Am J Physiol Heart Circ Physiol 314: H1289–H1297, 2018.
First published April 6, 2018; doi:10.1152/ajpheart.00286.2017.

RESEARCH ARTICLE | *Integrative Cardiovascular Physiology and Pathophysiology*

Vagal stimulation mimics preconditioning and postconditioning of ischemic myocardium in mice by activating different protection mechanisms

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Submitted 26 May 2017; accepted in final form 28 February 2018

Buchholz B, Kelly J, Muñoz M, Bernatené EA, Méndez Diodati N, González Maglio DH, Dominici FP, Gelpi RJ. Vagal stimulation mimics preconditioning and postconditioning of ischemic myocardium in mice by activating different protection mechanisms. *Am J Physiol Heart Circ Physiol* 314: H1289–H1297, 2018. First published April 6, 2018; doi:10.1152/ajpheart.00286.2017.—Vagal stimulation (VS) during myocardial ischemia and reperfusion has beneficial effects. However, it is not known whether short-term VS applied before ischemia or at the onset of reperfusion protects the ischemic myocardium. This study was designed to determine whether short-term VS applied before ischemia or at the onset of reperfusion reduces myocardial infarct size (IS), mimicking classic preconditioning and postconditioning. A second objective was to study the participation of muscarinic and nicotinic receptors in the protection of both preischemic and reperfusion stimulation. FVB mice were subjected to 30 min of regional myocardial ischemia followed by 2 h of reperfusion without VS, with 10-min preischemic VS (pVS), or with VS during the first 10 min of reperfusion (rVS). pVS reduced IS, and this effect was abolished by atropine and wortmannin. rVS also reduced IS in a similar manner, and this effect was abolished by the α_7-nicotinic acetylcholine receptor blocker methyllycaconitine. pVS increased Akt and glycogen synthase kinase (GSK)-3β phosphorylation. No changes in Akt and GSK-3β phosphorylation were observed in rVS. Stimulation-mediated IS protection was abolished with the JAK2 blocker AG490. rVS did not modify IL-6 and IL-10 levels in the plasma or myocardium. Splenic denervation and splenectomy did not abolish the protective effect of rVS. In conclusion, pVS and rVS reduced IS by different mechanisms: pVS activated the Akt/GSK-3β muscarinic pathway, whereas rVS activated α_7-nicotinic acetylcholine receptors and JAK2, independently of the cholinergic anti-inflammatory pathway.

NEW & NOTEWORTHY Our data suggest, for the first time, that vagal stimulation applied briefly either before ischemia or at the beginning of reperfusion mimics classic preconditioning and postconditioning and reduces myocardial infarction, activating different mechanisms. We also infer an important role of α_7-nicotinic receptors for myocardial protection independent of the cholinergic anti-inflammatory pathway.

cholinergic anti-inflammatory pathway; muscarinic receptor; myocardial ischemia-reperfusion; vagal nerve stimulation; α_7-nicotinic acetylcholine receptor

INTRODUCTION

In recent years, vagal nerve electrostimulation [vagal stimulation (VS)] has become a promising cardiac protective strategy (14). Indeed, VS has shown beneficial results in both acute myocardial infarction, with (3) or without reperfusion (12), and heart failure (14). These effects were observed in different species such as mice (12), rats (11), rabbits (3, 4), pigs (23), and dogs (26). In most studies, the ability of VS to reduce infarct size was demonstrated with prolonged stimulation, starting at the beginning of ischemia and maintained during reperfusion (11, 22, 23). This limits its potential clinical applicability and hinders insights into the molecular mechanisms involved in this protection.

The implementation of protection strategies in the first "golden" minutes of reperfusion is of great importance for the reduction of myocardial injury, and it has a powerful potential therapeutic application to the revascularization of patients with myocardial infarction (19). Although continuous VS could provide significant cardioprotective effects, even when initiated later during ischemia, it is not clearly effective after reperfusion (22, 23). Acute revascularization of an ischemic territory induces inflammatory cell infiltrates and increases the expression of proinflammatory cytokines (9). Several studies have suggested that VS may reduce infarct size by modulating the systemic anti-inflammatory response via activation of α_7-nicotinic acetylcholine receptors (α_7nAChRs) of the splenic cholinergic anti-inflammatory pathway (5, 7, 18). However, it is not known whether short-term VS at the onset of reperfusion can also modulate the systemic inflammatory response by this pathway and reduce lethal myocardial injury. The activation of intracellular pathways induced by surface receptors could represent a faster mechanism of myocardial protection, as is the

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case in classic myocardial postconditioning. While α_7nAChR expression in the sarcolemma of cardiomyocytes has been recently demonstrated (8), it is unknown whether these receptors participate in myocardial protection as they do in the protection of the brain by activation of the JAK2/STAT3 pathway (15). We hypothesized that a brief but continuous VS applied before ischemia (pVS) or at the beginning of reperfusion (rVS) would similarly reduce infarct size in mice, by activating different mechanisms: pVS working via the muscarinic Akt/glycogen synthase kinase (GSK)-3β pathway and rVS activating the nicotinic JAK2 pathway, without effects on systemic inflammation.

Therefore, our first objective was to determine whether VS mimics ischemic preconditioning and postconditioning in mice when applied briefly and continuously before myocardial ischemia or at the onset of the reperfusion, respectively. A second goal was to determine whether muscarinic acetylcholine receptor (mAChRs) or α_7nAChRs are, indeed, involved in the mechanisms of protection. The third goal was to determine whether the cholinergic anti-inflammatory pathway is involved in the protection mediated by α_7nAChR activation via rVS.

MATERIALS AND METHODS

Experiments were performed on male FVB mice (3–4 mo old) in accordance with the "Guiding Principles in the Care and Use of Laboratory Animals" of the Council of the American Physiological Society and with approval from the Animal Care Committee of the Faculty of Medicine of the University of Buenos Aires (protocol no. 1857/15).

Experimental model. Animals were randomly assigned to different experimental groups. They were anesthetized with pentobarbital sodium (90 mg/kg ip) and ventilated with a mixture of room air and oxygen using a tidal volume of 0.2 to 0.3 ml and a respiratory rate of 110 cycles/min. Anesthesia was maintained by intermittent intraperitoneal injections of pentobarbital sodium administered depending on requirements determined by the presence of superficial reflexes (5–15 $mg \cdot kg^{-1} \cdot h^{-1}$).

Regional myocardial ischemia was induced by the occlusion of the left anterior descending coronary artery using 8-0 polypropylene suture. Rectal temperature was controlled continuously using a digital thermometer (TCAT-2AC Controller-Physitemp) and was kept at 37°C with an infrared lamp (3, 4).

Vagal stimulation. The right cervical vagus nerve was stimulated with a bipolar electrode (MLA270 Stimulation Cable, AD Instruments) connected to a neurostimulator (Grass S44 stimulator). Stimulation consisted of rectangular electrical pulses of 0.1 ms, 10 Hz, and a variable intensity adjusted to each animal to obtain a reduction between 10% and 15% of heart rate (HR) (3, 4, 11).

Experimental protocols. The experimental protocols are shown in Figs. 1*A*, 2*A*, and 3*A*. Mice were subjected to 30 min of myocardial ischemia followed by 120 min of reperfusion [ischemia-reperfusion (I/R) groups]. To study the effects of VS, the right vagus nerve was stimulated for 10 min before ischemia (pVS groups) or during the initial 10 min of reperfusion (rVS groups). To assess the involvement of cholinergic receptors, the muscarinic blocker atropine or the α_7nAChR blocker methyllycaconitine (MLA) was administered during or 20 min before VS, respectively. To evaluate the involvement of the phosphatidylinositol 3′-kinase (PI3K) pathway, wortmannin was administered 15 min before VS. To determine the participation of JAK2, the blocker AG490 was administered 20 min before the onset of rVS. To rule out the involvement of a systemic anti-inflammatory component in rVS protection, mice were subjected to a dorsal vagus nerve section at a subdiaphragmatic abdominal level (DV-s) before their I/R protocol, either without VS (I/R + DV-s group) or with VS (rVS + DV-s group). In the final group, a splenectomy was performed before the onset of ischemia and rVS (rVS + Sp group).

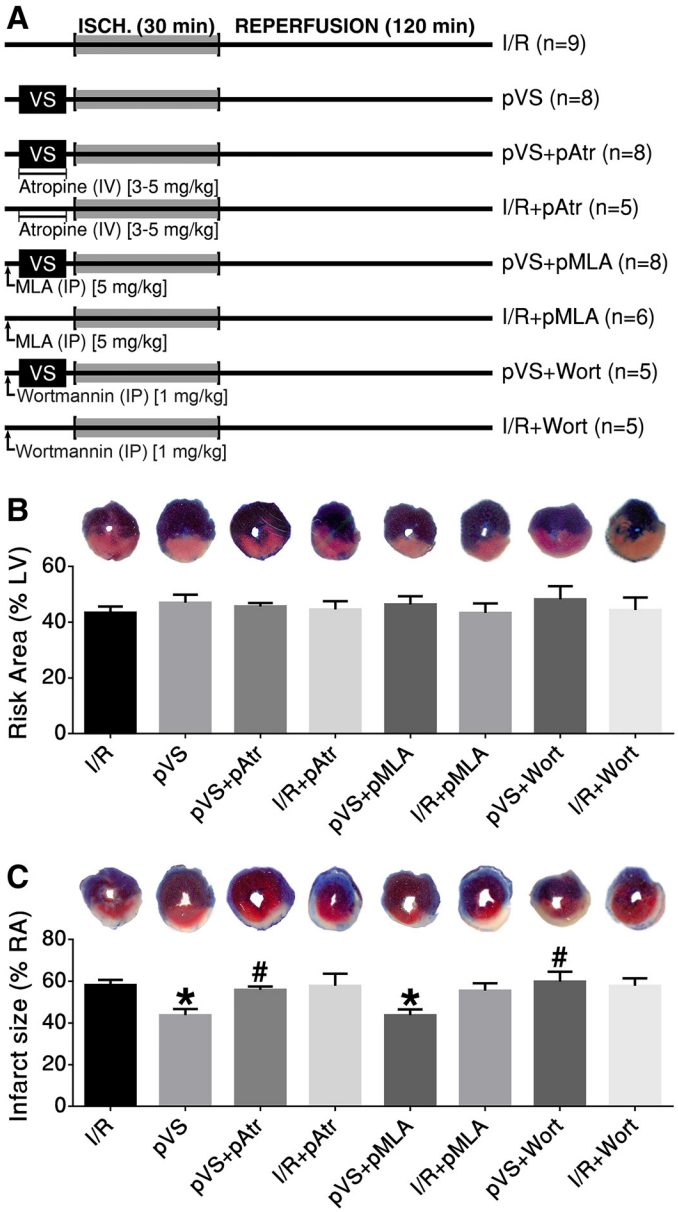

Fig. 1. Preischemic vagal stimulation (pVS) reduced infarct size through a muscarinic cholinergic-dependent mechanism. *A*: schematic design of experimental protocols. *B*: risk areas (RAs) were expressed as a percentage of the left ventricular (LV) area. There were no significant differences between groups. *C*: graph showing infarct sizes expressed as a percentage of the RA. pVS reduced myocardial infarct size. This myocardial protection was abolished by the muscarinic receptor antagonist atropine (Atr) and by the phosphatidylinositol 3′-kinase pathway blocker wortmannin (Wort) but not by the nicotinic receptor antagonist methyllycaconitine (MLA). Heart sections (*B* and *C*, *top*) were stained with Evans blue and 2,3,5-triphenyl-2H-tetrazolium chloride and are representative of RAs and infarct sizes of each group, respectively. VS, vagal stimulation; I/R, ischemia-reperfusion. $*P < 0.05$ vs. I/R; $\#P < 0.05$ vs. pVS.

Hemodynamic measurements. A fluid-filled catheter (1.5-Fr) was placed into the left ventricle (LV) through the right common carotid artery and connected to a pressure transducer (Deltram II, Utah Medical Products, Midvale, UT) to record LV pressure using a PowerLab 8/30 device (AD Instruments, Dunedin, New Zealand) and data acquisition and analysis software (LabChart version 8.0). Ventricular function was analyzed as previously described (3, 4).

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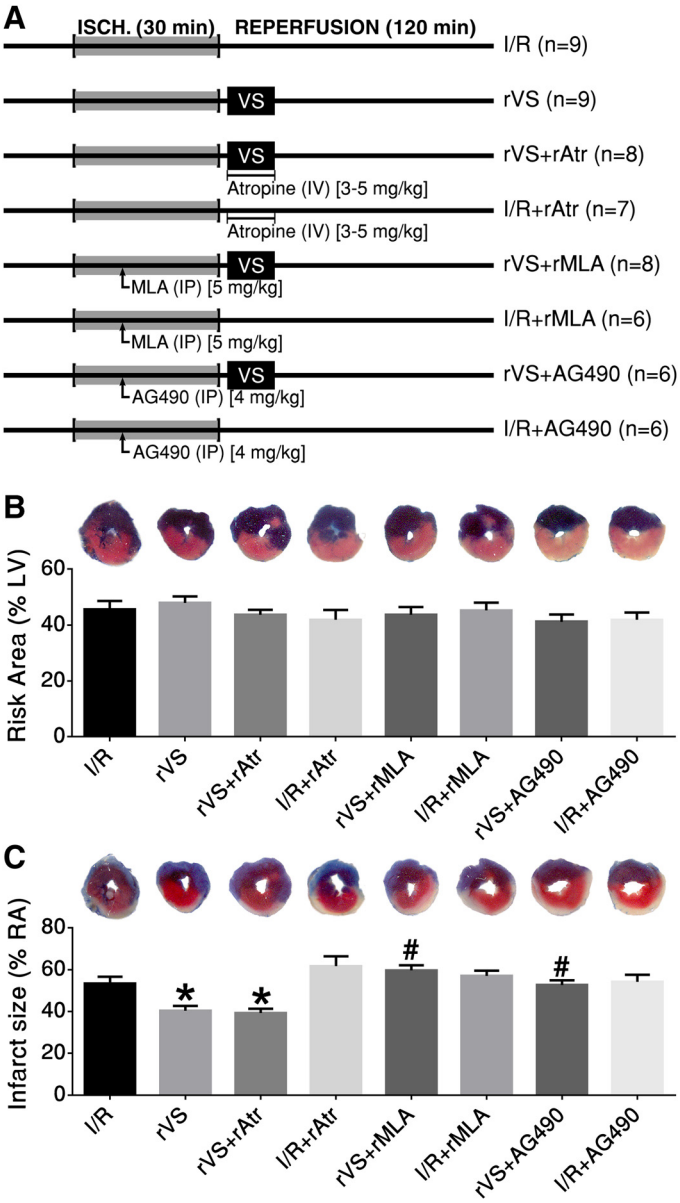

Fig. 2. Vagal stimulation (VS) during the first 10 min of reperfusion (rVS) reduced infarct size by a α_7-nicotinic acetylcholine receptor (nAChR) mechanism. *A*: schematic design of experimental protocols. *B*: risk areas (RAs) were expressed as a percentage of the left ventricular (LV) area. There were no significant differences between groups. *C*: graph showing infarct sizes expressed as a percentage of the RA. rVS reduced myocardial infarct size. This myocardial protection was abolished by the nicotinic receptor antagonist methyllycaconitine (MLA) but not by the muscarinic receptor antagonist atropine (Atr). Heart sections (*B* and *C*, *top*) were stained with Evans blue and triphenyltetrazolium chloride and are representative of RAs and infarct sizes of each group, respectively. I/R, ischemia-reperfusion. $*P < 0.05$ vs. I/R; $\#P < 0.05$ vs. rVS.

Measurement of infarct size. Two hours after reperfusion, mice were euthanized with an overdose of intraperitoneal pentobarbital sodium. The technical procedure and analysis of the area of risk and infarct size were performed as previously described (3, 4).

Tissue sample homogenization and cytokine quantification. Myocardial samples obtained 2 h after reperfusion from the risk area (ischemic tissue) and septum (nonischemic tissue) were homogenized in 50 mM Tris buffer (pH 8) containing 0.02% Triton X-100, 0.5 mg/ml EDTA, and 0.174 μg/ml PMSF using a tissue tearor homogenizer (PRO Scientific, Oxford, CT). Homogenates were then centrifuged for 10 min at 10,000 *g*, and the supernatants were stored at −70°C until use. Total protein concentration in the samples was measured using BCA protein assay reagent (Pierce Biotechnology, Rockford, IL).

Murine IL-6 and IL-10 from cardiac homogenates and plasma samples were quantified using noncompetitive ELISAs (BD Biosciences, San José, CA) following the manufacturer's instructions. Measurements were performed in sham, I/R, and rVS groups ($n = 7$ mice/group). Blood samples were obtained via venipuncture of the inferior vena cava (0.5−1 ml) 2 h after reperfusion, and they were anticoagulated by the addition of sodium heparin. Values were expressed as picograms per milligram of protein for homogenates and as picograms per milliliter for plasma samples.

Tissue solubilization and Western blot analysis. LV myocardial samples were taken from the risk area (ischemic tissue) after 15 min of reperfusion ($n = 5$ or 6 mice/group). Western blot analysis was performed as previously described (10) using the following primary antibodies: anti-Akt (1:2,000 dilution), anti-phospho-Akt (Ser^{473}, 1:1,000 dilution), anti-GSK-3β (1:3,000 dilution), anti-phospho-GSK-3β (Ser^{9}, 1:2,000 dilution), anti-phospho-ERK-1/2 (Thr^{202}/Tyr^{204}, 1:2,000 dilution), anti-ERK-1/2 (1:2,000 dilution), anti-JAK2 (Santa Cruz Biotechnology), and anti-phospho-JAK2 (Tyr^{1007}/Tyr^{1008}, 1:2,000). All reagents were obtained from Cell Signaling Technology (Danvers, MA) except for anti-JAK2 and anti-phospho-JAK2, which were obtained from Abcam (Cambridge, UK). Equal protein loading was confirmed by reprobing membranes with anti-GAPDH antibody (1:3,000 dilution, Abcam).

Statistical analysis. Data are presented as means ± SE. Comparisons among groups were carried out with one-way ANOVA followed by Bonferroni's post hoc test. Intragroup comparisons were analyzed by repeated-measures ANOVA. All statistical analyses were processed through GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA). Values of $P < 0.05$ were considered significant.

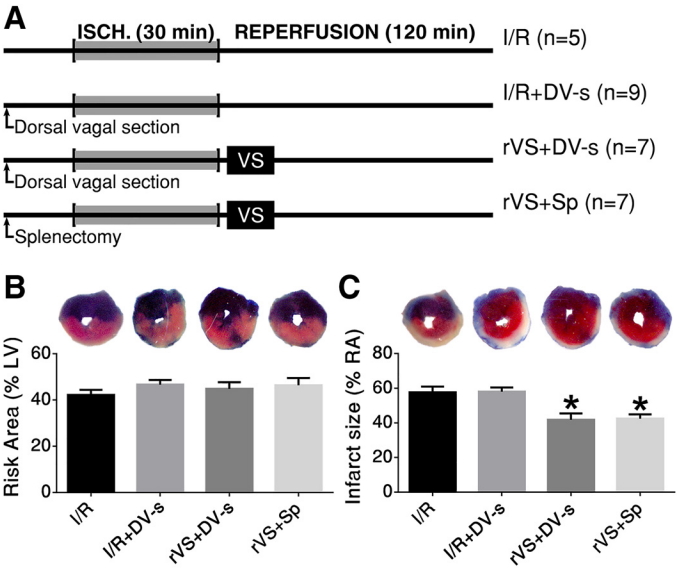

Fig. 3. The protection induced by vagal stimulation (VS) during the first 10 min of reperfusion (rVS) was not reversed by either dorsal vagal nerve section (DV-s) or splenectomy (Sp). *A*: schematic design of experimental protocols. *B*: risk area (RA) was expressed as a percentage of the left ventricular (LV) area. There were no significant differences between groups. *C*: graph showing infarct size expressed as a percentage of the RA. Heart sections (*B* and *C*, *top*) were stained with Evans blue and triphenyltetrazolium chloride and are representative of RA and infarct sizes of each group, respectively. $*P < 0.05$ vs. ischemia-reperfusion (I/R).

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RESULTS

Effects of preischemic VS on infarct size. VS reduced HR by 10 ± 2% in the pVS group, by 9 ± 1% in the pVS + MLA group, and by 10 ± 2% in the pVS + wortmannin group. This reduction in HR was abolished by atropine in the pVS + atropine group. Ischemia increased LV end-diastolic pressure, and pVS was not able to modify this deleterious effect (Table 1). The same effect on LV end-diastolic pressure was observed in all groups.

Figure 1 shows a reduction in infarct size in the pVS group (44 ± 3%) compared with the I/R group (58 ± 2%, $P < 0.05$). This protective effect of VS was reversed with administration of atropine (56 ± 2%, $P < 0.05$ vs. pVS) or wortmannin (60 ± 5%, $P < 0.05$ vs. pVS) but not with administration of the α_7nAChR blocker MLA (44 ± 3%, $P < 0.05$ vs. I/R). No significant differences were observed when the I/R group was compared with any of the groups without VS after administration of atropine (58 ± 6%), MLA (55 ± 4%), or wortmannin (58 ± 4%).

Effects of VS during reperfusion on infarct size. VS applied in reperfusion reduced HR by 9 ± 1%, 9 ± 1%, and 8 ± 1% in the rVS, rVS + MLA, and rVS + AG490 groups. This reduction in HR was blocked by atropine in the rVS + atropine group (Table 2).

Figure 2 shows that rVS reduced myocardial infarct size (40 ± 2%) compared with the I/R group (53 ± 3%, $P < 0.05$), and this beneficial effect was not reversed by atropine (39 ± 2.%). However, MLA and AG490 abolished the protection of VS during reperfusion (60 ± 2%, $P < 0.05$ vs. rVS; and 53 ± 2%, $P < 0.05$ vs. rVS, respectively). Administration of atropine, MLA, or AG490 in the groups subjected to ischemia and reperfusion without VS did not significantly modify infarct size compared with the I/R group (62 ± 5%, 57 ± 2%, and 54 ± 3%, respectively).

Relationship between the protective effects of VS on infarct size and the cholinergic anti-inflammatory pathway. Table 3 shows that rVS reduced HR by 10 ± 2% in the rVS + DV-s group and by 9 ± 2% in the rVS + Sp group.

The protective effect of rVS was not reversed by either abdominal vagus nerve section or splenectomy (42 ± 4% and 42 ± 3%, respectively, $P < 0.05$ vs. I/R, 58 ± 3%). Compared with the control group, laparotomy or vagus section (I/R + DV-s group) did not modify infarct size (58 ± 2%; Fig. 3).

Table 1. *LV function in experimental groups evaluated under basal conditions, during vagal stimulation, at preischemia, at the end of ischemia, and at different times of reperfusion*

	Basal	5 min/VS	Preischemia	30 min Ischemia	5 min Reperfusion	120 min Reperfusion
Heart rate, beats/min						
I/R	438 ± 11	441 ± 14	441 ± 12	463 ± 11	465 ± 8	456 ± 20
pVS	463 ± 12	414 ± 8	474 ± 9‡	473 ± 15	474 ± 16	513 ± 28
I/R + pAtr	440 ± 22	427 ± 18	444 ± 19	443 ± 4	456 ± 7	436 ± 14
pVS + pAtr	473 ± 14	505 ± 26	483 ± 14	493 ± 13	496 ± 15	503 ± 27
I/R + pMLA	435 ± 10	443 ± 10	441 ± 13	460 ± 19	462 ± 27	528 ± 20
pVS + pMLA	476 ± 16	435 ± 16	514 ± 18*‡	533 ± 24*†	545 ± 20*†	549 ± 16†
I/R + pWort	417 ± 3	446 ± 6	449 ± 22	499 ± 20	494 ± 28	555 ± 30
pVS + pWort	466 ± 15	430 ± 20	463 ± 12§	517 ± 15	526 ± 16†‡	579 ± 20
LV systolic pressure, mmHg						
I/R	94 ± 4	89 ± 4	91 ± 3	76 ± 3†	80 ± 3	87 ± 4
pVS	93 ± 4	92 ± 5	97 ± 7	80 ± 3	82 ± 3	81 ± 6
I/R + pAtr	109 ± 2	106 ± 4	103 ± 4	90 ± 4	93 ± 3	89 ± 3
pVS + pAtr	92 ± 5	84 ± 5	90 ± 5	79 ± 3	80 ± 4	74 ± 6†
I/R + pMLA	104 ± 4	106 ± 3	105 ± 4	79 ± 6	82 ± 5	97 ± 4
pVS + pMLA	107 ± 2	105 ± 4	97 ± 2	91 ± 3*†	93 ± 3*	94 ± 4†
I/R + pWort	100 ± 4	113 ± 9	120 ± 10	72 ± 5	73 ± 5	80 ± 8
pVS + pWort	112 ± 1	107 ± 6	103 ± 5	90 ± 7	92 ± 5	96 ± 6
LV end-diastolic pressure, mmHg						
I/R	3.6 ± 0.3	3.4 ± 0.3	3.5 ± 0.3	9.5 ± 1.1†	8.3 ± 1.1†	7.9 ± 1.3†
pVS	3.6 ± 0.5	5.4 ± 0.4*	4.5 ± 0.9	11.2 ± 1.6†	8.8 ± 1.2†	7.7 ± 2.0
I/R + pAtr	4.3 ± 0.4	4.2 ± 0.3	4.0 ± 0.3	11.5 ± 0.9†‡	8.1 ± 1.9	7.2 ± 1.6
pVS + pAtr	3.4 ± 0.4	3.6 ± 0.3	3.5 ± 0.4	10.9 ± 1.3†	9.1 ± 0.9†	5.8 ± 0.9§
I/R + pMLA	4.3 ± 0.8	4.6 ± 0.7	4.8 ± 0.9	14.1 ± 2.9	11.1 ± 2.3	6.7 ± 0.8
pVS + pMLA	3.9 ± 0.4	5.2 ± 0.6*	4.3 ± 0.3	9.6 ± 0.8†	9.0 ± 0.8†	8.3 ± 0.8†
I/R + pWort	5.1 ± 0.6	4.0 ± 0.4	4.3 ± 0.3	10.1 ± 3.7	5.5 ± 1.8	5.5 ± 0.3
pVS + pWort	2.4 ± 0.2	4.3 ± 0.4	2.9 ± 0.3	9.1 ± 1.5	6.6 ± 1.9	6.1 ± 1.2
$+dP/dt_{max}$, mmHg/s						
I/R	5909 ± 428	5747 ± 505	6023 ± 350	4745 ± 347	4564 ± 241	5016 ± 428
pVS	5222 ± 424	5099 ± 453	5789 ± 457	4382 ± 353	4462 ± 294	4472 ± 503
I/R + pAtr	7306 ± 245	7482 ± 420	7795 ± 577	6314 ± 260	6228 ± 207	6247 ± 363
pVS + pAtr	5404 ± 517	5121 ± 591	5481 ± 697	4708 ± 319	4490 ± 372	4116 ± 593†
I/R + pMLA	6730 ± 266	7092 ± 208	7203 ± 339	5398 ± 803	5350 ± 566	6940 ± 337
pVS + pMLA	7826 ± 402*	7551 ± 425	7284 ± 262	6734 ± 277*	6304 ± 377*†	6149 ± 415†
I/R + pWort	6649 ± 485	7451 ± 703	8022 ± 1237	4466 ± 516	4460 ± 598	5114 ± 462
pVS + pWort	7833 ± 122	6990 ± 211	6664 ± 398	6046 ± 223	5764 ± 175†	6196 ± 394

Values are expressed as means ± SE. LV, left ventricular; VS, vagal stimulation; I/R, ischemia-reperfusion; pVS, preischemic vagal stimulation; pAtr, preischemic atropine; pMLA, preischemic methyllycaconitine; pWort, preischemic wortmannin. *$P < 0.05$ vs. I/R; †$P < 0.05$ vs. basal; ‡$P < 0.05$ vs. 5 min/VS; §$P < 0.05$ vs. 30 min ischemia.

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Table 2. *LV function of experimental groups evaluated under basal conditions, at the end of ischemia, and at different times of reperfusion/VS*

	Basal	30 min Ischemia	5 min Reperfusion/VS	120 min Reperfusion
Heart rate, beats/min				
I/R	445 ± 4	464 ± 24	461 ± 16	465 ± 20
rVS	456 ± 11	474 ± 20	428 ± 13‡	517 ± 22†‡
I/R + rAtr	445 ± 31	437 ± 19	448 ± 9	432 ± 17
rVS + rAtr	467 ± 17	461 ± 26	470 ± 19	511 ± 19
I/R + rMLA	437 ± 10	466 ± 19	477 ± 23	516 ± 29†
rVS + rMLA	423 ± 11	483 ± 14†	440 ± 11	524 ± 23†
I/R + rAG490	422 ± 5	478 ± 13†	503 ± 17†	522 ± 14†‡
rVS + rAG490	458 ± 14	532 ± 31†	490 ± 32	578 ± 12*†
LV systolic pressure, mmHg				
I/R	101 ± 12	83 ± 10	87 ± 12	79 ± 1
rVS	98 ± 3	79 ± 2†	81 ± 3†	91 ± 3‡
I/R + rAtr	110 ± 6	80 ± 4†	85 ± 6†	84 ± 3†
rVS + rAtr	106 ± 4	73 ± 4†	74 ± 4†	87 ± 5†
I/R + rMLA	104 ± 1	84 ± 4†	87 ± 2†	93 ± 6
rVS + rMLA	96 ± 5	84 ± 4	82 ± 2	86 ± 5
I/R + rAG490	106 ± 1	84 ± 3†	90 ± 1†	82 ± 3†
rVS + rAG490	93 ± 4	81 ± 4†	77 ± 3†	92 ± 5
LV end-diastolic pressure, mmHg				
I/R	4.3 ± 1.2	9.1 ± 2.0†	8.2 ± 1.8†	8.5 ± 1.6†
rVS	4.2 ± 0.6	11.9 ± 1.4†	10.1 ± 1.3†	7.4 ± 1.2‡
I/R + rAtr	4.2 ± 0.3	11.5 ± 1.9†	10.9 ± 2.1†	8.6 ± 1.6
rVS + rAtr	4.1 ± 0.6	9.7 ± 1.0†	7.4 ± 1.6	8.6 ± 2.0†
I/R + rMLA	6.8 ± 1.8	12.1 ± 1.1†	10.5 ± 1.4	8.4 ± 1.2
rVS + rMLA	5.0 ± 1.0	13.1 ± 1.3†	9.4 ± 0.9†	7.8 ± 1.3‡
I/R + rAG490	4.4 ± 0.6	8.1 ± 1.6†	6.5 ± 1.1	5.6 ± 0.4
rVS + rAG490	4.2 ± 0.9	8.5 ± 1.1†	6.3 ± 0.6	4.9 ± 0.6‡
$+dP/dt_{max}$, mmHg/s				
I/R	7,427 ± 1,191	5,722 ± 830	5,762 ± 1,138	4,712 ± 379
rVS	5,958 ± 189	4,816 ± 346†	4,545 ± 425†	5,560 ± 321
I/R + rAtr	7,232 ± 422	5,339 ± 358	5,374 ± 353	5,633 ± 241
rVS + rAtr	7,089 ± 317	4,631 ± 615†	4,434 ± 435†	5,569 ± 593†
I/R + rMLA	6,930 ± 245	5,867 ± 552	5,809 ± 302	6,242 ± 643
rVS + rMLA	6,156 ± 462	5,805 ± 425	5,138 ± 295	5,535 ± 494
I/R + rAG490	7,565 ± 120	6,522 ± 341	6,706 ± 314*	5,716 ± 449†
rVS + rAG490	5,606 ± 337	6,044 ± 455	5,053 ± 316	6,610 ± 342

Values are expressed as means ± SE. LV, left ventricular; VS, vagal stimulation; I/R, ischemia-reperfusion; rVS, VS during the first 10 min of reperfusion; rAtr, reperfusion atropine; rMLA, reperfusion methyllycaconitine. *$P < 0.05$ vs. I/R; †$P < 0.05$ vs. basal; ‡$P < 0.05$ vs. 30 min ischemia.

There was an increase in IL-6 levels in the ischemic myocardium compared with the sham group (110 ± 14 vs. 28 ± 5 pg/mg tissue protein, respectively, $P < 0.05$; Fig. 4*A*). However, IL-6 levels were unaffected by rVS (128 ± 19 pg/mg). A similar result was observed in the nonischemic myocardium as well as in plasma samples. As shown in Fig. 4*B*, there was a decrease in the levels of the anti-inflammatory cytokine IL-10 at the end of reperfusion in the risk area of the I/R group compared with the sham group (8 ± 2 vs. 16 ± 4 pg/mg protein, respectively, $P < 0.05$). This change was again not reversed by rVS (9 ± 3 pg/mg). A similar result was observed in the nonischemic myocardium as well as in plasma samples.

Intracellular pathways involved in the protection conferred by preischemic and postischemic VS. Figure 5 shows an increase in the phosphorylation levels of Akt, GSK-3β, and ERK-1/2 after 30 min of ischemia and 15 min of reperfusion in the I/R group compared with the sham group ($P < 0.05$). Compared with the I/R group, pVS significantly increased Akt and GSK-3β phosphorylation levels ($P < 0.05$), and this effect was completely abolished with the administration of atropine. However, there were no significant differences in the phosphorylation levels of ERK-1/2 and JAK2 between the I/R, pVS, and pVS + atropine groups. The expression of these signaling proteins was not modified in any of the experimental conditions. Compared with the I/R group, rVS did not modify Akt, GSK-3β, or ERK-1/2 phosphorylation levels (Fig. 6). However, rVS increased JAK2 phosphorylation levels ($P < 0.05$ vs. sham). This effect was abolished in the presence of the α_7nAChR antagonist MLA (Fig. 6). Again, no differences were observed in the expression of protein levels of Akt, GSK-3β, ERK-1/2, and JAK2 between the different experimental groups.

DISCUSSION

The present study demonstrates that 10 min of VS applied before ischemia or at the onset of reperfusion similarly reduce myocardial infarct size, mimicking ischemic preconditioning and postconditioning, respectively. Importantly, the mechanisms involved in the reduction of infarct size seem to differ depending on the moment at which the vagus nerve is stimulated. In pVS, the protection appears to be mediated by activation of mAChRs and the intracellular PI3K/Akt and GSK-3β pathway. Conversely, with rVS, both α_7nAChRs and activation of the JAK2 intracellular pathway appeared to participate in the reduction of infarct size. This effect was independent of

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Table 3. *LV function of experimental groups evaluated under basal conditions, at the end of ischemia, and at different times of reperfusion/VS*

	Basal	30 min Ischemia	5 min Reperfusion/VS	120 min Reperfusion
Heart rate, beats/min				
I/R	461 ± 17	442 ± 28	457 ± 22	468 ± 15
I/R + DV-s	469 ± 12	507 ± 11	519 ± 13*	536 ± 23*
rVS + DV-s	500 ± 28	533 ± 22	476 ± 28	553 ± 21
rVS + Sp	446 ± 12	486 ± 10	442 ± 14	491 ± 31
LV systolic pressure, mmHg				
I/R	99 ± 6	81 ± 4	85 ± 3	83 ± 5
I/R + DV-s	95 ± 4	79 ± 3*	84 ± 3	84 ± 4*
rVS + DV-s	99 ± 4	81 ± 3*	78 ± 1*	77 ± 3*
rVS + Sp	101 ± 4	84 ± 5*	86 ± 4	90 ± 8
LV end-diastolic pressure, mmHg				
I/R	3.8 ± 1.2	11.5 ± 2.0*	9.5 ± 2.3*	6.1 ± 0.5
I/R + DV-s	3.5 ± 0.3	8.9 ± 1.6*	9.0 ± 1.9*	4.9 ± 0.4†
rVS + DV-s	4.1 ± 0.3	10.5 ± 1.0*	8 ± 1.6*	7.6 ± 1.7*
rVS + Sp	4.1 ± 0.6	9.0 ± 1.5*	10.2 ± 2.1*	5.8 ± 0.7†
$+dP/dt_{max}$, mmHg/s				
I/R	6,365 ± 497	5,449 ± 647	5,350 ± 513	5,088 ± 229
I/R + DV-s	6,020 ± 413	5,032 ± 235	4,886 ± 315*	5,159 ± 328
rVS + DV-s	6,448 ± 570	5,283 ± 513*	4,607 ± 233*	4,159 ± 455*
rVS + Sp	6,566 ± 268	5,584 ± 523	5,351 ± 528	5,858 ± 637

Values are expressed as means ± SE. LV, left ventricular; VS, vagal stimulation; I/R, ischemia-reperfusion; DV-s, dorsal vagus nerve section; rVS, VS during the first 10 min of reperfusion; Sp, splenectomy. *$P < 0.05$ vs. basal; †$P < 0.05$ vs. 30 min ischemia.

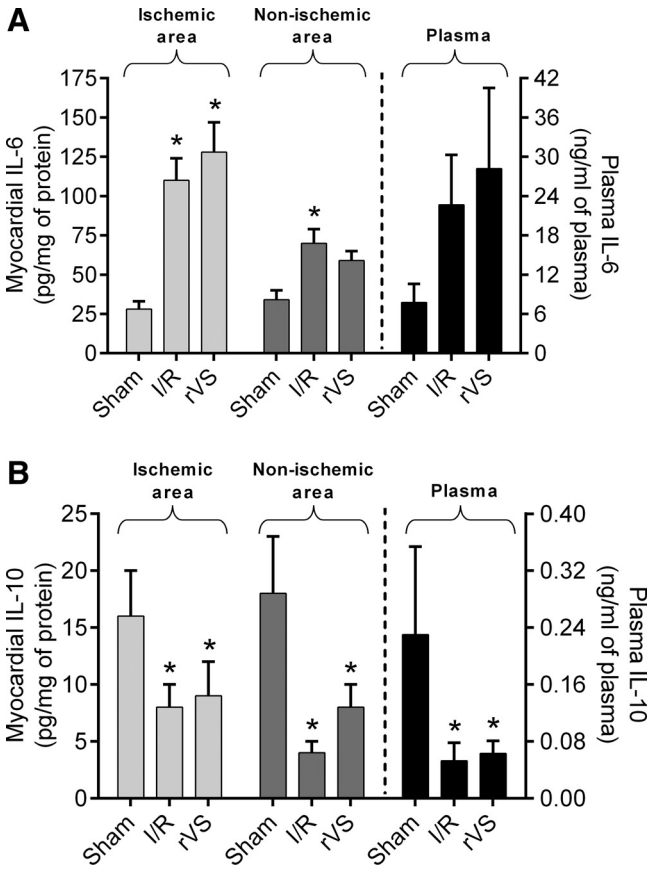

Fig. 4. Vagal stimulation (VS) during the first 10 min of reperfusion (rVS) does not affect IL-6 and IL-10 levels. Levels of cytokines in the ischemic myocardium, nonischemic myocardium (septum), and plasma were measured in sham, ischemia-reperfusion (I/R), and rVS groups 2 h after reperfusion. I/R increased IL-6 levels (*A*) and reduced IL-10 levels (*B*) in the myocardium and plasma, but rVS was not able to reverse these results (n = 7/group). *$P < 0.05$ vs. sham.

reductions of systemic and local inflammatory responses, suggesting local effects of these receptors on the myocardium.

The ability of acetylcholine to protect the myocardium by muscarinic activation of the ischemic preconditioning pathways was demonstrated in the isolated rabbit heart by Critz et al. (6) and also by our group (3). These findings strongly suggest that VS mimics preconditioning by modulating classical mAChR pathways and activation of Akt and GSK-3β.

It is difficult to determine whether the protection induced by pVS occurs during or after ischemia. Previous studies have observed that VS reduced infarct size in mice with myocardial ischemia without reperfusion (12), suggesting that this protection could occur throughout ischemia. In other studies, protective effects were also observed when VS was initiated before ischemia and continued during ischemia and part of the reperfusion in both in vivo (5, 27) and in vitro (11) experiments, both at the beginning (23) and during ischemia (22). However, the effects of VS applied selectively for a brief period of time in the first critical minutes of reperfusion are inconsistent. Intermittent VS applied right at the onset of reperfusion was reported to be unable to reduce infarct size in pigs (22). Conversely, another study in pigs showed that continuous VS applied in the last 5 min of ischemia and in the first 15 min of reperfusion decreased infarct size and no-reflow areas (25). This discordance could be ascribed, at least partially, to differences in the VS algorithm or intermittent VS applied at the onset of reperfusion versus continuous stimulation after the ischemic period, which could further complicate the interpretation of the effects of VS during reperfusion. In the present study, we demonstrate that in mice, 10 min of continuous VS at the onset of reperfusion reduces infarct size to a comparable extent as pVS. This discrepancy may be explained by differences in basal autonomic tone; our study was done in rodents characterized by a predominant sympathetic tone, in contrast to larger species such as pigs, where parasympathetic tone is predominant.

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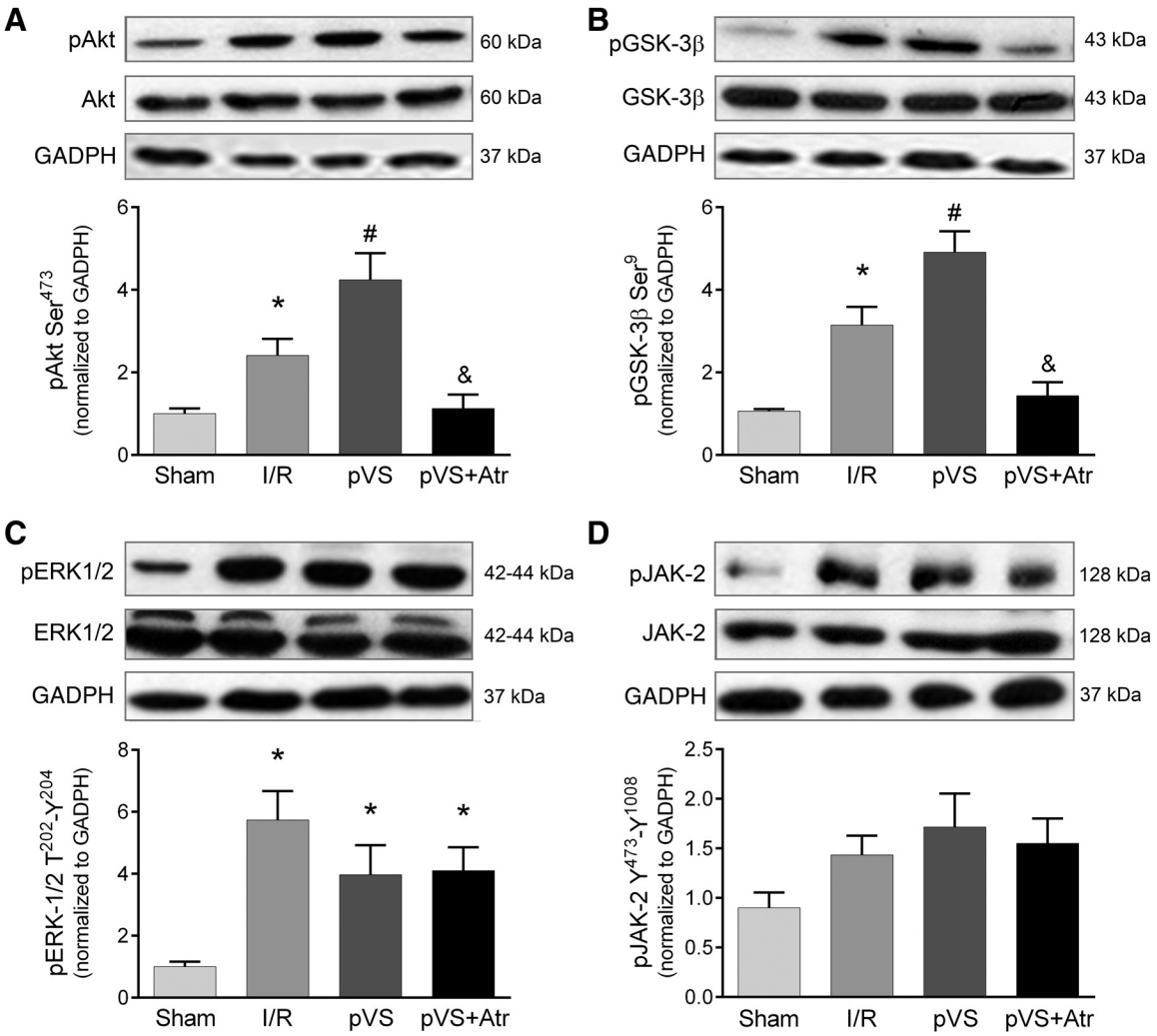

Fig. 5. Preischemic vagal stimulation (pVS) increases Akt and glycogen synthase kinase (GSK)-3β phosphorylation by a mechanism that involves muscarinic activation. Phosphorylation of Akt, GSK-3β, ERK-1/2, and JAK2 were evaluated in myocardial samples of sham, ischemia-reperfusion (I/R), pVS, and pVS + atropine (Atr) groups at 15 min of reperfusion. pVS increased Akt and GSK-3β phosphorylation but did not affect ERK-1/2 and JAK2 phosphorylation. No significant differences were observed in the expression of any of the studied proteins (n = 5 or 6/group). *$P < 0.05$ vs. sham; #$P < 0.05$ vs. sham and I/R; &$P < 0.05$ vs. pVS.

Bradycardia improves the relationship between the supply and demand of oxygen in the ventricular myocardium, favoring, in turn, the redistribution of blood flow in the ischemic canine heart (16). The reduction of experimental infarct size by VS-induced bradycardia has been demonstrated in a canine model of myocardial ischemia and reperfusion (1). Although we cannot rule out the possibility that decreased myocardial oxygen consumption secondary to bradycardia during VS contributes to the reduction in infarct size, a previous study has established that VS protects the myocardium independent of HR reduction (11). However, in the present experimental model of pVS, the stimulation phase was followed by a 5-min period without VS before the start of ischemia. VS could, indeed, achieve a direct effect on oxygen demand and coronary blood flow during reperfusion. However, we observed that blockade of α_7nAChRs reverses the protective effect on infarct size even when the VS-induced HR reduction is preserved.

The inflammatory response that occurs with reperfusion of ischemic myocardium is a potential target for therapeutic interventions (21). In a rat model, Calvillo et al. (5) observed that VS applied 5 min before ischemia until 5 min after the initiation of reperfusion reduced both infarct size and local and systemic inflammatory responses measured after 24 h of reperfusion. In pigs studied with a 20-min VS protocol, Uitterdijk et al. (25) found reduced macrophage and neutrophil myocardial infiltrates but did not detect lower levels of systemic TNF-α or IL-6 compared with a control group without VS. The ability of VS to modulate the systemic inflammatory response is based on the fact that vagal cholinergic activation reduces the cytokine production by macrophages and neutrophils in the spleen by the α_7nAChR pathway (5, 7, 18). However, in our study, VS for 10 min did not generate an anti-inflammatory response so as to protect the myocardium in the first "golden" minutes of reperfusion. The absence of a protective anti-inflammatory mechanism was confirmed because neither splenic parasympathetic denervation nor splenectomy eliminated the rVS protection. In addition, VS did not modify IL-6 and IL-10 levels in either plasma or myocardial tissue.

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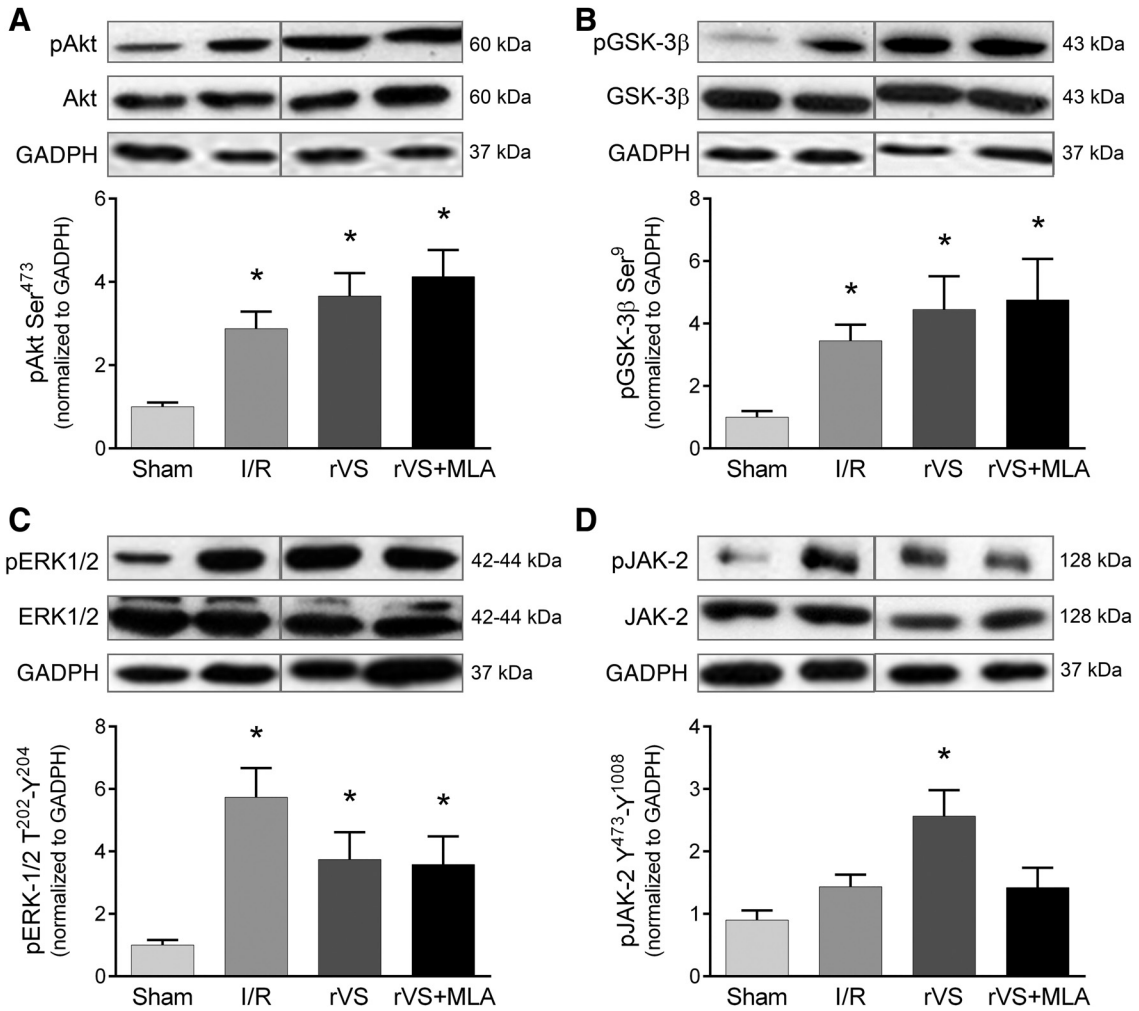

Fig. 6. Vagal stimulation (VS) during the first 10 min of reperfusion (rVS) does not significantly alter the phosphorylation of Akt, glycogen synthase kinase (GSK)-3β, and ERK-1/2 but does increase the phosphorylation of JAK2. Phosphorylation of Akt, GSK-3β, ERK-1/2, and JAK-2 was evaluated in myocardial samples of sham, ischemia-reperfusion (I/R), rVS, and rVS + methyllycaconitine (MLA) groups at 15 min of reperfusion. No significant differences were observed in the expression of any of the studied proteins (n = 5 or 6/group). *$P < 0.05$ vs. sham.

Although previous studies have confirmed that α_7nAChRs have essential roles in the cholinergic anti-inflammatory pathway (7, 27), our study demonstrates that α_7nAChRs contribute to myocardial protection independent of anti-inflammatory effects. The existence of α_7nAChRs in the heart was first reported in intracardiac parasympathetic neurons (20) and later in vascular smooth muscle cells and cardiomyocytes (8). In other tissues, there is significant evidence for a protective role of α_7nAChRs during ischemia and reperfusion, such as in the brain, where it occurs via the activation of the α_7nAChR-JAK2/STAT3 pathway (15). Interestingly, in the present study, we also inhibited the protective effect of VS in reperfusion by administration of a specific JAK2 blocker. The JAK2 pathway is important for intracellular protection during ischemic postconditioning (2, 13). Despite PI3K/Akt being one of the classic targets of JAK (17), our study revealed no increase in phosphorylation of Akt in rVS. Quite surprisingly, pVS activated Akt/GSK-3β through mAChRs, without any changes in JAK2 phosphorylation, which suggests an apparent dissociation between preischemic and postischemic protection pathways. Accordingly, the coexistence of two distinct signaling protective cascades during the trigger phase of ischemic preconditioning was observed: one used PI3K/Akt phosphorylation and the other involved the JAK/STAT3 pathway (24).

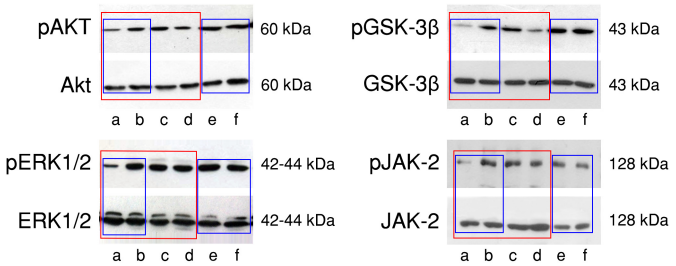

Fig. 7. Original uncropped and unadjusted blots corresponding to Figs. 5 and 6. Red boxes indicate the areas included in Fig. 5. Blue boxes indicate the areas included in Fig. 6. *Lane a*: sham; *lane b*: ischemia-reperfusion (I/R); *lane c*: preischemic vagal stimulation (pVS); *lane d*: pVS + preischemic atropine (pAtr); *lane e*: vagal stimulation during the first 10 min of reperfusion (rVS); *lane f*: rVS + preischemic methyllycaconitine (pMLA).

In conclusion, VS applied briefly and continuously before ischemia or at the onset of reperfusion reduced myocardial infarct size, mimicking classic preconditioning and postconditioning. However, the protection mechanisms appear to differ,

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since preischemic VS reduces infarct size by mAChR-mediated activation of the Akt/GSK-3β pathway and VS in reperfusion reduces infarct size by activation of the α_7nAChR and JAK2 pathway independent of local or systemic anti-inflammatory responses. The clinical relevance of these findings will depend on the application of VS in patients at the moment of coronary revascularization to reduce the reperfusion injury.

ACKNOWLEDGMENTS

Bruno Buchholz, Marina Muñoz, Eduardo A. Bernatené, Daniel H. González Maglio, Fernando P. Dominici, and Ricardo J. Gelpi are members of National Scientific and Technical Research Council of Argentina (Consejo Nacional de Investigaciones Científicas y Técnicas). Jazmín Kelly and Nahuel Méndez Diodati are doctoral fellows of Consejo Nacional de Investigaciones Científicas y Técnicas.

GRANTS

This work was supported by a research grant from Agencia Nacional de Promoción Científica y Tecnológica (PICT 2795), Universidad de Buenos Aires (20020130100557BA), and the National Scientific and Technical Research Council of Argentina (Consejo Nacional de Investigaciones Científicas y Técnicas, PIP 2014/012).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

B.B. and J.K. conceived and designed research; B.B., J.K., M.C.M., E.A.B., N.M.D., and D.H.G.M. performed experiments; B.B., J.K., M.C.M., D.H.G.M., and F.P.D. analyzed data; B.B., M.C.M., D.H.G.M., F.P.D., and R.J.G. interpreted results of experiments; B.B., J.K., E.A.B., and N.M.D. prepared figures; B.B. drafted manuscript; B.B., M.C.M., F.P.D., and R.J.G. edited and revised manuscript; R.J.G. approved final version of manuscript.

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