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Effect of Pyridostigmine on Heart Rate Recovery After Exercise in Trained Athletes and Sedentary Adults

Thomas Andrew Dewland
Yale University

EFFECT OF PYRIDOSTIGMINE ON HEART RATE RECOVERY AFTER EXERCISE IN TRAINED ATHLETES AND SEDENTARY ADULTS

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Thomas Andrew Dewland

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Abstract

EFFECT OF PYRIDOSTIGMINE ON HEART RATE RECOVERY AFTER EXERCISE IN TRAINED ATHLETES AND SEDENTARY ADULTS. Thomas A. Dewland, Stuart D. Katz. Section of Cardiology, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT.

Acetylcholinesterase inhibition with pyridostigmine has been previously studied in patients with congestive heart failure (CHF), but the effects of this medication on heart rate recovery after exercise and other indices of parasympathetic activity in populations with greater baseline vagal tone has not been fully characterized. Ten healthy, sedentary adults and ten aerobically trained athletes were enrolled in a prospective, double blind, randomized placebo controlled crossover trial. All study subjects were treated with a single dose of oral pyridostigmine 30 mg and matching placebo on separate days. Heart rate variability (HRV) at rest and heart rate recovery (HRR) at one minute after maximal cardiopulmonary exercise stress testing were measured. In sedentary adults, pyridostigmine significantly lowered resting heart rate (mean (SEM) 58.1 (2.4) beats/min versus 66.7 (4.0) beats/min, $p = 0.01$), increased HRR at one minute (45.1 (2.8) beats/min versus 40.7 (3.4) beats/min, $p = 0.02$), and lowered both resting mean arterial pressure (80.3 (2.0) mm Hg versus 84.3 (2.7) mm Hg, $p = 0.02$) and peak exercise mean arterial pressure (103.3 (3.1) mm Hg versus 108.8 (3.2) mm Hg, $p < 0.01$). In trained athletes, resting heart rate and HRR at one minute were unaffected by pyridostigmine dosing, although a significant increase in VO_2 max was observed with the study drug (54.8 (3.5) ml/kg/min versus 53.3 (3.6) ml/kg/min, $p = 0.02$). Pyridostigmine did not change indices of heart rate variability in either cohort. The difference in resting heart rate and HRR responses to pyridostigmine between athletes and sedentary controls likely reflects training induced modifications of the autonomic nervous system. The inability of acetylcholinesterase inhibition to affect

HRV in either sedentary adults or athletes further suggests the improved HRR previously observed in CHF patients treated with pyridostigmine is secondary to parasympathetic augmentation.

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Introduction

Activation of the sympathetic nervous system is a characteristic feature of heart failure.[1] As the myocardium fails to maintain adequate cardiac output, an escalated adrenergic response serves to increase heart rate, myocyte contractility, and blood volume in an effort to augment oxygen delivery to peripheral tissue. These compensatory mechanisms come at a biological cost; elevated afterload, electrolyte derangements, arrhythmias, volume overload, alterations in myocyte protein expression,[2] and myocyte apoptosis[3] are detrimental consequences of such autonomic nervous system (ANS) activation. A rise in plasma norepinephrine (NE) concentration, used as a rough estimate of this sympathetic enhancement, has been directly correlated with disease severity among patients with heart failure.[4] Indeed, experimental results indicate the plasma NE level is superior to hemodynamic variables in predicting mortality in this patient population.[5]

The increase in sympathetic activation with heart failure is accompanied by an attendant withdrawal of parasympathetic tone. First described in the early 1970's, this parasympathetic deficit was revealed by monitoring heart rate changes after pharmacologic autonomic manipulation. Heart failure patients demonstrated an attenuated rise in heart rate compared to healthy controls after blockade of autonomic input to the myocardium with successive doses of propranolol and atropine, indicating a severe impairment of parasympathetic restraint on the sinoatrial (SA) node.[6] Additional studies using noninvasive measurements of parasympathetic activity, including both time[7] and frequency[8] domain heart rate variability (HRV) analysis, have corroborated this finding. While the exact anatomical location of parasympathetic impairment is unknown, animal models of heart failure indicate a disruption in the transmission of vagal efferent activity at the parasympathetic ganglion.[9] As is the case with sympathetic activation, parasympathetic withdrawal has problematic implications. A reduction in parasympathetic tone is an independent negative prognostic indicator in

heart failure patients,[10] possibly because of the association between impaired parasympathetic activity and ventricular fibrillation.[11]

The Autonomic Nervous System and Heart Failure

Before the relative importance of autonomic imbalance was recognized, heart failure was understood solely in terms of hemodynamic perturbations to preload, afterload, and cardiac output. From this perspective, the pharmacologic alteration of pressure, volume, and flow were thought to be necessary and sufficient to improve prognosis. A study by Massie et al. in the mid 1980's was among the first to provide evidence that this model of heart failure was incomplete.[12] After characterizing the initial hemodynamic response seen in heart failure patients treated with the angiotensin-converting-enzyme (ACE) inhibitor captopril, this experiment then monitored these patients for three months to assess symptomatic improvement, exercise tolerance, heart size, and ejection fraction. The results demonstrated poor correlation between improvement of these clinical measurements and hemodynamic response to ACE inhibition.[12] Subsequent large scale clinical trials using vasodilators for the treatment of heart failure also raised questions regarding the association of hemodynamic benefit and disease improvement.

The Veterans Affairs Vasodilator-Heart Failure Trials (V-HeFT) was a three-armed study comparing the efficacy of prazosin and the combination of hydralazine and isosorbide dinitrate to placebo in the treatment of heart failure.[13] The trial investigators hoped to demonstrate a mortality benefit with vasodilator therapy. While prazosin was the most effective intervention at reducing blood pressure, it failed to confer an improvement in mortality at three years compared to placebo. The combination of hydralazine and isosorbide dinitrate reduced blood pressure to a lesser degree than prazosin, but this treatment led to a mortality risk reduction of 36% at three years when compared to placebo. These results were unexpected, as the medication with the greatest reduction in blood pressure did not exhibit the highest mortality benefit.

Similar observations were made when other vasodilator drugs were studied. Minoxidil[14] and nifedipine[15] *increased* mortality and heart failure deterioration, respectively, when compared to placebo, despite the ability of these drugs to reduce systemic vascular resistance and improve left ventricular function.

Treatment with positive inotropic medications produced equally dissatisfying results. The Prospective Randomized Milrinone Survival Evaluation (PROMISE) trial compared treatment with milrinone versus placebo in heart failure patients. The study found a 28% increase in all cause mortality and a 34% increase in cardiovascular mortality in the active treatment group when compared with placebo.[16] Collectively, these inotrope and vasodilator studies weakened the traditional hemodynamic model for heart failure by failing to demonstrate a benefit provided by pharmacologic improvements in preload, afterload, or contractility. Such results challenged the medical community to reexamine the pathophysiologic mechanisms underlying the progression of the disorder.

In light of the results generated from initial vasodilator and inotrope studies, more attention was given to the neurohormonal perturbations observed in heart failure patients. Researchers identified pathology caused by increased sympathetic tone and developed a greater appreciation for the cardiovascular stress induced by autonomic imbalance.[17] Large clinical trials utilizing pharmacologic blockade of the renin-angiotensin system (RAS) provided the first treatment-based evidence supporting the important causative role of neurohormonal imbalance in heart failure progression.

The Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS) examined the effect of the ACE inhibitor enalapril versus placebo on mortality in patients with class IV heart failure.[18] At six months, mortality was 26% in the enalapril cohort and 44% in the placebo group, representing a 40% reduction in mortality with ACE inhibition. These effects were again seen at one year, where a 31% reduction in mortality persisted in the enalapril-treated group. Similar results were found in the larger Studies of Left Ventricular Dysfunction (SOLVD) trial, which randomized patients

with ejection fractions less than or equal to 35% to treatment with enalapril or placebo.[19] Ninety percent of the patients in this trial were New York Heart Association functional class II or III, making them as a whole less impaired than those enrolled in the CONSENSUS trial. At an average follow up of 41 months, mortality was significantly reduced by 16% in those patients receiving the study drug compared to placebo.

Multiple lines of evidence suggest the major mechanism by which enalapril conferred benefit was through improvement in neurohormonal balance. In a follow-up Veterans Affairs Heart Failure Trial (V-HeFT II), treatment with enalapril was compared to hydralazine combined with isosorbide dinitrate. While both regimens were noted to lower blood pressure, only the ACE inhibitor had direct neurohormonal effects; enalapril lowered plasma levels of norepinephrine in study subjects while hydralazine in combination with isosorbide dinitrate did not.[20] At two years, patients treated with enalapril exhibited a lower mortality rate than those in the hydralazine and isosorbide dinitrate group.[21] Other research has also shown reduction in norepinephrine levels among heart failure patients treated with an ACE inhibitor.[22] In V-HeFT II, SOLVD, and CONSENSUS, patients with the highest initial levels of norepinephrine and angiotensin demonstrated the greatest reduction in mortality.[17, 19, 23] The previously mentioned early work of Massie and colleagues hinted at a disparity between clinical improvement and hemodynamic benefit in heart failure patients treated with captopril.[12]
Finally, studies examining vascular and myocyte physiology have linked the RAS and sympathetic nervous systems by implicating angiotensin II as a possible mediator of adrenergic augmentation. Elevated levels of this enzyme produce enhancement of α_1-adrenergic receptor function,[24] increased vasoconstriction,[25] and heightened plasma norepinephrine levels.[26] These study results collectively suggest restoration of autonomic balance is an important mechanism through which ACE inhibition confers mortality benefit.

The central role of neurohormonal activation in the pathogenesis of heart failure is further demonstrated by strong evidence supporting the efficacy of beta blockade in the treatment of the disease. In the late 1970's, a small, uncontrolled study reported reduced mortality and improved myocardial function in a group of 24 patients with class III and IV heart failure treated with beta blockers when compared to a symptomatically similar untreated cohort.[27] Initial attempts to replicate these findings in placebo controlled trials failed. One study, lasting less than four weeks, demonstrated reduced exercise tolerance and ventricular function in patients treated with acebutolol.[28] These negative results, likely due to the short time period over which individuals were followed, deterred further research in this field for a number of years. However, the efficacy of beta blockers was again tested in the mid 1990's amid mounting evidence demonstrating the harmful effects of sustained adrenergic stimulation of the myocardium.[29]

The first report of mortality reduction with beta blockade came when a double blind, placebo controlled trial demonstrated a decrease in mortality among heart failure patients treated with carvedilol from 7.8% to 3.2% at six months.[30] These results have subsequently been replicated in multiple randomized clinical trials with thousands of patients, all of which demonstrate an approximate 35% reduction in mortality with beta blocker treatment among patients with class II and III heart failure when compared with placebo.[31] While the neurohormonal changes associated with heart failure produce initially beneficial increases in cardiac output and peripheral perfusion, the mortality improvement observed with pharmacologic treatment of these imbalances suggests autonomic perturbations play a direct causative role in disease pathogenesis.

Assessment of Autonomic Function

Direct assessment of autonomic tone is not possible in humans.[32] Furthermore, resting heart rate is a poor estimation of parasympathetic activity because intrinsic properties of the SA node and sympathetic influences also contribute to its regulation.[33]

The autonomically mediated beat to beat heart rate change associated with respiration, known as respiratory sinus arrhythmia (RSA), has been studied as a method to gauge ANS function. The frequency at which the vagus nerve fires when an individual is at rest undergoes rhythmic fluctuations that are related to respiratory rate. Respiration initiates a complex series of feedback loops including pleural stretch reflexes, changes in cardiac filling secondary to alterations in intrathoracic pressure, and medullary neural interaction between the respiratory center and the nucleus solitarius of the vagus, all of which influence vagal firing. During inspiration, cardiac acceleration occurs secondary to decreases in vagal efferent activity. During expiration, vagal traffic is increased and the heart rate is slowed. Respiratory sinus arrhythmia, manifested as a slight fluctuation in adjacent beat to beat time intervals, is the end result of this alteration in vagal output caused by breathing. Heart rate variability (HRV) analysis is a tool used to quantify the beat to beat variation in heart rate secondary to RSA. Individuals with robust parasympathetic tone have abundant variation in successive R-R intervals, while those with decreased parasympathetic activity will lose such variation. After cardiac transplantation, HRV is severely reduced in the denervated heart.[34] Lack of variability is also evident in patients with autonomic imbalance secondary to heart failure and is a strong predictor of death among such patients.[10]

The various statistical techniques developed to standardize HRV analysis can be classified as either time or frequency domain measures. Time domain indices are an arithmetic quantification of differences in R-R interval duration. By examining changes in adjacent R-R intervals, the short-term variability in heart rate produced by efferent vagal input can be evaluated. Two common short-term time domain indices used to assess parasympathetic activity are the square root of the mean squared difference in adjacent R-R intervals (r-MSSD) and the percentage of pairs of adjacent R-R intervals differing by more than 50 milliseconds (pNN50).[35] Frequency domain measures of HRV are derived using fast Fourier transform analysis. Overall trends in the beat to beat fluctuation of heart rate are represented as a sum of component sine waves with

varying amplitude and frequency. This allows the rhythmic fluctuations in heart rate to be converted into a frequency spectrum and for a power value (squared amplitude) to be assigned to each frequency. A correlation between the individual components of the autonomic nervous system and peak frequency has been established by characterizing spectral changes induced through selective pharmacologic blockade of the sympathetic and parasympathetic nervous systems.[36] Power in the high frequency range (HF power, 0.15-0.40 Hz) represents changes in vagal firing secondary to respiration and is used to characterize parasympathetic modulation of heart rate. This association has been verified by demonstrating a predictable change in the frequency of HF spectral peak data with alteration of ventilation rate.[37] Additionally, HF power is severely attenuated with atropine administration, further implicating this measurement with vagal activity.[38] Time and frequency domain indices of parasympathetic activity highly correlate in healthy adults, suggesting HF power, r-MSSD, and pNN50 are all measurements of the same physiologic phenomenon.[35, 39] The origin of low frequency peaks (0.04 to <0.15 Hz) is more controversial, although current evidence links this data to baroreflex induced changes in heart rate mediated by both the sympathetic and parasympathetic systems.[36, 40]

It should be emphasized that these indices of HRV provide information regarding autonomic *fluctuation* and that they are not a direct quantification of autonomic tone.[41] Heart rate changes generating the HF power spectrum are secondary to oscillation in parasympathetic efferent activity. Increases or decreases in HF power therefore indicate alterations in vagal modulation. Such indices are widely used in research protocols because under stable physiologic conditions, modulation of autonomic activity likely reflects changes in absolute tone.[41, 42] Additionally, individual variation between absolute vagal tone and respiratory sinus arrhythmia, caused by differences in both respiratory parameters and respiratory-vagal coupling, limits the ability of HRV to predict differences in vagal tone between subjects.[32, 43] All interpretations of HRV data must take these considerations into account.

Heart Rate Recovery After Exercise

Interest in heart rate recovery (HRR) following an acute bout of exercise was heightened after three large studies from the Cleveland Clinic demonstrated impairment of normal chronotropic recovery to be a powerful predictor of mortality. Cole et al. followed over 2,400 patients referred for exercise thallium testing (63% of whom had no history of heart failure or coronary revascularization) for a mean follow-up time of 6 years.[44] Impaired HRR after exercise was defined as a reduction of 12 or less beats per minute (bpm) from peak heart rate to one minute of recovery. Patients exhibiting a blunted HRR response demonstrated a fourfold increase in overall mortality during the study period. This relationship persisted after controlling for possible confounding variables. In fact, impaired recovery was the strongest predictor of death in this study, surpassing age, sex, medications, and EKG findings in prognostic importance. HRR also remained predictive of death when measured as a continuous variable.[44]

The association between impaired HRR and mortality was verified in a second cohort of over 9,000 patients referred for exercise EKG testing, again demonstrating a fourfold increase in all cause mortality at a median of 5.2 years among individuals with an abnormal recovery value.[45] Additionally, HRR was shown to augment prognostic power when used in conjunction with the established Duke treadmill exercise score, indicating recovery data provides unique predictive information separate from exercise duration, EKG changes, and reproducibility of angina. A third large study examined heart rate recovery at two minutes after submaximal exercise in over 5,000 adults without evidence of cardiovascular disease. Using a HRR of less than or equal to 42 bpm at two minutes of recovery to categorize patients with an impaired chronotropic response, this study also confirmed abnormal HRR to be a strong predictor of poor prognosis.[46] The ability of HRR after exercise to predict mortality has subsequently been validated at other institutions and in different populations, including veterans, the elderly, and heart failure patients.[47-49]

The autonomic modulation of heart rate during and immediately after exercise provides insight into the association between impaired HRR and mortality. Using noninvasive techniques to quantify autonomic changes in heart rate, vagal activity has been shown to substantially decrease during early exercise and to rapidly increase during the first 1-2 minutes after exercise cessation.[50] Pharmacologic autonomic blockade studies demonstrate a gradual rise in sympathetic output during exercise, a gradual withdrawal following exercise cessation, and an impairment of HRR after exercise with the parasympatholytic atropine.[51, 52] These findings construct a model of autonomic heart rate modulation with physical exertion. As exercise is initiated, heart rate increases through a combination of early parasympathetic withdrawal and gradual sympathetic activation. Because parasympathetic tone predominantly controls heart rate at rest,[6, 53] it is estimated that parasympathetic reduction is the major contributor to heart rate increases up to 100 bpm, after which sympathetic activation accounts for the further rise.[41, 54] Despite early withdrawal of vagal tone, a reduced yet detectable level of parasympathetic activity exists at maximal exercise.[55] When exercise is stopped at peak exertion, an opposite cascade of events is initiated whereby parasympathetic tone is quickly increased and sympathetic tone slowly dissipates.[56] The magnitude of heart rate increase accompanying the parasympathetic withdrawal at early exercise has been shown to correlate with heart rate recovery at one minute, providing further evidence that both changes are mediated by the same mechanism (i.e. the parasympathetic nervous system).[57]

This model of autonomic heart rate regulation during exercise explains the association between impaired HRR and mortality. Since the decrease in heart rate during the first minute after exercise is nearly entirely vagally mediated, HRR can be understood as a simple, noninvasive tool to gauge stimulated parasympathetic tone and assess for autonomic disequilibrium. Identification of patients with impaired recovery after exercise is a means to distinguish individuals with the largest autonomic imbalance and the greatest risk for disease progression and death.

Pharmacologic Treatment of Autonomic Imbalance

Given the strong link between autonomic dysfunction and the pathogenesis of heart failure, substantial research has been directed at the pharmacologic manipulation of both sympathetic and parasympathetic tone. While reducing the damaging effects of sympathetic excess with beta blockade has become a cornerstone of therapy for this disorder, there are currently no approved pharmacologic treatment modalities that selectively improve parasympathetic response.

Multiple physiologic mechanisms are responsible for the end effects of parasympathetic nervous system activity. The myocardium is parasympathetically innervated by the vagus nerve (cranial nerve X). Efferent parasympathetic impulses originating in the brainstem travel along the preganglionic vagus neuron to the parasympathetic ganglion. At this junction, acetylcholine (ACh) is released from the preganglionic nerve and activates nicotinic receptors on the postganglionic neuron. This excites the postganglionic nerve, which conducts a neural impulse towards the myocardium. At the junction of the vagus and the SA node, the neurotransmitter acetylcholine is released from the cranial nerve and binds to muscarinic (M2 subclass) receptors on the node. This binding initiates a G protein linked cascade within the cells of the SA node, resulting in a decrease in heart rate, conduction velocity, and contractility. Acetylcholine is broken down into choline and acetic acid in the synaptic cleft by the enzyme acetylcholinesterase (AChE).

The complex pathway linking initial parasympathetic medullary activity to the myocardium provides multiple sites for potential pharmacologic vagal enhancement. Transdermal scopolamine, a tropane alkaloid, has been previously studied as a pro-cholinergic agent with possible clinical utility in the treatment of heart failure. This medication is thought to centrally increase the number of action potentials generated by medullary vagal-cardiac motornuclei.[58] In healthy subjects, scopolamine raises indices of parasympathetic tone, including average R-R interval, the standard deviation of the

R-R interval, and HF power.[58, 59] Additionally, scopolamine has been shown to increase time domain surrogates of parasympathetic tone in patients who have survived acute myocardial infarctions and in patients with moderate to severe congestive heart failure.[60-62] A major drawback to this intervention, however, is a peripheral anti-cholinergic effect seen when higher doses are administered,[63] raising concern about efficacy and safety when used in clinical practice.

Atropine sulfate is another agent previously studied in the context of parasympathetic modulation. Like scopolamine, atropine exhibits a dose-dependent effect on autonomic tone. Canine models indicate that the drug increases cardiac vagal efferent activity through a central mechanism, while high doses block muscarinic receptors at the sinus node and cause tachycardia.[64] At dosages less than 5 mcg/kg in humans, atropine decreases mean HR and increases HF power. Larger doses, however, reverse these trends and lead to increased heat rate and attenuated HF power.[65] Because of the opposing dose dependent properties of atropine and scopolamine, and because of the overall poor tolerance of scopolamine secondary to cholinergic side effects, research efforts have shifted to explore alternative pharmacologic methods of parasympathetic enhancement.[66]

For over sixty years, acetylcholinesterase inhibitors have been used for the treatment of the autoimmune neuromuscular disorder myasthenia gravis.[67] In this disease, muscular weakness is produced through the generation of autoantibodies against the acetylcholine receptor at the neuromuscular junction. Therapy with AChE inhibitors provides symptomatic relief for myasthenia gravis patients by binding to AChE and temporarily inactivating the enzyme. This inactivation increases the quantity of neurotransmitter available in the synaptic cleft, allowing for increased stimulation of the receptors unaffected by autoantibody blockage. Anti-AChE agents are eventually hydrolyzed by AChE, but at a slower rate than ACh.[68] The peripheral location of action of the AChE inhibitors contrasts with the centrally acting vagomimetic mechanism of the previously mentioned medications. It should be reiterated that while scopolamine

and atropine increase vagal firing, the AChE inhibitors instead augment *existing* parasympathetic tone though inhibition of neurotransmitter degradation. AChE inhibitors are appealing clinically because they do not inhibit parasympathetic activity when used in high concentrations and their effects can be reversed with atropine in the case of overdosing.[68]

Pyridostigmine is an orally available, FDA approved AChE inhibitor. After oral ingestion in fasting subjects, peak plasma concentrations are achieved between 90 and 150 minutes.[69] The elimination half life of the drug is on the order of 90-120 minutes and the bioavailability is 7.6%.[69] Since pyridostigmine is a charged molecule, it is not able to penetrate the blood brain barrier.[70] A single dose of 30 mg of pyridostigmine provides approximately 40% AChE inhibition.[71] The ability of pyridostigmine to produce a surplus of acetylcholine at the synapse of the vagus and the sinoatrial node makes it an appealing drug to further explore the effects of autonomic activity on myocardial performance.[68] At 30 mg the drug increases the duration of the mean R-R interval in healthy subjects at rest, while blood pressure and ventricular systolic function assessed by echocardiography remain unchanged.[66, 72] This dosage of oral pyridostigmine is well tolerated, with minor complaints of increased rhinorrhea and salivation occasionally noted.[66, 72, 73] Junctional bradycardias have been reported in patients being treated with high doses of pyridostigmine (>1000 mg/day) for myasthenia gravis, although such events occur with exceedingly low frequency and can be terminated with atropine administration.[74] The favorable safety profile and low incidence of side effects makes pyridostigmine an appealing potential therapy for the autonomic changes observed in heart failure.

Manipulation of HRR

With autonomic imbalance identified as the crucial link between impaired HRR and mortality, research efforts have begun to focus on methods to enhance chronotropic slowing after exercise. To date, improvements in HRR have been demonstrated with both pharmacologic therapy and exercise training. Initial interest in the medical manipulation of recovery focused on beta blockade. This class of agents garnered interest after propranolol was found to augment parasympathetic-associated components of the frequency domain HRV spectrum compared to placebo in patients who had previously suffered a myocardial infarction.[75, 76] Unfortunately, beta blockers have repeatedly failed to elicit an improvement in HRR in heart failure patients.[77, 78]

Recently, pyridostigmine has been shown to increase heart rate recovery after exercise in patients suffering from heart failure.[79] Twenty patients undergoing maximal exercise stress testing were treated in a crossover design with pyridostigmine and placebo to assess heart rate recovery at one minute after exercise cessation. The AChE inhibitor significantly increased heart rate recovery by five beats at one minute, suggesting the study drug was able to augment parasympathetic tone.[79] This research represents the first documented pharmacologic augmentation of heart rate recovery after exercise and invites more extensive exploration into the effects of pyridostigmine on heart rate recovery in populations with different autonomic profiles.

Exercise and the Heart

The overall benefits of exercise, especially relating to cardiovascular health, have been well known for decades. Reductions in blood pressure,[80] improvement in lipid profiles,[81] decreased insulin resistance,[82] and lower levels of atherogenic cytokines[83] have all been reported in physically conditioned subjects. More recently, the effect of exercise on autonomic tone has also been characterized.

As heart failure elicits a pathophysiologic perturbation of the autonomic nervous system, repeated bouts of exercise can be similarly viewed as an inciting process that

produces a change in autonomic balance. This change, however, occurs in a direction opposite of that seen during myocardial injury. Aerobically trained athletes have increased parasympathetic and decreased sympathetic activity at rest.[41, 84, 85] Clinically, this is manifest as a resting sinus bradycardia and increased heart rate variability.[84, 86, 87] This "training bradycardia" is likely to be only partially attributed to parasympathetic augmentation, as decreases in sympathetic tone and intrinsic heart rate associated with exercise training are also thought to play some role in heart rate reduction.[41, 88]

In a study conducted by Yamamoto et al., seven healthy subjects were enrolled in a six week aerobic exercise training program.[86] Prior to enrollment in the study, these subjects had a similar resting HR and HF power by frequency domain analysis of HRV at rest compared to a group of aged-matched sedentary controls. At the completion of the exercise training program, the trained group demonstrated a lower resting HR and increased HF power at rest, consistent with augmented parasympathetic output.[86] Other cross-sectional studies have compared HRV analysis between athletes and sedentary controls, confirming the finding of increased HF spectral power and therefore heightened parasympathetic modulation among trained subjects.[84, 89]

Effects of Exercise On HRR

Aerobically trained athletes exhibit increased HRR after exercise when compared to sedentary controls, an observation attributed to improved parasympathetic tone in such individuals.[78, 90] A small retrospective study identified improvement in HRR after maximal exercise in patients referred to cardiac rehabilitation following a cardiac event (MI, revascularization without infarction, or CABG) when compared to similar patients who did not enroll in the exercise program.[91] These results were confirmed in a prospective, uncontrolled experiment evaluating HRR in patients who underwent 12 weeks of cardiac rehabilitation.[92] Further evidence is provided by a small prospective randomized trial demonstrating improved HRR in patients post-CABG who participated in outpatient physical rehabilitation compared to a sedentary control group.[93]

Similarly, HRR after exercise has been increased in geriatric populations without heart disease through an eight week exercise program.[94] Other work in this area has shown heart rate decay in the first 30 seconds following exercise to be accelerated in well trained cross country skiers and attenuated in patients with chronic heart failure.[78]

Rationale for Present Study

A correlation between impaired HRR after exercise and mortality risk in heart failure patients has been identified.[44-46] With data implicating autonomic imbalance, and especially parasympathetic impairment, as the common mediator of these outcomes, research has focused on the pharmacologic amelioration of this autonomic derangement. The development of therapies aimed at sympathetic blockade has been rewarded by mortality reduction in heart failure patients and has demonstrated the treatment of ANS aberrations can affect outcome. To extend this hypothesis, investigation into parasympathetic augmentation must be further explored. While exercise has been shown to enhance vagal activity, pharmacologic intervention is particularly attractive because of the poor compliance associated with physical training programs and the presence of non-cardiac limitations to exercise experienced by many heart failure patients.

An improvement in HRR with pyridostigmine has been previously demonstrated in a population of heart failure patients.[79] However, unpublished data from this study revealed no difference in the time and frequency domain indices of HRV with pyridostigmine administration. Heart failure represents a state of parasympathetic impairment[6, 10] postulated to be secondary to decreased vagal efferent activity.[9] Canine models indicate this reduction in vagal tone causes a reactive cascade of molecular adaptations including a reduction in acetylcholinesterase levels at the junction of the vagus and SA node.[95] It is possible HRV was unchanged in heart failure patients treated with pyridostigmine because efferent vagal activity was too low and decreased

acetylcholinesterase levels were not sufficiently altered to produce a detectable HRV difference.

Healthy, sedentary subjects should have both increased vagal efferent traffic and higher concentrations of acetylcholinesterase than their heart failure counterparts, providing an experimental opportunity to further understand the effects of AChE inhibition on indices of parasympathetic activity. Aerobically trained athletes likewise have heightened parasympathetic activity compared to normal subjects,[84, 87] although definitive characterization of vagal efferent activity or acetylcholinesterase concentration in this population has not, to our knowledge, been accomplished. Since pyridostigmine augments existing vagal tone, it is plausible that treatment with this drug could heighten HRR and increase HRV in athletes compared to sedentary controls, who would in turn demonstrate a greater change in HRR with the study drug than heart failure patients. Exploring the effects of pyridostigmine on heart rate recovery and heart rate variability in both sedentary adults and aerobically trained athletes will allow further characterization of the autonomic differences between health and cardiovascular disease.

Hypothesis

The objective of this investigation is to observe the effects of acetylcholinesterase inhibition with pyridostigmine on parasympathetic activity in aerobically trained athletes and sedentary controls, as assessed by heart rate recovery following maximal exercise and heart rate variability at rest.

Hypotheses to be tested:

1) Administration of pyridostigmine will increase the heart rate recovery observed at one minute in both trained athletes and sedentary adults following maximal exercise compared to placebo.
2) The pyridostigmine mediated increase in heart rate recovery at one minute observed in sedentary controls following maximal exercise will be greater than the pyridostigmine mediated increase in heart rate recovery previously described in heart failure patients.[79]
3) The pyridostigmine mediated increase in heart rate recovery at one minute in trained athletes following maximal exercise will be greater than the pyridostigmine mediated increase in heart rate recovery in both the cohort of sedentary controls and in heart failure patients.
4) Pyridostigmine will increase HRV indices of parasympathetic tone, including pNN50, r-MSSD, and HF power, in normal controls and trained athletes, with greater changes evident in athletes.

Methods

Study Population

Sedentary Cohort

Six females and four males between the ages of 25-48 were enrolled in the sedentary cohort. These individuals had not participated in a regular exercise regimen for at least three months prior to enrollment. Subjects with a systolic blood pressure < 90 mm Hg, a resting heart rate < 50 beats per minute, a history of tobacco use, a history of bronchospasm or asthma, or any other chronic medical condition were excluded. Individuals taking cardioactive medications were also ineligible for study participation.

Trained Athlete Cohort

Seven males and three females between the ages of 22-47 were enrolled in the trained athlete group. Inclusion criteria for trained athletes consisted of a resting heart rate between 50-65 beats per minute and participation in ≥ 30 minutes of aerobic exercise at least five times per week for ≥ 3 months. Exclusion criteria were identical to those used for the sedentary cohort.

All twenty subjects had an unremarkable physical examination before testing. The study protocol was approved by the Yale University Human Investigation Committee. Subjects were informed of possible risks and provided written consent before participation.

Study Design

To compare the effect of a single oral dose of 30 mg of pyridostigmine versus placebo on heart rate recovery, a prospective, double blind, randomized crossover study design was employed. Participation in the study involved two symptom limited maximal

exercise tests performed 5-10 days apart. Recruitment and exercise stress testing of the two study cohorts occurred in a temporally sequential manner, such that all data from the sedentary group was collected before the athlete group was enrolled. All subjects were tested in the same laboratory under identical protocols.

Participants were instructed to abstain from caffeinated beverages and physical training in the 24 hours preceding each exercise test and from all food and beverages for the eight hours immediately before testing. Drug administration and maximal exercise testing were performed at the same time on both study days for each participant, although the time of day at which the experiment was conducted varied from patient to patient.

Study Drug

The study drug consisted of a single oral dose of pyridostigmine 30 mg (Mestinon®, ICN Pharmaceuticals, Costa Mesa, CA) or matching placebo. Pyridostigmine and placebo were administered in identical appearing oral tablets supplied by the Investigational Drug Service at Yale-New Haven Hospital. A separate blocked randomization allocation scheme was implemented for each cohort through the same service. Treatment order was unblinded after all participants in a cohort had completed both exercise tests.

Study Protocol

Baseline heart rate and blood pressure were taken before drug administration with subjects in a seated position ("baseline" data). After the study drug was administered, participants remained sedentary. Ninety minutes after drug administration, subjects entered a quiet, dark room controlled at 21° C and rested supine for 30 minutes while a Holter monitor (General Electric, Milwaukee, WI) recorded continuous EKG data. Immediately following this rest period, heart rate and blood

pressure were measured ("rest" data). Subjects then underwent maximal exercise testing 130 – 160 minutes after drug administration.

Maximal exercise testing was performed on a treadmill using a standard Bruce protocol. EKG data was recorded continuously (Cardiocontrol Cardioperfect MD, Netherlands), beginning with a one minute pre-exercise rest period during which the subject stood on the stationary treadmill. Electrocardiogram data collection was continued during exercise and throughout recovery. Breath by breath expired gas analysis (Medgraphics, St. Paul, Minnesota, USA) allowed for monitoring of oxygen uptake, carbon dioxide production, and ventilatory volume and rate during the one minute pre-exercise rest, exercise, and the first minute of recovery. Blood pressure was taken during the pre-exercise rest period, during each of the first four stages of the exercise protocol, and during the first minute of recovery. Excessive treadmill speed prohibited blood pressure assessment during the latter stages of exercise. Subjects were verbally encouraged to exercise until fatigue or dyspnea prevented further effort. At maximal exercise, VO_2 max was calculated by the median five of seven breath VO_2 values. After symptom limited peak work rate was attained, the treadmill was quickly stopped.

Immediately following maximal exercise, participants were helped to a stool placed on the stationary treadmill and heart rate recovery data was collected for five minutes with subjects in a seated position. Heart rate at maximal exercise and at each minute during recovery was calculated from the average of the R-R interval of four consecutive sinus beats taken from the EKG recording. Heart rate recovery was calculated by subtracting the recovery heart rate at the given time point from the heart rate at maximal exercise.

Holter Analysis

Thirty minutes of Holter data was recorded on a magnetic tape beginning ninety minutes after study drug administration while the subject rested supine in a darkened,

temperature controlled room. To ensure all analyzed data was obtained during supine rest, the first and last five minutes of each rest session was discarded. This yielded a twenty minute window of data for HRV examination. Each tape was digitally sampled and analyzed using a Marquette system (Milwaukee, WI) by technologists at the Yale-New Haven Hospital Holter Laboratory, then manually processed and edited. R-R interval data was generated and each beat was labeled as normal, ectopic, or noise. The ectopic beats and noise were removed from the data set and replaced with interpolated linear splines.

A fast Fourier transform was used to calculate power spectrum data, which was then corrected for attenuation due to windowing and sampling. The twenty minutes of data was segmented into sixteen overlapping four minute blocks, each offset from the previous by one minute. The power spectrum for each four minute window was integrated over the following frequency intervals as previously described:[96] ultralow frequency (ULF) <0.0033 Hz; very low frequency (VLF) 0.0033 to <0.04 Hz; low frequency (LF) 0.04 to <0.15 Hz; and high frequency (HF) 0.15 to 0.40Hz. Total power (TP) was generated from these measurements. The mean value for each frequency interval was calculated from the average of the corresponding sixteen data points. The maximal power for each frequency interval was derived from the four minute window with the largest value during each twenty minute block of R-R data used for analysis. As the data was highly skewed, a natural log transformation was performed to normalize the distribution. Time domain measurements, including pNN50 and r-MSSD, were calculated from the same twenty minutes of R-R interval data using custom-designed software (Dr. Forrester Lee, Yale University School of Medicine) based on standard methods described in the literature.

Data Analysis

All continuous variables are expressed as mean (SEM). The primary endpoint of the study was heart rate recovery (the difference between peak exercise heart rate and

heart rate recorded at one minute after peak exercise, derived from the average of the R-R interval of four consecutive sinus beats) following symptom limited maximal exercise testing after administration of pyridostigmine and placebo. Secondary analyses compared study drug administration to HRR at two minutes, changes in rest and peak exercise heart rate, mean arterial pressure, VO_2, minute ventilation, average resting R-R interval, and resting HRV data including pNN50, r-MSSD, HF power, LF power, VLF power, total power and the ratio of low frequency to high frequency power. Differences between pyridostigmine and placebo were analyzed in repeated measures analysis of variance models appropriate for the crossover design. Other clinical measurements were examined in relation to heart rate recovery using linear regression analysis. A two tailed p value < 0.05 was used to infer significance. Based on prior HRR data, a sample size of ten subjects was needed to provide 80% power to detect a 5 bpm change in heart rate recovery with placebo. Estimates of the effect of pyridostigmine versus placebo did not differ in models with or without a carryover term to adjust for treatment order. Therefore, we only report results from the simpler model without this term. All analyses were performed using the Intercooled Stata Statistics Package (version 8.0, StataCorp, College Station, TX).

The sedentary cohort was recruited and exercise tested by Ana Silvia Androne, M.D. Trained athletes were recruited and tested by the author. Data analysis from both cohorts was performed by the author.

Results

Ten subjects fulfilling entry criteria for the sedentary cohort were enrolled in the study and underwent maximal exercise testing between March and May 2003. An additional ten subjects meeting criteria for the trained athlete cohort were enrolled and exercise tested between June and August 2003. Table A lists the baseline clinical characteristics of both groups. The sedentary cohort tended to have a greater number of female participants than the trained cohort (6 versus 3) and was on average older. Trained athletes had a significantly lower resting heart rate compared to the sedentary controls. Resting blood pressure and weight did not differ between the two groups.

Effects of study drug at rest

Resting and exercise variables for both sedentary controls and trained athletes are described in Table B. Pyridostigmine significantly lowered resting heart rate in sedentary subjects when compared with placebo. The study drug also significantly lowered mean arterial pressure (MAP) in sedentary subjects.

While a trend towards decreased heart rate with study drug administration was noted in athletes, this observation was not statistically significant. Mean arterial blood pressure in athletes at rest was unaffected by drug dosing.

Resting respiratory rate, tidal volume, and VO_2 measurements did not differ with drug treatment in either cohort.

Effects of study drug on exercise performance

Pyridostigmine significantly lowered MAP at maximal exercise compared to placebo in sedentary controls. Oxygen consumption at maximal exercise was not different with study drug versus placebo in this cohort.

In athletes, MAP was not changed with drug administration. VO_2 max demonstrated a small but statistically significant increase with pyridostigmine compared to placebo.

Drug administration did not influence maximum heart rate, respiratory rate, tidal volume, or exercise duration in either study cohort.

Effects of study drug on heart rate recovery

Heart rate recovery data is summarized in Table C and graphically displayed for sedentary controls and athletes in Figures 1 and 2, respectively. Pyridostigmine significantly increased heart rate recovery at one minute compared to placebo in sedentary subjects. The study drug did not change heart rate recovery at two minutes.

Pyridostigmine demonstrated a slight but statistically insignificant increase in HRR compared to placebo in trained athletes. Drug administration did not change HRR at two minutes in the athlete cohort.

Regression analysis revealed no correlation between HRR at one minute and any baseline, resting, or exercise variable in either sedentary controls or trained athletes.

Effect of study drug on heart rate variability

Resting Holter EKG data was used to compute both frequency and time domain indices of heart rate variability. These values are summarized in Table D. Neither sedentary subjects nor athletes demonstrated a change in pNN50 or r-MSSD while resting supine after administration of the study drug compared to placebo. Correlating with this observation, HF power was not altered by pharmacologic intervention in either cohort. All other defined segments of the power frequency spectrum, including mean and max LF, VLF, TP, and LF/HF power, remained unchanged with study drug compared to placebo. Regression analysis in the sedentary control groups treated with placebo revealed very high correlation between pNN50 and HF power ($r = 0.748$, $p = 0.01$), pNN50 and r-MSSD ($r = 0.822$, $p = 0.004$), and r-MSSD and HF power ($r = 0.953$,

$p < 0.001$). Regression analysis in athletes treated with placebo also demonstrated high correlation between pNN50 and HF power ($r = 0.794$, $p = 0.006$), pNN50 and r-MSSD ($r = 0.759$, $p = 0.01$), and r-MSSD and HF power ($r = 0.925$, $p = 0.0001$).

Comparison between sedentary and athlete cohorts

Resting heart rate, measured two hours after drug administration, was significantly lower in the athletes compared to sedentary controls with placebo (53.9 (1.8) bpm athletes vs. 66.7 (4.0) sedentary, $p = 0.009$). Similarly, the average R-R interval measured by the Holter monitor during the 30 minute rest period was higher among athletes than controls with placebo (1168 (45) msec athletes versus 932 (33) sedentary, $p = 0.0005$). Resting mean arterial pressure, minute ventilation, and VO_2 did not differ significantly between cohorts with placebo administration.

Peak oxygen consumption (VO_2 max) was significantly higher among the trained athletes (53.3 (3.6) ml/kg/min athletes vs. 29.4 (6.2) sedentary, $p = 0.0001$). Minute ventilation was also significantly higher in athletes at peak exercise (122.5 (8.4) L/min athletes vs. 63.4 (5.6) sedentary, $p < 0.0001$). Neither HR at peak exercise nor mean arterial pressure at peak exercise differed between cohorts.

Mean heart rate recovery at one minute was greater among athletes compared to the sedentary cohort with placebo (46.5 (3.1) beats athletes vs. 40.7 (3.4) sedentary, $p = 0.220$), although this difference did not reach statistical significance. Athletes did demonstrate a statistically significant larger HRR at two minutes compared to sedentary subjects when treated with placebo (70.3 (3.2) beats athletes vs. 56.3 (3.6) sedentary, $p = 0.01$). Neither time nor frequency domain indices of parasympathetically mediated heart rate variability differed between study groups.

The effect of pyridostigmine versus placebo on primary and secondary endpoints did not differ due to training status, as all interaction terms were > 0.20.

Discussion

Inhibition of acetylcholinesterase with 30 mg of pyridostigmine lowered resting heart rate and increased heart rate recovery at one minute after maximal exercise in healthy, sedentary adults. In contrast, neither resting heart rate nor heart rate recovery at one minute was changed in aerobically trained athletes after pyridostigmine administration. The study drug did not influence indices of HRV in either cohort. Pyridostigmine decreased MAP in sedentary controls at both rest and maximal exercise. A small but significant rise in oxygen consumption at maximal exercise (VO_2 max) with pyridostigmine was observed among the athlete cohort. Athletes tended to have higher HRR values than the sedentary controls with placebo, although this difference did not reach statistical significance.

The magnitude of HRR after peak exercise exhibited by both athletes and sedentary controls in our study is consistent with values attained in other populations of trained and untrained individuals, respectively.[50, 55, 90, 97] The approximate four beat per minute improvement in HRR with pyridostigmine in the sedentary cohort is similar to the increase previously reported in heart failure patients after identical pyridostigmine dosing (27.4 (3.2) bpm with pyridostigmine versus 22.4 (2.4) bpm with placebo, $p < 0.01$).[79] The mean HRR values obtained at one minute with placebo were substantially higher for both athletes (46.5 (3.1)) and sedentary controls (40.7 (3.4)) compared to values recorded in heart failure patients using an identical protocol (22.4 (2.4)).[79]

Previous experiments examining the effect of AChE inhibition on heart rate in healthy subjects have reported a comparable heart rate reduction of 7-8 bpm after the administration of 30 mg of pyridostigmine.[66, 71, 72] Experiments using a pyridostigmine dose regimen of 30 mg every 8 hours have demonstrated both a rate reduction[98] or no change in heart rate at baseline compared to placebo.[65, 99]

The high correlation between pNN50, r-MSSD, and HF power described in this study has been reported in patients after myocardial infarction.[39] The replication of this finding in separate patient populations indicates a proper application of and internal consistency between the time and frequency domain analysis methods used in this experiment.

Prior research has also demonstrated a change in maximal oxygen consumption with pyridostigmine dosing. Patients with a history of exercise induced myocardial ischemia significantly increased VO_2 max with 45 mg of pyridostigmine compared to placebo.[100] Pyridostigmine blunted the heart rate response to exercise in this previous experiment, causing speculation that chronotropic slowing allowed for a delay in myocardial ischemia and higher peak VO_2.[100] However, an increase in maximal oxygen uptake was not observed in heart failure patients treated with 30 mg of pyridostigmine,[79] nor was it observed in untrained healthy subjects after receiving 45 mg of pyridostigmine.[101] Despite the pyridostigmine associated increase in maximal oxygen consumption among athletes in this present investigation, neither exercise duration nor maximal heart rate was affected by the study drug. Although the effect of pyridostigmine on myocardial contractility at maximal exercise has not been studied, 30 mg of the drug does not alter ventricular function at rest.[72] Therefore, the mechanism underlying this increase in VO_2 max is unclear. It is possible the small maximal oxygen consumption difference with study drug administration in athletes is the result of type I statistical error secondary to multiple comparisons.

AChE inhibition has also previously been associated with reductions in MAP. Pyridostigmine elicited a significant 5 mm Hg reduction in diastolic pressure during maximal exercise compared to placebo in hypertensive patients receiving beta blocker therapy.[99] As in our study, this pressure reduction was observed in the absence of a change in maximal heart rate. Contrasting with our findings, resting blood pressure was unaffected in this previous work.[99] The blood pressure reduction during exercise was speculated to be the result of a parasympathomimetic action of pyridostigmine on

peripheral vasculature,[99] but it is unclear whether the concomitant beta blockade played a role in this observation. The reduction in resting heart rate with pyridostigmine observed among sedentary controls in this study could have contributed to the decrease in resting blood pressure through a reduction in cardiac output. Previous studies in healthy, sedentary subjects revealed no blood pressure changes at rest with 30[66, 72] or 45 mg[101] of pyridostigmine.

There was a slight difference in average age between our athlete and sedentary control groups. Certain investigators have linked increasing age to impaired HRR after exercise, although this observation seems to be have been clouded by a discrepancy in both the amount and the intensity of physical activity among different age groups.[97] When athletes less than 30 years of age were compared with *similarly trained* athletes greater than 50 years old, HRR after exercise revealed no significant differences.[97] An overall decrement in HRV has been reported with increasing age,[102-104] although analysis of vagally mediated indices has produced contradictory results. Some experiments report no change in time domain parasympathetic indices,[102] while others have revealed decreases in HF power with aging.[103, 104] As in previous work with age and HRR, these studies did not control for physical activity differences between age groups. The average age of participants in prior studies was much higher than in our investigation, making their applicability to our study less certain. Our crossover design also permitted the intra-subject analysis of HRV data with and without pyridostigmine dosing, making absolute within-group changes in HRV with age irrelevant to the results obtained in this experiment.

We hypothesized the beat per minute HRR improvement at one minute with pyridostigmine would be greater in the sedentary group compared to heart failure patients. Since healthy adults presumably have heightened vagal efferent activity and greater levels of acetylcholinesterase compared to individuals with heart disease, we reasoned the sedentary controls would have more molecular substrate for pyridostigmine inhibition. For similar reasons, we also expected to see a greater

improvement in HRR with pyridostigmine among athletes compared to sedentary adults. Our data, however, does not confirm this proposition. Visual inspection of individual HRR values at one minute among the sedentary (Figure 1) and trained athlete (Figure 2) cohorts reveals overall differences in the data sets. HRR response to pyridostigmine was more homogenous in the sedentary controls than in the trained athletes. In both cohorts, individuals throughout the spectrum of placebo-associated HRR values experienced improvement in HRR with pyridostigmine. Trained subjects with large placebo-induced HRR values were still able to dramatically augment HRR with the study drug. In fact, one athlete increased HRR at one minute from an already high 53 beats with placebo to 72 beats with pyridostigmine. However, other trained athletes with large placebo-induced HRR values did not demonstrate recovery improvement with the AChE inhibitor. The ability of the study drug to improve HRR in a fraction of athletes with high baseline chronotropic slowing indicates an absolute HRR ceiling limiting study drug response cannot completely explain the lack of overall difference in HRR among athletes treated with pyridostigmine compared to placebo.

Only six of the ten athletes increased HRR with the study drug. It is possible two physiologically different subgroups were present within the trained athlete cohort. One subgroup was comprised of these six athletes who demonstrated a pharmacological response to pyridostigmine consistent with the HRR change observed in the sedentary control group. The second subgroup was populated with athletes unable to improve on an already maximized HRR. In these subjects, chronotropic recovery was at an *individual* physiologic ceiling during the unmedicated state. Pyridostigmine was not able to augment this value (Figure 3). Based upon our results, this physiologic ceiling for HRR must have substantial inter-individual variability. A ceiling effect may also account for the dramatic variation in HRR improvement observed among subjects in both cohorts who responded to the study drug. Individuals that exhibited only modest HRR improvement with pyridostigmine may have been near their maximal HRR ceiling with placebo. With study drug dosing, it is possible that they quickly reached this

ceiling and only marginally improved HRR. Others may have been able to dramatically increase HRR with pyridostigmine because they were far below their individual maximal HRR with placebo. A HRR ceiling may be secondary to a variety of feedback mechanisms, including central autonomic reflexes responsible for maintaining cerebral perfusion in the post-exercise state. Unfortunately, post hoc analysis was limited due to study size and was ultimately unsuccessful in identifying baseline (e.g. resting heart rate) or exercise variables that could predict individual study drug response. Consequently, we were unable to identify why certain individuals operated at this physiologic ceiling and why others demonstrated HRR improvement with the study drug.

Ten study participants were enrolled in each cohort to ensure both groups were sufficiently powered to detect a five beat per minute change in HRR with pyridostigmine. This HRR value was chosen because we expected to demonstrate an improvement in previous results attained in heart failure patients.[79] The lack of difference in HRR at one minute in trained athletes may represent a type II statistical error. The heterogeneity of athlete HRR responses to pyridostigmine increased the likelihood of this error.

Despite the widespread assumption that parasympathetic function is enhanced with aerobic physical training, changes in the parasympathetic regulation of heart rate due to repeated bouts of exercise have not been clearly described on a molecular level. Increased efferent vagal firing, altered neurotransmitter metabolism, changes in muscarinic receptor density, and modification of post-receptor signaling cascades could all play a role in the augmentation of parasympathetic control with exercise training. Experimental evidence suggests the various molecular components responsible for parasympathetic tone are part of a complex feedback network capable of adapting to perturbation. The reduction in vagal traffic caused by heart failure results in an increase in muscarinic receptors at the SA node,[105] G protein upregulation within the node,[106] and a reduction in acetylcholinesterase levels at the junction of the vagus and

SA node.[95] Similarly, patients with congenital deficiencies of acetylcholinesterase demonstrate reductions in ACh release at the skeletal neuromuscular junction.[107]

In light of the hypersensitivity to ACh that develops in heart failure, it is possible that an opposite desensitization to ACh occurs in athletes. Rat models demonstrate both increased levels of ACh in the atria[108] and a reduction in SA node sensitivity to this neurotransmitter[109] with training. Similar molecular adaptations in humans may explain the results observed in this investigation. The improvement in HRR among heart failure patients treated with pyridostigmine may reflect an underlying hypersensitivity to ACh, and therefore only small changes in neurotransmitter levels were needed to change chronotropic slowing. Conversely, the lack of HRR change with study drug administration in the athlete cohort might have been secondary to ACh desensitization and the inability of pyridostigmine to produce a sufficient increase in ACh to overcome postsynaptic changes. Sedentary controls also experienced HRR improvement with the study drug, possibly because their parasympathetic signaling cascade was sensitive enough to respond to pyridostigmine induced ACh concentration changes.

Athletes and sedentary subjects demonstrated significantly higher HRR values with placebo compared to previously reported values attained in heart failure patients.[79] Previous work exploring the interaction between the sympathetic and parasympathetic nervous systems may provide a novel explanation for the differences in heart rate recovery values observed between heart failure patients, sedentary controls, and trained athletes. Nerve stimulation studies performed in rabbits have demonstrated a nonlinear sigmoidal association between increased levels of sympathetic activity and changes in dynamic heart rate response to vagal stimulation.[110] As sympathetic activity is heightened, a given frequency of efferent vagal stimulation elicits larger reductions in heart rate. Individuals with heart failure have a higher level of sympathetic activation at rest when compared to healthy controls, reflected by increased basal catecholamine concentrations in the disease state.[111] However, heart failure patients are not able to

raise catecholamine levels to the same degree as healthy subjects during exercise.[111] Similarly, trained athletes are able to generate higher levels of catecholamines at peak exercise compared to healthy controls,[112] attributed to an increase in the secretory capacity of the adrenal medulla.[113] The trend towards higher HRR values observed in heart failure patients, healthy controls, and trained athletes might therefore be partially explained by the higher levels of sympathetic activation attained at peak exercise between these groups. The inability of beta blockade to significantly change heart rate recovery is likely because sympathetic enhancement of parasympathetic tone occurs at a central level, while these medications inhibit adrenergic response at the end organ. Parasympathetic activity, independent of sympathetic tone, also must play a role in the magnitude of this heart rate recovery because pyridostigmine is able to increase HRR in heart failure patients without changing peak exercise plasma catecholamine levels.[79] Since sympathetic tone is essentially maximized at peak exercise, an autonomic interdependency can also explain the large parasympathetically mediated drop in heart rate at exercise cessation and why such large changes in heart rate are not observed at rest with maneuvers designed to increase vagal firing.

We have therefore proposed three possible confounding mechanisms to explain differences in HRR between individuals and among various patient populations. The presence of a physiologic recovery ceiling likely limits HRR in certain individuals. Postsynaptic changes may also account for variation in HRR, as patients with dissimilar sensitivities to ACh will manifest different responses to pyridostigmine treatment. Finally, the level of sympathetic activation at maximal exercise is likely to affect HRR, as sympathetic tone appears to change the gain in parasympathetic output. The difference in HRR improvement with pyridostigmine, observed between study groups and among individuals within each group, likely results from a complex interaction between these mechanisms regulating parasympathetic control. As this experiment was conducted in humans, we had limited ability to quantify and control these variables.

The difference in placebo-induced one minute HRR values observed between athletes and controls did not reach statistical significance. This may reflect the inability of our selection criteria to establish two groups with sufficiently different levels of parasympathetic activity. It is also reasonable to assume parasympathetic tone has a heritable component that is variably amenable to exercise training. The correlation of HRV data among siblings supports such a genetic influence.[114, 115] Furthermore, previous studies examining HRR differences with physical conditioning enrolled trained individuals with higher mean VO_2 max values than the athletes in this investigation,[97] possibly indicating some athletes included in this study were not sufficiently well trained to manifest a significantly higher HRR than the sedentary controls.

While our study used exercise training characteristics and resting heart rate to differentiate the athlete and sedentary cohorts, previous studies examining HRV and physical conditioning have used VO_2 max or a combination of VO_2 max and physical activity to delineate these groups.[87, 116] A VO_2 max >55 ml/kg/min has been used to identify well trained athletes, while a VO_2 max < 40 ml/kg/min has been used to classify sedentary controls.[87] Others have used VO_2 max values of > 53 ml/kg/min for athletes and 34-42 ml/kg/min for controls.[116] These peak oxygen values approximate the results obtained for our athlete (52.5 ml/kg/min) and sedentary control (30.8 ml/kg/min) cohorts. There was some overlap in maximal oxygen consumption between the two cohorts (sedentary controls range 21.4 - 37.0 ml/kg/min, trained athletes range 30.7-66.8 ml/kg/min), likely reflecting the substantial genetic basis of VO_2 max.[117] Our athlete cohort also demonstrated a significantly lower resting heart rate and markedly increased minute ventilation at peak exercise compared to sedentary controls, consistent with inclusion criteria and training effects.

When enrolling athletes into our study, we did not specify nor formally record the form of aerobic exercise each individual practiced. As such, the trained athlete cohort contained individuals who participated in a variety of training activities, including running, cycling, and swimming. While differences in adrenergic receptor

densities have been reported between athletes with differing training modes,[118] other research has found no difference in HRV analysis between swimmers and runners with similar VO_2 max values.[119] We also did not question subjects regarding workout intensities. It has been previously proven that exercise intensity plays a greater role in improving maximal oxygen consumption than exercise duration.[120] It is plausible but unproven that short duration, high intensity "interval training" may differently alter autonomic tone compared to more sustained, lower intensity exercise. Our study design assumes all aerobic exercise augments autonomic tone in an analogous manner.

Athletes decreased their heart rate significantly faster than sedentary controls at two minutes of recovery with placebo administration. We examined HRR at two minutes because previous work using submaximal exercise testing associated impaired recovery at this time point with an increase in mortality.[46] Chronotropic slowing during the first two minutes of recovery is largely parasympathetically mediated, but the contribution of sympathetic withdrawal to slowing at this time point is less clear.[55, 78] The similarity in one minute recovery values between cohorts and the inability of pyridostigmine to prolong HRR at two minutes in either group suggests non-parasympathetic influences contributed to the disparity in HRR at two minutes observed between groups.

Similar to the HRR results, resting heart rate was not decreased with pyridostigmine in athletes, although this value trended in such a direction. Because the athletes had a low mean resting heart rate before the study drug (58 (1.6) bpm), it is possible the muscarinic receptors at the SA node were already saturated at rest and any increase in ACh concentration caused by pyridostigmine did not result in heart rate changes (Figure 3). Research in humans suggests heart rate reduction secondary to increases in parasympathetic tone eventually reaches a plateau. The direct linear relationship between vagal nerve stimulation and R-R interval levels off at stimulation frequencies above 25 Hz.[121] Postsynaptic desensitization secondary to training may also account for the lack of resting chronotropic change with pyridostigmine. Finally, if

only a small change in resting heart rate was afforded by AChE inhibition in athletes because of baseline receptor saturation or desensitization, this study may have been underpowered to detect such a difference.

Pyridostigmine significantly lowered resting heart rate by nine beats per minute compared to placebo in the sedentary control group. This suggests sufficient study drug dosing and appropriate timing of data collection relative to drug administration. The mean R-R interval measured by Holter monitor during the 30 minutes of supine rest was similarly increased in the sedentary cohort with pyridostigmine compared to placebo, again indicating vagal tone was affected by the study drug during HRV data acquisition.

Our data indicates pyridostigmine has no influence on time or frequency domain indices of autonomic function in both sedentary adults and athletes. We must concede it is possible this represents a type II error secondary to limited sample size. In sedentary controls treated with pyridostigmine, we observed a somewhat paradoxical reduction in heart rate without a change in the parasympathetically mediated indices of HRV compared to placebo. The pre-exercise respiratory rates for both cohorts with pyridostigmine and placebo were within the 0.15 – 0.40 Hz frequency window used in our HRV analysis to detect parasympathetically mediated respiratory sinus arrhythmia, indicating the lack of HRV change was not secondary to improper data acquisition.

Pyridostigmine has been associated with a decrease in the HF component of HRV.[65, 98] Some have questioned whether the drug has a bimodal effect on vagal tone similar to that seen with atropine or scopolamine, whereby peripheral cholinergic tone is augmented at low doses and central anticholinergic action is produced at higher doses.[98] A second study reporting HF power attenuation with pyridostigmine suggested the increase in ACh at the SA node due to the AChE inhibitor was sufficiently high to produce an exaggerated bradycardia while muting the difference in neurotransmitter levels between inspiration and expiration.[65] Because spectral analysis quantifies changes in vagal activity and not absolute parasympathetic tone, HF power was

reduced. This explanation for the decrease in HF power seen with pyridostigmine is more convincing than a dose dependent mechanism, as a bimodal hypothesis does not account for the simultaneous observation of decreased HF power and bradycardia. Neither of these previous investigations controlled for respiratory parameters while acquiring HRV data. The lack of correlation between the variability and rate measurements may have been confounded by changes in respiration between the two periods of EKG acquisition.[122]

Other studies have also revealed a divergence between HRV changes and heart rate modification, indicating these two variables are not representative of identical physiologic phenomena.[43, 122-125] Beta blockade, for instance, increased the average R-R interval in healthy adults but did not change HRV. This was explained by the ability of beta blockers to induce a sympatholytic reduction in heart rate without altering vagal efferent activity.[122] Changes in respiratory variables, including rate and tidal volume, have also been cited as a source of inconsistency between HRV and heart rate. When respiratory parameters are uncontrolled or altered, indices of HRV can change independently of heart rate.[122, 125] A respiratory induced shift in the distribution of vagal efferent activity throughout the cardiac cycle without a change in the overall efferent vagal firing rate could account for this observation. HRV can therefore be increased while constant vagal tone is preserved. Although alterations in respiratory parameters can account for HRV changes in the absence of chronotropic change, this mechanism does not account for the present observation of unchanged variability in the setting of heart rate slowing. Some have suggested the correlation between HRV and heart rate is lowered during drowsiness and sleep.[124] It is unclear how this would impact our measurements, as HRV data was obtained during a period of rest in a darkened room. Others have accounted for the dissociation between variability and rate changes by proposing the existence of two anatomically distinct vagal pathways originating in separate areas of the brainstem. Under this model, one vagal conduit regulates respiratory sinus arrhythmia while the other independently controls heart

rate.[124] Finally, there is data to suggest the relationship between parasympathetic tone and HRV is not linear.[123] Instead, increases in parasympathetic tone (and resultant decreases in heart rate) are met with a rise in HRV up to a point, after which HRV reaches a plateau level as tone and R-R interval continue to increase. Some individuals progress past the plateau point and develop a reduction in HRV with further increases parasympathetic activation, likely for saturation reasons described above.[123] This may explain why HRV remains unchanged or decreases in the setting of increased vagal tone.

We did not employ metronomic or volumetric methods to achieve respiratory synchronization during Holter data acquisition for HRV analysis. This omission would have the greatest impact on interindividual comparison of HRV data between athlete and sedentary controls. However, such analysis was not performed because of theoretical limitations previously discussed. Respiratory rate and tidal volume measured both immediately before and at peak exercise did not change with drug administration in either cohort. This evidence suggests respiratory variables during supine rest were also unchanged with drug administration and comparison of HRV data from the two study days within individuals using crossover analysis is appropriate. Furthermore, while some investigations indicate strict control of respiratory parameters is needed to correlate HRV with heart rate,[122] others imply respiratory synchronization may not improve the correlation between HRV and vagal tone within individuals.[43]

We propose an alternative explanation to account for the seemingly paradoxical heart rate reduction without HRV change seen in sedentary controls after pyridostigmine administration. A tonic increase in vagal tone provided by the study drug does not necessarily implicate changes in parasympathetic modulation.[42] Pyridostigmine theoretically increases the quantity of ACh at the SA node but does not alter vagal efferent firing. The lack of HRV change observed in this study could be explained if the drug increased the absolute amount of ACh in the nodal junction while

maintaining the relative *difference* in neurotransmitter present between inspiration and expiration (Figure 3).

The results of this investigation have several implications for the treatment of cardiovascular disease. The previously reported improvement in HRR with pyridostigmine in heart failure patients[79] has been replicated in sedentary adults. Unpublished data from the heart failure study revealed HRV indices were unchanged with pyridostigmine compared to placebo. As both HRV and HRR are thought to be measurements of parasympathetic activity, these results were somewhat contradictory. It was unclear if pyridostigmine augmented parasympathetic activity but did not change HRV because of pharmacologic mechanism of action, if pyridostigmine augmented parasympathetic activity but did not change HRV because of the specific autonomic changes present in heart failure, or if the pyridostigmine associated increase in HRR was due to non-parasympathetic influences. The replication of unchanged HRV with pyridostigmine in both sedentary controls and athletes, coupled with resting heart rate reduction and improved HRR in the controls, indicates a fundamental inability of pyridostigmine to influence heart rate variability. These findings then strengthen the conclusion that the improved heart rate recovery observed in heart failure patients was secondary to a pyridostigmine induced improvement in parasympathetic activity.

The inability of pyridostigmine to influence variability parameters indicates HRV may be an inadequate tool to assess benefit in future attempts to ameliorate the autonomic imbalance of heart failure with acetylcholinesterase inhibition. The capacity of vagomimetics such as scopolamine and atropine to augment HRV[59, 61] provides clinical verification that these medications alter vagal tone through a fundamentally different mechanism than pyridostigmine. A combined treatment approach utilizing both a vagomimetic and pyridostigmine may be appropriate and possibly synergistic in heart failure patients.

Pyridostigmine improved HRR in heart failure patients[79] but did not affect chronotropic slowing at one minute in trained athletes. These results provide indirect

evidence to support the presence of postsynaptic hypersensitization to ACh in cardiovascular disease and desensitization in trained athletes. If the difference in response between these two populations is indeed secondary to alterations in ACh sensitivity, this would imply heart failure patients with the most heightened sensitivity to ACh would derive the largest benefit with pyridostigmine treatment. Future efforts to provide a clinically accessible means of identifying postsynaptic neurotransmitter sensitivity may help identify heart failure patients most amenable to treatment with these medications.

To our knowledge, this investigation is the first to examine the effects of the acetylcholinesterase inhibitor pyridostigmine on aerobically trained athletes. It is also the first to study heart rate recovery after AChE inhibition in a population other than heart failure patients. In conclusion, heart rate recovery after maximal exercise is increased in healthy, sedentary adults after the administration of 30 mg of pyridostigmine but is unchanged in aerobically trained athletes. Indices of HRV, including pNN50, r-MSSD, and HF power, were unchanged with pyridostigmine compared to placebo in both cohorts.

Appendix

Table A. Baseline Clinical Characteristics of the Subjects, mean (SEM) range

	Sedentary Controls	Athletes	p Value
n (M/F)	10 (4/6)	10 (7/3)	0.18
Age (years)	34.8 (2.2) 25-48	27.1 (2.5) 22-47	0.03
Weight (kg)	70.5 (6.7) 50.0-118.1	71.0 (3.2) 54.0-87.0	0.94
Baseline HR (min^{-1})	70 (0.7) 64-72	58 (1.6) 51-64	< 0.01
Baseline MAP (mmHg)	82.8 (2.4) 73.3-96.6	87.6 (1.6) 77.0-93.7	0.12

Table B. Resting and Exercise Performance Variables, mean (SEM)

	Sedentary Controls			Athletes		
Resting Data	Placebo	Pyridostigmine	p Value	Placebo	Pyridostigmine	p Value
HR (min^{-1})	66.7 (4.0)	58.1 (2.4)	0.01	53.9 (1.8)	51.2 (2.3)	0.34
MAP (mmHg)	84.3 (2.7)	80.3 (2.0)	0.02	87.3 (1.2)	87.3 (2.4)	1.00
Respiratory Rate (min^{-1})	16.6 (2.3)	17.8 (1.5)	0.68	14.9 (1.2)	14.2 (0.8)	0.63
Tidal Volume (L)	0.8 (0.2)	0.6 (0.1)	0.30	0.7 (0.1)	0.7 (0.1)	0.79
Minute Ventilation (L/min)	12.1 (1.1)	10.5 (0.5)	0.21	10.3 (0.8)	10.4 (1.1)	0.93
VO_2 (ml/kg/min)	5.0 (1.0)	4.2 (0.6)	0.31	4.1 (0.3)	4.3 (0.4)	0.52
Maximal Exercise Data						
HR (min^{-1})	173.0 (3.9)	171.7 (4.6)	0.70	179.4 (2.6)	181.4 (2.4)	0.10
MAP (mm Hg)	108.8 (3.2)	103.3 (3.1)	< 0.01	106.5 (3.2)	105.6 (4.6)	0.74
Respiratory Rate (min^{-1})	37.5 (2.9)	34.9 (2.0)	0.46	50.1 (1.5)	49.4 (2.5)	0.81
Tidal Volume (L)	1.8 (0.2)	1.9 (0.2)	0.70	2.4 (0.1)	2.5 (0.1)	0.68
Minute Ventilation (L/min)	63.4 (5.6)	63.5 (5.5)	0.98	122.5 (8.4)	124.7 (9.6)	0.87
VO_2 Max (ml/kg/min)	29.4 (6.2)	28.5 (1.8)	0.28	53.3 (3.6)	54.8 (3.5)	0.02
Exercise Duration (sec)	670.4 (38.4)	619.6 (24.8)	0.25	1080.9(53.5)	1083.7(40.3)	0.93

Table C. Heart Rate Recovery, mean (SEM)

HRR 1 minute	Placebo	Pyridostigmine	p Value
Sedentary Controls (beats)	40.7 (3.4)	45.1 (2.8)	0.022
Athletes (beats)	46.5 (3.1)	50.4 (3.2)	0.163
HRR 2 minutes	Placebo	Pyridostigmine	p value
Sedentary Controls (beats)	56.3 (3.6)	59.0 (3.0)	0.198
Athletes (beats)	70.3 (3.2)	69.0 (3.7)	0.841

Figure 1. HRR in Sedentary Controls

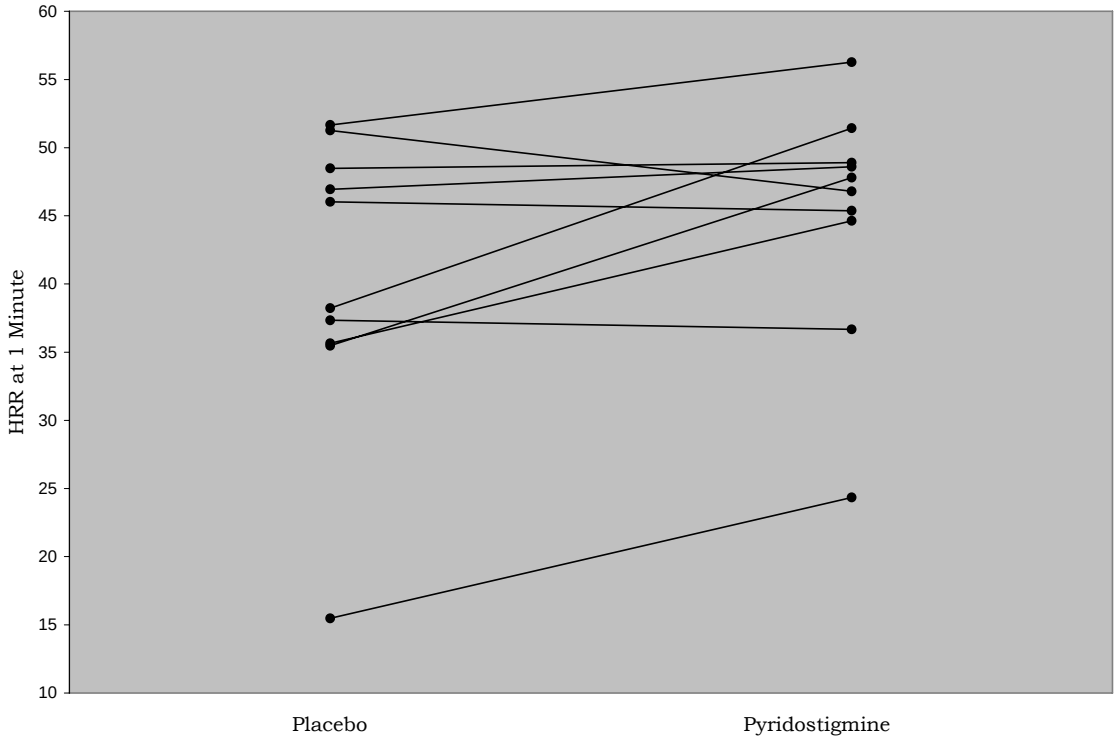

Figure 2. HRR in Trained Athletes

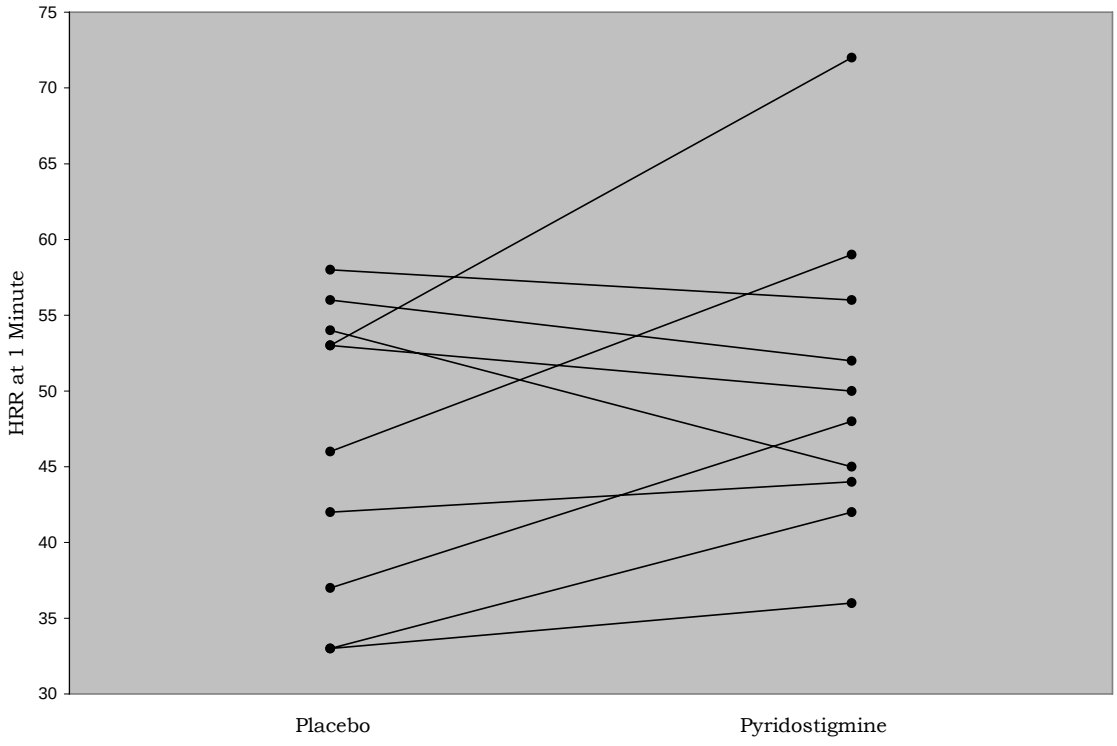

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Table D. Heart Rate Variability Analysis, mean (SEM)

Time Domain	Sedentary Controls			Athletes		
	Placebo	Pyridostigmine	p Value	Placebo	Pyridostigmine	p Value
pNN50	35.3 (5.5)	38.0 (8.4)	0.635	42.8 (5.0)	46.7 (5.3)	0.369
r-MSSD	66.8 (9.6)	78.5 (15.8)	0.202	83.8 (9.2)	81.1 (9.9)	0.806
Frequency Domain	**Sedentary Controls**			**Athletes**		
	Placebo	Pyridostigmine	p Value	Placebo	Pyridostigmine	p Value
HF mean	6.94 (0.30)	6.97 (0.50)	0.750	7.20 (0.20)	6.99 (0.26)	0.455
HF max	7.48 (0.33)	7.44 (0.48)	0.852	7.71 (0.19)	7.51 (0.26)	0.454
LF mean	7.10 (0.19)	7.11 (0.29)	0.810	7.47 (0.16)	7.28 (0.11)	0.303
LF max	7.74 (0.17)	7.77 (0.30)	0.817	8.20 (0.13)	7.92 (0.18)	0.277
VLF mean	7.24 (0.15)	7.55 (0.30)	0.358	7.95 (0.23)	7.82 (0.18)	0.442
VLF max	8.14 (0.16)	8.24 (0.33)	0.826	8.98 (0.26)	9.07 (0.27)	0.774
LF/HF	1.61 (0.36)	2.30 (0.92)	0.356	1.55 (0.22)	1.80 (0.41)	0.580
Mean TP	8.37 (0.17)	8.55 (0.31)	0.329	8.81 (0.18)	8.70 (0.16)	0.429
Max TP	8.87 (0.18)	8.92 (0.30)	0.681	9.54 (0.18)	9.51 (0.23)	0.917
RR mean (msec)	931.6 (32.7)	1003.7 (29.7)	0.003	1167.5 (45.0)	1198.5 (62.3)	0.627
RR max (msec)	974.0 (36.1)	1041.5 (30.1)	0.014	1213.0 (49.5)	1247.0 (68.7)	0.625

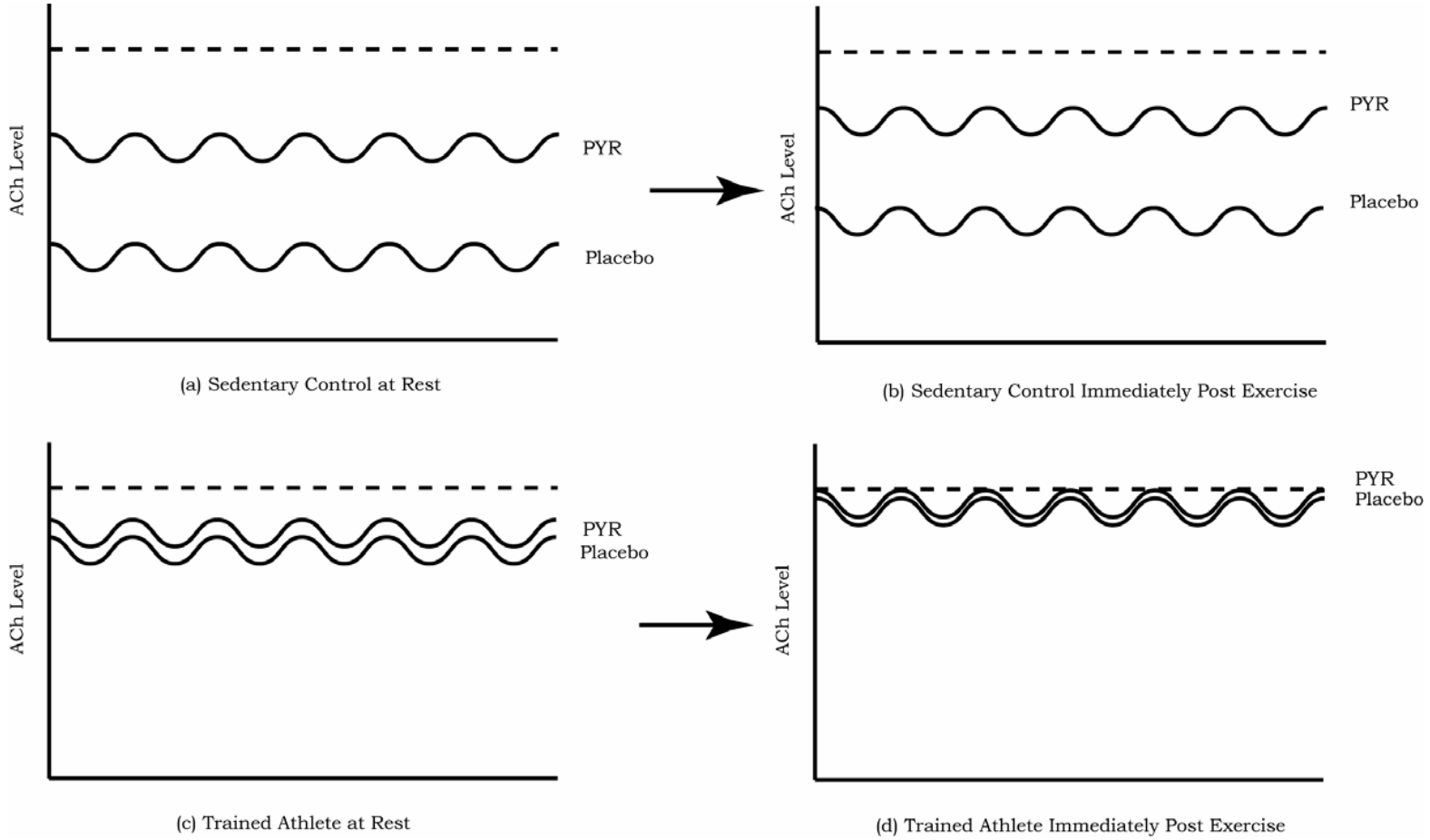

Figure 3. Proposed explanation for the effects of pyridostigmine observed in this experiment. The dotted line represents a hypothetical parasympathetic ceiling. (a) The oscillating ACh level at the SA node is due to alterations in efferent vagal activity secondary to respiration. This change in neurotransmitter concentration produces respiratory sinus arrhythmia and is quantified by HRV analysis. Pyridostigmine increases the absolute amount of neurotransmitter at the SA node, but does not affect the variation in ACh level with respiration. The higher ACh level results in increased parasympathetic tone and a reduction in heart rate among sedentary controls at rest. No change in HRV is detected because the variation in ACh level remains intact. (b) Overall vagal tone is increased during heart rate recovery. Pyridostigmine again augments this tone in sedentary subjects, resulting in increased HRR at one minute. (c) Pyridostigmine marginally enhances vagal tone in athletes at rest, but this affect is not sufficient to produce a statistically significant heart rate reduction. HRV is unchanged for reasons described above. (d) During HRR, vagal tone is increased to a physiologic ceiling in trained athletes. Pyridostigmine cannot raise vagal tone above this ceiling, and no change in HRR is seen.

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