Research Paper

Role of the parasympathetic nervous system in cardioprotection by remote hindlimb 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-induced injury and cardiac dysfunction, a phenomenon referred to as 'remote ischaemic preconditioning' (rIPC). This study addressed whether activation of sensory afferent nerves in the ischaemic hindlimb and vagal efferent nerves innervating the heart mediate rIPC.
- **What is the main finding and its importance?**
  Spinal cord section, bilateral vagotomy or blockade of muscarinic cholinergic receptors *in vivo* abolished rIPC and cardioprotection measured *in vitro*. Electrical stimulation of the vagus nerve induced cardioprotection, thus mimicking rIPC. The finding that sensory and parasympathetic neural mechanisms mediate rIPC confirms and extends previous results, with implications for translational studies in patients with coronary artery disease.

This investigation was designed to determine the participation of the vagus nerve and muscarinic receptors in the remote ischaemic preconditioning (rIPC) mechanism. New Zealand rabbits were anaesthetized, and the femoral artery was dissected. After 30 min of monitoring, the hearts were isolated and subjected to 30 min of global no-flow ischaemia and 180 min of reperfusion (non-rIPC group). The ventricular function was evaluated, considering the left ventricular developed pressure and the left ventricular end-diastolic pressure. In the rIPC group, the rabbits were subjected to three cycles of hindlimb ischaemia (5 min) and reperfusion (5 min), and the same protocol as that used in non-rIPC group was then repeated. In order to evaluate the afferent neural pathway during the rIPC protocol we used two groups, one in which the femoral and sciatic nerves were sectioned and the other in which the spinal cord was sectioned (T9–T10 level). To study the efferent neural pathway during the rIPC protocol, the vagus nerve was sectioned and, in another group, atropine was administered. The effect of vagal stimulation was also evaluated. An infarct size of 40.8 ± 3.1% was obtained in the non-rIPC group, whereas in rIPC group the infarct size decreased to 16.4 ± 3.5% ($P < 0.05$). During the preconditioning protocol, the vagus nerve section and the atropine administration each abolished the effect of rIPC on infarct size. Vagal stimulation mimicked the effect of rIPC, decreasing infarct size to 15.2 ± 4.7% ($P < 0.05$). Decreases in infarct size were accompanied by improved left ventricular function.

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DOI: 10.1113/expphysiol.2012.066217

We demonstrated the presence of a neural afferent pathway, because the spinal cord section completely abolished the effect of rIPC on infarct size. In conclusion, rIPC activates a neural afferent pathway, the cardioprotective signal reaches the heart through the vagus nerve (efferent pathway), and acetylcholine activates the ischaemic preconditioning phenomenon when acting on the muscarinic receptors.

(Received 23 March 2012; accepted after revision 31 July 2012; first published online 7 August 2012)
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Ischaemic preconditioning (IPC) is a cardioprotective strategy whereby prior brief episodes of ischaemia induce a state of protection against subsequent prolonged ischaemia–reperfusion (I/R; Murry *et al.* 1986). As IPC is not applicable in a clinical setting, a variant preconditioning stimulus called remote ischaemic preconditioning (rIPC) has received increasing attention. rIPC describes the phenomenon whereby transient regional ischaemia of a tissue at a distance from the target organ (heart) affords protection with the same efficacy as local IPC. Thus, brief episodes of I/R applied to the small intestine, kidney, liver or even hindlimb have been reported to reduce subsequent myocardial infarct size (Takaoka *et al.* 1999; Huda *et al.* 2005; Li *et al.* 2010).

The mechanisms underlying rIPC have not been fully elucidated. Previous studies have attributed the cardioprotective signal from the preconditioned remote organ to either a neural pathway (Lim *et al.* 2010) or a humoral pathway (Konstantinov *et al.* 2005), although both are not mutually exclusive. Gho *et al.* (1996) demonstrated that a reduction in myocardial infarct size, induced by brief I/R of the anterior mesenteric artery, could be reversed by hexamethonium (a ganglion blocker), supporting the neural pathway hypothesis. Other authors (Lim *et al.* 2010), in a model of hindlimb ischaemia, showed that rIPC protection requires a complete innervation of the lower limb. The hypothesis on a neural pathway was further developed, making allusion to the endogenous substances that are released by the remote preconditioned organ, such as adenosine (Steensrud *et al.* 2010), bradykinin (Schoemaker & van Heijningen, 2000) and calcitonin gene-related peptide (Wolfrum *et al.* 2005), that stimulate afferent nerve fibres. However, few studies (Jones *et al.* 2009; Basalay *et al.* 2012) describe the possible efferent neural pathway by which the signal reaches the heart from the remote organ and only one of them studied the possible role of the parasympathetic nervous system (Basalay *et al.* 2012).

The parasympathetic nervous system, through the vagus nerve, has been implicated in myocardial protection. Kawada *et al.* (2009) demonstrated that pre-ischaemic efferent vagal stimulation increases acetylcholine release and protects the heart from I/R injury.

We hypothesized that rIPC activates a neural pathway and that the cardioprotective signal reaches the heart via the vagus nerve. We also hypothesized that acetylcholine released by the nervous terminal of the vagus nerve could precondition the heart through the activation of muscarinic receptors. Thus, the objective of this work was to determine the possible participation of the vagus nerve and muscarinic receptors in the rIPC cardioprotective mechanism.

Methods

Ethical approval

The experiments were performed on 54 male New Zealand rabbits (1.8–2.5 kg). The procedures used in this study were approved by the Animal Care and Research Committee of the University of Buenos Aires and were in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication, Eight edition; 2010).

Surgical procedure

First, the rabbits were anaesthetized with pentobarbital (35 mg kg^{-1}, I.V.) and intubated for mechanical ventilation with a mixture of ambient air and oxygen. Second, a fluid-filled catheter was placed into the right common carotid artery and connected to a pressure transducer (Deltram II; Utah Medical Products, Midvale, UT, USA) to record the arterial pressure and calculate the heart rate. Third, the femoral artery was dissected and the animals were randomized into different experimental groups. Anaesthesia was maintained using 5–10 mg kg^{-1} h^{-1} I.V. pentobarbital when required.

After completion of the *in vivo* protocols described in the following paragraphs, the animals were killed by administration of pentobarbital (150 mg kg^{-1} I.V.) and each heart was rapidly excised and mounted on a Langendorff apparatus by the aortic root in less than 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; $CaCl_2$, 2.5; and glucose,

10; the pH was maintained at 7.2–7.4 by aerating the solution with a gas mixture 95% O_2–5% CO_2 at 37°C. Two electrodes were sutured and connected to a pacemaker with a constant heart rate of 200 beats min^{-1}.

A saline-filled latex balloon, connected via a catheter to a pressure transducer (Deltram II; Utah Medical Products), was inserted into the left ventricle (LV). The volume of the balloon was adjusted to an end-diastolic pressure of 8–10 mmHg. Coronary perfusion pressure was also recorded through a pressure transducer connected to the perfusion line. All hearts were perfused with constant flow.

Coronary flow was adjusted to obtain a coronary perfusion pressure of 70.5 ± 4.2 mmHg during the initial stabilization period. This flow was held constant throughout the experiment. The left ventricular developed pressure (LVDP) was calculated from the difference between peak systolic pressure and left ventricular end-diastolic pressure (LVEDP). Ventricular function was assessed at baseline during the first 30 min of reperfusion, although the hearts were reperfused for 180 min in order to measure infarct size.

Experimental groups (Fig. 1)

Group 1: non-rIPC ($n = 8$). Rabbits were anaesthetized and the femoral artery was dissected. After 30 min of monitoring, the hearts were excised and perfused according to the Langendorff technique. Myocardial infarction was induced by 30 min of global no-flow ischaemia followed by 180 min of reperfusion. We induced global no-flow ischaemia by abruptly decreasing the total coronary flow provided by the perfusion pump.

Group 2: rIPC ($n = 8$). First, the rabbits were anaesthetized and the femoral artery was dissected. The animals were remotely preconditioned by subjecting them to three cycles of hindlimb ischaemia (5 min) and reperfusion (5 min) by occlusion of the femoral artery with a vascular clamp. Second, in a Langendorff apparatus, they received the same protocol as that used in the non-rIPC group (30 min of global no-flow ischaemia followed by 180 min of reperfusion).

Group 3: rIPC with vagal section ($n = 8$). The same protocol as that used in the rIPC group was performed, but the left and right vagus nerves were sectioned at the midcervical level before starting the rIPC protocol.

Group 4: rIPC with atropine ($n = 6$). The same protocol as that used in the rIPC group was performed, but atropine sulfate (1.3–2 mg kg^{-1} I.V.), a muscarinic receptor blocker, was administered during the rIPC protocol.

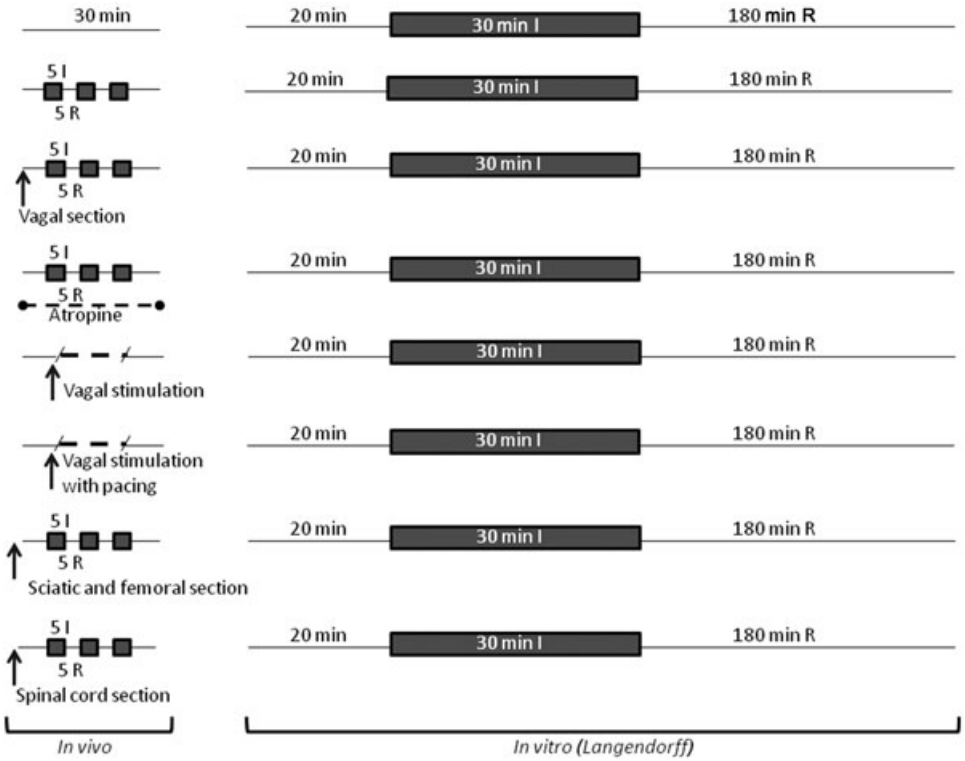

Figure 1. Schematic design of experimental protocols
Abbreviations: I, ischaemia; and R, reperfusion.

Group 5: vagal stimulation ($n = 5$). First, the rabbits were anaesthetized. The right vagus nerve was sectioned at the midcervical level and its distal end electrically stimulated for 10 min followed by 5 min of recovery without stimulation. Second, the hearts were excised and perfused according to the Langendorff technique. Myocardial infarction was induced by 30 min of global no-flow ischaemia followed by 180 min of reperfusion.

Group 6: vagal stimulation with pacing ($n = 4$). The same protocol as that used in the vagal stimulation group was performed, but during the electrical stimulation of the vagus nerve the heart was paced at a constant rate equal to 10% above baseline.

Group 7: rIPC with section of the femoral (F) and sciatic (S) nerves ($n = 10$). The same protocol as that used in the rIPC group was performed, but the femoral and sciatic nerves were sectioned before starting the rIPC protocol. The hearts were then excised and perfused according to the Langendorff technique. Myocardial infarction was induced by 30 min of global no-flow ischaemia followed by 180 min of reperfusion.

Group 8: rIPC with spinal cord section ($n = 5$). The same protocol as that used in the rIPC group was performed, but the spinal cord was sectioned between T9 and T10 before starting the rIPC protocol (Greenaway *et al.* 2001; Jones *et al.* 2009). In detail, rabbits were anaesthetized and bone markers identified at the thoracic vertebrae T9–T10 levels. Under optical magnification, the spinal cord between T9 and T10 was transected by a sharp knife, with limited bleeding. After that, the hearts were excised and perfused according to the Langendorff technique. Myocardial infarction was induced by 30 min of global no-flow ischaemia followed by 180 min of reperfusion.

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.

Infarct size measurement

After 3 h of reperfusion, the hearts were frozen and cut into 4 mm 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 sections were stained red and the infarct area remained unstained. Sections were scanned and measured (Image Pro Plus, version 4.5, MediaCybernetics, Rockville, MD, USA). The infarct size was expressed as a percentage of the left ventricular area. The demarcation of the infarct zone by tetrazolium staining is dependent on the loss of NADH and of dehydrogenases from the irreversibly damaged myocytes. In this context, Birnbaum *et al.* (1997) described that at least 3 h of reperfusion is needed to delineate infarct size by tetrazolium staining following 30 min of ischaemia in the rabbit.

Vagal stimulation

The right vagus nerve was sectioned at the midcervical level and its distal end was stimulated, using a bipolar silver electrode connected to a neurostimulator (Hugo Sachs Elektronik D7801, March-Hugstetten, Germany). The animals were subjected to rectangular electrical pulses of 0.1 ms, 10 Hz (the intensity of electrical stimulation was adjusted for each animal) in order to obtain a heart rate reduction between 10 and 20%. This stimulation protocol has been widely used by other authors (Kawada *et al.* 2001; Uemura *et al.* 2007).

Statistical analysis

Data are expressed as means ± SEM. Intergroup comparisons were carried out using one-way ANOVA followed by Student's unpaired *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. Comparisons between cardiac function measurements postmyocardial ischaemia *versus* baseline were performed by Student's paired *t* test.

Results

Table 1 shows *in vivo* measurement of heart rate and mean arterial pressure during application of the remote ischaemic preconditioning stimulus. There were no significant differences in the baseline values during the different interventions among groups. As expected, in the vagal stimulation group, there was a significant reduction of heart rate from 267 ± 15 to 223 ± 8 beats min^{-1} ($P < 0.05$) and there were no changes in mean arterial pressure. When the pre-ischaemic vagal stimulation (10 min) was performed at constant heart rate we did not observe changes in blood pressure (data not shown).

Interestingly, the rIPC also significantly reduced the heart rate, an effect that was reversed with the vagal section (Table 1). These results provide evidence for an increase in vagal tone in the group subjected to rIPC. It is important to mention that this effect was detected in the presence of pentobarbital as an anaesthetic agent, even though this drug has the ability to inhibit parasympathetic efferent activity.

Table 1. Changes in heart rate and mean arterial pressure during remote hindlimb ischaemic preconditioning protocol

Parameter	Group	Baseline	5 Isch. (1)	5 Rep. (1)	5 Isch. (2)	5 Rep. (2)	5 Isch. (3)	5 Rep. (3)
Heart rate (beats min^{-1})	Non-rIPC	275 ± 24	277 ± 25	276 ± 25	271 ± 23	273 ± 22	273 ± 21	266 ± 20
	rIPC	298 ± 10	294 ± 14	281 ± 12	281 ± 7	267 ± 13	272 ± 10	264 ± 10*
	rIPC with vagal section	310 ± 17	304 ± 19	305 ± 18	305 ± 20	302 ± 19	298 ± 21	300 ± 20
	rIPC with atropine	290 ± 19	290 ± 18	271 ± 21	271 ± 19	273 ± 15	278 ± 15	279 ± 16
	rIPC with F and S section	284 ± 10	283 ± 10	284 ± 10	285 ± 10	283 ± 11	284 ± 10	284 ± 10
	rIPC with spinal cord section	312 ± 26	316 ± 24	320 ± 22	319 ± 23	317 ± 24	319 ± 24	319 ± 23
Mean arterial pressure (mmHg)	Non-rIPC	88.7 ± 5.3	87.3 ± 5.3	85.2 ± 4.5	83.7 ± 5.7	87.5 ± 3.7	88.5 ± 5.1	85.1 ± 4.2
	rIPC	84.9 ± 5.4	82.1 ± 6.5	81.6 ± 2.7	82.9 ± 3.4	83.5 ± 4.9	81.1 ± 3.5	78.6 ± 1.9
	rIPC with vagal section	99.1 ± 1.5	95.7 ± 3.2	97.1 ± 2.5	94.9 ± 4.9	97.4 ± 1.7	97.1 ± 2.4	95.6 ± 1.7
	rIPC with atropine	83.6 ± 2.1	82.7 ± 8.6	82.7 ± 5.4	79.3 ± 0.1	81.1 ± 3.9	80.1 ± 3.7	79.6 ± 3.7
	rIPC with F and S section	92.1 ± 3.3	92.1 ± 2.8	90.6 ± 2.3	90.7 ± 3.5	91.7 ± 3.2	89.6 ± 2.9	89.6 ± 3.1
	rIPC with spinal cord section	85.9 ± 8.5	84.5 ± 8.4	85.1 ± 9.1	85.1 ± 9.4	84.5 ± 8.8	83.2 ± 8.6	80.7 ± 9.6

Abbreviations: F, femoral nerve; Isch., ischaemia; Rep., reperfusion; rIPC, remote ischaemic preconditioning; and S, sciatic nerve. The numbers in parentheses indicate the number of preconditioning cycles. *$P < 0.05$ *versus* baseline.

Afferent neural pathway

Figure 2 illustrates the infarct size after 30 min of global no-flow ischaemia and 180 min of reperfusion expressed as a percentage of the total left ventricular area. An infarct size of 40.8 ± 3.1% was obtained in the non-rIPC group, whereas in rIPC group the infarct size decreased to 16.4 ± 3.5% ($P < 0.05$ *versus* non-rIPC group). There was a trend for attenuation of the rIPC protection that was not statistically significant in the group with sectioned femoral and sciatic nerves (26.1 ± 5.2%). Interestingly, spinal cord section before starting the rIPC protocol completely abolished the effect of rIPC on myocardial infarction (43.8 ± 4.7%; $P < 0.05$ *versus* rIPC group).

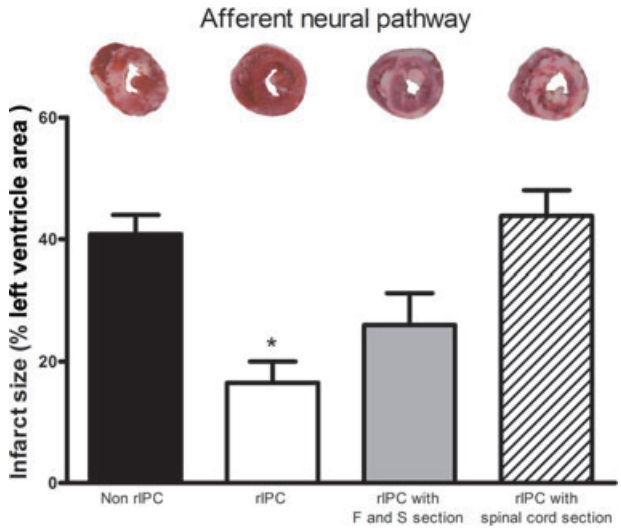

Figure 2. Infarct size expressed as a percentage of the total left ventricular area
Infarct size decreased significantly with remote ischaemic preconditioning (rIPC). The spinal cord section completely abolished the beneficial effect of rIPC. Abbreviations: F, femoral nerve; and S, sciatic nerve. *$P < 0.05$ *versus* non-rIPC and rIPC with spinal cord section.

Changes were seen in the LVDP (Fig. 3*A*) and LVEDP (Fig. 3*B*) at 30 min of reperfusion. In the non-rIPC group, LVDP recovered to 28.1 ± 3.4 mmHg at 30 min of reperfusion. In the rIPC group, LVDP reached 53.1 ± 5.8 mmHg ($P < 0.05$ *versus* non-rIPC group) at 30 min of reperfusion. The rIPC-induced improvement in systolic cardiac function (LVDP) was still observed after sectioning the femoral and sciatic nerves; however, sectioning of the spinal cord significantly attenuated the beneficial effect of rIPC, with LVDP reaching a value of 39.6 ± 12.1 mmHg at 30 min of reperfusion.

In the non-rIPC group, the LVEDP increased to 75.8 ± 4.8 mmHg at 30 min of reperfusion. Remote ischaemic preconditioning attenuated the increase of LVEDP, which reached a value of 32.4 ± 6.2 mmHg at 30 min of reperfusion ($P < 0.05$). This effect was abolished by spinal cord section but was not affected by sectioning the femoral and sciatic nerves (Fig. 3).

Efferent neural pathway

Figure 4 illustrates the infarct size after 30 min of global no-flow ischaemia and 180 min of reperfusion expressed as a percentage of the total left ventricular area. The vagal section and the administration of atropine each abolished

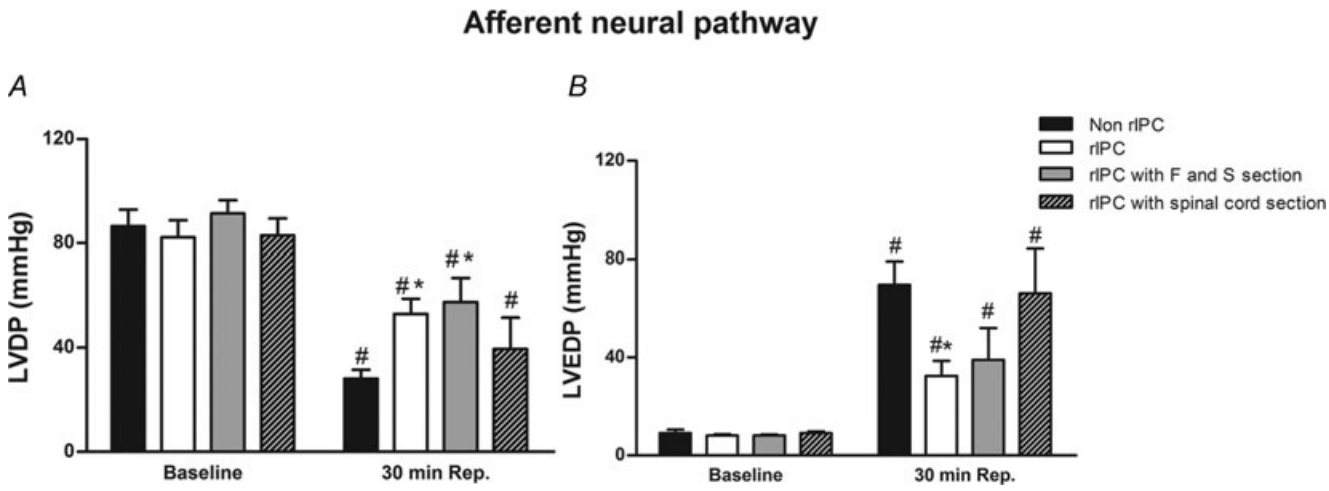

Figure 3. Left ventricular developed pressure (LVDP; *A*) and ventricular end-diastolic pressure (LVEDP; *B*) in the four studied groups
Remote ischaemic preconditioning improves the recovery of postischaemic systolic and diastolic ventricular function. Abbreviation: Rep., reperfusion. $^{*}P < 0.05$ *versus* non-rIPC; and $\#P < 0.05$ *versus* baseline.

the beneficial effect of rIPC, leading to an infarct size of 43.2 ± 4.8 and $37.7 \pm 3.1\%$, respectively ($P < 0.05$ *versus* the rIPC group). Interestingly, like rIPC, vagal stimulation decreased the infarct size to $15.2 \pm 4.7\%$ ($P < 0.05$ *versus* the non-rIPC group).

It is known that a decrease in heart rate can modify ischaemia–reperfusion injury. In order to dismiss this possibility, the rabbit hearts were paced during electrical stimulation of the vagus nerve. The results showed that vagal stimulation significantly decreases the infarct size to $17.2 \pm 5.3\%$ ($P < 0.05$ *versus* the non-rIPC group) independently of the heart rate.

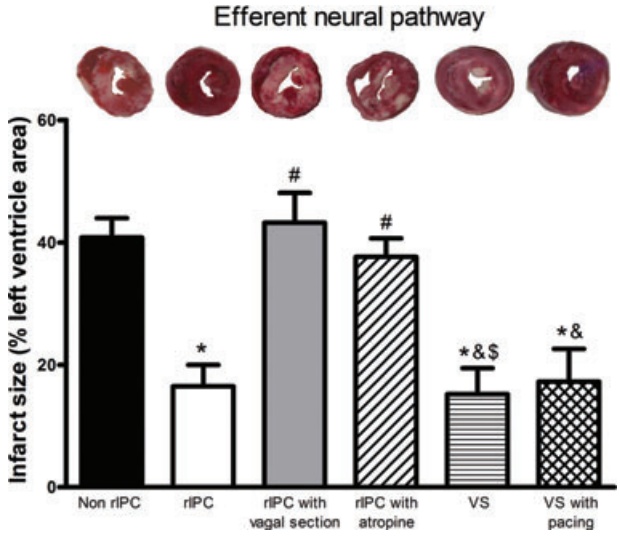

Figure 4. Infarct size expressed as a percentage of the total left ventricular area
Infarct size decreased significantly with rIPC and vagal stimulation (VS). The administration of atropine and vagal section each abolished the cardioprotective effect of rIPC. $^{*}P < 0.05$ *versus* non-rIPC; $\#P < 0.05$ *versus* rIPC; $\&P < 0.05$ *versus* rIPC with vagal section; and $\$P < 0.05$ *versus* rIPC with atropine.

Figure 5 illustrates changes in LVDP (Fig. 5*A*) and LVEDP (Fig. 5*B*) at baseline and at 30 min of reperfusion. As mentioned, in the rIPC group the LVDP reached a value of 53.1 ± 5.8 mmHg ($P < 0.05$ *versus* the non-rIPC group) at 30 min of reperfusion. Administration of atropine or sectioning the vagus nerves each abolished the beneficial effect of rIPC (LVDP of 39.1 ± 9.9 and 35.2 ± 10.1 mmHg, respectively). However, vagal stimulation, with or without pacing, mimicked the rIPC mechanism, with LVDP reaching a value of 63.1 ± 2.5 and 63.5 ± 4.6 mmHg, respectively. As shown, the LVEDP increased significantly after ischaemia–reperfusion in the non-rIPC group (75.8 ± 4.8 mmHg). This diastolic dysfunction was significantly attenuated in the rIPC group (LVEDP of 32.4 ± 6.2 mmHg). Vagal stimulation, with or without pacing, attenuated the increase of LVEDP, which reached a value of 15.3 ± 4.2 and 23.9 ± 7.7 mmHg, respectively, at 30 min of reperfusion ($P < 0.05$). Finally, the administration of atropine and the vagus section each abolished the beneficial effect of rIPC (LVEDP of 74.1 ± 19.1 and 63.5 ± 20.4 mmHg, respectively).

Discussion

The findings of this study confirm the hypothesis of the existence of a neural pathway in the rIPC mechanism and demonstrate that the cardioprotective signal reaches the heart through the vagus nerve (efferent pathway).

The administration of atropine abolished the protection, suggesting the participation of muscarinic receptors, which would be responsible for preconditioning the heart.

Previous studies have demonstrated that a neural pathway may be involved in the cardioprotection elicited by remote preconditioning of a non-cardiac organ. Ding *et al.* (2001) demonstrated that renal nerve section abolishes the cardioprotective effect induced by a preconditioning stimulus of renal ischaemia, providing strong supportive evidence of a neural pathway. These authors reported that during the renal preconditioning stimulus the renal afferent nerve discharge increased and that this enhanced neural activity could be abolished by a non-selective adenosine receptor blocker. Other authors (Lim *et al.* 2010) showed that rIPC induced by brief episodes of ischaemia and reperfusion applied to the limb requires both neural and humoral pathways to limit myocardial infarct size. Our results disagree in part with those published by Lim *et al.* (2010), because we found that after sectioning of the femoral and sciatic nerves there was a trend for attenuation of the rIPC protection that was not statistically significant. However, it is noteworthy that both femoral nerve section and sciatic nerve section probably did not produce a complete hindlimb deafferentation. We therefore performed another experiment with sectioning of the spinal cord (at T9–T10) in order to induce complete hindlimb deafferentation (Greenaway *et al.* 2001). This experiment allowed us to block the protective effect of rIPC completely, demonstrating that hindlimb rIPC involves an afferent neural pathway.

In another study, Jones *et al.* (2009) postulated that nociceptive stimulation of sensory nerves in the skin triggers a neurogenic signal that is transmitted via nerve fibres, causing the activation of a dorsal root reflex that also activates the spinal nerves higher in the spinal cord, ultimately leading to the activation of the cardiac sympathetic nervous system. We agree with that study, at least regarding the existence of a neural pathway. However, in contrast to Jones *et al.* (2009), we demonstrated involvement of the vagus nerve (efferent neural pathway) and muscarinic receptors in the rIPC cardioprotective mechanism, which suggests participation of the parasympathetic nervous system. It should be noted that in the present study we have not investigated the involvement of the sympathetic nervous system; therefore, it cannot be ruled out. Furthermore, the preconditioning stimulus was different; Jones *et al.* (2009) used a traumatic preconditioning stimulus, whereas we studied the effect of an ischaemic preconditioning stimulus. The possible differences between species (mouse *versus* rabbit) should also be considered.

In the present study, the rIPC and vagal stimulation decreased heart rate, thereby providing evidence for an increase in vagal tone. It is therefore possible that rIPC and vagal stimulation induced protection through a bradycardic effect, improving the relationship between myocardial oxygen supply and demand secondary to a favourable redistribution of the coronary flow. In order to rule out the role of the heart rate in the myocardial protection, we performed a set of experiments in which the heart rate was held constant by pacing during the vagal stimulation protocol. We found that vagal stimulation significantly reduced infarct size independently of the heart rate. These findings coincide with the work of Katare *et al.* (2009), in which it is shown that vagal stimulation significantly reduces the myocardial infarct size irrespective of the heart rate, thus confirming that

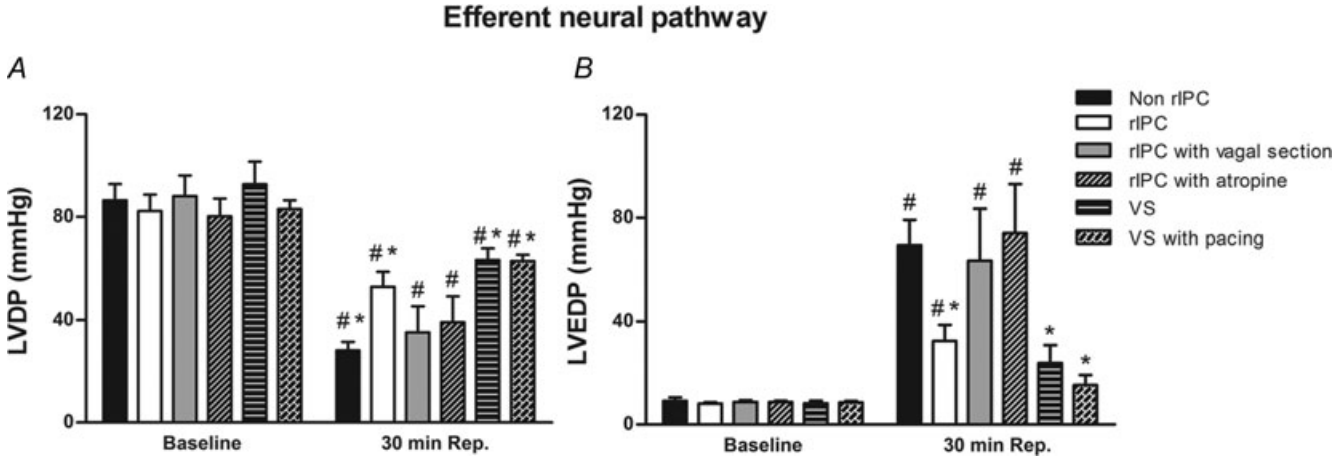

Figure 5. Left ventricular developed pressure (LVDP; *A*) and ventricular end-diastolic pressure (LVEDP; *B*) in the six studied groups
Remote ischaemic preconditioning and vagal stimulation (VS) with or without pacing improved the recovery of postischaemic systolic and diastolic ventricular function. $^{*}P < 0.05$ *versus* non-rIPC; and $\#P < 0.05$ *versus* baseline.

the protective effects induced by vagal stimulation are independent of its bradycardic effect. However, other authors have shown that an increase in the metabolic demand of the myocardium induced by tachycardia, in the absence of ischaemia, decreases the infarct size (Domenech *et al.* 1998).

On the contrary, it is known that the autonomic nervous system participates in the physiological and pathophysiological regulation of the mammalian heart. This regulation is established by sympathetic and parasympathetic cardiac nerves (Janes *et al.* 1986). In addition, there is evidence that neurons in the nucleus ambiguous and dorsal vagal nucleus innervate different subpopulations of intrinsic cardiac postganglionic neurons (Cheng *et al.* 2004). In particular, the autonomic nervous system, via the vagus nerve, has been implicated in phenomena of myocardial protection.

In this context we have recently described (Buchholz *et al.* 2012*a*,*b*) that vagal efferent stimulation, in certain conditions, coactivates the sympathetic nervous system together with an increase in plasma levels of catecholamines and myocardial consumption of oxygen, which, *in vivo*, increase the infarct size. However, when the vagus nerve is stimulated and the heart is then isolated, the beneficial effects of vagal stimulation are evidenced. In this sense, it is important to mention that in the present study myocardial ischaemia–reperfusion was induced *in vitro*, because our main objective was to study the myocardial infarction and ventricular function in the absence of extracardiac regulatory systems. Additionally, this model allowed us to exclude any effects of changes in heart rate and loading conditions.

As mentioned above, efferent vagal stimulation induces an increased release of acetylcholine, which could be one of the beneficial mechanisms of vagal stimulation in the context of myocardial ischaemia (Kawada *et al.* 2001). In the heart, acetylcholine is the principal neurotransmitter of the postganglionic fibres of the parasympathetic efferent pathways, and binding to the muscarinic receptors generates intracellular signals mediated by the G-protein-coupled receptor. Thus, in this work we demonstrated that vagal stimulation, performed before ischaemia, mimics the beneficial effect of rIPC on myocardial infarct size. Also, the bilateral vagotomy, which leads to the loss of the cardioprotection provided by rIPC, allows us to assert that rIPC could activate a neural pathway whereby the cardioprotective signal reaches the heart via the vagus nerve (efferent neural pathway). As increased vagal efferent nerve activity may lead to activation of nicotinic as well as muscarinic cholinergic receptors and release neuromodulators such as vasoactive intestinal polypeptide, we administered atropine during the rIPC protocol. The results show that the acetylcholine released by the vagus nerve endings induces the rIPC phenomenon by activating muscarinic receptors. Although the involvement of muscarinic receptors has been demonstrated in classic preconditioning (Oldenburg *et al.* 2002), the possible role of muscarinic receptors in rIPC has not been evaluated. Surprisingly, the administration of atropine did not increase the heart rate, as expected, perhaps due to interaction with the pentobarbital. However, it is clear that the dose used caused complete abolition of the protective effect of the rIPC.

In contrast, different studies have suggested that the reperfusion period should wash out a substance or humoral factor generated by the preconditioning ischaemia, which is then transported to the heart (Gho *et al.* 1996). Thus, recently Redington *et al.* (2012) showed that direct femoral nerve stimulation and C fibre stimulation by capsaicin are associated with the release of a blood-borne cardioprotective factor(s) similar to that associated with rIPC induced by transient limb ischaemia. Different experimental studies have attempted to identify the humoral factor involved in the rIPC mechanism and have suggested that endogenous substances such as adenosine (Pell *et al.* 1998), bradykinin (Schoemaker & van Heijningen, 2000), opioids (Patel *et al.* 2002) and endocannabinoids (Hajrasouliha *et al.* 2008) are released from the remote organ during the preconditioning ischaemia and are carried to the heart via the bloodstream, where they then activate intracellular pathways of cardioprotection. Despite this evidence, our data do not support this hypothesis. Although we cannot exclude the possible involvement of a humoral factor, it is clear that sectioning of the vagus nerve abolishes the rIPC-induced cardioprotection. In this sense, our results are in agreement with those obtained by Basalay *et al.* (2012), who demonstrated the involvement of the vagus nerve in the rIPC mechanism. In the present study, we have extended this knowledge because the administration of atropine abolished the protection, suggesting that acetylcholine released from vagal efferent nerves binds to muscarinic receptors on myocytes, which would be responsible for preconditioning the heart. Another difference from the study of Basalay *et al.* (2012) is that we detected myocardial protection secondary to rIPC, not only on infarct size but also on the postischaemic ventricular function.

The first description of rIPC (Przyklenk *et al.* 1993) shows that the transient ischaemia of one coronary artery territory reduces the effects of subsequent potentially lethal ischaemia in the territory of another coronary artery. It would be interesting to investigate whether the parasympathetic nervous system and the vagus nerve (efferent neural pathway) are involved in this intracardiac remote preconditioning, because this has not been studied in myocardial infarction, but only suggested in models of gastric injury (Brzozowski *et al.* 2004) to the best of our knowledge.

In conclusion, the present study demonstrates that the cardioprotection induced by rIPC involves an afferent neural signal, because the protection was lost after the spinal cord was sectioned. In addition, activation of efferent parasympathetic nerves and muscarinic cholinergic receptors is involved in rIPC, given that the cardioprotection was lost after sectioning of the vagus nerves and after administration of atropine. As rIPC could be an accessible procedure in clinical practice to render the heart resistant to ischaemia, further investigation is required to study cellular mechanisms in detail.

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Acknowledgements

This work was supported by a research grant from the National Agency for Scientific and Technological Promotion (ANPCyT, PICT 01071), the University of Buenos Aires (UBACyT M032), the National Council of Scientific and Technological Research (CONICET, PIP0197/2011) and Spanish Ministry of Science grant SAF 2008/03736. M. Donato and R. J. Gelpi are members of the National Council of Scientific and Technological Research (CONICET).