} channels in rIPC and observed a loss of the rIPC protective effect, thus demonstrating an important role of these channels in rIPC. The mK_{ATP} channel opening produces a higher K^+ influx to the mitochondria, thus decreasing the mitochondrial membrane potential and leading to an increase of H_2O_2 mitochondrial production (Kalogeris *et al.* 2014). In this sense, our study demonstrates that rIPC induces a higher mitochondrial H_2O_2 production before the myocardial ischaemia, and this effect was attenuated by inhibition of the NO production with L-NAME and with blockade of mK_{ATP} channels with 5-HD. In this regard, H_2O_2 could act as a second messenger of the rIPC protective signal. Besides, ROS production by the respiratory chain has been shown to activate mK_{ATP} channels (Tullio *et al.* 2013). Furthermore, ROS generated by the mitochondria could activate other sensitive redox enzymes, such as protein kinase Cε, which is one of the most important kinases participating in the mechanism of IPC (Fornazari *et al.* 2008). Finally, a higher H_2O_2 mitochondrial production before ischaemia would protect the heart against an exacerbated production of ROS during reperfusion (Krieg *et al.* 2003), being another important underlying mechanism of the protective effect of rIPC. A scheme combining our results and current knowledge regarding the intracellular mechanisms activated by rIPC in the heart is depicted in Fig. 5.

A limitation of the present study is that we have used an *in vitro* model to induce myocardial infarction. However, this was done in order to gain better control over different variables that could influence the results, such as heart rate, baseline contractile state and loading conditions. It would be interesting to validate these results in an *in vivo* model of ischaemia–reperfusion.

In conclusion, rIPC reduces the infarct size through a vagal pathway and a mechanism involving phosphorylation of Akt and subsequently eNOS, followed by mK_{ATP} channel opening and an increase in H_2O_2 production by the mitochondria. It is most important to note that our results clearly demonstrate that all these phenomena occur before the myocardial ischaemic insult, which is why they could act as triggers for rIPC, putting the heart into a preconditioned state.

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Additional information

Competing interests

None declared.

Author contributions

Conception or design of the work: M.D. and R.J.G. Acquisition or analysis or interpretation of data and drafting the work or revising it critically for important intellectual content: M.D., M.A.G., M.G., T.M., V.P., J.delM., C.H., M.R., P.E. and R.J.G. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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