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Subacute pyridostigmine exposure increases heart rate recovery and cardiac parasympathetic tone in rats

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Abstract

Heart rate recovery (HRR) describes the rapid deceleration of heart rate after strenuous exercise and is an indicator of parasympathetic tone. A reduction in parasympathetic tone occurs in patients with congestive heart failure, resulting in prolonged HRR. Acetylcholinesterase inhibitors, such as pyridostigmine, can enhance parasympathetic tone by increasing cholinergic input to the heart. The objective of this study was to develop a rodent model of HRR to test the hypothesis that subacute pyridostigmine administration decreases cholinesterase activity and accelerates HRR in rats. Ten days after implantation of radiotelemetry transmitters, male Sprague Dawley rats were randomized to control (CTL) or treated (PYR; 0.14 mg/ml pyridostigmine in the drinking water, 29 days) groups. Rats were exercised on a treadmill to record HRR, and blood samples were collected on days 0, 7, 14, and 28 of pyridostigmine administration. Total cholinesterase and acetylcholinesterase (AChE) activity in plasma was decreased by 32–43% and 57–80%, respectively, in PYR rats on days 7–28, while plasma butyrylcholinesterase activity did not significantly change. AChE activity in RBCs was markedly reduced by 64–66%. HRR recorded 1 min after exercise was higher in the PYR group on days 7, 14 and 28, and on day 7 when HRR was estimated at 3 and 5 min. Autonomic tone was evaluated pharmacologically using sequential administration of muscarinic (atropine) and adrenergic (propranolol) blockers. Parasympathetic tone was increased in PYR rats as compared with the CTL group. These data support the study hypothesis that subacute pyridostigmine administration enhances HRR by increasing cardiac parasympathetic tone.

Keywords

cholinesterase inhibitors; exercise model; exercise test; experimental animal models; parasympathetic nervous system; parasympathomimetic; pharmacology; rodents; subacute

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Introduction

The autonomic nervous system plays a major role in controlling cardiovascular functions. Neuroendocrine dysregulation, characterized by sympathetic over-activity and parasympathetic withdrawal, is an important determinant of mortality and morbidity in patients with congestive heart failure (CHF) (1). Cardiac autonomic function can be assessed in such patients by various tools, including the chronotropic response of the heart to both physiological stress and pharmacological blockade (2, 3), heart rate variability (HRV) analysis (4, 5), quantification of baroreflex sensitivity (6), plasma or coronary sinus catecholamine levels (7), HR turbulence (7), and others. In contrast to these methods, heart rate can be recorded noninvasively and inexpensively with the aid of commercially-available and automated equipment. Heart rate recovery (HRR) is the rapid deceleration of heart rate after strenuous exercise and can be easily determined (8). HRR after submaximal exercise approximates parasympathetic tone in human subjects (9–11). As an index of cardiac parasympathetic tone, HRR has clinical utility in predicting cardiovascular fitness, disease prognosis, and mortality in heart failure patients (12–16).

Some of the earliest clinical evaluations of heart rate recovery were performed by Perini and Imai and coworkers (10, 11). They observed that the early (within 1 min) recovery of heart rate after exercise is mediated by parasympathetic reactivation. Later, Pierpont and coworkers quantitatively linked HRR to parasympathetic function in healthy volunteers (17, 18). Shelter and Watanabe validated the use of HRR as a predictor of mortality in human subjects (13, 19). Despite the importance of HRR to human subjects, there are few studies that support the use of HRR to evaluate parasympathetic tone in rodent models. Barnard (1974) discussed the effect of training rats to near maximal heart rates and subsequent changes in HRR, but did not correlate HRR with changes in cardiac parasympathetic tone (20).

Acetylcholinesterase inhibitors, such as pyridostigmine, can increase parasympathetic activity by increasing acetylcholine levels at the vagal cholinergic nerve terminal that innervate cardiac tissue. Previous studies by Androne and co-workers demonstrated that short term pyridostigmine treatment augmented parasympathetic tone and accelerated HRR in stable CHF patients (21). Similarly, Serra *et al.* found that the chronotropic response to exercise in heart failure patients was reduced by the administration of pyridostigmine (22). There are currently few animal models that have reproduced these findings and could thus serve as a suitable model for further study of the relationship between the autonomic nervous system and heart rate recovery (23). The objectives of our study were therefore to develop a rodent exercise model of HRR and to use this model to test our hypothesis that subacute administration of pyridostigmine shortens HRR after submaximal exercise in normal rats by increasing cardiac parasympathetic tone.

Results

Pyridostigmine intake and *in vivo* and *in vitro* inhibition of cholinesterase activity

Pyridostigmine in tap water was stable in the dark at both room temperature and at 4° C, for up to 8 days. During the experimental period, water bottles containing either fresh water or

freshly-prepared pyridostigmine in water were replaced every 3 days. There were no significant differences in body weight gain (CTL, 55 ± 4 g; PYR, 51 ± 4 g, $P=0.53$) or water consumption (CTL, 100 ± 5 ml/kg/day; PYR, 106 ± 6 mL/kg/day, $P=0.47$) between the two groups. Pyridostigmine intake was estimated to be 15 ± 1 mg/kg/day in the PYR group.

Rat plasma contains relatively similar levels of both AChE and BChE, whereas rat RBCs primarily contain only AChE (24). Fig. 1A shows that the total ChE activity in plasma was decreased by 32%, 43% and 36% ($P=0.007$) in the PYR group relative to controls on days 7, 14 and 28, respectively. Fig. 1B shows the effects of PYR on plasma AChE activity, which was reduced by 57%, 80%, and 66% ($P<0.0001$) as compared to control rats on days 7, 14 and 28, respectively. In contrast, there was no significant reduction in plasma BChE activity at any time-point after beginning PYR administration (Fig. 1C). Similar to reductions in plasma AChE, AChE activity in RBCs was also markedly lower (64%, 66%, and 66%, $P<0.0001$, Fig. 1D) on days 7, 14, and 28 following the initiation of PYR administration. When compared to day 0, all the aforementioned enzyme activities were significantly lower on all subsequent days in the PYR group ($P<0.0001$ for total ChE, plasma AChE and RBC AChE, and $P<0.01$ for plasma BChE). These *in vivo* data suggested that AChE was more sensitive to inhibition by PYR as compared with BChE. Signs of cholinergic toxicity (involuntary movements and SLUD (salivation, lacrimation, urination and defecation) signs were not observed in either the CTL or PYR group.

We also evaluated *in vitro* sensitivities of AChE and BChE to inhibition by PYR. Figure 2 shows that the IC_{50} (95% confidence interval) for PYR was relatively similar for AChE in both RBC (1.02×10^{-7} M; $0.91 - 1.1 \times 10^{-7}$) and plasma (1.76×10^{-7} M; $1.65 - 1.89 \times 10^{-7}$). In contrast, BChE in plasma was markedly (more than an order of magnitude) less sensitive to *in vitro* inhibition by PYR, with a mean IC_{50} of 2.3×10^{-6} M ($2.16 - 2.5 \times 10^{-6}$).

Heart rate recovery

As shown in Fig. 3A, basal HR was similar between groups ($P>0.28$). Fig. 3B shows that the maximum or peak HR at the maximum running speed of 20 m/min was significantly lower in PYR rats as compared to pretreatment values on days 7, 14 and 28 ($P<0.01$). Pyridostigmine administration accelerated HRR as compared with CTL rats, at 1, 3 and 5 minutes after the end of exercise e.g., HRR1, HRR3 and HRR5 (Figs. 4A, B and C respectively). The HRR1 in PYR rats was significantly higher than in the CTL group on days 7, 14 and 28 ($P=0.02$, interaction term, Fig. 4A). However, the HRR3 was significantly higher in the PYR rats on day 7, but not on days 14 or 28 ($P=0.01$, interaction term Fig. 4B). Similarly, on day 7 but not on days 14 or 28, HRR5 was significantly higher in the PYR group as compared with CTL ($P=0.02$, Fig. 4C).

Autonomic tone and intrinsic heart rate

Figure 5 shows that parasympathetic tone was increased in the PYR rats as compared to the CTL group, as indicated by a greater bradycardic response (17% decrease in HR) after propranolol administration ($P<0.001$). In contrast, there was no difference in sympathetic tone (28% vs. 29% increase in HR, $P=0.99$) between the treatment groups after atropine

administration. Intrinsic HR was 374 ± 9 vs. 368 ± 8 beats/min and 349 ± 10 vs. 352 ± 11 beats/min ($P=0.53$) in the CTL and PYR groups on days 27 and 29, respectively (data not shown). Basal HR was also similar between the groups on day 27 (338 ± 12 and 324 ± 10 beats/min in CTL and PYR groups, respectively, $P=0.4$); however, on day 29, basal HR was significantly lower in the PYR group (350 ± 7 vs. 321 ± 9 beats/min in CTL and PYR groups, respectively $P=0.02$; data not shown).

Discussion

Heart rate recovery describes the duration of time over which the heart rate decelerates after moderate to heavy exercise and is dependent on the dynamic relationship between the parasympathetic and sympathetic systems (25). During and after exercise, parasympathetic and sympathetic control of the heart adjusts according to metabolic demands. Savin and colleagues (26) studied cardiac function in normal patients with and without selective autonomic blockers and proposed that sympathetic withdrawal contributes more substantially to this decrease in heart rate soon after exercise, whereas the parasympathetic system predominates later in the recovery phase. However, recent studies have instead proposed that parasympathetic reactivation occurs faster than sympathetic withdrawal, thus playing a major role in the early deceleration of the heart after the termination of exercise (11, 17, 27–29). Our study was designed to explore the relative contributions of the sympathetic and parasympathetic systems to heart rate recovery following forced running exercise in rats.

Barnard and coworkers reported relatively few differences in HRR among different ages of rats exercised until exhaustion (20). To the best of our knowledge, there is no other published literature on HRR in rats for comparison. In order to develop an exercise model of HRR and the cardiac neuroendocrine system in rats, we modified an exercise protocol from a previous study by Copp and coworkers (30). Using this exercise model, we determined that subacute administration of pyridostigmine in rats does indeed accelerate heart rate recovery (Fig. 4). Pyridostigmine can modify parasympathetic control of cardiac function by inhibiting the enzymatic breakdown of acetylcholine at vagal nerve terminals. This leads to enhanced activation of the muscarinic receptors, affecting cardiac muscle contraction and heart rate. A single dose of pyridostigmine in CHF patients led to an increase in heart rate recovery recorded at 1 min after the termination of exercise (21). In 2007, Dewland et al showed that in sedentary adults, pyridostigmine decreased resting heart rate and accelerated post-exercise heart rate recovery at 1 min, yet had no significant effect on trained athletes (31). The study by Dewland and colleagues described post-exercise heart rate recovery as an index of cholinergic signaling in the sinoatrial nodal junction. Similarly, Serra *et al.* reported that repeated pyridostigmine administration accelerated HRR and other hemodynamic parameters in human CHF patients (22). Our study also demonstrated that subacute pyridostigmine administration (28 days in the drinking water) accelerated heart rate recovery in rats (Fig. 4). The results from our study thus generally agree with and reflect those previously reported in humans. However, basal heart rate did not differ significantly between treatment groups in our study (Fig. 3), contrasting with an earlier study performed in rats with induced heart failure (32). This discrepancy may be due to physiological differences between the normal rats used in the current study as compared to those with heart failure. In

heart failure rats, a positive chronotropic condition occurs as a consequence of the withdrawal of the parasympathetic and stimulation of the sympathetic systems (32). As an indirect parasympathomimetic drug, pyridostigmine enhances cardiac vagal tone, thus a greater negative chronotropic effect might be expected in rats with heart failure as compared with a smaller magnitude negative chronotropic effect in normal rats. This smaller magnitude effect, along with day to day variability, may also explain why mean basal HR was not significantly lower in PYR rats as compared to CTL rats, both before exercise and on day 27, just prior to pharmacological estimation of autonomic tone, but was significantly lower on day 29, just before pharmacological manipulations.

Soares et al reported that 0.14 mg/mL pyridostigmine in the drinking water of rats resulted in an average pyridostigmine intake of 31 mg/kg/day (33). These investigators reported that this concentration of pyridostigmine led to approximately 40% inhibition of plasma acetylcholinesterase activity after a seven day treatment period (33). Although the average pyridostigmine intake in our study was estimated to be only 15 mg/kg/day, AChE inhibition after seven days of drug administration was 57% and 64% in rat plasma and RBCs, respectively (Fig. 1). Other studies used the same pyridostigmine dosing conditions in rats as Soares et al and the resulting dose rate was also higher than that administered in our study (32, 34–36). The relatively lower intake of pyridostigmine by our rats may be attributed to higher water intake by the young rats used in the above listed studies, as compared to the older rats used in our study (37). Moreover, when compared to 28% cholinesterases inhibition by pyridostigmine in HRR studies performed in human subjects and patients with cardiovascular diseases (38, 39), the level of inhibition of acetylcholinesterases were generally higher in our study (Fig. 1). The higher dose rate of pyridostigmine in our study may have contributed to the substantial acceleration of HRR (Fig. 4).

We noted substantial differences in the magnitude of AChE and BChE inhibition following *in vivo* PYR exposure (Fig. 1). We therefore evaluated the relative *in vitro* sensitivity of AChE and BChE to inhibition by pyridostigmine. In both the RBC and plasma, pyridostigmine was a more potent *in vitro* inhibitor of AChE as compared to BChE, confirming that pyridostigmine has greater inhibitory effect on AChE than on BChE (Fig. 2). A previous report indicated that *in vitro* measurements of human and rat AChE may also show differences in sensitivity to pyridostigmine (40). The slight difference of *in vitro* sensitivity of AChE in plasma to pyridostigmine vs AChE in RBCs that was noted in our study may be due to differences in PYR metabolism in the plasma vs RBC fractions. Pyridostigmine is a carbamate acetylcholinesterase enzyme inhibitor, and some carbamate anticholinesterases are metabolized by plasma carboxylesterases (41). Thus, there may be more stoichiometric binding sites for pyridostigmine in the plasma as compared with the RBCs, and thus less inhibition of AChE in plasma, despite similar concentrations of pyridostigmine. Pyridostigmine may also bind to plasma albumin (42), decreasing its unbound concentration that is available to inhibit plasma enzymes.

Subacute administration of pyridostigmine increased parasympathetic tone but had no apparent effect on sympathetic tone (Fig. 5) in the present study. In contrast, in a previous study of heart failure rats (32), the authors reported that parasympathetic tone was increased and sympathetic tone was decreased by pyridostigmine administration, with the latter

possibly due to the effect of pyridostigmine on cholinergic transmission in the sinoatrial node. However, in both heart failure rats from this previous study and in the normal rats used in our current study, intrinsic heart rate did not differ between the PYR and CTL groups. Pharmacological blockade of the sympathetic and parasympathetic nervous systems by the administration of selective blocking agents are useful for assessing autonomic tone (29, 43–45), but the specific manner in which these agents are administered and the subsequent data manipulation can affect the conclusions. Lataro et al subtracted HR_{basal} from HR_{atropine} to estimate parasympathetic tone, and then subtracted that value from $HR_{\text{propranolol}}$ to estimate sympathetic tone (32). However, after subtracting the basal HR from the HR_{atropine} or $HR_{\text{propranolol}}$, the intrinsic HR still influences HR and confounds the interpretation of relative effects of the autonomic system. We proposed that changes in autonomic tone could be better evaluated by subtracting the intrinsic HR from HR_{atropine} to determine sympathetic tone, then subtracting that value from $HR_{\text{propranolol}}$ to estimate parasympathetic tone (Equations 1 and 2). This approach may exclude the influence of intrinsic activity while individually isolating the parasympathetic and sympathetic contributions. A similar approach was taken in rats by Ichige et al. where sympathetic tone was calculated as ‘HR after atropine minus intrinsic HR’ and parasympathetic tone as ‘HR after atenolol minus intrinsic HR’ (46). However, the most important finding regarding pharmacological blockade in the current study was confirmation that the anticipated parasympathomimetic effect of pyridostigmine administration correlated with accelerate heart rate recovery in rats. Therefore, modulation of heart rate recovery in rats is potentially a useful model with relevance to common human measures of autonomic tone.

Material and methods

Optimization of experimental methods

Preliminary data were collected from eight, 8–9 week-old, male Sprague Dawley rats weighing 275–300 g (Charles River, Wilmington, MA). The goal of the pilot study was to optimize surgical and data collection procedures and to estimate the number of animals needed to test the study hypothesis. This preliminary assessment showed that intraperitoneal implantation of the radiotelemetry transmitter device with an intradermal suture pattern led to a faster recovery as compared to subcutaneous implantation with simple interrupted sutures. In the pilot study, exercise performance and heart rate were assessed at increasing treadmill speeds to a maximum rate of 25 m/min. Rats initially ran at a speed of 5 m/min at a 10% grade for 10 min. Thereafter, the treadmill was held at a constant grade while the speed was increased by 5 m/min every 5 min until the rat was unable or unwilling to maintain pace with the treadmill belt (modified from (30)). The heart rate at exercise failure was deemed the maximal heart rate. The target submaximal HR was 80% of the maximal HR and was achieved at a speed of 20 m/min. From the pilot study (data not shown), a sample size of eight rats per treatment group was calculated to be sufficient to detect a treatment difference of 44 beats/min in HRR at 1 minute after termination of exercise, with a power of 80% and an alpha of 0.05.

Experimental animals

Sixteen male, 8–9 week-old, Sprague Dawley rats weighing 275–300 g were purchased from Charles River (Wilmington, MA). Only one gender was selected in order to avoid any potential sex-related differences in responses to drug administration. The rats were housed individually and maintained on a 12-hour light-dark cycle in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International)-accredited facility. Rats were given *ad libitum* access to water and standard laboratory rodent chow. All animal experiment protocols were reviewed and approved by the Oklahoma State University Institutional Animal Care and Use Committee.

Surgical implantation of radiotelemetry device

After one week of acclimatization, a small animal radiotelemetry transmitter device (CTA-F40, Data Sciences International, St. Paul, MN) was surgically implanted under isoflurane general anesthesia. The surgical protocol followed was adapted from the recommendations of the radiotelemetry unit manufacturer (ETA, CTA, EA, or CA Device Surgical Manual, Data Sciences International, St. Paul, MN). A single dose of morphine (5 mg/kg, IM) was administered before surgery. Meloxicam (2 mg/kg, bid, SQ) was administered to all rats for three days after surgery and enrofloxacin (Baytril®, 5 mg/kg, bid, SQ) for five days after the surgery. The rats recovered uneventfully.

Exercise protocol and measurement of heart rate

Data were collected by telemetry, using a DSI PhysioTel® Receivers – RPC-1 (Data Sciences International, St. Paul, MN), and DataQuest ART (Advanced Research Technology) Version 4.0 software (Data Sciences International, St. Paul, MN). This allowed the continuous collection of electrocardiograms from conscious rats.

After recovering from surgery, each individual rat was trained daily to run on the treadmill, over a three day period. After the training period, the rats were exercised as described above and HR data were collected by telemetry. For collection of data, the treadmill was inclined to a 10% grade and the speed of the treadmill was increased by 5 m/min every 3 min until reaching a speed of 20 m/min, which was maintained for 6 min. The treadmill was then stopped and the rats were allowed to rest on the treadmill for 5 min.

Pyridostigmine treatment

After recovery from the surgery, rats were randomly divided into pyridostigmine treated (PYR) and control (CTL) groups (n=8 per group). The PYR group received 0.14 mg/mL (33–35) of pyridostigmine dissolved in drinking water, whereas the CTL rats received only water, both for 29 days. Stability of pyridostigmine in water was confirmed by serial quantification using mass spectrometry. Pyridostigmine (0.14 mg/mL) in water was stable in both ambient and cold (4° C.) temperatures for up to 8 days.

Assessment of heart rate recovery

Heart rate recovery (beats/min) was determined by subtracting the heart rate at one, three and five minutes after exercise from the maximum heart rate achieved at a speed of 20 m/

min. The HRR at 1, 3, and 5 minutes after termination of exercise were termed HRR1, HRR3 and HRR5. Heart rate recovery data were collected on days 0 (pre-treatment), 7, 14 and 28 after initiating PYR administration.

Acetylcholinesterase and butyrylcholinesterase activity

Blood samples were collected from the retro-orbital plexus, under isoflurane general inhalation anesthesia, on days 0, 7, 14 and 28 for determining blood cholinesterase (ChE) activities. An aliquot of whole blood was collected, and plasma and red blood cells (RBCs) were separated by centrifugation ($5000 \times g$, 2 min). The RBCs were washed by resuspension in phosphate buffered saline solution, centrifuged as before, and the supernatant was discarded. The washing step was repeated 3 times. Plasma and washed RBCs were then stored at -80°C until analysis. Acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and total ChE activity were measured in the plasma, whereas only AChE activity was measured in RBCs, using a radiometric method with [^3H]acetylcholine iodide (1 mM final concentration) as the substrate and incubation at 26°C (47). Twenty μL of tissue (plasma or RBC) were incubated for one minute with 60 μL of 50 mM potassium phosphate buffer, pH 7, with and without the tested enzyme inhibitor. One mM of [^3H]acetylcholine iodide substrate was then added and incubated for 30 seconds. The reaction was stopped by adding 100 μL of acidic 'stop solution' prepared by adding 9.45 grams of chloroacetic acid, 2 grams of sodium hydroxide and 11.6 grams of sodium chloride to 100 ml of deionized water. Once the reactions had been stopped, 5 ml of organic scintillation cocktail was added to each vial. Organic scintillation cocktail for the cholinesterase assay consisted of 0.5% (w/v) 2, 5-diphenyloxazole, 0.03% (w/v) 1, 4-bis [5-phenyl-2-oxazolyl] benzene and 10% (v/v) isoamyl alcohol in toluene. The vials were capped and vortexed for 5–10 seconds and were then counted in a Tri-Car 2810 TR (PerkinElmer, Waltham, MA). To determine total cholinesterase activity, plasma samples with no selective inhibitor were assayed in the enzyme reaction. To estimate BChE activity, the specific acetylcholinesterase inhibitor 284c51 (1, 5-bis[allyldimethylammoniumphenyl] pentane-3-dibromide), 2 μM , Sigma-Aldrich (St. Louis, MO, USA) was added 1 minute prior to adding substrate. AChE activity was defined as the difference in acetylcholine hydrolysis between total ChE and BChE. Preliminary assays were carried over 30 sec to 30 min incubation times, to determine incubation times that resulted in linear rates of substrate hydrolysis.

To determine the *in vitro* sensitivity of cholinesterases to PYR, blood was collected from a different set of three naïve rats, and plasma and RBCs were separated as before and stored at -80°C . To determine the *in vitro* sensitivity of plasma BChE to pyridostigmine, all reactions contained 284c51 (2 μM) and either vehicle (50 mM potassium phosphate buffer, pH 7) or one of a range of concentrations of pyridostigmine (0.0003 to 30 μM) in the vehicle. To determine *in vitro* sensitivity of plasma AChE to pyridostigmine, all samples contained the butyrylcholinesterase-selective inhibitor ethopropazine (Sigma-Aldrich; 10 μM dissolved in 50 mM potassium phosphate buffer, pH 7 containing 1% ethanol) and either vehicle or one of a range of concentrations of pyridostigmine. In all cases, reactions were pre-incubated at 26°C for 30 minutes before adding substrate and measuring residual activity.

Pharmacological determination of autonomic tone

Autonomic tone was assessed in freely moving, conscious rats. On day 27, basal heart rate (HR_{basal}) was recorded for 10 minutes. Atropine sulphate (Henry Schein, Dublin, Ohio, USA) was injected intraperitoneally, at a dose of 2 mg/kg (48–50) to block parasympathetic input to the heart. The HR recorded 15 min after atropine injection was defined as HR_{atropine} , i.e., HR where cholinergic inputs have been blocked. Propranolol (4 mg/kg, IP, West-Ward Pharmaceutical Corp., Eatontown, NJ, USA) was then administered and HR was again recorded 15 min later and defined as $HR_{\text{intrinsic}}$, i.e., HR with both parasympathetic and sympathetic innervation blocked. Sympathetic tone was estimated using HR_{atropine} and $HR_{\text{intrinsic}}$ as below in equation 1.

$$\text{Sympathetic tone} = \frac{HR_{\text{atropine}} - HR_{\text{intrinsic}}}{HR_{\text{intrinsic}}} * 100 \quad \text{Equation 1}$$

Where, $HR_{\text{atropine}} = HR_{\text{sympathetic}} + HR_{\text{intrinsic}}$

Similarly, on day 29, after recording HR_{basal} for 10 min, propranolol was administered as described above (48–50). The HR that was recorded at 15 min after propranolol injection was termed $HR_{\text{propranolol}}$ and reflected the parasympathetic and intrinsic activity of the SA node. Atropine was administered at a dose of 2 mg/kg and the HR was recorded 15 min later for determination of $HR_{\text{intrinsic}}$. The parasympathetic tone was calculated using $HR_{\text{propranolol}}$ and $HR_{\text{intrinsic}}$, as shown below in equation 2.

$$\text{Parasympathetic tone} = \frac{HR_{\text{propranolol}} - HR_{\text{intrinsic}}}{HR_{\text{intrinsic}}} * 100 \quad \text{Equation 2}$$

Where, $HR_{\text{propranolol}} = HR_{\text{parasympathetic}} + HR_{\text{intrinsic}}$

Statistical analysis

All data are presented as the mean $\pm$ SEM. Comparison of the basal HR and maximum HR were performed using a two way repeated measures ANOVA followed by Dunnett's test for multiple comparisons. Similarly, HRR and ChE enzyme activity were analyzed using a repeated measures two way ANOVA followed by Sidak's (to compare between treatment groups) and Dunnett's (to compare day 7, 14 and 28 with day 0) test for multiple comparisons. For measurements that were obtained only once (i.e., sympathetic tone, parasympathetic tone, intrinsic HR, weight gain, and water intake), a student's t-test was selected. The level of significance was set at $\alpha < 0.05$. *In vitro* inhibition data were fitted to a nonlinear curve to calculate the IC_{50} by using nonlinear curve fitting of inhibitor dose response function in GraphPad prism 6[®] (GraphPad Software, Inc., La Jolla, CA).

Conclusion

The data support our hypothesis that subacute PYR dosing enhances HRR by increasing cardiac parasympathetic tone in rats. Post-exercise HRR can be used to assess autonomic tone and thus can serve as an indicator of cardiovascular fitness. The present model of HRR after submaximal exercise should be suitable for future studies in rats with heart failure.

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Fig. 1.

Cholinesterase activity in CTL and PYR rats. The plasma total cholinesterase (A), plasma acetylcholinesterase (B) and plasma butyrylcholinesterase (C) and red blood cell acetylcholinesterase activity (D) were analyzed on days 0, 7, 14 and 28 of pyridostigmine administration. Data are presented as the mean $\pm$ SEM. * $P < 0.05$ compared to CTL group, † $P < 0.05$ compared with day 0.

Fig. 2.

In vitro inhibition of red blood cell AChE, plasma AChE and BChE activity by pyridostigmine. Tissues were pre-incubated with pyridostigmine for 30 minutes at 26°C prior to adding substrate and measuring residual activity. Enzyme activity was expressed as mean percent of control activity in the absence of inhibitor.

Fig. 3.

Basal and maximum heart rate. The basal heart rate (A) before exercise and maximum heart rate (B) at the maximum running speed of 20 m/min was measured on days 0, 7, 14 and 28 during pyridostigmine administration. Data are presented as the mean $\pm$ SEM. $\dagger P < 0.05$ compared with day 0.

Fig. 4.

Heart rate recovery after exercise. The 1, 3 and 5 min (A, B, and C) HRR was measured on days 0, 7, 14 and 28 during pyridostigmine administration. Data are presented as the mean $\pm$ SEM. * $P < 0.05$ compared to CTL group, † $P < 0.05$ compared with day 0.

Fig. 5. Modulation of autonomic tone represented by the alteration in heart rate following pharmacological intervention in PYR and CTL groups. See equations 1 and 2 for definitions of vagal and sympathetic tone. Data are presented as the mean $\pm$ SEM. * $P < 0.05$ compared to CTL group.