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Changes in the loading conditions induced by vagal stimulation modify the myocardial infarct size through sympathetic-parasympathetic interactions

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Abstract In a previous research, we described that vagal stimulation increases the infarct size by sympathetic co-activation. The aim of this study was to determine if hemodynamic changes secondary to the vagal stimulation are able to activate sympathetic compensatory neural reflexes, responsible for increasing the infarct size. A second goal was to determine if intermittent vagal stimulation avoids sympathetic activation and reduces infarct size by muscarinic activation of the Akt/glycogen synthase kinase 3 β (GSK-3β) pathway. Rabbits were subjected to 30 min of regional myocardial ischemia and 3 h of reperfusion without vagal stimulation, or the following protocols of right vagus nerve stimulation for 10 min before ischemia: (a) continuous vagal stimulation and (b) intermittent vagal stimulation (cycles of 10 s ON/50 s OFF). Continuous vagal stimulation increased the infarct size (70.7±4.3 %), even after right vagal section (68.6±4.1 %) compared with control group (52.0±3.7 %, $p<0.05$). Bilateral vagotomy, pacing, and esmolol abolished the deleterious effect, reaching an infarct size of 43.3±5.1, 43.5±2.1, and 46.0 ±4.6 % ($p<0.05$), respectively. Intermittent stimulation reduced the infarct size to 29.8±3.0 % ($p<0.05$ vs I/R). This effect was blocked with atropine (50.2±3.6 %, $p<0.05$). Continuous vagal stimulation induced bradycardia and increased the loading conditions and wall stretching of the atria. These changes provoked the co-activation reflex of the sympathetic nervous system, observed by the rise in plasmatic catecholamine levels, which increased the infarct size. Sympathetic co-activation was abolished by continuous vagal stimulation with constant heart rate or parasympathetic deafferentation. Intermittent vagal stimulation attenuated the sympathetic tone and reduced the infarct size by the muscarinic activation of the Akt pathway and GSK-3β inhibition. Continuous stimulation only phosphorylated Akt and GSK-3β when esmolol was administered.

Keywords Myocardial infarction · Autonomic nervous system · Vagal stimulation · Catecholamines

Introduction

The autonomic imbalance is deleterious for the myocardium, and this effect is associated with a worse outcome of cardiovascular diseases [4, 11, 30]. One of the strategies that have been used in recent times to modify this imbalance is electrical vagus nerve stimulation [31]. The beneficial effects of vagal stimulation have been demonstrated for acute myocardial ischemia as well as in heart failure in experimental animal models [14, 18, 36]. However, the mechanisms by which vagal stimulation produces its protective effects are not yet entirely known. One of the main mechanisms attributed to vagal stimulation would be its ability to antagonize the sympathetic system. In this sense, Li et al. [18] demonstrated that vagal stimulation increases the survival in rats with heart failure. This benefit is associated with a reduction of the catecholamine plasmatic levels. Calvillo et al. [3] showed that vagal stimulation performed during ischemia significantly reduces the infarct size by nicotinic receptor activation. Furthermore, our previous research unexpectedly showed that

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right vagus nerve stimulation, performed under certain conditions, increases the infarct size by sympathetic co-activation [1]. However, we did not demonstrate the mechanisms by which vagal stimulation can co-activate the sympathetic system and increase the infarct area. In the present study, we propose the hypothesis that the bradycardia produced by vagal stimulation prolongs the duration of the left ventricular diastolic filling, increasing the left ventricle end diastolic pressure and the atrial pressure. These hemodynamic changes would activate sympathetic compensatory neural reflexes that would be responsible for the increase in the infarct size. This would rely on the fact that it has been demonstrated that the stimulation of the right vagus nerve can depolarize the atrial stretch receptors by increasing atrial pressure [27, 37]. However, these modifications have not been studied in the context of ischemia/reperfusion injury and neither if this impact directly on the infarct size.

Moreover, multiple cellular mechanisms could contribute to the protection of vagal stimulation in ischemic heart disease, but the effects of sympathetic co-activation on these intracellular mechanisms were not demonstrated. Tong et al. [33] observed that ischemic preconditioning activates the phosphatidylinositol-3-kinase(PI3K) and inhibits glycogen synthase kinase 3 β (GSK-3β), reducing the infarct size. GSK-3β is an enzyme involved in the ischemia/reperfusion injury [22] and its inhibition by phosphorylation at the time of reperfusion has been shown to enhance cell survival and reduce the infarct size by activation of the mitochondrial ATP-sensitive K + channels (mKATP) before ischemia [32]. Although it has been demonstrated that muscarinic stimulation is capable of activating PI3K/Akt [17], the role of the Akt/GSK-3β pathway is not known in the context of sympathetic-parasympathetic interaction in pre-ischemic vagal stimulation.

Therefore, the first aim was to determine if hemodynamic modifications that accompany vagal stimulation are responsible for the co-activation of the sympathetic nervous system, and as a consequence, of the non-activation of the Akt/GSK-3β pathway and the increase in the infarct size. If the co-activation of the sympathetic nervous system is responsible for the deleterious effect of vagal stimulation, then performing a protocol of electrical stimulation without effects over this branch of the autonomic system could be beneficial. Thus, a second goal was to study if vagal stimulation, applied intermittently, avoids this co-activation and produces protective effects on the infarct size, activating the Akt/GSK-3β pathway.

Material and methods

Experimental model

The experiments were performed on 54 male New Zealand rabbits (2.0 to 2.6 kg). Animals were randomly assigned to different experimental groups. They were anesthetized with pentobarbital (35 mg/kg) and intubated for mechanical ventilation with a mixture of room air and oxygen. Anesthesia was maintained using pentobarbital, administered intravenously depending on requirements determined by the presence of corneal reflex (5–15 mg/kg/h).

Then, a left thoracotomy was performed, followed by pericardiectomy to expose the surface of the heart. Regional myocardial ischemia was induced by ligating a prominent branch of the left coronary artery using a curved needle and 5-0 polyester suture. The suture was adjusted by interposing a small plastic tube which, once the ischemic period is completed, can be easily released, allowing reperfusion. The presence of myocardial ischemia was confirmed by the appearance of regional pallor on the left ventricular surface. Rectal temperature was continuously measured using a digital thermometer, keeping it at 38–39 °C using a heating pad [1].

Vagal stimulation

The right vagus nerve was isolated at a cervical level to be stimulated using a silver bipolar electrode connected to a neurostimulator (Hugo Sachs Elektronik D7801). Rectangular electrical pulses of 0.1 ms, 10 Hz, and variable intensity adjusted to each animal to obtain a reduction between 10 and 20 % of heart rate (HR) were applied. This stimulation protocol has been widely used [1, 15, 35].

Experimental protocols (Fig. 1)

1 Ischemia/reperfusion (I/R) (n=10): After a stabilization period, myocardial ischemia was induced by left coronary artery ligation for 30 min followed by 180 min of reperfusion.
2 Vagus nerve stimulation (VNS) (n=7): After stabilization, the right vagus nerve was stimulated for 10 min. After 5 min of recovery, ischemia and reperfusion were performed similarly to the I/R group.
3 VNS/right vagus nerve section (RS) (n=11): The same protocol of the previous group was performed but the right vagus nerve was stimulated efferently after being sectioned at middle cervical level.
4 VNS/RS + pacing (PAC) (n=6): The right vagus nerve was stimulated efferently after being sectioned but the heart rate was kept constant with an external pacemaker during the whole period of vagal stimulation. Afterward, myocardial ischemia and reperfusion were performed similarly to the I/R group.
5 VNS/bilateral vagus nerve section (BS) (n=7): The protocol of the second group was repeated, but vagal stimulation was performed after the section of both vagus nerves at middle cervical level.

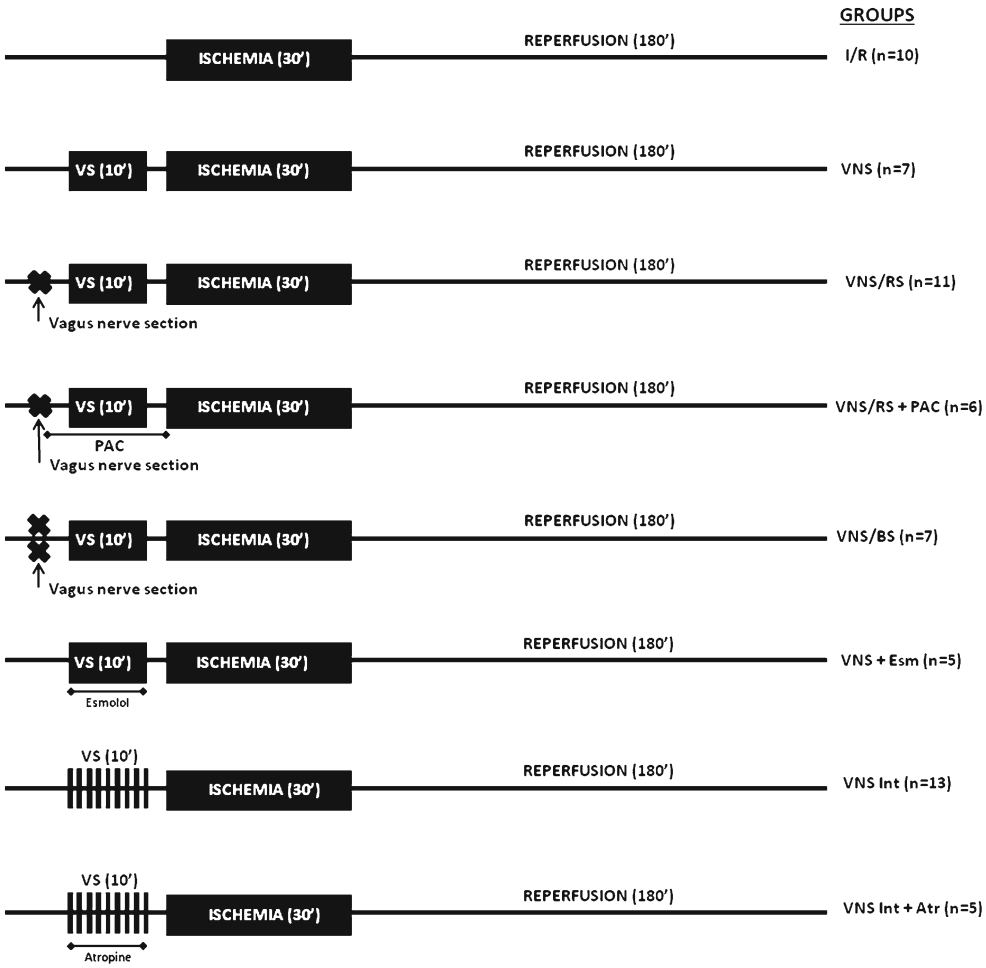


Fig. 1 Schematic design of experimental protocols. *VS* vagal stimulation, *I/R* ischemia/reperfusion group, *VNS* vagus nerve stimulation group, *RS* right vagus nerve section, *BS* bilateral vagus nerve section, *PAC* pacing, *Atr* atropine, *Esm* esmolol, *Int* intermittent

6 VNS + esmolol (Esm) (n=5): The same protocol of the second group was performed but during vagal stimulation, esmolol, a selective β1 adrenergic blocker, was administered intravenously. Esmolol was given as an initial dose of 3 mg/kg within 1 min, followed by a 10-min infusion at a rate of 500 μg/kg/min [9].

7- VNS Int (n=13): The right vagus nerve was stimulated without being sectioned but intermittently, with cycles of 10 seconds of stimulation (ON period) followed by 50 s without stimulation (OFF period), during 10 minutes before ischemia. Afterwards, myocardial ischemia and reperfusion were performed.

8- VNS Int + Atr (n=5): The same protocol used in the previous group was repeated, but atropine, a muscarinic blocker, was administered endovenously only during the vagal stimulation period (1.3 to 2.0 mg/kg) [1, 8].

Additionally, groups I/R, VNS, VNS Int, and VNS Int + Atr were repeated but the animals were sacrificed at 5 min of reperfusion, and left ventricular tissue samples were taken from the ischemic area for the Western blot studies (n=3–5, per group).

Hemodynamic measurements

A fluid-filled catheter was placed into the left ventricle (LV) through the right common carotid artery and connected to a pressure transducer (Deltram II, Utah Medical System, Midvale, UT, USA), which allowed recording left ventricular pressure. Ventricular function was analyzed considering the left ventricular systolic pressure (LVSP, mmHg), the maximal rate of rise of left ventricular pressure LV+dP/dt_{max} (mmHg/s), the +dP/dt_{max}/LVEDP index (/s) [7], and the left ventricular end-diastolic pressure (LVEDP, mmHg) [24]. The above mentioned variables were recorded in real time on a computer with a 16-Analog-Input Multifunction DAQ (National Instruments; NI PCI-6013) and software for this purpose. All hemodynamic variables were evaluated from the beginning of stabilization until the end of reperfusion. In another group of animals, the left atrial pressure was registered through a fluid-filled catheter connected to a pressure

transducer and the peak pressure of the "a" and "v" waves was measured.

Echocardiography

Rabbits were anesthetized with pentobarbital (35 mg/kg) and placed in dorsal decubitus. Echocardiography was performed using a 10-MHz linear ultrasound transducer (Acuson Sequoia C512). We took 2D-guided motion mode measurements of the left atrial area in apical four-chamber view. The measurements were performed at baseline, at 10 min of continuous vagal stimulation and in the "ON" period of intermittent vagal stimulation.

Measurement of infarct size

After the end of the reperfusion period, the animals were euthanized with an overdose of sodium pentobarbital and the coronary artery was religated. The ascending thoracic aorta was immediately cannulated and infused with a solution of Evans blue 1 % in order to determine the risk area (area not stained with Evans blue) in 4 mm sections. The risk area was expressed as a percentage of the total area of the left ventricular wall. These sections were then incubated in a solution of 2,3,5-triphenyltetrazolium chloride (TTC) 1 %, at pH 7.8 and 37 °C for 20 min and then fixed in 10 % formaldehyde for 24 h. Finally, digital images were obtained and analyzed using computerized planimetry (Image Analyzer, Image-Pro Plus, version 6.0). Infarct size was expressed as a percentage of the risk area [1].

Catecholamine assay

Samples of arterial blood were taken from the femoral artery before and after vagal stimulation to measure plasmatic norepinephrine and epinephrine in all groups (n=4–7, per group). Also, to observe the effects of continuous preischemic vagal stimulation on the behavior of plasmatic norepinephrine throughout the experiment, additional blood samples were taken at 30 min of ischemia and at 5, 60, and 180 min of reperfusion in the I/R and VNS groups. Norepinephrine and epinephrine were purified from plasma sampling using boric acid gel extraction as previously reported [10]. The mixture was shaken for 10 min, centrifuged at 10,000g for 1 min, and the gel was washed four times with distilled water. Then, norepinephrine and epinephrine were eluted from acid boric gel by shaking with 100 mL of 0.75 mol/L acetic acid for 1 min. Concentration of norepinephrine and epinephrine in the supernatant were measured by HPLC-EC using a Phenomenex Luna 5 mm, C18, 250×4.60-mm column and an LC-4C electrochemical detector with glassy carbon electrode (BAS, West Lafayette, IN, USA). The working electrode was set at +0.65 V with respect to an Ag/AgCl reference electrode. The mobile phase contained 0.76 mol/L $NaH_2PO_4 \cdot H_2O$, 0.5 mmol/L EDTA, 1.2 mmol/L 1-octane sulphonic acid, and 2 % methanol. Limit of quantification of norepinephrine and epinephrine was 50 and 70 pg/ml, respectively [1].

Western blot

Left ventricular myocardial samples were taken from the risk area, after 5 min of reperfusion to measure total and phosphorylated levels of Akt and GSK-3β protein in the I/R, VNS, VNS + Esm, VNS Int, and VNS Int + Atr groups (n=3–5, per group).

Left ventricular myocardial protein samples were separated by SDS-PAGE and transferred to a PVDF membrane. The samples were then incubated for 1 h at room temperature in a blocking buffer containing 5 % non-fat milk and 5 % BSA. Blots were then incubated overnight at 4 °C with rabbit monoclonal anti-Akt (1:1,000) (Cell Signaling Technology), rabbit monoclonal anti-Ser473 phospho-Akt (1:1,000) (Cell Signaling Technology), rabbit polyclonal anti-GSK-3β (1:1,000) (Santa Cruz Biotechnology Inc.), and rabbit monoclonal anti-Ser9phospho-GSK-3β (1:300) (Cell Signaling Technology). The blots were then incubated with goat anti-rabbit (1:15,000) (Cell Signaling Technology) at room temperature for 1 h. Afterward, they were rinsed with TBST. Blots were developed using the enhanced chemiluminescence method (Pierce) according to the manufacturer's instructions. Relative levels of Akt, phospho-Akt, GSK-3β and phospho-GSK-3β were quantified by densitometric analysis using Image Gauge 4.0 software (Fujifilm). Given that some protein proteolysis can occur during ischemia, the loading control was performed quantitating the total protein levels in the membrane dyed with Ponceau Red 0.1 %.

Statistical analysis

Data are expressed as mean±standard error of the mean (SEM). Inter-group comparisons were carried out using one-way analysis of variance (ANOVA) and the Bonferroni test. The data comparisons were not significant unless the corresponding p value was less than 0.05. The intra-group comparisons were analyzed by repeated measures ANOVA. Catecholamine data were compared by paired Student's t test. Statistical analysis and figures were performed using the GraphPad Prism 5.0 software.

Results

Table 1 shows the values of different hemodynamic and left ventricular function variables, evaluated at a baseline during vagal stimulation, pre-ischemia, at the end of the ischemia,

and at 60 and 120 min of reperfusion. There were no differences between groups at the beginning of the protocols. As expected, vagal stimulation reduced heart rate in approximately 20 % in the VNS, VNS/RS, VNS/BS, and VNS + Esm groups. This effect was reverted at 5 min of recovery (pre-ischemia). During ischemia, LVEDP significantly increases in all groups. This increase was reverted during reperfusion in all groups, except in VNS and VNS/RS + PAC. A significant reduction of LV+dP/dt_{max} was observed during vagal stimulation in the VNS + Esm group.

Figure 2a–c shows the changes on the heart rate and atrial pressure, measured previous to ischemia. The reduction of heart rate, induced by continuous vagal stimulation (Fig. 2a) in the VNS/RS group, was associated with an increase of the values of the "a" (Fig. 2b) and "v" waves of the left atrial pressure (Fig. 2c), and with an increase of the area of the left atrium (Fig. 2d) measured by echocardiography. In the VNS Int group, a transitory reduction of the heart rate during the 10 s of stimulation (ON period) was observed. This was associated with periodic and concordant changes on the "a" wave of the left atrial pressure, without significant modifications on the "v" wave. This lack of change in the "v" wave of atrial pressure during intermittent stimulation is consistent with the absence of changes in the area of the left atrium.

Figure 3 shows the LVEDP (Fig. 3a) and +dP/dt_{max}/LVEDP (Fig. 3b) during vagal stimulation. In the VNS/RS group, continuous vagal stimulation produced an increase of LVEDP and a reduction of the contractile state evaluated by the +dP/dt_{max}/LVEDP ratio. Changes on both variables were not observed when heart rate was kept constant using a pacemaker. The increase of LVEDP and the reduction of the contractility were only observed during the 10 s of stimulation in the VNS Int group. Changes in the heart rate, the LVEDP, and the "a" wave of the left atrial pressure in the group subject to intermittent vagal stimulation plotted during only 10 s of an "ON" stimulation period are shown in Fig. 3c. A significant increase of the pressures and a drop of the heart rate that returns to the baseline value during the 50 s of the "OFF" period are shown. The changes in the HR, left atrial pressure, LVEDP, and contractility, observed in the groups VNS and VNS/BS, were similar to the VNS/RS group (data not shown).

Also, there is a positive correlation between the changes in the left atrial pressure and the LVEDP, evaluated during vagal stimulation (R^2=0.836) (Fig 4a). The same way, LVEDP was positively correlated with the infarct size (R^2=0.443) (Fig. 4b).

The effects of vagal stimulation on the infarct size are shown in Fig. 5. Continuous vagal stimulation significantly increases the infarct size in the VNS group and the VNS/RS group to 70.7±4.3 and 68.6±4.1 %, respectively, compared with the I/R group (52.0±3.7 %, p<0.05) (b). This increase is not observed when both vagus nerves were sectioned (43.3±5.1 %), when the animals were paced (43.5±2.1 %), or with the adrenergic blockade by esmolol (46.0±4.6 %). This data suggests that vagal afferents and β1 adrenergic receptors could be involved in a deleterious reflex activation to the myocardium. On the contrary, intermittent vagal stimulation reduced the infarct size to a 29.8±3.0 % (p<0.05 vs I/R), despite that animals have both vagus nerves intact. Also, the blockade of muscarinic receptors with atropine abolished the intermittent vagal stimulation protective effect (50.2±3.6 %, p<0.05 vs VNS Int).

In Fig. 6, the behavior of plasmatic norepinephrine in groups I/R and VNS is shown. There are no significant changes neither in norepinephrine levels during ischemia nor in reperfusion, compared to baseline levels of the I/R group. Continuous vagal stimulation significantly increased the norepinephrine levels by 250 % (p<0.05 vs baseline). It was reduced at 5 min of recovery and remained near baseline levels during ischemia and reperfusion. Figure 7 shows the changes in the concentration of plasmatic norepinephrine and epinephrine at baseline conditions and at 10 min of vagal stimulation. Continuous vagal stimulation increases the norepinephrine 97.9±36.5 % and the epinephrine 27.5±9.2 % (p<0.05 vs baseline) levels in the VNS group. In the VNS/RS group, norepinephrine is increased a 33.6±17.1 % (p<0.05 vs baseline) and epinephrine 81.2±49.7 % (NS). Vagal stimulation in animals with bilateral vagotomy does not significantly modify the catecholamine plasmatic levels. On the contrary, continuous vagal stimulation performed in animals kept with a constant heart rate, reduces the levels of norepinephrine to 39.2±4.8 %, and epinephrine to 43.7±22.4 % (p<0.05 vs baseline). A similar effect was obtained with intermittent vagal stimulation in which norepinephrine is reduced to 62.9 ±12.4 % and epinephrine to 60.0±9.0 % (p<0.05 vs baseline).

Figure 8 shows the effects of vagal stimulation on the phosphorylation of Akt and GSK-3β enzymes, assessed at 5 min of reperfusion. Continuous vagal stimulation phosphorylates residue serine 473 of Akt and residue serine 9 of GSK-3β, only when the adrenergic blocker esmolol is administered. Conversely, intermittent stimulation induces phosphorylation of Akt and GSK-3β. These effects are blocked by the administration of atropine.

Discussion

In a previous study, we demonstrated that right vagus nerve pre-ischemic stimulation increases the myocardial infarct size by co-activation of the sympathetic nervous system [1].

The results of the present study increase this knowledge and strongly suggest that changes in the myocardial loading conditions, secondary to vagal stimulation, are the responsible mechanism for the increase of the infarct size. In detail, vagal stimulation produced the expected bradycardia, increasing the diastolic ventricular filling time and also the atrial and

Table 1 Hemodynamic and left ventricular function variables of the different groups studied, evaluated at basal conditions, during vagal stimulation, at pre-ischemia, at the end of ischemia, and at different times of reperfusion

	Groups	Baseline	Vagal stimulation	Pre-ischemia	30 min ischemia	60 min reperfusion	120 min reperfusion
HR (beat/min)	I/R	264±10	261±10	262±9	252±9	246±11	253±9
	VNS	285±11	230±10#†	280±10	274±10	258±7	256±9
	VNS/RS	279±5	222±5#	278±4	269±6	260±5	264±6
	VNS/BS	285±6	224±6#†	279±10	268±10	257±11*&	258±11*
	VNS/RS + PAC	258±15	294±18*	298±18*	273±14	263±13	275±15
	VNS + Esm	289±9	210±4#	266±6	284±10	272±12	265±13
	VNS Int	270±6	238±8#†	264±6	261±5	257±4	259±6
	VNS Int + Atr	289±7	280±12	291±9	283±7	277±7	270±14
LVSP (mmHg)	I/R	103±3	104±2	104±3	90±4$	84±4$	85±4$
	VNS	107±3	108±3	105±4	91±4	88±3$	87±3$
	VNS/RS	101±4	102±4	101±4	89±4$	82±4$	87±4$
	VNS/BS	107±5	104±5	104±5	94±5$	90±4$	92±3$
	VNS/RS + PAC	99±4	90±6	87±4	84±3	83±2	85±5
	VNS + Esm	106±5	95±8	102±8	93±5	93±5	90±6
	VNS Int	97±4	98±3	97±3	89±4	84±4$	81±4$
	VNS Int + Atr	91±5	91±3	94±4	81±3$	79±5	72±9$
LVEDP (mmHg)	I/R	2.1±0.4	2.0±0.3	2.1±0.4	8.9±1.5$	4.4±0.6 Δ	5.4±1.1$ Δ
	VNS	2.5±0.3	3.8±1.0	2.4±0.3	8.5±2.4*	4.1±1.8	4.9±2.3
	VNS/RS	2.0±0.2	3.5±0.5	2.3±0.3	7.8±1.2$	4.2±0.9 Δ	5.0±0.9* Δ
	VNS/BS	1.9±0.2	3.9±0.8	1.9±0.4	7.9±2.0$	4.4±1.1	3.7±0.8 Δ
	VNS/RS + PAC	2.3±0.3	2.0±0.3	1.7±0.2	4.5±1.1$	3.1±0.8	3.4±0.7
	VNS + Esm	1.7±0.3	4.4±0.3*	2.8±0.3	4.0±0.5*	1.6±0.3 Δ	2.0±0.4 Δ
	VNS Int	2.4±0.3	3.7±0.2	2.6±0.2	7.9±1.2$	5.3±1.4*&	4.0±0.6 Δ
	VNS Int + Atr	1.7±0.3	1.5±0.3	1.6±0.4	3.8±0.6$	1.9±0.3 Δ	2.2±0.5 Δ
LV+dP/dt$_{max}$ (mmHg/s)	I/R	5,938±474	6,156±432	6,200±389	4,400±406$	3,793±491$	3,801±436$
	VNS	6,868±610	6,028±571	6,429±701	4,945±430*	4,278±403*&	3,968±329$
	VNS/RS	6,717±522	5,649±456	6,620±456†	4,415±276$	3,369±210$	3,606±259$
	VNS/BS	5,954±508	5,565±364	5,937±469	4,776±429*&	3,903±308$	3,933±299$
	VNS/RS + PAC	5,417±526	4,153±383	4,318±364	4,159±368	3,563±339	3,524±341
	VNS + Esm	5,211±594	2,560±473* °	4,558±788	4,195±497	3,997±448	3,787±483
	VNS Int	5,749±332	5,369±348	5,432±315	4,472±259$	3,562±269$ Δ	3,274±235$ Δ
	VNS Int + Atr	5,464±355	4,436±242	4,497±235	3,722±287*	3,405±285*	2,987±518$

Values are means±SEM

HR heart rate, *LVSP* left ventricular systolic pressure, *LVEDP* left ventricular end-diastolic pressure, $+dP/dt_{max}$ maximum first derivative of left ventricular pressure

*$p<0.05$ vs baseline; #$p<0.05$ vs all points of the group; &$p<0.05$ vs pre-ischemia; $ $p<0.05$ vs baseline, vagal stimulation, and pre-ischemia; Δ $p<0.05$ vs 30 min ischemia; †$p<0.05$ vs VNS/RS + PAC; °$p<0.05$ vs I/R

ventricular volumes with the consequent increase of the respective pressures. These changes in the loading conditions produce the stretching of the ventricular and atrial wall, activating afferent vagal pathways that lead to sympathetic compensatory neural reflexes that would be responsible for the increase of the infarct size. Intermittent vagal stimulation does not increases pressures and loading conditions, and decreases the levels of plasmatic catecholamines, which suggest a reduction of the sympathetic tone [18] (Fig. 9).

Consistent with our data, it is known that the increase of the sympathetic system tone is deleterious, particularly in the context of cardiovascular diseases [5, 6]. However, we only observed an increase in plasmatic catecholamine levels prior to ischemia. With the aim of demonstrating that a brief and transient increase of sympathetic activity is capable of generating a more prolonged damage, we administered esmolol (an ultra-short action β adrenergic blocker) only during the period of pre-ischemic stimulation. The β adrenergic blockade abolished the deleterious effects of continuous vagal

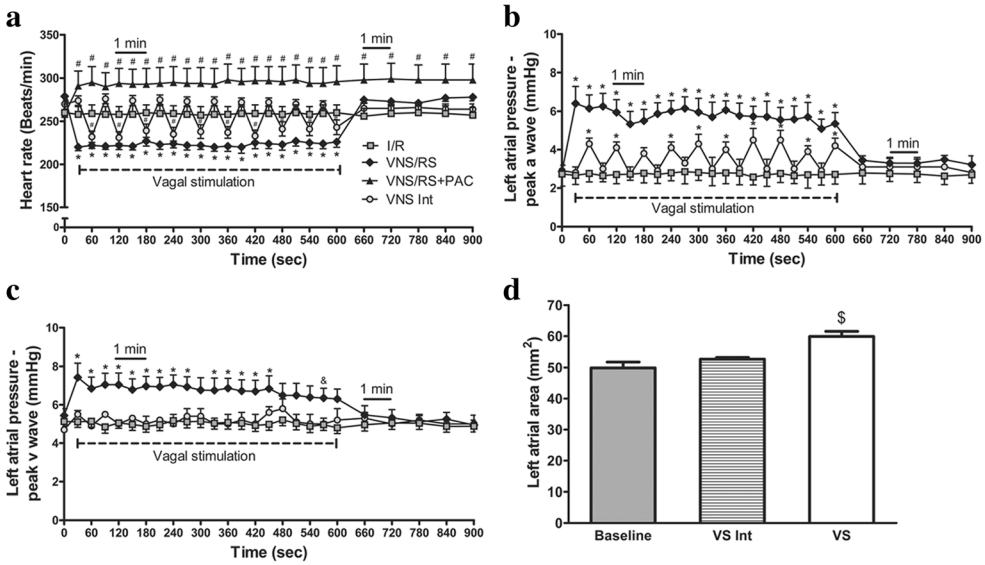


Fig. 2 Changes on the hemodynamic variables during the 10 min of vagal stimulation and the 5 min of recovery can be observed. Continuous vagal stimulation significantly reduced the heart rate, which recovered completely after 5 min without stimulation (**a**). In the group with pacing, heart rate was 13 % above the baseline level and was not reduced by vagus nerve stimulation. In the VNS Int group, heart rate was significantly reduced during the "ON" period and it was completely recovered in the "OFF" period. Continuous vagal stimulation generated a significant increase of the atrial pressure "a" wave (**b**). An intermittent behavior is observed in the VNS Int group. The atrial pressure "v" wave behaves similarly to the "a" wave in the group with continuous stimulation. However, no changes are observed in the VNS Int group (**c**). **d** An increase of the left atrial area during continuous vagal stimulation in the VNS/RS group measured by echocardiography is observed. On the contrary, no increase in the atrial area was observed during intermittent stimulation in the VNS Int group. *Min* minutes, *Sec* seconds, *VS* continuous vagal stimulation, *VS Int* intermittent vagal stimulation. *$p<0.05$ vs baseline and I/R; #$p<0.05$ vs baseline; &$p<0.05$ vs I/R; \$$p<0.05$ vs baseline and VS Int

stimulation. These results are consistent with our previous work, in which we have observed that the deleterious effect of continuous vagal stimulation on infarct size was completely abolished with the administration of a prolonged action β adrenergic receptor blocker, such as atenolol and the depletion of catecholamines with reserpine [1]. Furthermore, previously, we have also demonstrated that the sympathetic co-activation increases the infarct size and the myocardial oxygen

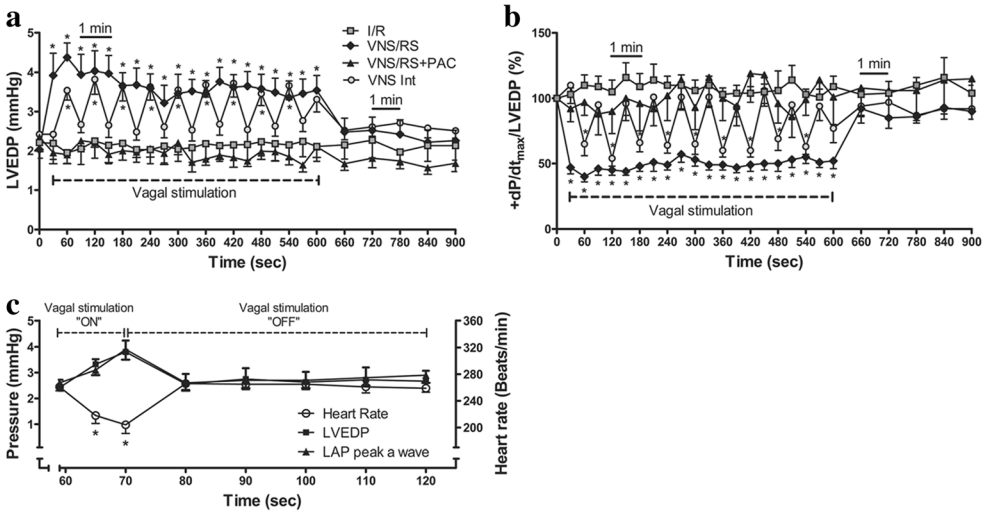


Fig. 3 The changes of LVEDP and the ratio +dP/dtmax/LVEDP are observed during the 10 min of stimulation and the 5 min of recovery. In the VNS/RS group, continuous vagal stimulation generated a significant increase of LVEDP that was recovered at the end of the stimulation (**a**). An intermittent behavior is observed in the VNS Int group. No significant changes are observed in the I/R and VNS/RS + PAC groups. A similar behavior is observed in contractility (**b**). **c** Hemodynamic modifications produced with intermittent vagal stimulation in a 1-min stimulation cycle are observed. During the "ON" period, a reduction of the heart rate at 5 and 10 s of stimulation is observed. This reduction of the heart rate is accompanied by an increase of LVEDP and the atrial pressure "a" wave. All variables are recovered completely and stay stable in the next 50 s of the period in which there is no stimulation (OFF). *LVEDP* left ventricular end diastolic pressure, *LAP* left atrial pressure, *Min* minutes, *Sec* seconds. *$p<0.05$ vs baseline and I/R

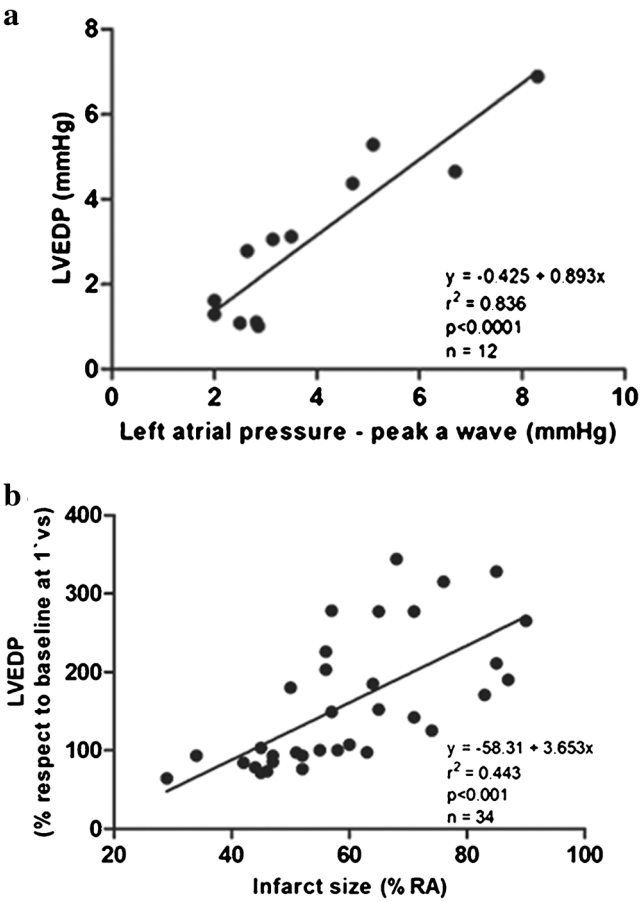


Fig. 4 **a** The lineal and positive correlation between LVEDP and the peak "a" wave of the left atrial pressure in the VNS/RS group is observed. **b** The correlation between the infarct size and the percentage increase of LVEDP in groups I/R, VNS/RS, and VNS Int. The values of LVEDP and left atrial pressure in both panels were measured 1 min after starting vagal stimulation. *LVEDP* left ventricular end diastolic pressure, *RA* risk area, *VS* vagal stimulation

consumption [1]. This association evidences the importance of the autonomic nervous system as a regulator of conservative oxygen reflexes at a cardiac level, similarly to what has been well described in other organs like the brain [29]. Taken together, our results strongly support the association between sympathetic stress and infarct size, although further studies to demonstrate more mechanisms could be necessary to the better comprehension of this complex phenomenon.

In the present study, the protection conferred by intermittent vagal stimulation could be associated with its capacity to reduce the catecholamine plasmatic levels. Interestingly, in the group with a constant heart rate where continuous stimulation was applied, the levels of catecholamines were reduced, but the infarct size was not modified in comparison with the group I/R. These data allows us to conclude that the deleterious effect of continuous vagal stimulation is produced by the co-activation of the sympathetic system as nervous reflexes. This would include vagal afferents, since the section of both vagus nerves avoids the increase of catecholamines and the infarct size. We also observe that the reduction of plasmatic catecholamines is not enough to confer protection. When vagal stimulation was performed at constant heart rate, plasmatic catecholamines significantly decreased; however, we did not observe a decrease in the infarct size similarly to intermittent stimulation. Therefore, it is possible that this protection is related to the capacity of vagal electrical stimulation of inducing the liberation of Ach in the interstitial space [39], although this was not measured in the present study. Thus, we show for the first time that vagal stimulation, applied only for a short time before ischemia, in an in vivo model is capable of taking the myocardium to a "preconditioning-like" state. This is in agreement with the actual concepts that involve the cholinergic muscarinic pathway in the protective stimulus of remote ischemic preconditioning [8, 21]. Moreover, it has been shown that brief exogenous administration of ACh to the isolated heart trigger the preconditioning through different signal transduction pathways [26].

In this sense, we have evaluated the expression and phosphorylation of the Akt and GSK-3β enzymes as possible mechanisms involved in the protection conferred by preischemic intermittent vagal stimulation. It is known that GSK-3β participates in different cellular processes, including ischemia/reperfusion injury [22]. It has also been demonstrated that the inhibition of GSK-3β by phosphorylation at the time of reperfusion enhances cell survival and reduces the infarct size by activation of the mKATP channel before ischemia [32]. Similarly, the phosphorylation of GSK-3β by activation of the PI3K-Akt pathway is capable of reducing the infarct size both in preconditioning [12] as in ischemic postconditioning [2]. In addition, in transgenic mouse models, Zhai et al. [38] demonstrated that the inactivation of GSK-3β during reperfusion reduces the infarct size. Since the major changes that determine the severity of reperfusion injury occur during the early reperfusion, we performed measurements of Akt at 5 min of reperfusion. The results show that preischemic intermittent vagal stimulation induces Akt and GSK-3β phosphorylation in the early reperfusion, reducing the infarct size by mechanisms involving muscarinic receptors. As expected, the status of serine 9 phosphorylation in GSK-3β coincided with that of Akt phosphorylation, suggesting that Akt may control the activity of GSK-3β in the intermittent vagal stimulation [38]. Interestingly, continuous vagal stimulation was only capable of phosphorylating Akt and GSK-3β when β1 adrenergic receptors were blocked. Therefore, this data suggests that continuous vagal stimulation increases the infarct size as a consequence of sympathetic stress and the non-activation of the Akt/GSK-3β pathway (Fig. 9).

Experimental and clinical studies suggest that the antagonistic action of the parasympathetic system over the sympathetic is one of the main beneficial effects of vagal stimulation [18, 31, 34]. However, we hypothesize that the interaction

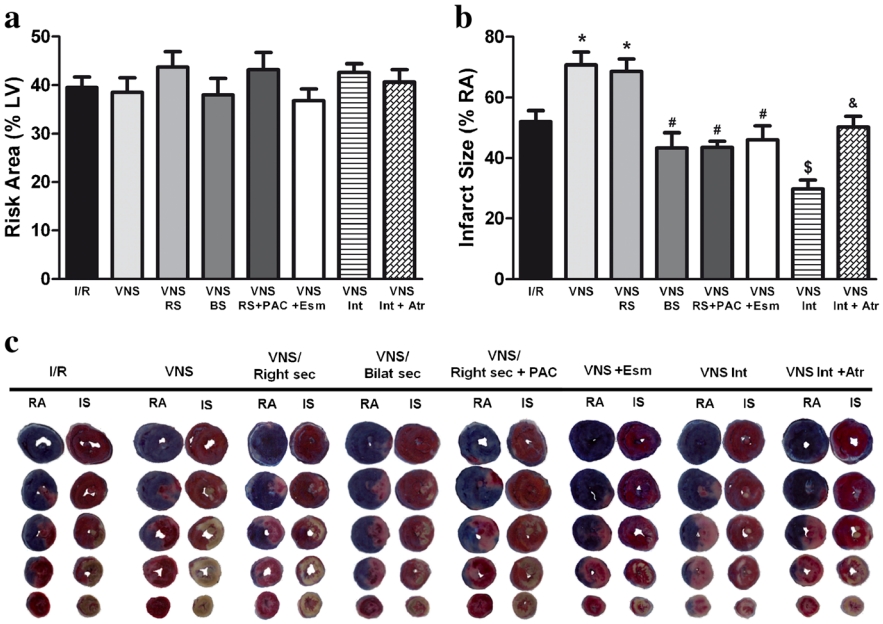


Fig. 5 **a** The risk areas of every studied group expressed as a percentage of the left ventricle area are observed. There are no significant differences between groups. **b** The graph shows the infarct sizes expressed as a percentage of the risk area. Continuous vagal stimulation in the VNS and VNS/RS groups increases the infarct size. This effect is blocked by bilateral vagus section, the application of a pacemaker, or the administration of esmolol. Intermittent vagal stimulation reduces the infarct size, a protective effect blocked by atropine. *Below*, we can see representative images of each group (**c**). These images correspond to the heart sections dyed with Evan's blue (*images on the left*) or TTC (*images on the right*). *LV* left ventricle, *RA* risk area, *IS* infarct size. *$p<0.05$ vs I/R; #$p<0.05$ vs VNS and VNS/RS; \$$p<0.05$ vs I/R and VNS Int + Atr

between both systems will depend on the conditions in which vagal stimulation is applied. The plasmatic catecholamine levels in our study suggest a synergic action between both systems during continuous vagal stimulation, and an antagonistic action in intermittent vagal stimulation. We also show evidence that the increase of LVEDP and the pressure and area of the left atrium,

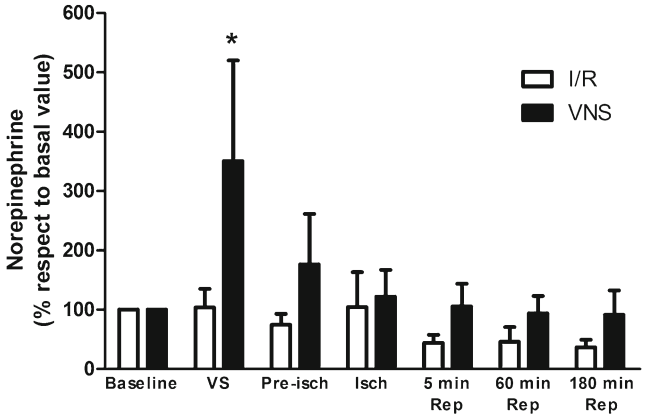
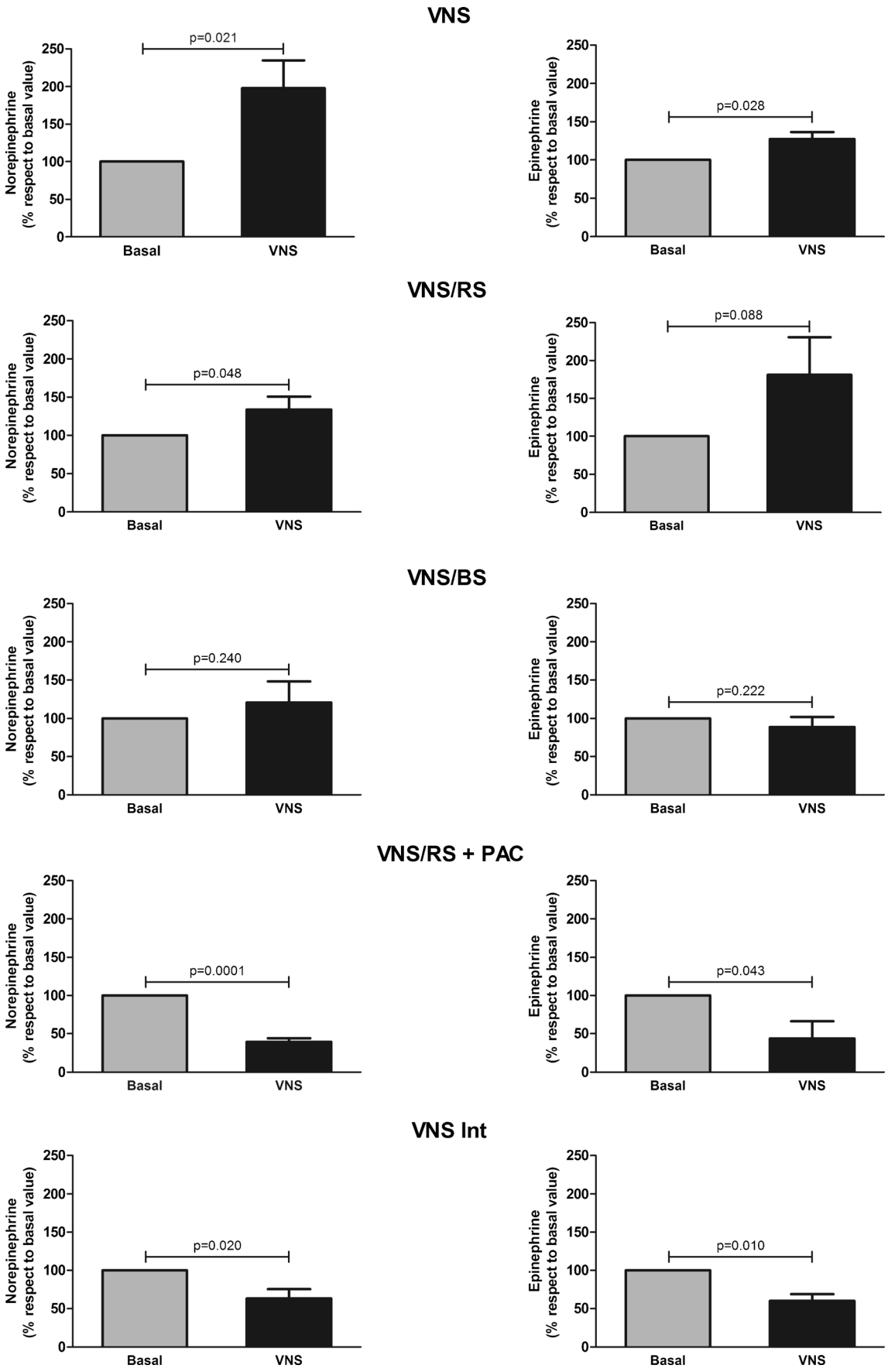


Fig. 6 Plasmatic levels of norepinephrine, evaluated at baseline condition, during vagal stimulation, previous to the onset of ischemia, during ischemia, and in three times of reperfusion in groups I/R and VNS. No significant modification of norepinephrine during the whole experimental protocol in the I/R group were observed. Continuous vagal stimulation increases the norepinephrine levels only before ischemia. There are no significant differences in the pre-ischemic and reperfusion times evaluated in the VNS group compared to the I/R group. Values are expressed as a percentage of the baseline level. *VS* vagal stimulation, *Isch* ischemia, *Rep* reperfusion. *$p<0.05$ vs baseline

consequence of the induced bradycardia by continuous vagal stimulation, could be responsible for the activation of the sympathetic compensatory nervous system reflex mediated by the stretching of the wall of the mentioned chambers. Even though this activation has been well described during the increase of atrial volume by administration of intravascular liquid [27, 28], in the stretching of the atrium walls with an intracavitary balloon [16, 19] or during vagal stimulation [37], at least to our knowledge, it had not been demonstrated that these hemodynamic changes could modify the infarct size.

In the atrium, there are type A nervous receptors that depolarize with the atrial pressure "a" wave, and type B receptors that depolarize with the atrial pressure "v" wave [20]. We find that continuous vagal stimulation increases both the "a" and the "v" waves of the left atrial pressure curve. When intermittent stimulation is performed, the "a" wave only increases during the 10 s of the ON period of vagal stimulation. Therefore, it is possible that this would not be enough to activate the sympathetic stimulating reflex, since type A receptors have a latency period of a few seconds until their activation [20, 28]. In this stage, the response is an initial inhibition of the sympathetic system followed by its activation [16]. Also, during intermittent vagal stimulation, the "v" wave and the left atrial volume do not change and this could contribute to the inhibitory action of vagal stimulation on the sympathetic system. This is based on findings of studies that shown a strong association between the increase of pressure of the "v" wave and sympathetic activation [20]. Undoubtedly, the duration of the stimulation seems to play a


VNS
Norepinephrine (% respect to basal value)
p=0.021
Basal
VNS
Epinephrine (% respect to basal value)
p=0.028
Basal
VNS
VNS/RS
Norepinephrine (% respect to basal value)
p=0.048
Basal
VNS
Epinephrine (% respect to basal value)
p=0.088
Basal
VNS
VNS/BS
Norepinephrine (% respect to basal value)
p=0.240
Basal
VNS
Epinephrine (% respect to basal value)
p=0.222
Basal
VNS
VNS/RS + PAC
Norepinephrine (% respect to basal value)
p=0.0001
Basal
VNS
Epinephrine (% respect to basal value)
p=0.043
Basal
VNS
VNS Int
Norepinephrine (% respect to basal value)
p=0.020
Basal
VNS
Epinephrine (% respect to basal value)
p=0.010
Basal
VNS

◀ **Fig. 7** Plasmatic concentration of norepinephrine and epinephrine evaluated before and after vagal stimulation in the groups VNS, VNS/RS, VNS/BS, VNS/RS + PAC, and VNS Int. Values are expressed as a percentage of the baseline level. Continuous vagal stimulation increases the catecholamine plasmatic levels in the VNS and VNS/RS groups. No significant changes of catecholamines in the group with bilateral vagal section were observed. The levels of norepinephrine and epinephrine were reduced in the VNS/RS + PAC and VNS Int groups

fundamental role, at least in our experimental model. A vagal stimulation superior to 10 s could extend the increase in the "a" wave pressure and even overcome the latency period of the type A receptors and at the same time, increase atrial volume and "v" wave pressure to levels that could depolarize type B receptors [16, 20, 27, 28]. This way, both phenomenons could lead to sympathetic activations in more extended vagal stimulations. Even though the activation of ventricular receptors is often associated to inhibitory responses, we cannot discard that under the effects of vagal stimulation they participate in the generation of sympathetic reflexes [25], since we have observed a continuous increase of LVEDP during the whole period of continuous vagal stimulation.

Some authors have demonstrated a lower incidence of arrhythmias induced by ischemia in animals subjected to vagal stimulation [23]. On the other hand, Li et al. [18] observed a significant increase in survival and improvement in post-infarction left ventricular remodeling in rats with chronic heart failure subjected to chronic intermittent vagal stimulation. Although these authors assessed the infarct size, they did not find differences in its size among the studied groups. Uemura et al. achieved a reduction in infarct size in a model of ischemia and reperfusion in rabbits [36]. However, in this study, reperfusion was allowed for 8 weeks and vagal stimulation was applied throughout ischemia and during the first 3 days of reperfusion. On the other hand, Katare et al. [14] have shown a reduction in infarct size in a model of regional ischemia of efferent vagus nerve stimulation for 3 h, but these authors did not carry out reperfusion but applied VS throughout ischemia and performed the experiment in mice, instead of rabbits. The same authors noted that VNS during a preischemic period prevented the reperfusion injury at the risk area through the preservation of mitochondrial function. However, they used an isolated heart model with absence of

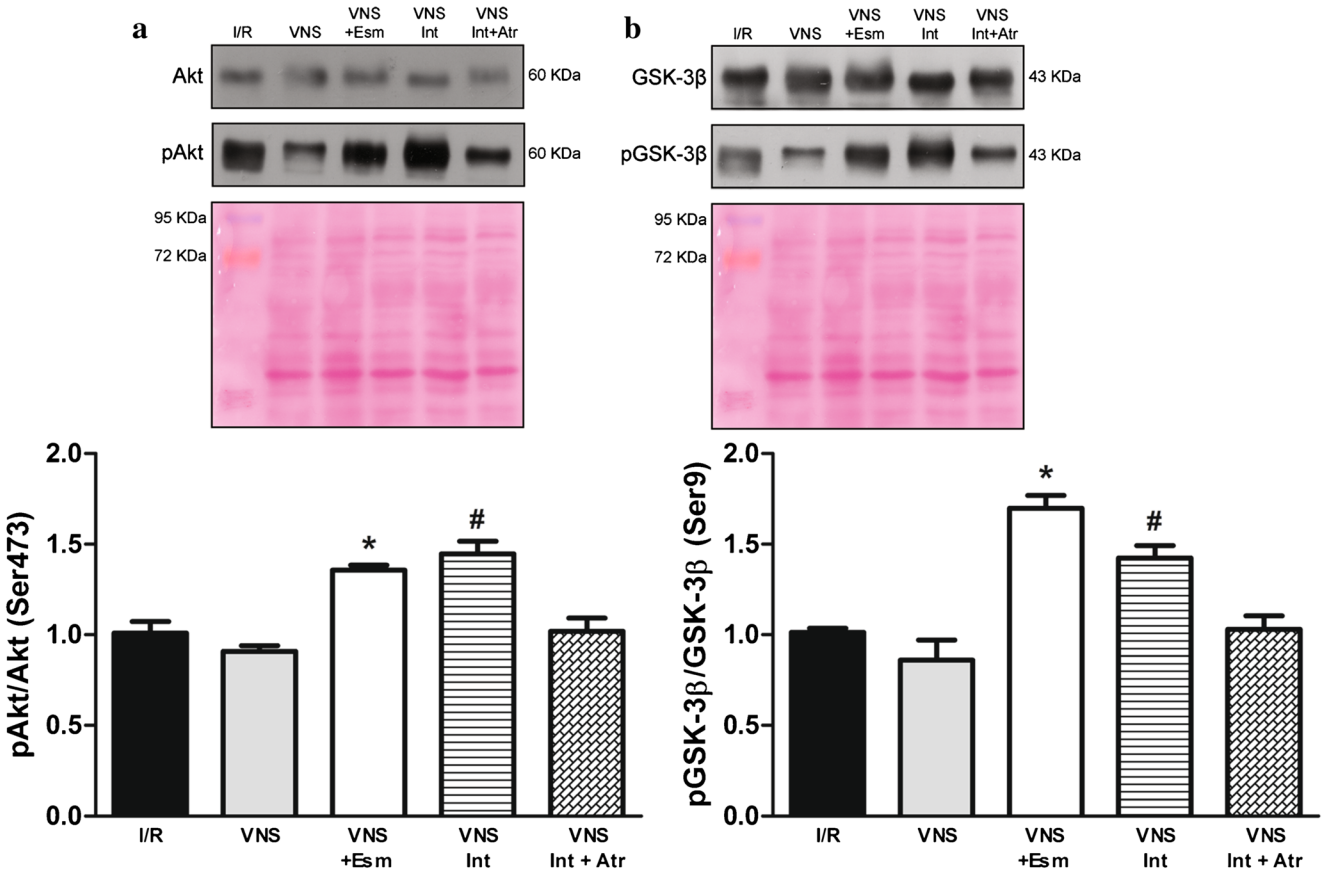


Fig. 8 Phosphorylation of Akt (**a**) and GSK-3β (**b**) proteins in the I/R, VNS, VNS + Esm, VNS Int, and VNS Int + Atr groups. Continuous vagal stimulation was only capable of phosphorylating Akt and GSK-3β in the presence of esmolol. An increased phosphorylation of Akt and GSK-3β is observed in the group with intermittent vagal stimulation. These effects were blocked with the administration of atropine. In all Western blotting experiments, data were normalized with individual total protein levels. There were no differences in the protein load according to the membranes stained with Ponceau Red. p^* $p<0.05$ vs I/R and VNS; #$p<0.05$ vs I/R and VNS Int + Atr

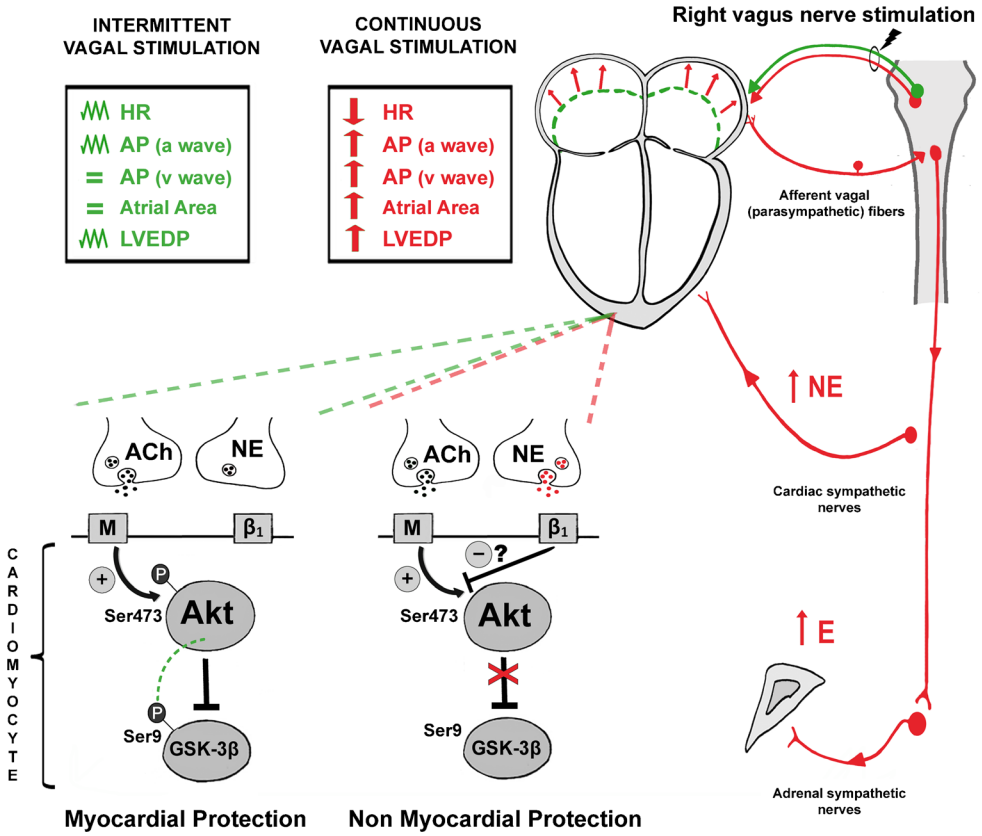


Fig. 9 Schematic illustration of the neural and intracellular pathways activated as a consequence of continuous (*red*) and intermittent (*green*) vagal stimulation. Intermittent vagal stimulation produces intermittent changes in the heart rate, the "a" wave of the atrial pressure and the LVEDP, without modifying the "v" wave of neither the atrial pressure nor the atrial area assessed by echocardiography. This type of stimulation avoids the sympathetic co-activation, generating a predominance of the parasympathetic tone. Acetylcholine, released in cardiac vagal nervous terminals, activates muscarinic receptors located in the cardiomyocyte plasma membrane, phosphorylating Akt and GSK-3β enzymes. As a consequence of the activation of these pathways, the infarct size is reduced. Conversely, continuous vagal stimulation produces a sustained and significant reduction of heart rate and a sustained increase of the "a" and "v" waves of the atrial pressure curve, LVEDP, and atrial area. This increase in the pressures and the atrial area should produce stretching of the walls, which is ultimately responsible for the co-activation of compensatory sympathetic reflexes. This is evidenced by an increase in the levels of plasmatic catecholamines. β1 adrenergic activation somehow interferes with the muscarinic effects on the Akt/GSK-3β pathway. This way, the myocardial protection conferred by the intermittent stimulation is not produced. *HR* heart rate, *AP* atrial pressure, *LVEDP* left ventricular end diastolic pressure, *ACh* acetylcholine, *NE* norepinephrine, *E* epinephrine, ⩙ cyclical variations of HR, AP, and LVEDP

extracardiac regulatory systems, such as baroreflex or the sympathoadrenal system, excluding any effects of extracardiac regulatory mechanisms on cardiac and subcellular functions [13].

In conclusion, the results of the present study demonstrate that the effects of vagal stimulation on the infarct size strongly depend on the sympathetic-parasympathetic interaction. Continuous vagal stimulation co-activates the sympathetic system and increases the infarct size by changes in the cardiac chamber volumes. This increase in the sympathetic stress and the non-activation of the Akt/GSK-3β pathway lead to an increase on the infarct size. On the contrary, vagal stimulation performed intermittently antagonizes the sympathetic system and reduces the infarct size by cholinergic muscarinic activation and the phosphorylation of the Akt and GSK-3β enzymes. Given that vagal stimulation is an intervention used for the treatment of neurological and cardiovascular diseases, this would contribute to improve its beneficial effects and to reduce the possibility of unwanted outcomes.

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Ethical standards The procedures used in this study were approved by the Animal Care and Research Committee of the University of Buenos Aires (Protocol # 2004/11) 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).

Conflict of interest The authors declare that they have no conflict of interest.

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