Cholinergic stimulation with pyridostigmine protects myocardial infarcted rats against ischemic-induced arrhythmias and preserves connexin43 protein

Fernanda Machado Santos-Almeida,¹ Henrique Girão,² Carlos Alberto Aguiar da Silva,¹ Helio Cesar Salgado,¹ and Rubens Fazan, Jr.¹

¹Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil; and ²Institute of Biomedical Imaging and Life Sciences, School of Medicine, University of Coimbra, Coimbra, Portugal

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MYOCARDIAL INFARCTION (MI) is a key component of the burden of cardiovascular disease and one of the leading causes of death in high- or middle-income countries (49, 61). Epidemiological evidence establishes that 50% of MI patients do not survive the first hour after MI before receiving any medical support (37) and that ventricular arrhythmias cause most of these deaths (60). These observations are corroborated by experiments in animal models of MI (25, 28, 44). Myocardial ischemia and MI are often accompanied by a marked autonomic imbalance that is characterized by sympathovagal overactivity (38, 60) and vagal attenuation (54). Previous studies have shown that autonomic alterations may precede the MI (27, 29, 30). It is well established that sympathetic overactivity is associated with a higher risk of cardiovascular events (38, 60). Nevertheless, reduced vagal drive to the heart is an independent risk factor for life-threatening arrhythmias and sudden cardiac death (21).

It has been argued that parasympathetic activation may have beneficial implications after MI (13). Previous studies have demonstrated that electrical stimulation of the vagal nerve improves myocardial performance, reduces the incidence of arrhythmias, restores cardiac autonomic control, and extends survival in experimental models of MI (36, 64). Therefore, the increase in parasympathetic function may be a reliable therapy for the prevention of MI outcomes. However, vagal nerve stimulation is an invasive procedure. Pharmacological agents that augment ACh availability at the neuroeffector junction may have beneficial effects, and these agents should be considered an alternative therapy for MI. Pyridostigmine (PYR), a reversible anticholinesterase inhibitor that does not cross the blood-brain barrier and acts particularly in the peripheral synaptic cleft, may be useful in this context (55). PYR induced bradycardia when administered to healthy humans for 1–2 days (15, 41). PYR reduced heart rate (HR) (9) and ventricular arrhythmia density (5) in patients with heart failure. These findings suggest that PYR can modulate the cardiac sympathovagal balance under physiological conditions and in patients who have suffered MI.

Another electrocardiographic abnormality that is commonly elicited by MI, and strongly associated with a higher incidence of ventricular arrhythmias, is the lengthening of the QT interval. PYR decreased the QTc interval in patients with MI (9, 11) and reduced the incidence of cardiac arrhythmias (5).

Arrhythmias are often caused by an impairment of electrical impulse propagation through gap junctions, which consist of intercellular channels that are formed by transmembrane proteins known as connexins, which allow electrical coupling between cardiomyocytes (4, 20). Connexin (Cx)43 is particularly abundant in cardiac ventricles (32, 40). Previous studies have shown that the total amount of Cx43 is reduced after heart ischemia (4), MI (45), and heart failure (47). However, cardiac cholinergic stimulation, both in vitro (62) and in vivo (4), prevented the degradation and dephosphorylation of Cx43. Therefore, it is reasonable to speculate that the antiarrhythmic effect already observed in infarcted humans treated with PYR (5) could be due, at least in part, to a role of ACh in cardiomyocyte membrane stability.

Therefore, we hypothesize that the early arrhythmogenic phase of MI could be offset by enhancing ACh availability in...
the heart via acetylcholinesterase inhibitor administration. There are no reports of the effect of PYR during the early phase of MI. Therefore, we proposed to investigate the effects of acute PYR administration on the hemodynamics, electrocardiographic alterations, and incidence of arrhythmias during the first 4 h after MI. Moreover, we studied the Cx43 content in hearts subjected to MI to investigate the possible mechanisms behind the antiarrhythogenic effect of PYR.

METHODS

All experimental procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals [National Institutes of Health (NIH) Publication No. 85-23, Revised 1985]. The Committee of Ethics in Animal Research of the Medical School of Ribeirão Preto, University of São Paulo, approved the experimental protocols (protocol no. 177/2011).

Animals

Experiments were performed in adult male Wistar rats (280–350 g) that were maintained on a 12:12-h light-dark cycle and housed individually with free access to food and water.

Protocols

Protocol 1: cardiac autonomic tone and acetylcholinesterase activity. Preliminary experiments (data not shown) were performed to evaluate the cardiovascular effects of different doses of PYR. This initiative led us to use 0.25 mg/kg because this dose of PYR was enough to determine slight bradycardia (decrease of HR of ~10–15% from baseline) without eliciting significant hemodynamic changes. Rats were anesthetized with urethane [ethyl carbamate (1 g/kg ip), Sigma Chemical, St. Louis, MO] and placed on a heating pad (Insight Equipments) to maintain core body temperature between 36 and 37°C. Animals were instrumented with subcutaneous electrodes for ECG recordings and polyethylene catheters (Intramedic, Clay Adams, Par- sippany, NJ) into the femoral artery and vein for arterial pressure (AP) recordings and drug administration, respectively. Electrodes were connected to a differential amplifier (CL-615422-1, Gould Instruments Systems, Valley View, OH) to record ECGs, and the arterial line was connected to a pressure transducer (MLT0380/D, AD Instruments, Sydney, NSW, Australia). ECG and AP were continuously sampled (500 Hz) using an IBM/PC equipped with an analog-to-digital interface (DI-720 Datq System, Akron, OH). After basal recordings, rats received an intravenous injection of saline (n = 16) or PYR (0.25 mg/kg, Valeant Pharmaceuticauks, Campinas, São Paulo, Brazil, n = 18). One hour after the administration of saline or PYR, rats were treated with the cardiac autonomic receptor blockers methylatropine (1 mg/kg iv) and propranolol (2 mg/kg iv) at intervals of 15 min to indirectly assess cardiac parasympathetic and sympathetic autonomic tones. The doses of methylatropine and propranolol used in the present study are well accepted as effective blockers of cardiac muscarinic and β-adrenergic receptors in rats (17, 23, 56). Half of the rats in each group (saline or PYR) received the autonomic blockers in the opposite order, i.e., propranolol followed by methylatropine. A distinct small group of rats treated with saline (n = 4) or PYR (n = 4) had a sample of arterial blood withdrawn to measure plasmatic acetylcholinesterase activity to confirm the anticholinesterase effect of PYR.

ACETYLCHOLINESTERASE ACTIVITY. Blood samples (300 µl) were collected from the arterial catheter before and 1 h after saline or PYR treatment. Blood samples were collected in vials containing 30 µl EDTA (0.1 M, Sigma-Aldrich). Enzymatic assays were performed using an adaptation of the colorimetric method as previously described by Alves-Amalar et al. (3). The velocity of the reaction for each sample was determined in duplicate, and the results are expressed as arbitrary units per minute per milliliter of plasma.

Protocol 2: MI. Initially, animals were anesthetized and instrumented as previously described for protocol 1. After 20 min of basal recordings, animals were endotracheally intubated and supplied by a mechanical ventilator (model 680, Harvard Apparatus rodent respirator, Holliston, MA) attached to a system that continuously measured expired CO2 (Micro Capstar End-tidal CO2 analyzer, CWE). An extensive MI was produced according to the method previously described by Johns and Olson (24). Control rats had the heart exteriorized, but the coronary ligation was not performed (sham surgery, n = 6). The heart was returned to the chest cavity, and the thorax incision was closed with sutures. After coronary ligation, rats were randomized to groups that received, intravenously, PYR (n = 14) or saline (n = 14) 10–15 min after MI. All variables were continuously recorded for 4 h after MI. Mechanical ventilation was withdrawn between the first and second hour after coronary ligation, when animals were able to breathe spontaneously with an end-tidal CO2 below 6%.

Measurement of the Ischemic Risk Zone

Hearts were excised after the experiments were finished, and Evans blue dye (0.5%) was retrograde flushed through the ascending aorta at a flow rate of 18 ml/min to demarcate the ischemic risk zone, i.e., the myocardial area that the blue dye did not reach (Fig. 1). Hearts were transversely cut into six slices of uniform thickness (2 mm) using a custom-made, equally spaced blade slicer. Slices were digitally scanned, and the extension of ischemic risk zones was measured in each slice using the public domain software NIH ImageJ (developed by NIH and available on the internet site http://rsb.info.nih.gov/nih-image/). All rats used in the present study showed an ischemic risk zone involving >35% of the circumference of left ventricular wall.

Evaluation of Cx43 Levels

We conducted this protocol separately from hemodynamic and arrhythmia experiments because the experimental preparation of Evans blue dye injections for risk area assessment did not allow us to perform immunoblot assays of excised hearts. We prepared and analyzed eight infarcted hearts and five hearts from sham-operated control subjects for each group.

Hearts from groups of rats subjected or not to MI were excised and stored at −80°C. The immunoblot analysis for Cx43 was made as previously described elsewhere (6).

Data Analysis

AP, ECG, and expired CO2 recordings were analyzed offline. Beat-by-beat-based series of mean AP (MAP) and HR (calculated from RR interval changes) were generated.
The HR changes elicited by methyl-atropine and propranolol were measured, and the results were considered indexes of vagal and sympathetic cardiac tonic influence, respectively. HR in the presence of both autonomic blockers, i.e., without autonomic influences, was considered the intrinsic pacemaker HR, i.e., HR without the influence of cardiac autonomic drive (19).

Segments of ECG with 2,000 beats each were selected from entire recordings of each rat that was submitted to MI from the basal period (before MI) and 30, 60, 120, 180, and 240 min after MI. Each segment was visually inspected to detect and quantify premature ventricular beats (PVBs). Moreover, ECGs recorded right after MI (between 30 and 60 min) and between 180 and 240 min were processed using the LabChart module for ECG Analysis (LabChart, AD Instuments) to measure changes in the PR interval and QT segment corrected by the RR interval (QTc; Bazett formula: QT interval/RR interval). The computer software automatically detected both the onset and end of each ECG wave. Nevertheless, a visual inspection was performed to verify and correct any automatic misdetection.

**Statistical Analysis**

Data are reported as means ± SE. Differences in the sympathovagal balance, plasmatic acetylcholinesterase activity, and extent of the ischemic risk zone were compared between groups using Student’s t-test. Hemodynamics and electrocardiographic variables were compared among the three groups using two-way ANOVA adjusted for repeated measures followed by a Newman-Keuls comparison test. The incidence of PVBs between infarcted rats was adjusted for repeated measures followed by a Newman-Keuls comparison test. The incidence of PVBs between infarcted rats was compared among groups using one-way ANOVA followed by a Newman-Keuls comparison test. Differences were considered significant at P values of <0.05.

**RESULTS**

**Cardiac Autonomic Tone**

Basal HR was 438 ± 8 beats/min (N = 34). HR was unaltered (435 ± 11 beats/min, n = 16) 1 h after saline administration, but PYR produced marked bradycardia (387 ± 7 beats/min, n = 18). MAP was similar in rats treated with PYR or saline (87 ± 3 and 91 ± 5 mmHg). Table 1 shows HR responses to methyl-atropine (vagal tone) and propranolol (sympathetic tone) and intrinsic pacemaker HR 60 min after PYR or saline. PYR produced smaller sympathetic and higher parasympathetic cardiac tone. Intrinsic HR was similar between the groups.

**Acetylcholinesterase Activity**

Plasma acetylcholinesterase activity was reduced by 12 ± 9% (P < 0.05) 60 min after PYR administration, whereas it was not affected by saline.

**MI**

The occlusion was performed in the left coronary artery, and the area of ischemia (ischemic risk zone) was located primarily in the anterior wall of the left ventricle (Fig. 1). The MI size was similar in rats treated with saline (47 ± 4%) or PYR (50 ± 4%).

**Hemodynamics**

Figure 2 shows MAP and HR changes after acute sham surgery or MI in rats treated with PYR or saline. MI elicited a prompt fall in MAP that was not affected by PYR. Nontreated rats showed a gradual rise in HR after MI. Infarcted treated rats also showed a marked bradycardia after PYR, similar to healthy rats from protocol 1. Nevertheless, PYR-elicited bradycardia recovered slowly and reached baseline levels at the end of the protocol.

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**Table 1. HR responses 1 h after the administration of saline or PYR**

<table>
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<th>Saline</th>
<th>PYR (0.25 mg/kg)</th>
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<tr>
<td>HR, beats/min</td>
<td>435 ± 11</td>
<td>387 ± 10*</td>
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<tr>
<td>n</td>
<td>16</td>
<td>18</td>
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<tr>
<td>Sympathetic tone, change in beats/min</td>
<td>-69 ± 7</td>
<td>-31 ± 8*</td>
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<tr>
<td>n</td>
<td>6</td>
<td>8</td>
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<tr>
<td>Vagal tone, change in beats/min</td>
<td>44 ± 5</td>
<td>86 ± 7*</td>
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<tr>
<td>n</td>
<td>10</td>
<td>10</td>
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<tr>
<td>Intrinsic pacemaker HR, beats/min</td>
<td>412 ± 6</td>
<td>410 ± 7</td>
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<td>n</td>
<td>16</td>
<td>18</td>
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n, Number of rats/group. Shown are values of heart rate (HR) 1 h after the administration of saline or pyridostigmine (PYR; 0.25 mg/kg). Additionally, the HR response to propranolol (sympathetic tone) and methyl-atropine (vagal tone) and HR after both autonomic blockers were administered (intrinsic pacemaker HR) are shown. *P < 0.05 compared with saline.
with PYR or saline. MI induced a lengthening of the QTc (30 min) and at the end of the protocol (240 min) in rats treated with saline (n = 14). The Kaplan-Meier plot shows the incidence of PVBs after MI in rats treated with saline (n = 14) or PYR (n = 4). *P < 0.05 compared with sham rats; †P < 0.05 compared with PYR-treated rats.

**Electrocardiographic Variables**

Table 2 shows changes in the QTc interval shortly after MI (30 min) and at the end of the protocol (240 min) in rats treated with PYR or saline. MI induced a lengthening of the QTc interval in nontreated rats but not in rats that received PYR. The basal PR interval was 50 ± 1 ms (n = 28), and no change was found after MI in rats treated with PYR (50 ± 1 ms, n = 14) or saline (46 ± 3 ms, n = 14).

Arrhythmias, such as self-limited periods of ventricular tachycardia and ventricular fibrillation, were often observed during the first 30 min after coronary ligation. However, multifocal isolated PVB was the main arrhythmic phenomenon observed after 30 min in both groups with MI. The incidence of PVBs after MI in PYR-treated and nontreated rats is shown in Fig. 3. Acute PYR treatment reduced the incidence of PVB after MI.

**Cx43 in MI hearts**

We determined the amount of Cx43 using Western blot analysis in the left ventricles of animals subjected to MI in the presence or absence of PYR. Animals subjected to sham surgery were used as controls. The results obtained showed that MI induced a remarkable reduction in total Cx43, which was partially prevented by PYR (Fig. 4).

**DISCUSSION**

This is the first study showing the hemodynamic effects of intravenous administration of PYR in healthy and MI rats. In healthy animals, PYR induced bradycardia without affecting AP, increased cardiac parasympathetic tone, and reduced sympathetic tone. In MI rats, PYR induced also bradycardia, whereas it prevented lengthening of the QTc interval, reduced the incidence of PVBs, and prevented Cx43 reduction in the infarcted ventricle.

As a reversible anticholinesterase agent that acts predominantly in peripheral synapses, PYR has been widely used in the treatment of myasthenia gravis (8). However, authors have recently examined other potential beneficial effects of this drug in some pathophysiological conditions, such as MI and heart failure (5, 8, 9). It has been documented that the acetylcholinesterase inhibitor donepezil improved cardiac function in rats with heart failure (43). However, due to its ability to cross the blood-brain barrier and high affinity for brain acetylcholinesterase, donepezil and other anticholinesterase agents may cause undesirable side effects involving their action on the central nervous system, for instance, dizziness, nausea, and insomnia (39, 51). The most prominent effect of PYR in the present study was a prompt bradycardia that was observed immediately after its administration in healthy and infarcted rats. Nevertheless, the magnitude of the HR fall (10–15% of baseline) was not sufficient to cause important circulatory changes that would affect the infarcted rats.

**Pyridostigmine and Cardiac Sympathovagal Balance in Healthy Anesthetized Rats**

HR responses to cardiac autonomic receptor blockers showed that acute PYR administration in urethane-anesthetized rats effectively elicited a marked shift in the cardiac sympathovagal balance toward the vagal modulation with a decrease in sympathetic tone. There is evidence suggesting that ACh can act on muscarinic receptors located near the presynaptic sympathetic nerve terminals, inhibiting norepinephrine release (35, 57), decreasing efferent sympathetic activity to the heart, and further contributing to reduce chronotropic cardiac activity. Studies from our laboratory have shown that chronic administration of PYR in rats subjected to MI improved the baroreflex sensitivity, increased vagal modulation, and reduced sympathetic tone without changing the intrinsic pacemaker HR (33,
showed a lengthening of the QTc interval, and PYR prevented (52). In the present study, infarcted rats treated with saline of life-threatening arrhythmias and cardiac sudden death (1, 22, 53). The sinus node fires at its intrinsic rate in the absence of any sympathetic or parasympathetic drive (19), and, in this study, the intrinsic pacemaker HR was not different between groups.

**Hemodynamics After MI**

The hypotension observed immediately after acute MI is consistent with previous studies of MI in rats (25, 46) and has been attributed to an impairment of myocardial performance due to the acute infarct (31, 46, 48). Surprisingly, a similar fall in AP was observed in sham-operated rats. During the surgical procedures for either real or sham MI, the heart is strongly manipulated, and we do believe that this procedure might lead to an acute impairment in myocardial performance, explaining the observed hypotension. PYR did not affect the hypotension after MI but elicited a prompt bradycardia, which slowly recovered over time. We do believe that the short half-life of PYR (1.5–2 h) (55) may explain the return of HR to its previous baseline. Several studies have shown that PYR elicited bradycardia in healthy subjects (10, 14, 42) and infarcted patients (5, 9). However, to our knowledge, few studies have evaluated the hemodynamic effects of acute PYR administration, e.g., over several hours or up to 1 wk (12, 53). No changes in HR or AP (12, 53) were observed in response to PYR. These differences may be due to different methodological approaches, such as the means of administration and/or doses. On the other hand, consistent with the present results, PYR administration in drinking water during 4 wk induced significant bradycardia in rats with heart failure (33). Moreover, infarcted rats not treated with PYR showed a progressive increase in HR that reached a plateau 3–4 h after coronary ligation. Tachycardia after acute MI is consistent with previous experimental (28) and clinical (60) findings. This tachycardia might be due to sympathetic overactivity (38, 60) or cardiac vagal withdraw after MI (26).

**Electrocardiographic Responses**

It is well established that MI leads to a lengthening of the QTc interval after MI (2, 22), which is considered a predictor of life-threatening arrhythmias and cardiac sudden death (1, 22, 52). In the present study, infarcted rats treated with saline showed a lengthening of the QTc interval, and PYR prevented the lengthening of this interval without affecting the PR interval. These findings are consistent with reports from Castro et al. (9, 10), who showed that PYR shortened the QTg interval of healthy subjects and patients with MI. Moreover, PYR reduced the QTg interval during recovery from maximal exercise in ischemic heart disease (11).

Few studies have evaluated the antiarrhythmic effect of vagal stimulation on MI. In this study, the administration of PYR 10 min after MI reduced the incidence of PVBs. The pharmacological enhancement of cholinergic activity to the heart using PYR also reduced the incidence of PVBs in patients with heart failure (5) and arrhythmias but no history of MI (59). Zimmerman et al. (65) suggested that the antiarrhythmic effect of PYR could be partially related to a reduction of the refractory ventricular period, which was shorter in humans after a single dose of PYR.

There is evidence showing that vagal nerve stimulation reduced the incidence of ventricular fibrillation in conscious dogs subjected to cardiac ischemia (58) and that it increased the ventricular fibrillation threshold in hearts from rabbits subjected to coronary artery ligation (7). Furthermore, it has been argued that this antiarrhythmic effect of parasympathetic activity could occur because preventing Cx43 degradation in infarcted hearts, in turn, prevents the cell-to-cell electrical uncoupling of ventricular myocytes (4).

**Cx43 in Hearts Subjected to MI**

There is evidence showing that an enhanced vagal drive to the heart via vagal nerve stimulation (4) or direct cardiac pharmacological stimulation with ACh (62) prevented reductions in Cx43. Therefore, we speculated that ACh plays a role in Cx43 quantity and function. We previously demonstrated that degradation of gap junctions can occur via endocytosis or autophagy, through mechanisms based on the prior ubiquitination of Cx43 (6, 18). Therefore, it is conceivable to suggest that PYR is hampering the MI-induced reduction of Cx43 content by interfering with its degradation pathways. Although Zhao et al. (63) previously reported that ACh protects against hypoxia/reoxygenation injury in H9C2 cells by promoting autophagy through the AMP-activated protein kinase pathway, it is likely that PYR prevents detrimental autophagy degradation involving other players, such as Beclin1. However, additional studies are required to elucidate this issue. Moreover, it appears that the beneficial antiarrhythmic role of ACh does not depend on its bradycardic effect. Brack et al. (7) showed a reduced incidence of arrhythmias without bradycardia in rabbit hearts. These findings corroborate our results as we showed that PYR induced significant bradycardia up to 2 h after treatment but that the antiarrhythmic effect persisted up to 4 h after MI.

**Conclusions and Perspectives**

First, we investigated the effects of acute PYR treatment on cardiac autonomic tone in healthy urethane-anesthetized rats and its effects on hemodynamic and ECG parameters during the first 4 h after coronary artery ligation. PYR reduced the sympathetic tone and increased the vagal tone to the heart. Furthermore, the main results demonstrated that PYR reduced the incidence of arrhythmias in infarcted rats and preserved Cx43 in treated rats. Therefore, PYR could modulate the metabolism of connexins (components of gap junctions) through the increase of tissue ACh, which could be involved in its antiarrhythmic effect.

PYR could be beneficial during the acute phase of MI because it reduced some risk factors for sudden cardiac death after MI, such as QTg lengthening and the incidence of arrhythmias. However, more studies are required to clarify the role of ACh in Cx43 degradation pathways.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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