EFFECTS OF PENTOXIFYLLINE ON EXERCISING SKELETAL MUSCLE VASCULAR CONTROL IN RATS WITH CHRONIC HEART FAILURE

by

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Abstract

Both cardiac and peripheral vasculature dysfunction likely contribute, in part, to elevations in TNF-α and exercise intolerance in chronic heart failure (CHF). The pharmaceutical TNF-α synthesis suppressor pentoxifylline (PTX) reduces plasma [TNF-α] and improves left ventricular (LV) function in CHF rats, but the effects of PTX on skeletal muscle blood flow (BF) and vascular conductance (VC) during exercise are unknown. We tested the hypothesis that PTX would elevate skeletal muscle BF and VC at rest and during submaximal treadmill exercise in CHF rats (coronary artery ligation). CHF rats received i.p. injections of 30 $mg \cdot kg^{-1} \cdot day^{-1}$ of PTX (CHF+PTX, n=13) or saline (CHF, n=8) for 21 days. Mean arterial pressure (MAP) and BF (radiolabeled microsphere infusions) were measured at rest and during treadmill exercise (20 m/min, 5% grade). Myocardial infarct (MI) size was not different between groups (CHF: 37±4, CHF+PTX: 37±3% of LV wall; $p>0.05$). Resting and exercising MAP was greater in CHF+PTX compared to CHF ($p<0.05$ for both). At rest, total hindlimb skeletal muscle BF and VC were not different between groups ($p>0.05$). However, during exercise PTX increased total hindlimb BF (CHF: 83±9, CHF+PTX: 114±8 $ml \cdot min^{-1} \cdot 100g^{-1}$, $p<0.05$) and VC (CHF: 0.75±0.08, CHF+PTX: 0.88±0.06 $ml \cdot min^{-1} \cdot 100g^{-1} \cdot mmHg^{-1}$, $p<0.05$). Furthermore, exercising BF was increased in 21, and VC in 11, of the 28 individual hindlimb muscles or muscle parts with no apparent fiber-type specificity. Thus, PTX administration augments skeletal muscle BF and VC during locomotory exercise in CHF rats, which carries important therapeutic implications for CHF patients.

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Abbreviations Used

CHF, chronic heart failure; LV, left ventricle; O_2, oxygen; TNF-α, tumor necrosis factor-alpha; NO, nitric oxide; iNOS, inducible nitric oxide synthase; IL-1, interleukin-1; PTX, pentoxifylline; LVEDP, left ventricular end diastolic pressure; LV dp/dt, left ventricular delta pressure delta time; BF, blood flow; VC, vascular conductance; MI, myocardial infarction; $\dot{V}O_2$, oxygen uptake; $\dot{V}CO_2$, carbon dioxide production; CO_2, carbon dioxide; RSNA, renal sympathetic nerve activity

Dedication

To my parents, thank you for instilling in me the importance of education and the value of hard work. Without your love and constant support I would not have been able to achieve all that I have during my college career. And to my loving husband, thank you for always encouraging me to strive for excellence and to never settle for work that is less than my best. The passion you exude for your work inspires me, and without your bolstering support I would not have realized my true potential.

Love always, Gabi

Chapter 1 - Introduction

Chronic heart failure (CHF) is a multifaceted clinical disorder hallmarked by left ventricular (LV) dysfunction and cardiac output insufficiency. Reductions in cardiac output prompt an exaggeration in sympathetic nervous system activity, which initially attempts to maintain arterial blood pressure but precipitates progressive multiple-organ system dysfunction and impaired peripheral oxygen (O_2) transport (Reviewed by Poole *et al*., 2012). Specifically, CHF results in enhanced sympathetically-mediated vasoconstriction and reduced peripheral vasomotor control which alters the spatial (Musch & Terrell, 1992; Miyazaki *et al*, 2007) and temporal (Richardson *et al*., 2003) matching of skeletal muscle O_2 delivery relative to O_2 demand during exercise. Impairments in peripheral O_2 transport lead to exercise intolerance and a reduced quality of life.

One potential mediator of the impaired O_2 transport in CHF patients is elevation of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α). TNF-α is elevated in both cachectic and non-cachectic CHF patients and plasma TNF-α concentration ([TNF-α]) correlates positively with disease severity thereby constituting a powerful prognostic indicator (Levine *et al*., 1990; Milani *et al*., 1996). Elevations of TNF-α in autonomic regulatory regions of the brain result in sympathoexcitation via alterations in superoxide and nitric oxide (NO)-mediated signaling in CHF (Guggilam *et al*., 2011). In the heart, TNF-α up regulates inducible NO synthase (iNOS), induces negative inotropic effects and reduces cardiac myocyte contractility (Finkel *et al*., 1992; Balligand *et al*., 1993). In the periphery, TNF-α, in conjunction with interleukin-1 (IL-1), increases expression of adhesive molecules and associated receptors on vascular endothelial cells (Reviewed by Ceconi *et al*., 1998) and contributes to impaired skeletal

muscle structure and function through promotion of catabolism and inhibition of contractile function (Li *et al*., 2001).

Given the negative cardiovascular effects of TNF-α in CHF, the phosphodiesterase inhibitor pentoxifylline (PTX) represents a powerful therapeutic strategy based on its ability to down regulate TNF-α gene transcription and suppress TNF-α synthesis (Doherty *et al*., 1991). In a clinical setting, PTX administration in CHF patients improved LV ejection fraction and New York Heart Association (NYHA) functional classification due, at least in part, to reductions in plasma [TNF-α] in addition to increased exercise capacity (Sliwa *et al*., 2004). In CHF rats, PTX administration also reduces [TNF-α] and improves LV function as evidenced by reductions in LV end-diastolic pressure (LVEDP; Guggilam *et al*., 2008). In addition, PTX administration lowers circulating levels of norepinephrine and epinephrine and attenuates renal sympathetic nerve activity (Guggilam *et al*., 2007). However, whether PTX-induced improvements in LV function and reductions in sympathoexcitation translate to increases in blood flow (BF) within and among active skeletal muscle during exercise in CHF subjects remains unknown. This is crucial information given that improvements in BF and O_2 delivery during exercise may improve exercise capacity and quality of life in CHF patients.

The purpose of the present investigation was to determine the effects of chronic PTX administration on active skeletal muscle BF distribution during locomotory exercise in CHF. Specifically, we tested the hypothesis that 21 days of PTX administration in CHF rats would elevate hindlimb skeletal muscle blood flow and vascular conductance (VC) during submaximal treadmill running.

Chapter 2 - Literature Review

Recent medical advances now allow individuals to survive a cardiovascular event, however many experience reduced quality of life after recovery. These advancements have contributed to an increased incidence and prevalence of individuals living with cardiovascular disease and heart failure (Antunes-Correa *et al*., 2011). There has been a call to action for health professionals to investigate recovery, rehabilitation, and treatment options for heart disease and CHF patients.

CHF and Exercise Intolerance

The cardiovascular system has crucial decisions to make about how to distribute cardiac output to various organs and tissues in the body. Interaction between the sympathetic nervous system and locally mediated muscle and tissue vasodilation plays an important role in this distribution, and these two systems work together to deliver adequate BF to active and inactive tissues while maintaining mean arterial pressure (MAP; DiCarlo *et al*., 1996). CHF is a complex clinical disorder with the core perturbation being central cardiac dysfunction, specifically reduced cardiac output. Sympathetic nerve activity is upregulated as a part of the body's response to the reductions in cardiac output caused by myocardial damage. While it initially serves to maintain cardiac output, chronic sympathetic activation contributes to a cascade of organ and tissue dysfunction throughout the body (Reviewed by Poole *et al*., 2012). Additionally, the NO pathway, a major contributor to skeletal muscle vasodilation, is also impaired in CHF (Hirai *et al*., 1995).

Patients with CHF experience reduced maximal oxygen uptake and exercise capacity, and experience rapid fatigue when completing activities of daily living (Marcinkiewicz *et al*., 2000; Antunes-Correa *et al*., 2011). These deleterious effects are the result of reduced skeletal muscle

BF, O_2 transport, and NO bioavailability (Reviewed by Poole *et al*., 2012). Reductions in skeletal muscle BF affect the body's ability to meet the O_2 demand of the working muscle. This is due in part to slower O_2 uptake kinetics and an impaired O_2 diffusion capacity (Richardson *et al*., 2003). Additionally, reductions in skeletal muscle BF appear to be fiber-type dependent, given that O_2 transport and microvascular O_2 pressure recovery to slow-twitch muscle fibers is selectively reduced (Hirai et al., 1995; McDonough *et al*., 2004). Slow twitch muscle fibers are responsible for many of the activities of daily living, explaining the decreased exercise capacity and rapid fatigue experienced by patients with CHF (McDonough *et al*., 2004).

Immune Activation in CHF

CHF is associated with immune system activation, which includes elevated circulating pro-inflammatory cytokines, specifically plasma TNF-α (Levine *et al*., 1990). TNF-α is secreted by macrophages as an immune response mediating the effects of a physiologic disturbance, (in CHF, ischemia in cardiac muscle tissue), furthering the inflammatory response (Doherty *et al*., 1991). Circulating concentrations of pro-inflammatory cytokines are correlated with severity of disease, making them important prognostic indicators (Levine *et al*., 1990).

Elevated [TNF-α] facilitates functional derangement in CHF via perturbations in sympathetic nerve activity, cardiac muscle tissue, and peripheral vascular control. Guggilam *et al*. (2011) determined that TNF-α upregulation in autonomic regulatory regions of the brain, including the hypothalamus and paraventricular nucleus, contributes to sympathoexcitation in CHF due to alterations in NO-mediated signaling. TNF-α expression is also elevated in cardiac tissue, which leads to reductions in cardiac myocyte contractility via upregulatin of iNOS (Balligand *et al*., 1993; Finkel *et al*., 1992). Endothelial dysfunction including increased adhesive molecule expression (Reviewed by Ceconi *et al*., 1998) and impaired skeletal muscle

structure and function (Li *et al*., 2001) is another result of TNF-α upregulation in CHF ultimately affecting exercise tolerance and quality of life in these patients.

TNF-α: Effects of Direct Inhibition

Etanercept is a soluble recombinant TNF-α antagonist that inactivates TNF-α when binding, thus preventing TNF-α from binding with its cell membrane receptor (Mann *et al*., 2004). Due to the negative impact immune activation and elevated pro-inflammatory cytokines have in CHF a clinical trial was conducted to investigate the effects of TNF-α receptor inhibition in CHF patients using etanercept. It was thought that blocking the action of TNF-α would improve functional status of CHF patients, however the study was terminated early due to lack of benefits seen in the participants. The exact mechanism for this lack of improvement, and in certain cases worsening of functional status, is not known. Nevertheless this study demonstrates that inhibition of TNF-α at the receptor site is ineffective in the condition of heart failure.

Effects of Pentoxifylline (PTX) Administration

PTX is a methyl-xanthine derivative and a competitive non-specific phosphodiesterase inhibitor used regularly in the treatment of peripheral vascular disease because of it vasodilatory affects in the vasculature (McNamara *et al*., 1998). PTX has also been shown to downregulate TNF-α synthesis via downregulation of TNF-α gene transcription. Importantly, PTX also attenuates circulating plasma [IL-12], [catecholamine], [C-reactive protein] as well as other pro-inflammatory cytokines in CHF (Guggilam *et al*., 2007; 2008; Reviewed by Shaw *et al*., 2009).

Guggilam *et al*. investigated the link between immune activation and neurohumoral excitation in CHF employing PTX (2007; 2008). In this study CHF rats were treated with either PTX or a vehicle for 5 weeks. CHF rats had a reduced left ventricular function compared to that of healthy rats prior to treatment; however, treatment with PTX prevented further decline in

cardiac pump function in the CHF rats. Plasma [TNF-α] and [catecholamine] in the PTX-treated group were reduced to values near that of the healthy rats (Guggilam *et al*., 2007). Additionally PTX administration also resulted in reduced renal sympathetic nerve activity and Nox2 expression in the CHF rats (Guggilam *et al*., 2007). This study demonstrates the role pro-inflammatory cytokines play in the sympathoexcitation apparent in CHF, and the important implications of targeting cytokines through therapeutic strategies.

Administration of PTX in CHF patients has also demonstrated beneficial results. Bahrmann *et al*. (2004) examined the effects of PTX on LV function and circulating pro-inflammatory cytokines in CHF patients over a 6-month period. PTX had beneficial effects on LV function, O_2 uptake and exercise time in patients with ischemic and hypertensive cardiomyopathy (Bahrmann *et al*., 2004). Importantly, other clinical trials involving PTX treatment in CHF patients have resulted in similar outcomes (i.e. improvements in LV function, exercise time, and NYHA functional class; Skudicky *et al*., 2001; Bahrmann *et al*., 2004; Sliwa *et al*., 2004). Interestingly, the underlying mechanisms through which PTX downregulates TNF-α and produces these central cardiac improvements are still not clearly understood.

Summary & Future Research

It is known that CHF results in impaired peripheral vascular control (Hirai *et al*., 1995), reduced skeletal muscle BF and aerobic capacity (Musch & Terrell, 1992). It is also known that less capillaries support RBC flux in CHF, impairing oxygen delivery (Richardson *et al*., 2003; McDonough *et al*., 2004). CHF is associated with elevated circulating [TNF-α], which plays a role in both central cardiac and peripheral dysfunction (Levine et al., 1990; Finkel *et al*., 1992; Balligand *et al*., 1993; Reviewed by Ceconi *et al*., 1998). The above-mentioned perturbations contribute to the exercise intolerance and rapid fatigue that CHF patients experience. Exercise

training is a safe and effective therapeutic strategy for both middle aged and older CHF patients (Antunes-Correa *et al*., 2011). However, because of the severe exercise intolerance seen in many CHF patients drug therapies lend well to initially improving dysfunction. Direct inhibition of TNF-α at the receptor site via etanercept has been shown to be ineffective in CHF patients (Mann et al., 2004). Conversely, improvements in LV function and reductions in plasma [TNF-α] have been seen with PTX treatment in CHF rats and patients (Skudicky *et al*., 2001; Bahrmann *et al*., 2004; Sliwa *et al*., 2004; Guggilam *et al*., 2007; 2008). Unveiling the specific mechanism through which PTX provides these improvements is certainly important and warrants further investigation. Presently, it is not know whether PTX-induced improvements in CHF translate to increases in skeletal muscle BF or VC in active or inactive muscles or muscle parts, prompting the current investigation.

Chapter 3 - Methods

Animals

Twenty-one male Sprague Dawley rats (body mass: 492±43, age: ~6 months, Charles River Laboratories, Wilmington, MA) were used in the present investigation. All rats were housed in accredited facilities and kept on a 12:12 light-dark cycle, with food and water provided ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of Kansas State University and were conducted in accordance with institutional and National Institutes of Health guidelines.

Myocardial Infarction Procedure

Myocardial infarction (MI) was induced in all rats via left main coronary artery ligation (Musch & Terrell, 1992). To begin, rats were anesthetized with a 5% isoflurane (balance O_2) mixture and intubated for mechanical ventilation with a rodent respirator (Harvard Model 680; Harvard Instruments, Holliston, MA) for the length of the surgical procedure. The heart was accessed through a left thoracotomy in the fifth intercostal space. The left main coronary artery was ligated with 6-0 silk suture approximately 1-2 mm distal to the edge of the left atrium. The incision was then closed, and ampicillin (50 mg/kg i.m.) was administered to reduce risk of infection. The analgesic agents bupivacaine (1.5 mg/kg s.c.) and buprenorphine (0.01-0.05 mg/kg i.m.) were administered and isoflurane anesthesia and mechanical ventilation ceased. All rats were then extubated and monitored closely for ≥6 hours for development of cardiac arrhythmias and signs of undue distress (i.e., labored breathing, etc.) with care administered as needed. Rats were also monitored daily (i.e., appetite, weight loss/gain, gait/posture, etc.) consistent with an intensive 10-day post-operative plan carried out in conjunction with the university veterinary staff.

Intervention Protocol

21 days following the MI procedure rats were assigned randomly to either the experimental group (CHF+PTX, n=13), or control (CHF, n=8) group. The CHF+PTX group received pentoxifylline once daily for 21 days (30 mg/kg in 0.3 ml saline i.p.) whereas the CHF group received saline (0.3 ml i.p.).

Performance Testing

All rats were subjected to exercise performance testing on days 19 and 20 of the 21-day intervention period (i.e. 40-41 days following MI procedure). Rats were first acclimatized to the treadmill for 5 consecutive days prior to exercise tests of endurance capacity and peak O_2 uptake ($\dot{V}O_2$ peak), which were completed in random order on consecutive days.

The endurance capacity of each rat was measured using a progressive exercise test in which each animal ran initially at a speed of 25 m/min up a 5% grade for 15 min. Subsequently, the treadmill grade was held constant while the speed was increased incrementally by 5 m/min every 15 min until the rat was unable/unwilling to maintain pace with the treadmill belt despite manual bursts of high-pressure air aimed at the hindlimbs. At the end of each test, exhaustion was confirmed by an attenuation of the rats' righting reflex. Our laboratory has demonstrated previously that time-to-exhaustion with this protocol is highly accurate and reproducible within each animal (Copp *et al*., 2009).

$\dot{V}O_2$ peak for each rat was determined as described previously for our laboratory (Copp et *al*., 2009). Briefly, each rat was placed in a custom made metabolic chamber designed to fit into one stall on the treadmill and utilized standard techniques originally described by Brooks and White (1978) for determining $\dot{V}O_2$ and carbon dioxide (CO_2) production ($\dot{V}CO_2$). Gas analysis measurements were made in real-time via online CO_2 and O_2 analyzers (CO_2: model CD-3A; O_2:

model S-3A/I; AEI Technologies, Pittsburgh, PA) set in series. The analyzers were calibrated before and after each exercise test using precision-mixed gases that spanned the expected range of gas concentrations based on previous investigations. Each rat ran initially in the metabolic chamber at a speed of 25 m/min (5% incline) for 2-3 minutes. Subsequently, the speed of the treadmill was increased to 40 m/min for an additional 2-3 minutes. Thereafter, the treadmill speed was increased progressively in a ramp-like manner by ~5-10 m/min every minute until the rat was unable or unwilling to keep pace with the treadmill belt. $\dot{V}O_2$ peak was recorded as the $\dot{V}O_2$ at which the rat was no longer able/willing to run. Peak $\dot{V}CO_2$ was also recorded and the respiratory exchange ratio (RER, $\dot{V}CO_2/\dot{V}O_2$) was calculated. Criteria for a successful test were the observation of a change in gait indicative of exhaustion immediately preceding the termination of the test (Copp *et al*., 2009), and/or no further increase in $\dot{V}O_2$ despite continued increases in treadmill speed.

Surgical Instrumentation

The final experimental protocol was initiated immediately following the 21-day intervention period (i.e. ~42 days after MI procedure). All rats were anesthetized initially with 5% isoflurane-O_2 gas mixture. The carotid artery was cannulated and a 2-French catheter-tipped pressure transducer (Millar Instruments, Houston, TX) was advanced into the LV for measurement of diastolic pressures and LV delta pressure/delta time (dp/dt). Due to technical complications LVEDP was not determined in 1 of the 13 CHF+PTX rats. Upon completion of the measurement the transducer was removed and the carotid artery was re-cannulated with a catheter (PE-10 connected to PE-50, Intra-Medic polyethylene tubing, Clay Adams Brand, Becton, Dickinson and Company, Sparks, MD) for measurement of heart rate (HR), mean

arterial pressure (MAP), and arterial blood sampling. A second catheter (PE-10 connected to PE-50) was placed in the caudal (tail) artery, as described previously (Musch & Terrell, 1992). Both catheters were tunneled subcutaneously to the dorsal aspect of the cervical region and exteriorized through a puncture wound in the skin. Incisions were closed, anesthesia was terminated, and the animal was given >1 hour to recover.

Radiolabeled Microsphere Infusion

Following the instrumentation each rat was placed on the treadmill. The tail artery catheter was connected to a 1-ml plastic syringe that was connected to a Harvard infusion/withdrawal pump (model 907, South Nattick, MA). The carotid artery catheter was connected to a pressure transducer set at the same level as the rat for continuous measurement of MAP and HR. Exercise was initiated, and the speed of the treadmill was increased progressively during the next 30 seconds to a speed of 20 m/min (5% grade). The rat then ran steadily for another 2.5 minutes. After 3 minutes of total exercise time, blood withdrawal from the tail artery catheter was initiated at a rate of 0.25 ml/min. Simultaneously, MAP and HR were measured via the carotid artery catheter. The carotid artery catheter was then disconnected from the pressure transducer and ~0.5-0.6 X 10^6 microspheres with 15 μm diameter (^{57}Co or ^{85}Sr, in random order, Perkin Elmer Life and Analytical Sciences, Waltham, MA) were injected into the aortic arch via the carotid artery catheter to determine regional blood flow. After the microsphere infusion a blood sample (~0.3 ml) was taken from the carotid artery catheter for measurement of blood gases, pH, hematocrit, and blood lactate concentrations ([lactate]). Subsequently (~30 seconds after the microsphere infusion), blood withdrawal from the tail artery catheter was stopped and exercise was terminated. After a >30 minute recovery period, MAP and HR were measured as the rat sat quietly on the treadmill. A second microsphere infusion (differently labeled from the

first infusion) and blood sampling procedure were then performed exactly as described above during exercise to determine BFs at rest. This strategy (exercise followed by rest) minimizes the potential for blood loss to affect the exercise response and facilitates "resting" measurements that do not reflect the pre-exercise anticipatory response (Armstrong *et al*., 1989).

Determination of Morphological Characteristics, Regional BF and VC

Following the experimental protocol rats were euthanized promptly using sodium pentobarbital overdose (≥100 mg/kg infused i.a. into the carotid artery catheter). The thorax was opened and placement of the carotid artery catheter was confirmed. The lungs were excised and weighed to determine lung/body mass ratio for each animal. The heart was removed; the right ventricle (RV) was separated from the LV and septum, and both tissues were weighed and normalized to each animal's body mass. To determine LV infarct size, an incision was made through the interventricular septum, from the base to the apex of the LV, and a digital photograph of the endocardium was taken. The image was printed, and endocardial infarct surface area was determined by planimetry. Internal organs and individual muscles and muscle portions of the hindlimb were identified and excised. Upon removal, tissues were blotted, weighed, and placed immediately into counting vials.

Radioactivity of each tissue was determined using a gamma scintillation counter (Packard Auto Gamma Spectrometer, model 5230, Downers Grove, IL). Tissue BF was then calculated using the reference sample method (Musch & Terrell, 1992) and expressed in $ml \cdot min^{-1} \cdot 100g^{-1}$. Adequate mixing of the microspheres was confirmed for each rat by demonstrating a <15% difference in BF to the right and left kidney and/or to the right and left hindlimb musculature. VC was calculated by normalizing BF to MAP and expressed as $ml \cdot min^{-1} \cdot 100g^{-1} \cdot mmHg^{-1}$

Blood Sample and Cytokine Analysis

Pre-PTX and post-PTX administration blood samples were taken from the sub-orbital plexus using a glass capillary pipette. This sampling method was implemented to avoid acute elevation of cytokine concentrations post-surgical instrumentation. Pre-PTX blood samples were taken prior to group assignment, and post-PTX blood samples were taken prior to catheterization on the day of the final experimental protocol. Approximately 0.8 ml of blood was drawn into heparinized sample tubes, and centrifuged at 6000 g at 4°C for 6 minutes, plasma was extracted and frozen immediately at -80°C for later analysis of plasma cytokine concentrations. Circulating [TNF-α] was quantified in the plasma by using a commercially available rat TNF-α enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer instructions (Abcam, Cambridge, MA). Due to technical complications [TNF-α] in 1 of the 13 CHF+PTX rats was not determined.

Statistical Analysis

Cardiac morphological and hemodynamic measurements were compared using unpaired Student's t-tests. Plasma [TNF- α] variance was compared using a *post hoc* F-test. All other data were compared within (rest vs. exercise) and between (CHF vs. CHF+PTX) groups using mixed 2-way ANOVAs and Student-Newman-Keuls *post hoc* tests where appropriate. Significance was set at $p<0.05$ and values are expressed as mean ± SEM.

Chapter 4 - Results

Effects of PTX on plasma [TNF-α]

Due to large sample variability in plasma [TNF-α] among rats the large absolute differences between CHF and CHF+PTX rats came close but did not reach statistical significance ($p=0.052$). However, the CHF+PTX group demonstrated significantly less variance in post-intervention plasma [TNF-α] compared with CHF ($p<0.05$, Figure 1).

Effects of PTX on Cardiac Indices and Exercise Performance

MI size and hemodynamic and morphological indices of cardiac function for CHF and CHF+PTX rats are presented in Table 1. The LVEDP/MI size ratio was significantly lower in CHF+PTX compared with CHF ($p<0.05$, Figure 2). There was no difference in endurance capacity between groups (CHF: 18±5, CHF+PTX: 19±2 min, $p>0.05$). $\dot{V}O_2$ peak was not different ($p>0.05$) between CHF (64±7, RER: 1.08± 0.03) and CHF+PTX (66±3 ml $O_2 \cdot kg^{-1} \cdot min^{1}$, RER:1.02±0.02) groups.

Effects of PTX on HR, MAP, arterial [O_2], saturation, and [lactate]

The effects of PTX on resting and exercising HR and MAP are displayed in Figure 3. CHF+PTX rats had higher MAP at rest and during exercise compared to CHF ($p<0.05$ for both). HR was not different between groups at rest but was greater in CHF+PTX compared to CHF rats during exercise ($p<0.05$). There were no differences in resting (CHF: 95±2, CHF+PTX: 97±1%, $p>0.05$) or exercising (CHF: 95±2, CHF+PTX: 96±1%, $p>0.05$) arterial O_2 saturation (and, therefore, calculated arterial [O_2]) between groups. Blood [lactate] was not different between groups at rest (CHF: 1.1±0.4, CHF+PTX: 0.9±0.1 mmol/L) or during exercising (CHF: 4.5±1.5, CHF+PTX: 4.7±0.7 mmol/L).

Effects of PTX on hindlimb BF and VC at rest and during exercise

There were no differences in resting total hindlimb BF or VC between CHF and CHF+PTX rats (Figure 4). Moreover, there were no differences in resting BF or VC to any individual hindlimb muscle or muscle part between groups (Table 2). In contrast, exercising total hindlimb skeletal muscle BF and VC were significantly elevated in CHF+PTX compared to CHF rats (Figure 4). Specifically, exercising BF was higher in 21 of 28 and VC was higher in 11 of 28 individual hindlimb muscles or muscle parts in CHF+PTX compared to CHF rats with no apparent muscle fiber-type composition or function specificity (Table 3).

Effect of PTX on renal and splanchnic BF and VC at rest and during exercise

Renal and splanchnic BF and VC values are presented in Table 4. Blood flow to the adrenals, spleen, and large intestine were higher at rest in CHF+PTX rats compared to CHF ($p<0.05$) whereas only resting adrenal and splenic VC was higher in CHF+PTX rats ($p<0.05$). There were no differences in renal or splanchnic organ BF and VC during exercise between groups ($p>0.05$).

Chapter 5 - Discussion

The principal novel finding of the present investigation is that PTX administration (30 $mg \cdot kg^{-1} \cdot day^{-1}$ for 21 days) in CHF+PTX rats elevated hindlimb skeletal muscle BF and, therefore, O_2 delivery during submaximal treadmill exercise compared to CHF. The significant improvements in O_2 delivery were associated with enhanced cardiac function, increased VC, and elevated systemic driving pressure for bulk O_2 delivery (i.e. increased MAP). However, despite the significant cardiovascular improvements PTX administration had no effects on endurance capacity or $\dot{V}O_2$peak.

Mechanisms of PTX-induced BF elevations

Whole-body exercise prompts sympathetically-mediated increases in cardiac output and peripheral blood flow redistribution toward working skeletal muscle. Within active skeletal muscle vascular beds blood flow increases via mechanical, humoral, and metabolic vasomotor control signals. CHF patients and animals evidence lower skeletal muscle blood flow and O_2 delivery for a given level of exercise compared to healthy subjects consequent to lower cardiac output, exaggerated sympathetic vasoconstriction and impaired vascular endothelial function (Reviewed by Poole et al., 2012). Importantly, elevations in TNF-α have been implicated, in part, as one mechanism underlying each of the above-mentioned perturbations (Cunnion *et al*., 1989; Reviewed by Ceconi *et al*., 1998) and it has been shown that plasma [TNF-α] correlates positively with CHF severity (Levine *et al*., 1990).

Previous studies have shown that TNF-α administration in cancer patients can induce cardiac enlargement and pulmonary congestion, as well as reduce myocardial contractility, leading to cardiomyopathy (Hegewisch *et al*., 1990; Finkel *et al*., 1992). In the present investigation blockade of TNF-α production in CHF+PTX rats with the phosphodiesterase

inhibitor PTX increased cardiac contractility (i.e. increased LV dp/dt) and lowered LVEDP and the lung/body mass ratio (suggesting less pulmonary congestion consequent to improvement in LV contractile function). These marked improvements in cardiac function were evident despite similar MI size between the CHF and CHF+PTX group and are consistent with previous investigations (Sliwa *et al*., 2004; Guggilam *et al*., 2007; 2008).

CHF-induced cardiac dysfunction, particularly during exercise, challenges the ability of the cardiovascular system to regulate MAP. In this regard, the higher resting and exercising MAP values observed herein following PTX administration are similar to that observed in healthy rats (Pfeifer *et al*., 2001; Ferguson *et al*., 2013) and likely reflect improvements in cardiac function as discussed above. Interestingly, at rest it appears that PTX-induced elevations in MAP are facilitated by increased SV, as HR and organ and hindlimb muscle VC (i.e. the inverse of systemic vascular resistance) were unchanged. Conversely, during exercise the 21% higher MAP is consistent with increases in both SV and HR (given HR increased only 8%), which occurred despite significant increases in VC (and therefore lower systemic vascular resistance). Additionally, the elevated exercising HR response seen in CHF+PTX rats is consistent with reversal of the chronotropic incompetence associated with CHF. This reversal has also been achieved through exercise training (Musch *et al*., 1989).

PTX administration in CHF+PTX rats increased exercising hindlimb skeletal muscle BF (↑38%) and, therefore, O_2 delivery compared to CHF. This increase occurred concomitant with a higher cardiac output (as opposed to peripheral redistribution, as suggested by similar or increased kidney and splanchnic organ BFs between groups), elevated systemic driving pressure for bulk O_2 delivery (i.e. higher MAP), as well as modified peripheral vascular control (i.e. 18% higher VC). In regards to the latter, TNF-α participates in functional derangement of endothelial

cells, which includes activation of iNOS (Reviewed by Ceconi *et al*., 1998), formation of adhesive molecules (Reviewed by Ceconi *et al*., 1998; Suschek *et al*., 1993), and decreased endothelial nitric oxide synthase (eNOS) mRNA due to its increased rate of degradation (Blum & Miller, 2001). Stosić-Grujicić *et al*. (2001) demonstrated that PTX administration results in reduced local iNOS expression in intraislet and endothelial cells. This suggests that PTX administration could be increasing exercising BF through reductions in iNOS, thus lessening the systemic inflammatory response, and enhancing local NO-mediated vasodilation via increased eNOS expression and/or function. Moreover, PTX administration reduces renal sympathetic nerve activity (RSNA) (Guggilam *et al*., 2007; 2008) consistent with the notion that PTX administration may be exerting a central effect, whereby BF is increased due to less sympathetically-meditated vasoconstriction. Further investigation into the effects of PTX on NO bioavailability, reactive O_2 species (ROS) regulation, and sympathetic nerve activity during exercise may elucidate the specific mechanisms of PTX-induced BF elevations in CHF+PTX rats evident in the present investigation. Given the lack of muscle fiber-type specific exercising BF and VC increases found herein and the fiber-type specificity of vasomotor signals including NO (Hirai *et al*., 1994), sympathetic vasoconstriction (Behnke *et al*., 2011), and functional sympatholysis (Thomas *et al*., 1994), it is likely that PTX modified multiple vascular control pathways.

Clinical Implications

In the present investigation PTX administration increased hindlimb skeletal muscle BF and VC but did not change exercise endurance capacity or $\dot{V}O_2$ peak between groups. Those exercise performance values are consistent with severe CHF (Musch *et al*., 2002). However, the improved indices of cardiac function following PTX administration in the CHF+PTX group

more closely resemble moderate CHF (Diederich *et al*., 2002). The relatively short 21 day PTX treatment period could potentially play a role in the lack of improvement in exercise performance, similar to the delayed reversal of impaired vasodilation seen in CHF patients post-heart transplantation (Sinoway *et al*., 1988). However, the present data are also consistent with the fact that PTX administration improves LV function but not exercise performance in CHF patients (Skudicky *et al*., 2001; Bahrmann *et al*., 2004; Sliwa *et al*., 2004) and suggests that PTX may not improve the ability of the active skeletal muscle to utilize the increased O_2 delivery during exercise. For example, alterations in capillary geometry (Xu *et al*., 1998), fewer capillaries supporting red blood cell flux at rest and during contractions (Kindig *et al*., 1999; Richardson et al., 2003), a reduced capillary-muscle fiber ratio (Xu *et al*., 1998; Wüst *et al*., 2012), and altered microvascular O_2 pressure kinetics (Diederich *et al*., 2002) as well as intrinsic skeletal muscle abnormalities including lower oxidative enzyme activity (Arnolda *et al*., 1991; Delp *et al*., 1997), mitochondrial dysfunction (Wüst *et al*., 2012) and impaired excitation-contractile function (Poole-Wilson *et al*., 1988; Musch *et al*., 2002; Helwig *et al*., 2003; Perreault *et al*., 2003;) have been reported in CHF. The absence of PTX-induced improvements in any of these mechanisms may potentially explain the lack of improvements in exercise performance despite higher skeletal muscle BF in CHF+PTX rats found herein. For this reason, further investigation into the effects of PTX on capillary hemodynamics and microvascular O_2 pressure kinetics during muscle contraction, as well as PTX administration combined with therapies that may ameliorate structural and functional vascular and metabolic derangements (i.e. exercise training) and/or reduce the O_2 cost of exercise (i.e. nitrate supplementation, Jones *et al*., 2012), may prove most efficacious in improving exercise tolerance and quality of life in CHF patients.

Experimental Considerations

In the present investigation PTX administration reduced [TNF-α] variance in CHF+PTX rats, and resulted in close-to-significant reductions in [TNF-α] (~70%↓, p=0.052), compared with CHF. Guggilam *et al.* (2007; 2008) reported lower tissue-bound TNF-α expression in the LV and paraventricular nucleus, as well as circulating [TNF-α] following PTX administration. It is important to note that our investigation differed from Guggilam et al. (2007; 2008) in that the present sample size was smaller, blood sampling was done at rest not in response to an endotoxin or stress stimulus, and 21 days were allowed (post-MI procedure) for development of CHF prior to the intervention (Fishbein *et al*., 1978). This 21-day time period may account for initial elevations in plasma [TNF-α] seen in pre-PTX blood sampling (Guggilam *et al*., 2007). Furthermore, post-PTX plasma [TNF-α] values in CHF+PTX rats resembled closely pre-PTX administration values. Conversely, in CHF rats plasma [TNF-α] appear to increase and become more variable over the same time period. Importantly, our present data is consistent with the complex nature of the TNF-α system previously described by Ferrari *et al*. (1995) as well as several studies where PTX administration in CHF patients did not reduce circulating [TNF-α] (Skudicky *et al*., 2001; Bahrmann *et al*., 2004). The complexity of TNF-α bioassays and the effects of soluble TNF-α receptors (sTNF-Rs) on TNF-α activity as well as the conditions of our sampling are potential explanations for [TNF-α] variability found herein. The present PTX dose (30 $mg \cdot kg^{-1} \cdot day^{-1}$) as well as administration route (i.p.) was chosen because of the successful results (i.e. reductions in RSNA, ROS, [TNF-α], and improved LV function) seen both centrally and peripherally by Guggilam et al. (2007; 2008). Although those authors administered PTX for a 5-week period, it is noteworthy that we were able to see major cardiovascular effects of the drug after our 21-day administration protocol.

Summary and Conclusion

The present study is the first to investigate the effects of PTX administration on skeletal muscle BF and VC at rest and during submaximal treadmill exercise in CHF rats. PTX administration (30 $mg \cdot kg^{-1} \cdot day^{-1}$ for 21 days) improved LV function and elevated skeletal muscle BF and VC in CHF rats. These improvements were associated with PTX-induced elevations in MAP (similar to values observed in healthy rats) and reductions in plasma [TNF-α] variance compared with CHF. Although the specific mechanisms responsible for the increases in skeletal muscle BF and VC remain unclear, the improvements in both cardiac and peripheral vasculature function have crucial implications for CHF patients.

Table 1. *Morphological and hemodynamic characteristics of CHF and CHF+PTX rats.*

	CHF	***CHF+PTX***
MI, %	37±4	37±3
LVEDP, mmHg	24±3	16±2*
RV/body mass, mg/g	0.77±0.08	0.72±0.04
Lung/body mass, mg/g	7.7±1.2	5.2±0.6*
LV dp/dt, mmHg/s	5663±502	6783±277*

MI, myocardial infarct size; LVEDP, left ventricular end diastolic pressure; LV dp/dt, left ventricular delta pressure/delta time. CHF: n=8; CHF+PTX: n=13 (LVEDP and LV dp/dt reflect n=12) *$p<0.05$ versus CHF rats.

Table 2**.** *Effects of PTX on resting blood flow (ml·min^{-1}·100 g^{-1}) and vascular conductance (VC, ml·min^{-1}·100 g^{-1}·mmHg^{-1}) to the individual muscles or muscle parts of the rat.*

	Blood flow		***VC***	
	CHF	***CHF+PTX***	***CHF***	***CHF+PTX***
Ankle extensors				
Soleus	72±14	111±19	0.72±0.17	0.92±0.17
Plantaris	13±4	11±2	0.12±0.04	0.09±0.01
Gastrocnemius, red	21±6	41±10	0.22±0.07	0.34±0.09
Gastrocnemius, white	9±2	9±1	0.08±0.02	0.07±0.01
Gastrocnemius, mixed	8±2	13±2	0.08±0.02	0.11±0.02
Tibialis posterior	11±2	14±2	0.11±0.02	0.12±0.01
Flexor digitorum longus	12±2	19±3	0.12±0.02	0.15±0.03
Flexor halicus longus	9±1	10±1	0.09±0.03	0.08±0.01
Ankle flexors				
Tibialis anterior, red	22±11	24±6	0.19±0.10	0.20±0.05
Tibialis anterior, white	13±5	14±2	0.12±0.05	0.11±0.01
Extensor digitorum longus	10±2	19±3	0.10±0.02	0.08±0.01
Peroneals	12±2	15±2	0.12±0.02	0.13±0.02
Knee extensors				
Vastus intermedius	46±11	82±15	0.47±0.12	0.66±0.11
Vastus medialis	12±3	17±2	0.12±0.03	0.14±0.02
Vastus lateralis, red	27±12	43±8	0.26±0.11	0.35±0.06
Vastus lateralis, white	10±3	10±1	0.10±0.03	0.08±0.01
Vastus lateralis, mixed	12±4	13±1	0.11±0.03	0.11±0.01
Rectus femoris, red	17±6	24±5	0.15±0.06	0.18±0.04
Rectus femoris, white	10±2	13±2	0.09±0.02	0.10±0.01
Knee flexors				
Biceps femoris anterior	6±1	8±1	0.06±0.01	0.06±0.01
Biceps femoris posterior	7±2	10±2	0.07±0.02	0.08±0.02
Semitendinosus	11±3	15±3	0.10±0.03	0.12±0.02
Semimembranosus, red	11±3	12±3	0.11±0.03	0.10±0.02
Semimembranosus, white	9±2	8±1	0.09±0.02	0.07±0.01
Hip adductors				
Adductor longus	106±17	135±12	0.99±0.17	1.09±0.10
Adductor magnus & brevis	11±3	12±2	0.10±0.03	0.10±0.02
Gracilis	11±3	16±3	0.11±0.03	0.13±0.03
Pectinius	18±4	25±3	0.15±0.04	0.20±0.03

Data are mean±SEM. CHF: n=8; CHF+PTX: n=13.

Table 3. *Exercising individual hindlimb skeletal muscle and muscle part blood flow (ml·min^{-1}·100g^{-1}) and VC (ml·min^{-1}·100g^{-1}·mmHg) for CHF and CHF+PTX groups.*

	Blood flow		***VC***	
	CHF	***CHF+PTX***	***CHF***	***CHF+PTX***
Ankle extensors				
Soleus	257±25	258±20	2.33±0.24	2.00±0.15
Plantaris	148±23	209±17*	1.36±0.21	1.62±0.12
Gastrocnemius, red	298±22	361±56	2.69±0.20	2.79±0.41
Gastrocnemius, white	36±10	45±4	0.36±0.12	0.35±0.03
Gastrocnemius, mixed	118±10	156±14*	1.08±0.11	2.21±0.10
Tibialis posterior	95±17	114±19	0.89±0.19	0.89±0.14
Flexor digitorum longus	54±17	108±19*	0.54±0.19	0.85±0.15*
Flexor halicus longus	50±10	88±7*	0.47±0.10	0.68±0.05*
Ankle flexors				
Tibialis anterior, red	251±29	304±22*	2.27±0.27	2.36±0.17
Tibialis anterior, white	81±12	112±7*	0.72±0.08	0.87±0.05*
Extensor digitorum longus	38±6	62±6*	0.36±0.06	0.48±0.05*
Peroneals	101±11	141±15*	0.91±0.09	1.09±0.11
Knee extensors				
Vastus intermedius	301±23	353±25*	2.71±0.22	2.76±0.21
Vastus medialis	138±20	182±15*	1.24±0.19	1.41±0.11
Vastus lateralis, red	238±32	361±29*	2.13±0.26	2.80±0.23*
Vastus lateralis, white	13±3	23±2*	0.12±0.03	0.18±0.02*
Vastus lateralis, mixed	116±12	141±12*	1.04±0.12	1.10±0.11
Rectus femoris, red	220±24	247±18	1.96±0.20	1.93±0.14
Rectus femoris, white	91±10	98±6	0.82±0.09	0.78±0.05
Knee flexors				
Biceps femoris, anterior	15±3	28±4*	0.14±0.03	0.22±0.03*
Biceps femoris, posterior	67±9	83±7*	0.60±0.08	0.65±0.05
Semitendinosus	33±6	60±5*	0.30±0.05	0.47±0.04*
Semimembranosus, red	92±16	126±16*	0.84±0.15	0.98±0.12
Semimembranosus, white	16±3	32±5*	0.14±0.03	0.25±0.04*
Hip adductors				
Adductor longus	254±40	297±27	2.31±0.36	2.29±0.19
Adductor magnus & brevis	64±13	92±11*	0.57±0.11	0.71±0.08
Gracilis	21±5	57±8*	0.15±0.05	0.45±0.06*
Pectinius	27±7	59±9*	0.25±0.07	0.46±0.07*

(Table 3 caption)

Data are mean±SEM. CHF: n=8; CHF+PTX: n=13. *$p<0.05$ versus CHF.

Within CHF 24, and within CHF+PTX 28, of 28 hindlimb muscles and muscle parts demonstrated elevated exercising blood flow and VC above rest ($p<0.05$) (exceptions: gracilis, pectinius, white portions of the semimembranosus and vastus lateralis for CHF group)

Table 4**.** *Renal and splanchnic organ blood flow (ml·min^{-1}·100g^{-1}) and VC (ml·min^{-1}·100g^{-1}·mmHg) at rest and during exercise for CHF and CHF+PTX groups.*

	At rest				***During exercise***			
	Blood flow		***VC***		***Blood flow***		***VC***	
	CHF	***CHF+PTX***	***CHF***	***CHF+PTX***	***CHF***	***CHF+PTX***	***CHF***	***CHF+PTX***
Right kidney	542±30	576±38	5.26±0.36	4.68±0.33	379±56	350±48	2.87±0.42	2.67±0.35
Left kidney	534±32	556±33	5.19±0.35	4.53±0.30	373±61	341±48	2.72±0.42	2.59±0.34
Stomach	108±18	139±20	0.89±0.16	1.14±0.18	53±12	61±9	0.35±0.08	0.46±0.06
Adrenals	431±59	683±62*	4.04±0.72	5.52±0.51*	267±52	388±50	1.91±0.36	2.97±0.36
Spleen	254±46	398±51*	2.09±0.23	3.19±0.39*	62±16	78±18	0.43±0.13	0.59±0.13
Pancreas	139±21	166±25	1.19±0.19	1.35±0.19	93±26	113±23	0.75±0.18	0.86±0.16
Sm. intestine	377±60	460±41	3.11±0.57	3.77±0.37	220±42	238±24	1.50±0.30	1.83±0.17
Lg. intestine	168±25	223±24*	1.41±0.16	1.82±0.20	112±31	120±19	0.75±0.20	0.92±0.14
Liver[1]	16±3	27±7	0.16±0.04	0.22±0.05	14±3	25±5	0.10±0.02	0.19±0.04

Data are mean±SEM. [1]Denotes arterial not portal blood flow. CHF: n=8; CHF+PTX: n=13. *p<0.05 versus CHF.

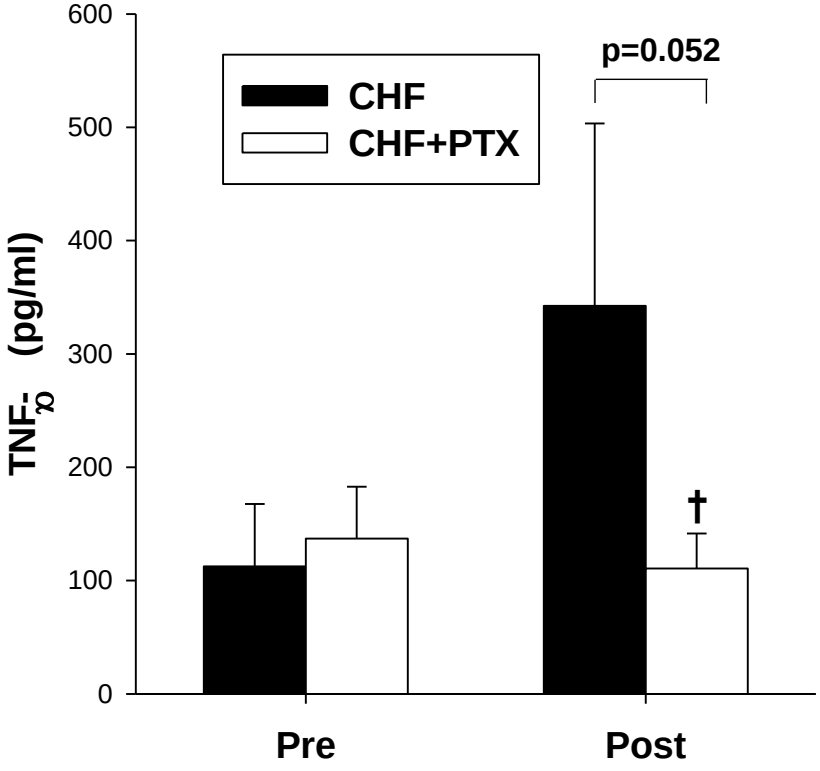

Figure 1. Effects of PTX administration on plasma [TNF-α] pre and post PTX administration. CHF: n=8; CHF+PTX: n=12. Data are mean±SEM. Absolute difference in post intervention plasma [TNF-α] did not reach statistical significance (p=0.052). † F-test revealed a significant reduction in [TNF-α] variance in CHF+PTX rats.

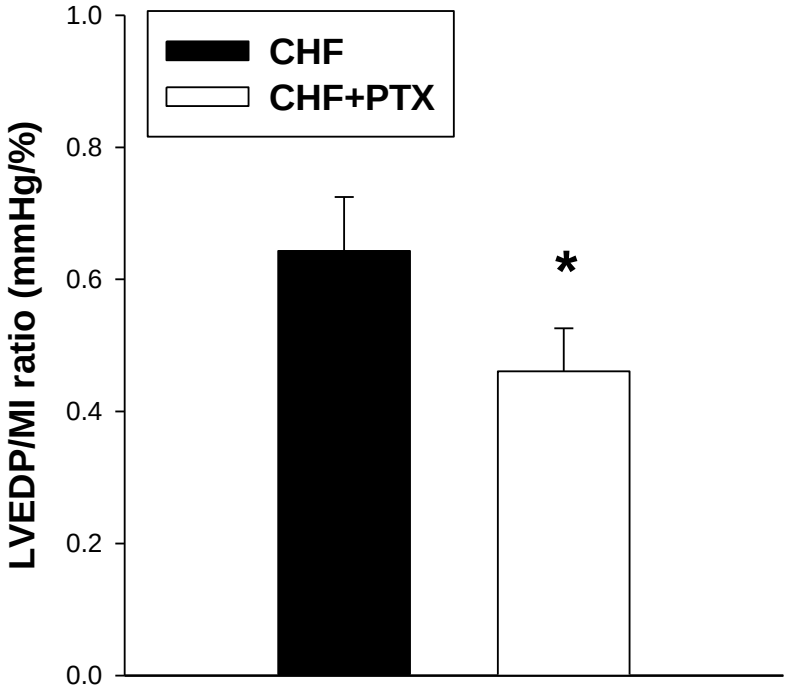

Figure 2. Effects of PTX administration on LVEDP/MI ratio. CHF: n=8; CHF+PTX n=12. Data are mean±SEM. *p<0.05 versus CHF.

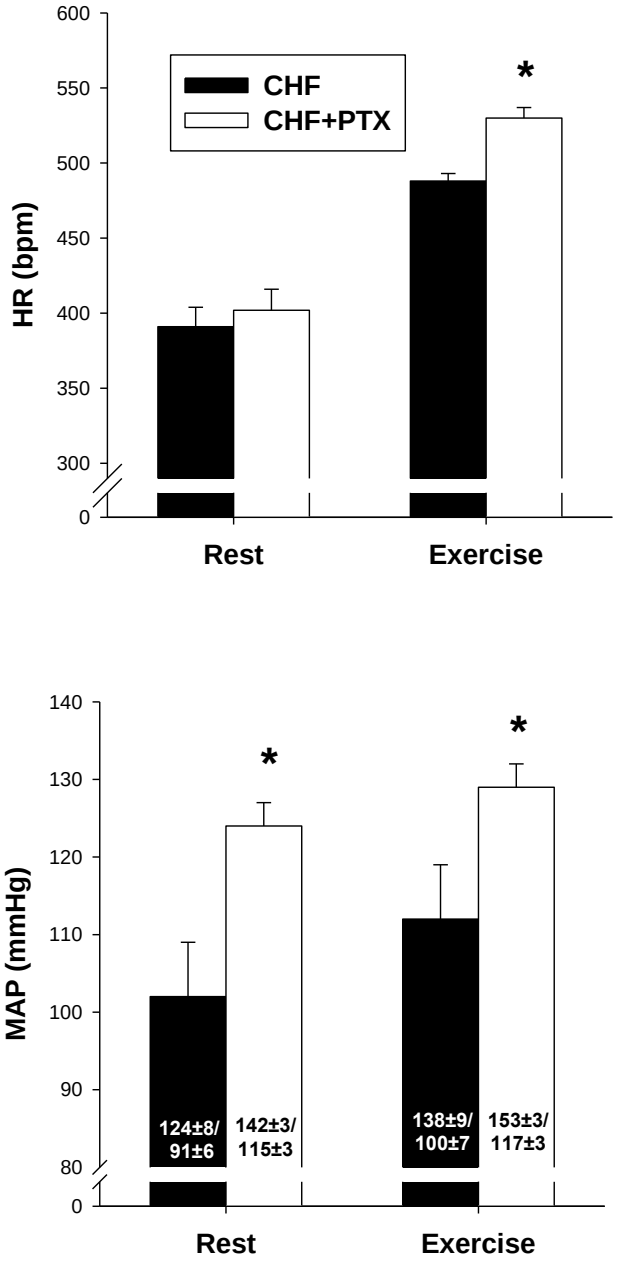

Figure 3. Effects of PTX administration on MAP and HR. MAP and HR measured at rest and during submaximal treadmill exercise. Within CHF and CHF+PTX conditions exercise MAP and HR were significantly different from rest ($p<0.05$). Systolic/diastolic pressure is depicted within MAP bars and are all significantly different between CHF and CHF+PTX groups. CHF: n=8; CHF+PTX: n=13. Data are mean±SEM. *$p<0.05$ versus CHF.

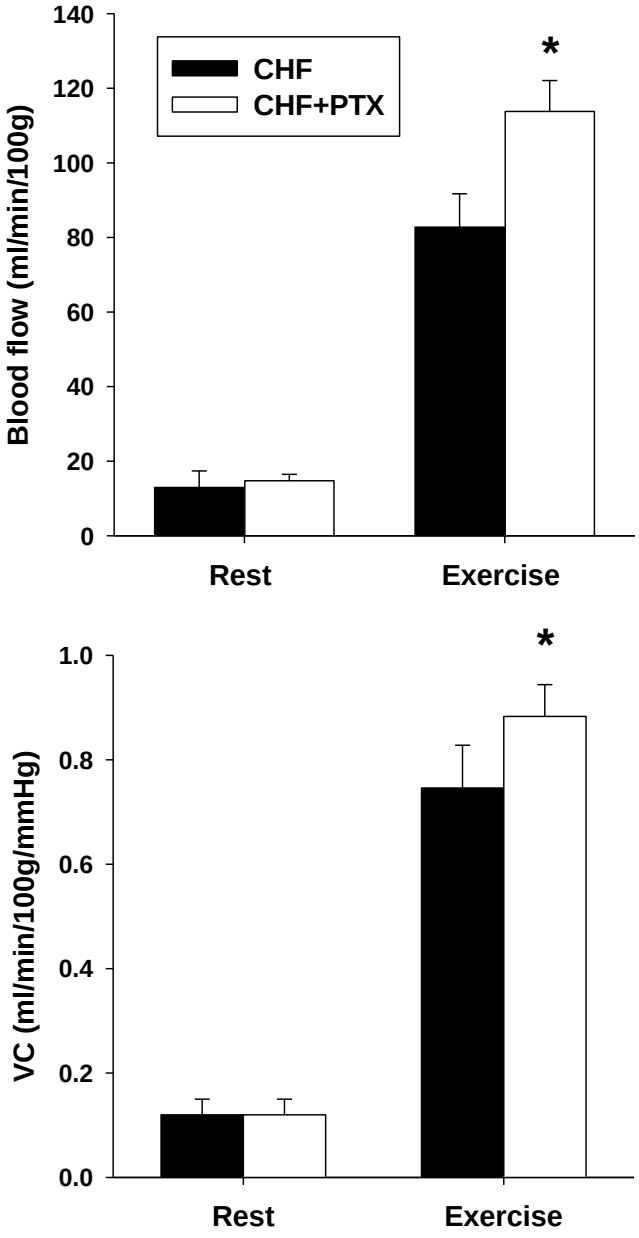

Figure 4. Total hindlimb muscle BF and VC at rest and during exercise for CHF and CHF+PTX rats. Within CHF and CHF+PTX groups exercising BF and VC were significantly different ($p<0.05$) from rest. CHF: n=8; CHF+PTX: n=13. Data are mean±SEM. *$p<0.05$ versus CHF.

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