ORIGINAL ARTICLE

Remote Mechanisms of Myocardial Protection

Mecanismos de protección miocárdica a distancia

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ABSTRACT

Background: Remote ischemic preconditioning (rIPC) has been suggested to reduce infarct size through the activation of a parasympathetic neural pathway. However, the intracellular mechanisms responsible for this protection remain unclear.
Objective: The aim of this study was to describe some of the intracellular protective signals activated at the cardiac level by rIPC prior to myocardial ischemia.
Methods: Isolated rat hearts were subjected to 30 minutes of global ischemia and 120 minutes of reperfusion (I/R). In a second group, before the isolation of the heart, a rIPC protocol (three cycles of left femoral artery ischemia/reperfusion) was performed, followed by the I/R protocol. Additionally, four experimental groups were studied, in which prior to the rIPC protocol a bilateral cervical vagotomy [VS (vagal section)] was performed or atropine (muscarinic receptor blocker), L-NAME (NO synthesis inhibitor), and 5-HD (mK^+_{ATP} channel blocker) was administered, respectively. Infarct size and eNOS phosphorylation were measured in I/R, rIPC, and VS groups. Finally, mitochondrial H_2O_2 production was assessed.
Results: Remote ischemic preconditioning significantly decreased infarct size and this effect was abolished by VS and atropine, L-NAME, and 5-HD treatments. Furthermore, rIPC increased eNOS phosphorylation and this effect was abolished by VS. Finally, rIPC increased the mitochondrial H_2O_2 production, and this effect was also abolished by VS.
Conclusions: Remote ischemic preconditioning activates a muscarinic vagal pathway involving eNOS phosphorylation, opening of mitochondrial mK^+_{ATP} channels, and the production of mitochondrial H_2O_2.

Key words: Myocardial Infarction - Myocardial Protection - Ischemic Preconditioning

RESUMEN

Introducción: Se ha propuesto que el precondicionamiento isquémico remoto (PCr) reduce el tamaño del infarto a través de la activación de una vía neuronal parasimpática. Sin embargo, los mecanismos intracelulares responsables de esta protección no se conocen completamente.
Objetivo: Describir algunas de las señales intracelulares activadas a nivel cardíaco por el PCr antes de la isquemia miocárdica y que participan en la protección.
Material y métodos: Corazones aislados de ratas fueron sometidos a 30 minutos de isquemia global, seguidos de 120 minutos de reperfusión (I/R). En un segundo grupo, antes del aislamiento del corazón se realizó un protocolo de PCr (tres ciclos de isquemia/reperfusión en la arteria femoral izquierda); una vez finalizado, se repitió el protocolo del grupo I/R. Adicionalmente, se estudiaron cuatro grupos experimentales, en los que antes del PCr se realizó una vagotomía cervical bilateral [SV (sección vagal)] o se administró atropina (bloqueante de los receptores muscarínicos), L-NAME (inhibidor de la síntesis de NO) y 5-HD (bloqueante de los canales mK^+_{ATP}), respectivamente. Se midieron el tamaño del infarto y la fosforilación de la eNOS en los grupos I/R, PCr y SV. Por otro lado, se midió la producción mitocondrial de H_2O_2.
Resultados: El PCr redujo significativamente el tamaño del infarto y este efecto fue abolido por la SV y con los tratamientos con atropina, L-NAME y 5-HD. Además, el PCr incrementó la fosforilación de la eNOS y este efecto fue abolido por la SV. Finalmente, el PCr produjo un aumento de la producción de H_2O_2 mitocondrial, hecho que también fue abolido con la SV.
Conclusiones: El PCr activa una vía muscarínica vagal, que involucra la fosforilación de la eNOS, la apertura de los canales mK^+_{ATP} de la mitocondria y un aumento de la producción de H_2O_2 mitocondrial.

Palabras clave: Infarto del miocardio - Protección miocárdica - Precondicionamiento isquémico

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Abbreviations

CCP	Classical preconditioning	**mK^+_{ATP}**	mitochondrial ATP-dependent potassium channels
CPP	Coronary artery perfusion pressure	**NO**	Nitric oxide
eNOS	Endothelial nitric oxide synthase	**ROS**	Reactive oxygen species
5-HD	5-hydroxydecanoate	**rIPC**	Remote ischemic preconditioning
I/R	Ischemia/reperfusion	**VS**	Vagal section
L-NAME	NG-nitro-L-arginine methyl ester		

INTRODUCTION

Remote ischemic preconditioning (rIPC) is a strategy by which brief ischemia/reperfusion (I/R) cycles performed in a tissue or remote organ protect the heart against the damage produced by an episode of prolonged myocardial ischemia. Several authors have described the cardioprotective signal transfer from the peripheral organ to the heart as the result of humoral factors, neural pathways or neurohumoral interaction. (1) In this regard, we established that rIPC activates a neural pathway and that the signal reaches the heart through the vagus nerve. Thus, acetylcholine released from vagal nerve endings, preconditions the heart by activating muscarinic receptors. (2) However, in this work we did not study remotely triggered signal transductions involved in the intracellular rIPC mechanism. According to some authors (1, 3) rIPC activates a signaling mechanism similar to that of classical preconditioning (CP), while others show that cardioprotection provided by rIPC follows a different pattern (4). Heusch (5) describes some of the signal transductions involved in different forms of ischemic preconditioning. In his review, the author mentions that there are still several unresolved issues when studying the events of myocardial protection, among them the absence of a temporary description of the participant cardioprotective signals. Several years ago, Downey et al. proposed a classification of signals involved in CP, using a logical/causal sequence of events that coincide with the time sequence of the preconditioning protocol used. (6,7) Thus, these authors describe an "activating" factor released during preconditioning I/R cycles which acts as a stimulus to activate "mediators" transmitting the cardioprotective signal during prolonged myocardial ischemia to an "end-effector" which is responsible for attenuating irreversible injury during ischemia and early reperfusion. Following this reasoning, several studies evaluated the intracellular signals involved in rIPC cardioprotection; (8-10) however, in these works the temporal sequence of activation signals has not been considered. Therefore, the aim of this study was to determine some of the transduction signals acting, before prolonged myocardial ischemia, as rIPC stimuli. The involvement of muscarinic receptors, endothelial nitric oxide synthase (eNOS) and mitochondrial K^+_{ATP} channels (mK^+_{ATP}) were specifically addressed.

METHODS

Experiments were performed on male Wistar rats (200 to 250 g.).

Rats were anesthetized with sodium pentobarbital (35 mg/kg) and intubated for ventilation with a mixture of ambient air and oxygen using a Harvard respirator (Model 683). Then, the femoral artery of the left hind limb was dissected and the animals were randomized into different experimental groups.

After completing the in vivo protocols (see below) the animals were euthanized with sodium pentobarbital (150 mg/kg) and each heart was quickly removed from the animal and mounted by the root of the aorta in a perfusion system for isolated organ, according to the Langendorff technique. The hearts were perfused with Krebs-Henseleit buffer solution containing: 118.5 mM NaCl, 4.7 mM KCl, 8 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM Mg SO_4, 1.5 mM $CaCl_2$ and 10 mM glucose, at pH 7.2-7.4). Temperature was kept constant at 37 ° C, and the buffer was bubbled with carbogen gas (95% O_2-5% CO_2). Two electrodes were connected to a pacemaker to maintain a constant heart rate of 250 beats per minute. A latex balloon filled with saline solution, connected to a pressure transducer (Deltram II, Utah Medical System), was introduced in the left ventricle. The balloon volume was adjusted to achieve left-ventricular end-diastolic pressure between 8 and 10 mmHg. Coronary perfusion pressure (CPP) was also recorded with a pressure transducer connected to the perfusion line. Coronary flow was adjusted to obtain a CPP of 70.5±4.2 mmHg during the initial stabilization period.

Experimental protocols (Figure 1)

- Group 1 (I/R, n=8): Rats were anesthetized and the femoral artery in the left hind limb was dissected. After 30 min follow-up, the animals were euthanized and the hearts removed and perfused according to the Langendorff technique. After 20 min stabilization, they underwent 30 min of global ischemia followed by 120 min of reperfusion.
- Group 2 (rIPC; n=9): Group 1 protocol was repeated, but prior to myocardial ischemia a rIPC protocol (3 cycles of occlusion/reperfusion of the left femoral artery) was performed.
- Group 3 [vagal section (VS); n=7]: Group 2 protocol was repeated, but prior to rIPC bilateral vagotomy was performed at the cervical level.
- Group 4 (rIPC + Atropine; n=6): Group 2 protocol was repeated, but rats were treated with atropine (muscarinic receptor blocker; attack-dose of 2mg/kg and maintenance at 1mg/Kg/h) during the rIPC protocol.
- Group 5 [rIPC + L-NAME (NG-nitro-L-arginine methyl-ester); n=8]: Group 2 protocol was repeated, but rats were treated with L-NAME (eNOS inhibitor, 100 μM) during the rIPC protocol.

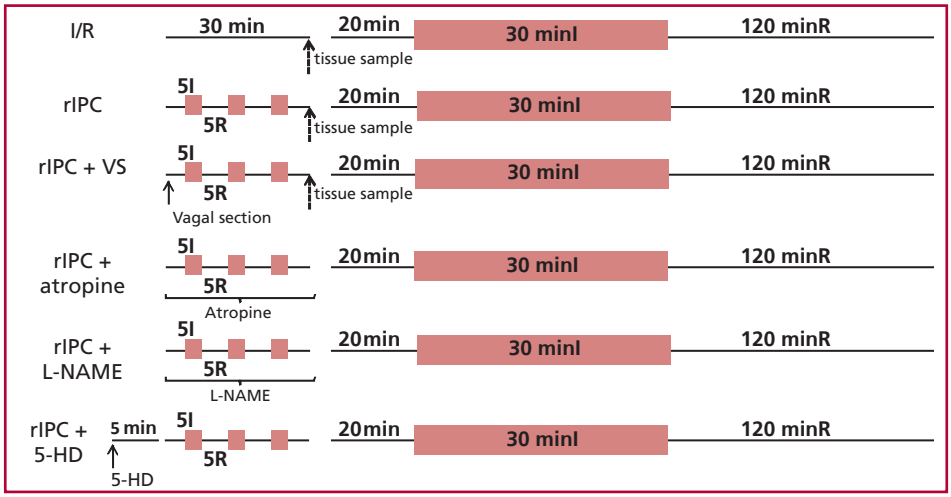

Fig. 1. Schematic diagram of experimental protocols. I/R: Ischemia/reperfusion. rIPC: Remote ischemic preconditioning. I: Ischemia. R: Reperfusion. VS: Vagal section. L-NAME: NG-nitro-L-arginine methyl ester. 5-HD: 5-hydroxydecanoate.

- Group 6 [rIPC + 5-HD; (5-hydroxydecanoate); n=5]: Group 2 protocol was repeated but rats received a dose of 5-HD (potassium channel blocker, 100 μM) 5 min prior to rIPC.

Infarct size measurement

At the end of the protocols the hearts were cut into transverse sections and incubated in % 2,3,5-triphenyl-tetrazolium chloride solution during 20 min. With this technique, viable tissue stains red while the unstained area corresponds to the infarction area. Sections were scanned and the infarcted areas were measured using computerized planimetry (image analyzer: Image Pro Plus, version 4.5). Infarct size was expressed as percentage of the left ventricular surface.

Western blot

Left ventricular tissue samples were homogenized in 3 vol (w/v) buffer cooled on ice consisting of: 150 mM NaCl, 50 mM Trizma HCl, 1% (v/v) sodium deoxycholate, 1 mM EGTA, 1mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium pervanadate (all reagents from Sigma Aldrich, St. Louis, USA) and a protease inhibitor cocktail at 4%, (Roche Hertfordshire, UK), at pH 8.0, and they were centrifuged at 10,000 rpm for 10 min at 4 ° C. The supernatant protein was measured with the Lowry method using bovine serum albumin as standard solution and was resuspended in 2X SDS sample buffer (62.5 mM Tris-HCl buffer, pH 6.8 containing 2% (w/v) SDS, 25% (w/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.01% (w/v) bromophenol blue) and heated for 5 min at 95 ° C. An equal amount of proteins (80 mg) was loaded in 8% SDS-PAGE and transferred to nitrocellulose membranes. After blocking with PBS 3% nonfat milk buffer (w/v) for 1 h, the membranes were incubated overnight at 4 ° C with the corresponding primary antibodies: rabbit anti-eNOS (1:750 dilution, Cell Signalling Technology, Danvers, MA, USA) and phosphorylated eNOS in its 1177 serine residue in the I/R, rIPC and VS groups, immediately after the rIPC protocol. The blots were hybridized with a horseradish peroxidase-conjugated secondary antibody (dilution 1: 5000, Santa Cruz Biotech, Inc. Dallas, TX, USA). Complexes were visualized by chemiluminescent detection (Pierce ECL Western Blotting Substrate). The membranes were stripped and re-incubated with phospho-eNOS antibody (1:750 dilution (Cell Signalling Technology, Danvers, MA, USA). Densitometry analysis of the bands was performed using Image J software (NIH, Bethesda, Maryland, USA). Protein band densities were normalized by eNOS content.

Mitochondrial isolation

Heart mitochondria were isolated from tissue homogenates (n=4 per group) by differential centrifugation in a RC5C Sorvall centrifuge (Sorvall, Buckinghamshire, England). Heart samples were washed and minced in STE buffer [250 mM sucrose, 5 mM Tris-HCl and 2 mM EGTA (pH 7.4)]. A brief digestion was performed in STE buffer supplemented with 0.5% (w/v) fatty-acid-free BSA, 5 mM $MgCl_2$, 1 mM ATP and 2.5 IU/ml proteinase, bacterial type XXIV. After 4 min at 4 ° C, samples were homogenized in 1:10 STE buffer with a glass Potter-Elvejhem homogenizer and centrifuged at 8000 g for 10 min. The resulting pellet was resuspended in STE buffer and centrifuged at 700 g during 10 min. The sediment was discarded and mitochondria were precipitated from the supernatant by two centrifugation steps at 8000 g for 10 min each. Finally, the pellet was washed, rinsed and resuspended in 500 ml of STE buffer. The whole procedure was performed at 0-4° C. The purity of isolated mitochondria was evaluated by determining lactate dehydrogenase activity; only mitochondria with less than 5% impurity were used. Protein concentration was measured by the Lowry assay using BSA as standard curve.

Mitochondrial H_2O_2 production

Mitochondrial H_2O_2 production rate was evaluated by the Amplex red/horseradish peroxidase assay, the previously described HRP method. 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 buffer (pH 7.2). Amplex Red (25 mM) was followed by oxidation in the presence of HRP (0.5 U/ml), malate (2 mM) and glutamate (5 mM) as mitochondrial respiration substrates. After an initial stabilization period, newly isolated heart mitochondria were added (0.25 mg protein/ml) to the reaction mixture. Resorfurin formation from the reaction of H_2O_2 and Amplex Red oxidation by HRP was measured in a Perkin Elmer LS 55 fluorescence spectrometer (Perkin Elmer, Waltham, MA, USA) at 563 nm (excitation) and 587 nm (emission). Catalase suppressed the increase in fluorescence, indicating that changes in fluorescence are due to the formation of H_2O_2. Controls in the absence of isolated mitochondria or HRP indicate that non-specific probe oxidation is minimal (<1%). A calibra-

tion curve was performed with standard H_2O_2 solutions. The mitochondrial H_2O_2 production rate was expressed as nmol/min/mg protein.

Statistical analysis:

Data are expressed as mean±standard error of the mean. Comparisons between groups were made by one-way analysis of variance (ANOVA) followed by T tests with p value adjusted for multiple comparisons (Bonferroni test). Data comparisons were considered significant when the corresponding p value was less than 0.05/k, where k represents the number of comparisons.

Ethical considerations

The study procedures were approved by the Institutional Animal Care and Use Committee of the University of Buenos Aires (Protocol 2948/10) in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH), USA.

RESULTS

Figure 2 shows that a 30-minute episode of myocardial ischemia resulted in infarct size of 52.31% ±3.31% and that rIPC reduced infarct size to 31.29%±2.65% ($p < 0.05$). Both bilateral vagotomy as treatment with atropine eliminated the protective effect of rIPC on infarct size (53.26%±4.29% and 51.10%±3.84%, respectively). The same effect was observed in animals treated with L-NAME and 5-HD, in which infarct size was 49.11%±1.87% and 53.08%±4.29%, respectively.

Figure 3 shows the changes observed in eNOS phosphorylation at the Ser-1177 residue before myocardial ischemia. A significant increase in eNOS phosphorylation was induced by rIPC, and this effect was eliminated by bilateral VS.

It has been suggested that release of reactive oxygen species (ROS) could play an important role in ischemic preconditioning. (11) Therefore, we evaluated the rate of H_2O_2 production in isolated mitochondria of left ventricular tissue samples retrieved before myocardial ischemia. Figure 4 (Panel A) shows a representative tracing during the initial stabilization period and after the addition of mitochondria isolated from non-preconditioned rats and of animals subjected to a rIPC protocol. It can be seen that rIPC significantly increased mitochondrial H_2O_2 production and release. Thus, mitochondrial H_2O_2 concentration increased by 27% in rats subjected to rIPC compared to the non-IPC group (I/R: 1.3±0.1 nmol H_2O_2/min/mg of protein, $p < 0.05$).

DISCUSSION

Findings of the present study reinforce the hypothesis of a vagal cholinergic muscarinic pathway for rIPC and demonstrate that at the heart level intracellular signals are activated involving eNOS phosphorylation, K^+_{ATP} channel opening and release of mitochondrial H_2O_2. All these events occur before myocardial ischemia, and could therefore be considered as rIPC triggers.

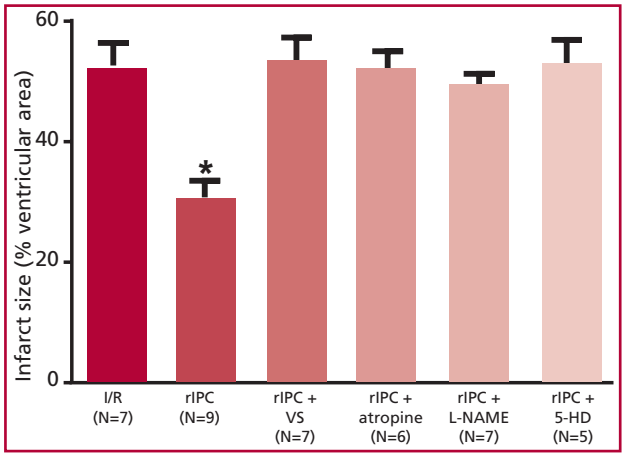

Fig. 2. Infarct size as percent of left ventricular area. I/R: Ischemia/reperfusion. rIPC: Remote ischemic preconditioning. L-NAME: NG-nitro-L-arginine methyl ester. 5-HD: 5-hydroxydecanoate. * p<0.05 vs. the other groups.

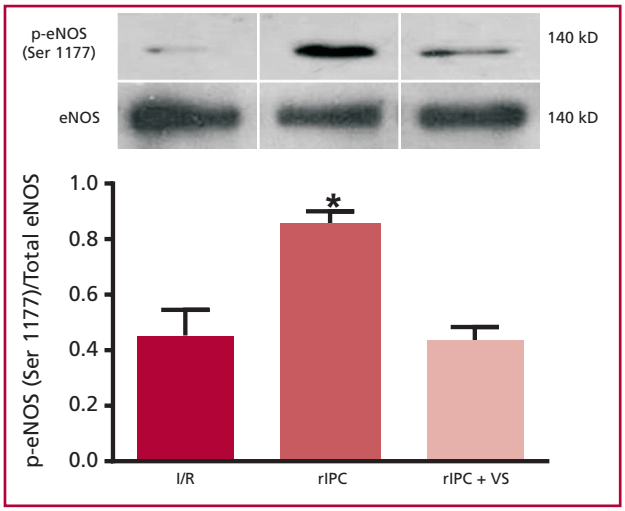

Fig. 3. Phosphorylated eNOS expression (Ser 1177) in left ventricular tissue at the end of the rIPC protocol I/R: Ischemia/reperfusion. rIPC: Remote ischemic preconditioning. VS: Vagal section. * p<0.05 vs. the other groups.

Different authors assessed the possible intracellular mechanisms involved in rIPC, particularly the participation of the RISK (12) and STAT 5 pathway. (13) However, in all these works, the participation of these enzymatic complexes was studied in very dissimilar times: before myocardial ischemia (14), early reperfusion (12) and late reperfusion (15) and also using different animal species, (2, 16-17) making it difficult to establish comparisons. On the other hand, the results of our study extend this knowledge by demonstrating that the muscarinic receptor is responsible for triggering the protection. In addition, the rIPC activation pathway at the cardiac level involves eNOS; since L-NAME administration abolished the protective effect. In the same sense, rIPC induced eNOS phosphorylation before myocardial ischemia in preconditioned

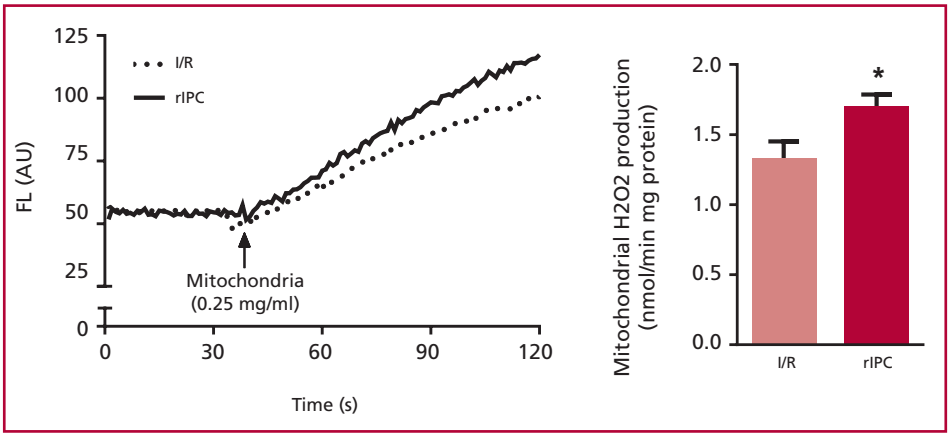

Fig. 4. Panel A shows a representative tracing during the initial stabilization period and after the addition of isolated mitochondria from non-preconditioned rats and from animals subjected to a rIPC protocol. Panel B shows that the mitochondrial H_2O_2 production rate was significantly increased by 27% in rIPC rats compared with the non-preconditioned group (I/R: 1.3±0.1 nmol H_2O_2/min/mg de protein; p <0.05). I/R: Ischemia/reperfusion. rIPC: Remote ischemic preconditioning

hearts. The role of nitric oxide (NO) in the rIPC mechanism is complex because it could participate at the tissue or organ level where the preconditioning stimulus originates or at the cardiac level as part of the signals that lead to cardioprotection. In the latter case, it is well known that the infusion of substances capable of increasing NO bioavailability turn the heart into a preconditioned state. (18) In our case, as we have mentioned, we show that there is cardiac eNOS phosphorylation, immediately after finishing the rIPC protocol. In addition, the increase in NO production is able to act directly on the mitochondria by inhibiting the respiratory chain and promoting ROS production. In fact, in our study we show that rIPC induces higher production of mitochondrial H_2O_2.

Different authors have shown that rIPC or diazoxide administration causes opening of mK^+_{ATP}; (19) for this reason we administered 5-HD (mK^+_{ATP} channel blocker) and observed the loss of rIPC protective effect. Opening of mK^+_{ATP} causes significant mitochondrial changes. Once mK^+_{ATP} channels open, K+ flow into the mitochondria increases and the mitochondrial membrane potential decreases. This condition would protect the heart against exacerbated ROS production during reperfusion (11) and would constitute the underlying mechanism of rIPC protection. As suggested, (20) mitochondrial ROS production could contribute to mK^+_{ATP} opening. Consequently, H_2O_2 could act as a messenger of the rIPC protective signal.

Moreover, ROS generated by mitochondria could activate other redox sensitive enzymes, including PKCε which is one of the major kinases involved in the CP mechanism. (21).

CONCLUSIONS

In conclusion, rIPC reduces infarct size using a vagal muscarinic pathway and a mechanism involving eNOS phosphorylation, mK^+_{ATP} channel opening and mitochondrial H_2O_2 release. All these phenomena occur prior to myocardial ischemia, thus acting as rIPC "stimuli".

Conflicts of interest

None declared. (See authors' conflicts of interest forms in the website/Supplementary material).

REFERENCES

1. Hausenloy DJ, Yellon DM. Remote ischaemic preconditioning: underlying mechanisms and clinical application. Cardiovasc Res 2008;79:377-86. http://doi.org/cfvgn2
2. Donato M, Buchholz B, Rodríguez M, Pérez V, Inserte J, García-Dorado D, et al. Role of the parasympathetic nervous system in cardioprotection by remote hindlimb ischaemic preconditioning. Exp Physiol 2013;98:425-34. http://doi.org/bcxv
3. Lim SY, Hausenloy DJ. Remote ischemic conditioning: from bench to bedside. Front Physiol 2012;3:27. http://doi.org/bcxw
4. Heinen NM, Pütz VE, Görgens JI, Huhn R, Grüber Y, Barthuber C, et al. Cardioprotection by remote ischemic preconditioning exhibits a signaling pattern different from local ischemic preconditioning. Shock 2011;36:45-53. http://doi.org/fm6d2z
5. Heusch G. Molecular basis of cardioprotection: signal transduction in ischemic pre-, post-, and remote conditioning. Circ Res 2015;116:674-99. http://doi.org/bcxx
6. Yellon DM, Downey JM. Preconditioning the myocardium: from cellular physiology to clinical cardiology. Physiol Rev 2003;83:1113-51. http://doi.org/bcxz
7. Downey JM, Krieg T, Cohen MV. Mapping preconditioning's signaling pathways: an engineering approach. Ann N Y Acad Sci 2008;1123:187-96. http://doi.org/bdh8dh
8. Li SJ, Wu YN, Kang Y, Yin YQ, Gao WZ, Liu YX, et al. Noninvasive limb ischemic preconditioning protects against myocardial I/R injury in rats. J Surg Res 2010;164:162-8. http://doi.org/b6s7dv
9. Breivik L, Helgeland E, Aarnes EK, Mrdalj J, Jonassen AK. Remote postconditioning by humoral factors in effluent from ischemic preconditioned rat hearts is mediated via PI3K/Akt-dependent cell-survival signaling at reperfusion. Basic Res Cardiol 2011;106:135-45. http://doi.org/ds7226
10. Wolfrum S, Schneider K, Heidbreder M, Nienstedt J, Dominiak P, Dendorfer A. Remote preconditioning protects the heart by activating myocardial PKCepsilon-isoform. Cardiovasc Res 2002;55:583-9. http://doi.org/bh7xjp
11. Kalogeris T, Bao Y1, Korthuis RJ1. Mitochondrial reactive oxygen species: a double edged sword in ischemia/reperfusion vs preconditioning. Redox Biol 2014;2:702-14. http://doi.org/bcx2
12. Hausenloy DJ, Iliodromitis EK, Andreadou I, Papalois A, Gritsopoulos G, Anastasiou-Nana M, et al. Investigating the signal transduction pathways underlying remote ischemic conditioning in the porcine heart. Cardiovasc Drugs Ther 2012:87-93. http://doi.org/fx9hfc
13. Heusch G, Musiolik J, Kottenberg E, Peters J, Jakob H, Thielmann M. STAT5 activation and cardioprotection by remote isch-

emic preconditioning in humans: short communication. Circ Res 2012;110:111-5. http://doi.org/d6dmxm
14. Li J, Xuan W, Yan R, Tropak MB, Jean-St-Michel E, Liang W, et al. Remote preconditioning provides potent cardioprotection via PI3K/Akt activation and is associated with nuclear accumulation of β-catenin. Clin Sci (Lond) 2011;120:451-62. http://doi.org/dcrj6m
15. Dow J, Bhandari A, Simkhovich BZ, Hale SL, Kloner RA. The effect of acute versus delayed remote ischemic preconditioning on reperfusion induced ventricular arrhythmias. J Cardiovasc Electrophysiol 2012;23:1374-83. http://doi.org/bcx3
16. Brandenburger T, Huhn R, Galas A, Pannen BH, Keitel V, Barthel F, et al Remote ischemic preconditioning preserves Connexin 43 phosphorylation in the rat heart in vivo. J Transl Med 2014;27;12:228.
17. Konstantinov IE, Li J, Cheung MM, Shimizu M, Stokoe J, Kharbanda RK, et al. Remote ischemic preconditioning of the recipient reduces myocardial ischemia-reperfusion injury of the denervated donor heart via a Katp channel-dependent mechanism. Transplantation 2005;27;79:1691-5. http://doi.org/bvpm3j
18. Sun J, Aponte AM, Kohr MJ, Tong G, Steenbergen C, Murphy E. Essential role of nitric oxide in acute ischemic preconditioning: S-nitros(yl)ation versus sGC/cGMP/PKG signaling? Free Radic Biol Med 2014;54:105-12. http://doi.org/bcx4
19. Yao Z, Gross GJ. Role of nitric oxide, muscarinic receptors, and the ATP-sensitive K+ channel in mediating the effects of acetylcholine to mimic preconditioning in dogs. Circ Res 1993;73:1193-201. http://doi.org/bcx5
20. Fornazari M, de Paula JG, Castilho RF, Kowaltowski AJ. Redox properties of the adenoside triphosphate-sensitive K+ channel in brain mitochondria. J Neurosci Res 2008;86:1548-56. http://doi.org/b848bm
21. Penna C, Rastaldo R, Mancardi D, Raimondo S, Cappello S, Gattullo D, et al. Post-conditioning induced cardioprotection requires signaling through a redox-sensitive mechanism, mitochondrial ATP-sensitive K+ channel and protein kinase C activation. Basic Res Cardiol 2006;101:180-9. http://doi.org/dccrfg