Increase in parasympathetic tone by pyridostigmine prevents ventricular
dysfunction during the onset of heart failure

Renata M. Lataro,1 Carlos A. A. Silva,1 Rubens Fazan, Jr.,1 Marcus A. Rossi,2† Cibele M. Prado,2
Rosely O. Godinho,3 and Helio C. Salgado1

Departments of 1Physiology and 2Pathology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil and 3Division of Cellular Pharmacology, Department of Pharmacology, Federal University of São Paulo, São Paulo, Brazil

Submitted 25 February 2013; accepted in final form 8 August 2013

Lataro RM, Silva CA, Fazan R Jr, Rossi MA, Prado CM, Godinho RO, Salgado HC. Increase in parasympathetic tone by pyridostigmine prevents ventricular dysfunction during the onset of heart failure. Am J Physiol Regul Integr Comp Physiol 305: R908–R916, 2013. First published August 28, 2013; doi:10.1152/ajpregu.00102.2013.—Heart failure (HF) is characterized by elevated sympathetic activity and reduced parasympathetic control of the heart. Experimental evidence suggests that the increase in parasympathetic function can be a therapeutic alternative to slow HF evolution. The parasympathetic neurotransmission can be improved by acetylcholinesterase inhibition. We investigated the long-term (4 wk) effects of the acetylcholinesterase inhibitor pyridostigmine on sympathovagal balance, cardiac remodeling, and cardiac function in the onset of HF following myocardial infarction. Myocardial infarction was elicited in adult male Wistar rats. After 4 wk of pyridostigmine administration, per os, methylatropine and propranolol were used to evaluate the cardiac sympathovagal balance. The tachycardic response caused by methyl- atropine was considered to be the vagal tone, whereas the bradycardic response caused by propranolol was considered to be the sympathetic tone. In conscious HF rats, pyridostigmine reduced the basal heart rate, increased vagal, and reduced sympathetic control of heart rate. Pyridostigmine reduced the myocyte diameter and collagen density of the surviving left ventricle. Pyridostigmine also increased vascular endothelial growth factor protein in the left ventricle, suggesting myocardial angiogenesis. Cardiac function was assessed by means of the pressure-volume conductance catheter system. HF rats treated with pyridostigmine exhibited a higher stroke volume, ejection fraction, cardiac output, and contractility of the left ventricle. It was demonstrated that the long-term administration of pyridostigmine started right after coronary artery ligation augmented cardiac vagal and reduced sympathetic tone, attenuating cardiac remodeling and left ventricular dysfunction during the progression of HF in rats.

vagal stimulation; anticholinesterase agent; cardiac function; cardiac remodeling; autonomic imbalance

HEART FAILURE (HF) is a frequent outcome of myocardial infarction that is characterized by general adrenergic activation and parasympathetic withdrawal, which contributes to the progression of the disease (6, 19, 49). Derangement of the vagal control of the heart rate (HR) becomes apparent at a very early stage of left ventricle (LV) dysfunction (7, 30) and is closely associated with negative outcomes in patients with HF after myocardial infarction (6). Indeed, the activation of the parasympathetic nervous system may have beneficial implications in the development of HF (17). Chronic vagal nerve stimulation enhanced the long-term survival of HF rats through the prevention of contractile dysfunction and cardiac remodeling (40). In dogs, chronic vagus nerve stimulation attenuated the outcomes of HF and improved the cardiac autonomic control reflected by increased HR variability, increased baroreflex sensitivity, and reduced plasma norepinephrine levels (73). In HF patients, vagus nerve stimulation performed during a 6-mo period was associated with significant improvements in NYHA class, quality of life, a 6-min walk test, the LV ejection fraction, and LV systolic volumes (17). Therefore, the increase in parasympathetic function seems to be a therapeutic alternative to improve HF outcomes by acting on the autonomic control of the heart and improving cardiac remodeling and function.

Because efferent vagal nerve activity uses acetylcholine as the neurotransmitter, drugs that augment acetylcholine availability at the neuromuscular junction may be expected to have an effect similar to electrical stimulation. Anticholinesterase agents prevent the hydrolysis of acetylcholine by acetylcholinesterase at sites of cholinergic transmission, which prolongs the availability of acetylcholine in the cholinergic nerve endings and thereby increases the efficiency of cholinergic transmission (67). Anticholinesterase agents have been used in experimental designs aiming to enhance the parasympathetic influences on the heart (19). Donepezil, an anticholinesterase agent that crosses the blood-brain barrier, was administered during a 6-wk period and prevented LV dysfunction and neurohumoral activation in HF rats (48).

On the other hand, pyridostigmine (PYR) is a reversible anticholinesterase agent that does not cross the blood-brain barrier and acts specifically in the peripheral synaptic chelot (67). PYR increased HR variability in normal rats (63), healthy humans (47), and HF patients (5). In healthy humans, PYR given during a 1- or 2-day period produced bradycardia (20, 46) without modifying cardiac function (18). In HF patients, PYR increased HR recovery after exercise (2), reduced the HR and ventricular arrhythmia density (5), and improved autonomic and hemodynamic profiles during dynamic exercise (62). This suggests that PYR has a modulator effect in the cardiac sympathovagal balance in normal and HF patients. We hypothesize that the administration of PYR right after coronary artery ligation augments cardiac vagal tone and attenuates cardiac remodeling and LV dysfunction during the progression of HF in rats. Hence, it is proposed in the current study to investigate the effects of the long-term (4 wk) administration of PYR on sympathovagal balance, cardiac remodeling, and cardiac function in the onset of HF following myocardial infarction in rats.

METHODS

The experimental protocols used in the current study were reviewed and approved by the Committee of Ethics in Animal Research of the School of Medicine of Ribeirão Preto, University of São Paulo, SP, Brazil (Protocol no. 1477/2007). Experiments were carried out in...
adult male Wistar rats supplied by the Animal Facility of the School of Medicine of Ribeirão Preto, University of São Paulo, SP, Brazil. The animals were housed individually with free access to food and water and were maintained on a 12-h light-dark cycle. Experimental heart failure and pyridostigmine treatment. HF was produced by myocardial infarction according to the method described by Pfeffer et al. (53). Briefly, rats (250–300 g, 7–8 wk old) were anesthetized with ketamine (50 mg/kg ip; União Química Farmacêutica Nacional S/A, Embu-Guáçu, SP, Brazil) and xylazine (10 mg/kg ip; Hertzape Caliê Sadie animal S/A, Juatuba, MG, Brazil), endotracheally intubated, and mechanically ventilated with room air. A left thoracotomy was performed to expose the heart. The pericardium was opened, and the heart was exteriorized. The left anterior descending coronary artery was ligated between the pulmonary artery outflow tract and the left atrium with polyester suture (4-0; Ethicon, São José dos Campos, SP, Brazil). The heart was returned to the chest cavity, and the thorax incision was closed. Control rats underwent similar surgical procedure without coronary ligation (n = 20). The infarct size was confirmed by postmortem examination, and only rats with an infarct size >40% of the LV wall were involved in the study. Overall, 103 rats were submitted to coronary artery ligation as follows: 55 rats did not survive the surgery, 8 rats were excluded due to infarct size <40%, and 40 rats were used in the study. After coronary ligation, rats were randomized to groups that received PYR administration and groups that did not. Pyridostigmine bromide (Valeant Farmacêutica do Brasil, Campinas, SP, Brazil) was prepared every day and was given in the drinking water (0.14 mg/ml) starting right after the myocardium infarction. Soares et al. (63) described that this dose of pyridostigmine bromide (0.14 mg/ml) given in drinking water during 7 days produced an average inhibition of 40% on plasma acetylcholinesterase activity compared with pretreatment values. Body weight and water consumption were measured every day during the 4-wk study protocol. Arterial pressure and heart rate measurement. Four weeks after myocardial infarction, or sham operation, the rats were anesthetized (tribromoethanol, 250 mg/kg ip; Sigma-Aldrich) and instrumented with polyethylene catheters (Intramedic, Clay Adams, Parsippany, NJ) into the femoral artery and vein for direct measurement of arterial pressure (AP) and drug administration, respectively. The catheters were exteriorized on the back of the neck, between the scapulae of the rat. After this procedure, the animals were allowed to recover in individual cages for 48 h. On the day of the experiment, the rats were taken to the recording room at least 30 min before the beginning of the experiment, and a quiet environment was maintained to avoid any stress. The arterial catheter was connected to a pressure transducer (Statham, P23 XL, Valley View, OH). Pulsaassist AP was continuously sampled (2 kHz) using an IBM/PC equipped with an analog-to-digital interface (220, Dataq, Akron, OH). Pulsaassist AP recordings were analyzed by computer software designed to detect inflection points of a periodic wave (Advanced CODAS, Dataq Instruments). Beat-by-beat time series of systolic, diastolic, and mean arterial pressure (MAP) were generated. HR was measured from successive diastolic pulse intervals. Experimental protocol. All recordings were carried out in conscious freely moving rats during approximately 1 h. Basal AP was recorded during 30 min followed by measurement of the autonomic tone (vagal and sympathetic) by means of methylethropine and propranolol (12). The HR measured when both autonomic blockers were administered was considered the intrinsic HR (iHR). The doses of methylethropine and propranolol were used as described previously (16, 21). The vagal tone was measured in a group of rats after the administration of a bolus of a solution with methylethropine (2 mg/kg iv; Sigma-Aldrich). The difference between the HR calculated at the end of the 15-min period after methylethropine administration and basal HR was considered to be the vagal tone. Fifteen minutes after the administration of methylethropine, a bolus of a solution with propranolol (4 mg/kg iv; Sigma-Aldrich) was given to the rats, and the AP was continuously recorded during another 15-min period for assessment of iHR. The sympathetic tone was measured in another group of rats after the administration of a bolus of a solution with propranolol (4 mg/kg iv). The difference between the HR calculated at the end of the 15-min period after propranolol administration and the basal HR was considered to be the sympathetic tone. Fifteen minutes after the administration of propranolol a bolus of a solution with methylethropine (2 mg/kg iv) was given to the rats, and the AP was continuously recorded during another 15-min period for assessment of iHR. After the hemodynamic recordings, the rats were killed by a pentobarbital sodium overdose (Rohofarma Indústria Farmacêutica, Hortolândia, SP, Brazil). The hearts and lungs were rapidly removed, rinsed in ice-cold 0.9% NaCl solution and weighed. The hearts were collected for morphological analysis (infarct size, myocyte size, and interstitial collagen density). Morphological analysis. The hearts were cut in the short axis, fixed in phosphate-buffered 10% Formalin, and submitted to paraffin inclusion. Each block was serially cut at 6 μm from the midventricular surface. The sections were stained with hematoxylin and eosin or picrosirius red. For morphometric and infarct size analyses the hearts stained with hematoxylin and eosin were used. The infarct size was measured using the public-domain software NIH ImageJ (developed by National Institutes of Health and available on the internet site http://rsh.info.nih.gov/nih-image/). Infarct size was calculated by dividing the length of the infarcted area by the total circumference of the LV and expressed as a percentage (55). The minor diameter of myocytes in the surviving LV was measured using video microscopy Leica Qwin (Leica Imaging Systems, Cambridge, UK). Approximately 30 values were obtained per rat, and the mean value was calculated. To estimate the volume fraction (%) of collagen in picrosirius red-stained sections, quantitative examination of the surviving LV was carried out on a medium-power light-microscopic field. For each heart, 15 fields per rat were randomly selected and analyzed using a Leica Qwin software (Leica Imaging Systems). The mean value was subsequently calculated. Cardiac function analysis. This protocol was performed in separate groups of anesthetized (pentobarbital sodium) animals. Four weeks after myocardial infarction or sham operation, rats were anesthetized with pentobarbital sodium (40 mg/kg ip). A micropipet pressure-volume (P-V) catheter (SPR-838, Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the LV as described elsewhere (51). After stabilization, the signals were continuously recorded using the P-V conductance system (MPVS, Millar Instruments) coupled to a PowerLab/4SP (AD Instruments, Sydney, NSW, Australia) attached to an IBM/PC. The LV end-diastolic pressure (EDP), maximal slope of the systolic pressure increment (+dP/dt) and diastolic pressure decrement (−dP/dt), relaxation time constant (τ), ejection fraction (EF), stroke volume (SV), end-systolic volume (ESV), end-diastolic volume (EDV), and cardiac output (CO) were calculated using a cardiac P-V analysis program (PVAN Ultra 1.1, Millar Instruments) followed by corrections of cuvette and saline calibrations as described elsewhere by Pacher et al. (51). At the end of the experiment, the heart was collected, and the ventricles were cut transversely into two parts. The apical portion was fixed in phosphate-buffered 10% Formalin and stained with hematoxylin and eosin for infarcted size analysis. The noninfarcted myocardium from the remaining basal part was frozen in liquid nitrogen and subjected to Western blot analysis to determine the vascular endothelial growth factor (VEGF) protein level. Western blot analysis. Tissue samples were homogenized in protein extraction buffer containing protease inhibitors (Sigma-Aldrich). Protein concentration was determined using a Bradford Assay Kit (Bio-Rad Laboratories, Hercules, CA). Each sample of 50 μg was separated by SDS-PAGE 10%, transferred to a nitrocellulose membrane, blocked with 5% nonfat milk in TBS-T overnight, and then incubated with mouse anti-VEGF antibodies (1: 500, Santa Cruz Biotechnology, Santa Cruz, CA) with 5% nonfat milk overnight. After washing was completed, the membranes were incubated for 40 min with secondary antibodies (anti-mouse; 1:5,000) conjugated to peroxidase. The pro-
teins were detected by chemiluminescence using an ECL kit (Hybond-ECL, Amersham Pharmacia Biotech, Amersham, UK). The images were captured by a ChemiDoc XRS System (Bio-Rad Laboratories) and analyzed by the NIH ImageJ software. GAPDH was used as a loading control.

Acetylcholinesterase activity. A blood sample (300 μl) was collected from the rat tail before myocardial infarction and after 4 wk of PYR or water intake. Blood samples were collected in vials containing 30 μl of EDTA (0.1 M, Sigma-Aldrich). After centrifugation (3,000 g, 4°C) for 20 min, plasma was collected and kept at −20°C until the determination of acetylcholinesterase activity. Enzymatic assays were performed using an adaptation of the colorimetric method (24), as described by Alves-Amaral et al. (1). Plasma samples (10 μl) were incubated in 96-well microplates with 0.01 M 5,5′-dithio-bis-(2-nitrobenzoic acid, DTNB, Sigma-Aldrich) and the excess of the substrate (acetylthiocholine, 0.075 M, Sigma-Aldrich) in 0.2 mM phosphate buffer, pH 8.0 at 30°C, in the presence of the selective inhibitor of butyrylcholinesterase, tetraisopropylpyrophosphoramide (IsoOMPA 10−3 M, Sigma-Aldrich). Generation of the reaction product was followed in a microplate reader (BioTek FL600, BioTek Instruments, Winooski, VT) at 405 nm for 60 min at 3-min intervals. The maximum velocity (Vmax) of the reaction for each sample was determined in duplicate and expressed as arbitrary units per minute per milliliter of plasma.

Statistical analysis. All data are presented as the means ± SE. Comparisons of the general characteristics of the animals studied, basal AP, HR, sympathetic tone, vagal tone, iHR, VEGF, morphological, hemodynamic and cardiac function parameters, and water and pyridostigmine intake were performed using one-way ANOVA. In case of lack of variance homogeneity a log transformation was employed to reach this ANOVA assumption. The Student-Newman-Keuls test was used for post hoc comparisons. Averages of acetylcholinesterase activity were compared using the one-way ANOVA for repeated measures followed by the Student-Newman-Keuls posttest. The level of significance was set at P < 0.05.

RESULTS

General characteristics of control and HF rats with or without pyridostigmine treatment. Representative heart sections, the general characteristics and basal hemodynamics (MAP and HR) of control, and HF rats with or without PYR treatment used for measuring the autonomic tone are shown in Fig. 1. Control rats display normal LV wall thickness without damage of the myocardium. However, an extensive scar can be observed in the LVs of HF and HF+PYR rats. Body weight was similar in the three groups. HF rats exhibited greater heart and lung weights than control rats. The infarct size was similar between HF rats with PYR treatment and those without. Control and HF rats treated with PYR showed almost the same MAP value as those without PYR treatment. However, basal HR was elevated in HF rats but was reduced by PYR.

Cardiac autonomic tone and intrinsic heart rate. HF rats displayed reduced vagal tone and elevated sympathetic tone compared with control animals (Fig. 2). The greater tachycardic response caused by methylatropine in HF+PYR rats indicates an augmented vagal tone compared with HF rats (Fig. 2). On the other hand, the smaller bradycardic response caused by propranolol in HF+PYR rats indicates a less sympathetic tone compared with HF rats (Fig. 2). Moreover, the combined administration of methylatropine and propranolol shows that PYR did not affect the iHR of HF rats (control: 377 ± 5; HF: 392 ± 6; HF+PYR: 394 ± 8 beats/min). The administration of methylatropine and/or propranolol did not affect the AP.

Myocyte size, interstitial fibrosis, and VEGF protein. The minor diameter of the myocytes (Fig. 3), collagen density (Fig. 4), and VEGF protein level (Fig. 5) from the surviving LV of HF rats was significantly greater compared with control rats. In HF rats, both minor diameter of the myocytes (Fig. 3) and collagen density (Fig. 4) were reduced by PYR. However, PYR increased the VEGF protein expression in the surviving LVs of HF rats (Fig. 5).

Hemodynamic and cardiac function. The infarct size of HF rats treated with PYR (56 ± 2%) was almost the same as those from HF rats without PYR treatment (59 ± 2%) when used in the hemodynamic and cardiac function studies. Table 1 shows the weight, hemodynamic parameters, and the indices of sys-

\[
\begin{array}{ccc}
\text{Control} & \text{HF} & \text{HF+PYR} \\
\text{(n=13)} & \text{(n=12)} & \text{(n=14)} \\
\text{BW (g)} & 474 ± 12 & 447 ± 14 & 435 ± 11 \\
\text{HW (mg)} & 1355 ± 31 & 1536 ± 33* & 1589 ± 51* \\
\text{LW (mg)} & 1299 ± 60 & 1804 ± 90* & 1617 ± 80* \\
\text{HW/BW ratio (mg/g)} & 2.9 ± 0.1 & 3.5 ± 0.01* & 3.6 ± 0.1* \\
\text{LV/BW ratio (mg/g)} & 2.5 ± 0.1 & 3.9 ± 0.2* & 3.5 ± 0.2* \\
\text{Infarct Size (%LV)} & - & 57 ± 1 & 53± 2 \\
\text{MAP (mmHg)} & 103 ± 2 & 103 ± 3 & 94± 4 \\
\text{HR (bpm)} & 335 ± 5 & 388 ± 11* & 347 ± 4 † \\
\end{array}
\]
tolic and diastolic function from P-V relations in anesthetized rats. The parameters obtained from P-V analysis confirmed that rats submitted to myocardial infarction developed HF. These rats exhibited remarkable increases in EDP, ESV, and $\tau$. In addition, they also showed conspicuous reduction in SV, EF, CO, $+dP/dt$, and $-dP/dt$ compared with control rats (Table 1). PYR increased the SV, EF, CO, and $+dP/dt$ in HF+PYR rats.

Fig. 2: A: representative heart rate (HR) series from a control (light gray lines) and HF rats with (solid lines) or without PYR treatment (dark gray lines) that received methylatropine (top) and propranolol (bottom). B: tachycardic response caused by methylatropine (top) and bradycardic response caused by propranolol (bottom) in control and HF rats with or without PYR treatment. Values are presented as the means ± SE; HR, heart rate; the number inside the bar represents the number of animals of the respective group. *$P < 0.05$ compared with the control group; †$P < 0.05$ compared with the HF group.

Fig. 3: Photomicrographs and bar graphs of the minor diameter of myocytes from the left ventricle of control and HF rats with or without PYR treatment. Values are presented as the means ± SE; the number inside the bar represents the number of animals of the respective group. Bar = 50 $\mu$m. *$P < 0.05$ compared with the control group; †$P < 0.05$ compared with the HF group.
Pyridostigmine intake and acetylcholinesterase activity. Water consumption was similar among the three groups studied (control: 55 ± 2; HF: 51 ± 1; HF+PYR: 54 ± 1 ml/day). PYR intake was 21 mg·kg·day⁻¹ for HF rats. The effect of PYR on plasma acetylcholinesterase activity is shown in Fig. 6. Plasma acetylcholinesterase activity decreased 42% in HF rats treated with PYR compared with pretreatment values.

**DISCUSSION**

This study shows that chronic administration (per os) of the acetylcholinesterase inhibitor PYR reduced HR, increased cardiac vagal tone, and reduced sympathetic tone. Likewise, PYR attenuated the cardiomyocyte hypertrophy, the collagen expression in the noninfarcted myocardium, and the decline of the systolic LV function during the progression of HF following myocardial infarction.

The current study is in line with previous observations (6, 22, 34, 54, 75) and demonstrates elevated cardiac sympathetic tone and attenuated parasympathetic tone in conscious HF rats.

**Table 1. Hemodynamic parameters and indices of left ventricle systolic and diastolic function derived from pressure-volume relations in anesthetized control and HF rats with or without PYR treatment**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HF</th>
<th>HF+PYR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, g</td>
<td>526 ± 40</td>
<td>448 ± 20</td>
<td>453 ± 20</td>
</tr>
<tr>
<td>EDP, mmHg</td>
<td>7 ± 0.5</td>
<td>16 ± 3*</td>
<td>13 ± 1*</td>
</tr>
<tr>
<td>ESV, µl</td>
<td>155 ± 20</td>
<td>240 ± 20+</td>
<td>199 ± 16</td>
</tr>
<tr>
<td>EDV, µl</td>
<td>253 ± 22</td>
<td>280 ± 21</td>
<td>256 ± 20</td>
</tr>
<tr>
<td>SV, µl</td>
<td>98 ± 2</td>
<td>41 ± 6*</td>
<td>62 ± 4†</td>
</tr>
<tr>
<td>EF, %</td>
<td>40 ± 4</td>
<td>15 ± 2*</td>
<td>28 ± 4†</td>
</tr>
<tr>
<td>CO, ml/min</td>
<td>36 ± 4</td>
<td>13 ± 2*</td>
<td>20 ± 2*†</td>
</tr>
<tr>
<td>+dP/dt, mmHg/s</td>
<td>10,111 ± 523</td>
<td>5,114 ± 355*</td>
<td>6,437 ± 249†</td>
</tr>
<tr>
<td>−dP/dt, mmHg/s</td>
<td>−11,370 ± 810</td>
<td>−3,941 ± 255*</td>
<td>−4,598 ± 244*</td>
</tr>
<tr>
<td>τ, ms</td>
<td>12 ± 1</td>
<td>22 ± 3*</td>
<td>25 ± 2*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7. EDP, end-diastolic pressure; ESV, end-systolic volume; EDV, end-diastolic volume; SV, stroke volume; EF, ejection fraction; CO, cardiac output; +dP/dt and −dP/dt, maximal slope of the systolic increment and diastolic decrement of ventricular pressure, respectively; τ, relaxation time constant; HF, heart failure; PYR, pyridostigmine. *P < 0.05 compared with the control group; †P < 0.05 compared with the HF group.
without change in the iHR, which might explain the tachycardia exhibited by this group of animals. A lower iHR in patients with HF may indicate impaired sinus node function (50). However, HF rats treated with PYR did not show any cardiac autonomic abnormality. HF rats treated with PYR showed HR, vagal tone, and sympathetic tone quite similar to those observed in control rats, indicating that PYR prevented the sympathovagal imbalance, the usual outcome observed during the development of HF. The increase in cardiac parasympathetic tone in HF rats produced by PYR may be due to the improvement of cholinergic neurotransmission in the sinus node. The pharmacological effect of PYR might be attributed to the inhibition of acetylcholinesterase, which was 42% less than control values, with consequent accumulation of endogenous acetylcholine in the vicinity of the nerve endings in the heart, producing effects similar to vagal nerve stimulation (19, 67), for instance, bradycardia.

The increase in parasympathetic tone in HF rats treated with PYR was accompanied by a significant decrease in the cardiac sympathetic tone. The neural control of the heart is exerted by the tonic interaction between the sympathetic and parasympathetic arms of the autonomic nervous system (60). It has previously been proposed that parasympathetic stimulation is able to inhibit the sympathetic drive (38). Acetylcholine can act on muscarinic receptors situated in sympathetic presynaptic nerve endings inhibiting the release of norepinephrine (4, 41, 45). The interaction between the sympathetic and parasympathetic nervous system may also be partially modulated by second messengers in cardiac cells (49), where cholinergic stimulation may inhibit the increase of intracellular cAMP (72), inhibiting the adrenergic signaling cascade (38).

It has been documented that on the sinoatrial node the parasympathetic modulation predominates over the sympathetic activity (28, 58, 71). This response is known as “accentuated antagonism” (38). When a vagal electrical stimulation is applied simultaneously to a sympathetic electrical stimulus, there is a reduction in HR similar to that observed in the presence of vagal stimulation alone (37). Moreover, in determining the amount of circulating norepinephrine in a dog’s coronary sinus, there was a reduction of this neuromediator when the vagus nerve was activated simultaneously with the sympathetic nerve (36). The results from the current study suggest that the parasympathetic improvement produced by PYR in HF rats was able to suppress the augmented sympathetic drive to the heart. The level of sympathetic activity is closely related to the severity of symptoms seen in HF (13), and the reduction of cardiac sympathetic tone can provide beneficial influences in the prognosis of this disease.

One of the consequences of myocardial infarction is the remodeling of the surviving myocardium, which is characterized by both interstitial fibrosis (70) and hypertrophic growth of cardiac myocytes and capillary rarefaction (3). This remodeling may affect the LV function and consequently HF development and the prognosis for survival (52, 70). In the present study, PYR attenuated the collagen accumulation and the myocyte hypertrophy in the surviving LV in HF rats. Sympathetic hyperactivity can contribute to morphological changes and functional deterioration of the myocardium. Adrenergic activation increases the heart wall stress, which accelerates the tissue injury process (42), and directly affects the myocardium through the catecholamine cardiotoxic effects, inducing myocardial necrosis and capillarization disturbance (11, 57, 69). Recent study showed that PYR was effective in preventing the hypertrophic effect of isoproterenol in neonatal cardiomyocytes maintained in culture (56). HF rats that received PYR showed reduced sympathetic cardiac tone, which may have helped to attenuate the cardiac remodeling.

Moreover, the cardiac morphological alterations produced by PYR can also be associated with the reduction of the HR. It is well known that tachycardia increases the myocardial oxygen consumption and reduces the coronary perfusion time, decreasing the myocardial oxygen supply, contributing to tissue injury and the subsequent HF (75). The reduction of the HR leads to decreased myocardial oxygen consumption and increased diastolic period of the cardiac cycle, which enhances the coronary perfusion time, increasing both myocardial perfusion and oxygen supply (8, 65). These effects could have an antiremodeling action following the improvement of the surviving myocardial energetic balance (44).

HR reduction in HF rats is associated with angiogenesis in postinfarcted hearts (10, 35). Previous studies have shown that drug-induced bradycardia enhances vascularity and coronary reserve, preserving the function of the surviving myocardium (10, 35). This angiogenic response is dependent on VEGF (35, 74). It has been documented that after myocardial infarction the VEGF is increased in the surviving myocardium (29, 39, 64, 66) as observed in the current study. Despite the increase of VEGF in HF rats, previous studies have shown that neovascularization in the surviving myocardium is not able to maintain the adequate tissue perfusion (32, 33). In the present study, PYR augmented the VEGF protein level, suggesting increased angiogenesis in the surviving myocardium. The expansion of the capillary bed in the affected vasculature of the heart can prevent cardiac remodeling and support better pumping capacity (10, 35).

The current results indicate that the increase in the cardiac vagal tone produced by PYR attenuated the LV dysfunction in HF rats. Studies have shown that chronic electric vagal stimulation improves the cardiac function in HF rats (40) and dogs (73). Okazaki et al. (48) also observed an increase in the LV contractility after the chronic administration of the anticholinesterase agent donepezil in HF rats. The beneficial effects of PYR in cardiac function in HF rats could be related to the
prevention of modifications in the LV structure, i.e., a reduction of both collagen accumulation and myocyte hypertrophy in the surviving LV and a likely angiogenesis improvement. Furthermore, there is in vitro evidence that sympathetic stimulation induces apoptosis in cardiac myocytes, contributing to myocardial dysfunction (14). Muscarinic receptor stimulation produced by carbachol can prevent the apoptosis induced by norepinephrine (15), suggesting that muscarinic activation can improve myocardial function by this mechanism (49). Reduction in the diameter of cardiomyocytes from HF rats that received PYR was not accompanied by heart weight reduction. The presence of greater number of surviving myocytes in the viable myocardium from HF rats treated with PYR indicates that more myocytes have been spared. Thus the higher number of cells in the viable LV may be due to the reduction of apoptosis. Nevertheless, further studies are required to confirm this notion.

The EF values measured in control rats using the P-V catheter were lower (40%) compared with values obtained by echocardiography, i.e., around 80% for a normal rat and less than 40% for the HF rat. It has been shown in the literature that both methods, echocardiography and pressure-volume conductance, provide highly correlated ($r = 0.95$) different values for EF (25, 31). However, the EF observed in the current study, 40% for control rats and 15% for HF rats, are lower when compared with other studies that used the P-V conductance method, and observed values between 52 and 59% in control rats and between 27 and 36% in HF rats (26, 31, 51, 61).

In our laboratory Sabino et al. (59) showed that PYR was able to prevent the reduced baroreflex sensitivity, cardiac parasympathetic tone, and iHR in HF rats, 6–7 wk after coronary artery ligation. However, PYR did not prevent the decrease of basal AP and cardiac performance (59). In the current study the effects of the acetylcholinesterase inhibitor PYR on sympathovagal balance, cardiac remodeling, and cardiac function were investigated in the onset of HF, i.e., 4 wk following myocardial infarction. These data are in line with those from Sabino et al. (59) concerning an increase in the cardiac parasympathetic tone. However, in contrast to Sabino et al. (59) observations, the current study demonstrated decreased cardiac sympathetic tone and improvement of the LV dysfunction combined with attenuation of the cardiac remodeling in HF rats treated with PYR. Therefore, the difference between the current study and the previous from Sabino et al. (59) could be attributed to the difference in time course, i.e., 4 versus 6–7 wk postmyocardial infarction.

Nevertheless, the current study was the first to show that PYR was able to reduce the cardiac sympathetic activation and attenuate the decline in systolic LV function and the cardiac remodeling in HF rats. Previous studies showed that physical and pharmacological enhancement of parasympathetic function decreased sympathetic activation and improved the cardiac function in HF animals (40, 48, 73). However, these studies did not describe alterations in cardiac remodeling, attenuation of cardiomyocyte hypertrophy, and collagen expression in the noninfarcted myocardium, as described in the present study.

In conclusion, long-term (4 wk) administration of PYR started right after coronary artery ligation augmented cardiac vagal and reduced sympathetic tone. Likewise, PYR attenuated the increase in cardiomyocyte hypertrophy, the collagen expression in the noninfarcted myocardium, and the decline in systolic LV function during the progression of HF.

**Perspectives and Significance**

HF is characterized by a chronic sympathovagal imbalance that contributes to the pathophysiology and progression of the disease. It has been demonstrated that a high level of parasympathetic activation provides cardioprotection by means of several potential mechanisms. Even though vagus nerve stimulation appears to be safe and well tolerated by patients (17), the pharmacological approach of parasympathetic activation for treatment of HF has the advantage of being noninvasive and appeared to be quite efficient.

**ACKNOWLEDGMENTS**

The authors thank Maria Elena Rial, from the Department of Pathology of the School of Medicine of Ribeirão Preto (University of São Paulo), for the excellent histological support; Dr. Francisco Silveira Guimarães from the Department of Pharmacology of the School of Medicine of Ribeirão Preto (University of São Paulo) and Guillermo Andrey Ariza Traslaviña from the Department of Physiology of the School of Medicine of Ribeirão Preto (University of São Paulo) for the statistical analysis assistance.

**GRANTS**

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


