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Effects of chronic heart failure on neuronal nitric oxide synthase-mediated control of microvascular O_2 pressure in contracting rat skeletal muscle

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Key points

- Nitric oxide (NO) is an important vasodilatory signalling molecule that regulates O_2 pressure within the skeletal muscle microvasculature (P_{O_2mv}). In healthy subjects, NO is derived from two principal NO synthase (NOS) isoforms: neuronal NOS (nNOS) and endothelial NOS (eNOS).
- Chronic heart failure (CHF) results in peripheral vascular dysfunction that is attributed, in part, to impaired NO function. This NO-mediated impairment is attributed generally to eNOS dysfunction. It is unknown if nNOS-mediated regulation of P_{O_2mv} function is impaired in CHF.
- Our present results demonstrate that skeletal muscle blood flow reductions and P_{O_2mv} alterations during contractions observed following nNOS inhibition in healthy rats are markedly attenuated or absent in CHF rats, which is indicative of impaired nNOS function.
- Identification of the mechanisms underlying impaired microvascular function in CHF is an important step in the development of treatments designed to improve CHF-induced skeletal muscle microvascular pathology.

Abstract Chronic heart failure (CHF) impairs nitric oxide (NO)-mediated regulation of the skeletal muscle microvascular O_2 delivery/$\dot{V}_{O_2}$ ratio (which sets the microvascular O_2 pressure, P_{O_2mv}). Given the pervasiveness of endothelial dysfunction in CHF, this NO-mediated dysregulation is attributed generally to eNOS. It is unknown whether nNOS-mediated P_{O_2mv} regulation is altered in CHF. We tested the hypothesis that CHF impairs nNOS-mediated P_{O_2mv} control. In healthy and CHF (left ventricular end diastolic pressure (LVEDP): 6 ± 1 *versus* 14 ± 1 mmHg, respectively, $P < 0.05$) rats spinotrapezius muscle blood flow (radiolabelled microspheres), P_{O_2mv} (phosphorescence quenching), and $\dot{V}_{O_2}$ (Fick calculation) were measured before and after 0.56 mg kg^{-1} I.A. of the selective nNOS inhibitor *S*-methyl-L-thiocitrulline (SMTC). In healthy rats, SMTC increased baseline P_{O_2mv} (Control: 29.7 ± 1.4, SMTC: 34.4 ± 1.9 mmHg, $P < 0.05$) by reducing $\dot{V}_{O_2}$ ($\downarrow$20%) without any effect on blood flow and speeded the mean response time (MRT, time to reach 63% of the overall kinetics response, Control: 24.2 ± 2.0, SMTC: 18.5 ± 1.3 s, $P < 0.05$). In CHF rats, SMTC did not alter baseline P_{O_2mv} (Control: 25.7 ± 1.6, SMTC: 28.6 ± 2.1 mmHg, $P > 0.05$), $\dot{V}_{O_2}$ at rest, or the MRT (Control: 22.8 ± 2.6, SMTC: 21.3 ± 3.0 s, $P > 0.05$). During the contracting steady-state, SMTC reduced blood flow ($\downarrow$15%) and $\dot{V}_{O_2}$ ($\downarrow$15%) in healthy rats such that P_{O_2mv} was unaltered (Control: 19.8 ± 1.7, SMTC: 20.7 ± 1.8 mmHg, $P > 0.05$). In marked contrast, in CHF rats SMTC did not change contracting steady-state blood flow, $\dot{V}_{O_2}$, or P_{O_2mv} (Control: 17.0 ± 1.4, SMTC: 17.7 ± 1.8 mmHg, $P > 0.05$). nNOS-mediated control of skeletal muscle microvascular function is compromised in CHF *versus*

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healthy rats. Treatments designed to ameliorate microvascular dysfunction in CHF may benefit by targeting improvements in nNOS function.

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Abbreviations: ACh, acetylcholine; CHF, chronic heart failure; eNOS, endothelial nitric oxide synthase; HR, heart rate; iNOS, inducible nitric oxide synthase; LV, left ventricle; LV dp/dt, left ventricular change in pressure over change in time; LVEDP, left ventricular end diastolic pressure; MAP, mean arterial pressure; MRT, mean response time; L-NAME, N^G-nitro-L-arginine-methyl-ester; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; O_2, oxygen; P_{O_2mv}, microvascular partial pressure of oxygen; RV, right ventricle; SMTC, *S*-methyl-L-thiocitrulline; TD, time delay; VC, vascular conductance; $\dot{V}_{O_2}$, oxygen consumption.

Introduction

The ability of the skeletal muscle microvasculature to modulate O_2 delivery relative to O_2 demand both spatially and temporally during contractions is a fundamental feature of integrated cardiorespiratory function. In healthy subjects, the skeletal muscle microvascular O_2 delivery/$\dot{V}_{O_2}$ balance (which sets the P_{O_2mv} and represents the pressure head driving blood-myocyte O_2 flux according to Fick's law of diffusion) at rest and during contractions is dependent critically upon adequate NO bioavailability (Ferreira *et al.* 2006*b*; Hirai *et al.* 2010) derived via constitutively expressed neuronal (Type I) and endothelial (Type III) NOS isoforms. The contribution of inducible NOS (iNOS, Type II) in young healthy individuals is expected to be minimal.

CHF is a clinical syndrome marked by perturbations in peripheral vascular (Longhurst *et al.* 1976) and metabolic (Massie *et al.* 1987) regulation which contribute to compromised P_{O_2mv} control (Diederich *et al.* 2002; Copp *et al.* 2010*a*) and exercise intolerance (Nicoletti *et al.* 2003; Poole *et al.* 2012). Impairments in NO-mediated function explain, at least in part, CHF-induced reductions in bulk contracting muscle O_2 delivery (Katz *et al.* 1996) as well as derangements in peripheral skeletal muscle spatial (Hirai *et al.* 1995) and temporal (Ferreira *et al.* 2006*a*) O_2 delivery relative to O_2 demand. For example, non-selective NOS inhibition with L-NAME lowers contracting skeletal muscle P_{O_2mv} to a greater extent in healthy *versus* CHF rats revealing reduced NO bioavailability and impaired NO-mediated microvascular function in CHF (Ferreira *et al.* 2006*a*). Given the pervasive reports of compromised endothelial function in CHF subjects (Kaiser *et al.* 1989; Katz *et al.* 1992), the tacit presumption has been that those reductions in NO bioavailability reflect principally eNOS dysfunction. Recently, however, selective nNOS inhibition with *S*-methyl-L-thiocitrulline (SMTC) (Furfine *et al.* 1994; Wakefield *et al.* 2003) reduced healthy rat skeletal muscle blood flow and $\dot{V}_{O_2}$ revealing a novel role for nNOS-derived NO specifically in the regulation of P_{O_2mv} at rest and during contractions (Copp *et al.* 2011). Whether this nNOS-derived NO regulation of P_{O_2mv} is altered in CHF is unknown. However, Thomas *et al.* (2001) reported that the attenuation of sympathetic vasoconstrictor activity in contracting hindlimb muscles is impaired (i.e. less sympathetic vasoconstrictor amelioration is present) in CHF *versus* healthy rats. Considering that this 'functional sympatholysis' emanates predominately from nNOS-derived NO (Thomas *et al.* 2001), indirect evidence supports that nNOS function is reduced in CHF and may therefore contribute to the impaired NO-mediated microvascular function, although this conclusion has not been tested specifically. Clouding this issue are inconsistent reports that nNOS protein expression may be increased (Rush *et al.* 2005) or unchanged (Thomas *et al.* 2001; Rush *et al.* 2005) in CHF rats and humans compared to healthy counterparts. Moreover, the possibility of CHF-induced NOS uncoupling (reviewed by Munzel *et al.* 2005) signifies that altered nNOS protein levels in CHF may not necessarily match changes (or the lack thereof) in nNOS expression. Therefore, direct assessment of the presence and extent of compromised nNOS-mediated P_{O_2mv} regulation in CHF is warranted. This information is critical considering that elucidation of the mechanisms responsible for impaired O_2 transport and $\dot{V}_{O_2}$ in CHF may lead to new therapeutic treatment modalities.

The purpose of the present investigation is to determine if the nNOS-derived NO control of skeletal muscle blood flow, $\dot{V}_{O_2}$, and the dynamic blood flow/$\dot{V}_{O_2}$ ratio (i.e. P_{O_2mv}) during contractions is compromised in CHF *versus* healthy rats. We tested the hypothesis that CHF rats would demonstrate blunted or absent P_{O_2mv} alterations following nNOS inhibition with SMTC when compared to healthy rats.

Methods

Ethical approval

All experimental protocols described herein were approved by the Institutional Animal Care and Use

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Committee (IACUC) of Kansas State University and conducted in agreement with the guidelines established by the National Institutes of Health and *The Journal of Physiology* (Drummond, 2009). Novel experiments in healthy rats ($n = 6$, 4 P_{O_2mv}/blood flow and 2 force production/ACh) were added to previously reported data from healthy animals ($n = 15$, Copp *et al.* 2011) to give the present healthy rat data. This strategy was required to cohere with the IACUC mandate to avoid unnecessary animal killing.

Animals

A total of 40 male Sprague–Dawley rats (~6 months old, Charles River Laboratories, Boston, MA, USA) were used in the present investigation. All rats were housed in accredited facilities and maintained on a 12:12 h light–dark cycle with food and water available *ad libitum* for the duration of the experimental protocol.

Surgical procedures

Myocardial infarction was induced in CHF rats via left main coronary artery ligation (Musch & Terrell, 1992). To begin, CHF rats were anaesthetized with a 5% isoflurane (Butler Animal Health Supply, Elk Grove Village, IL, USA)–95% O_2 (Linweld, Inc., Dallas, TX, USA) mixture and intubated for mechanical ventilation with a rodent respirator (Harvard Model 680; Harvard Instruments, Holliston, MA, USA) for the duration 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^{-1} I.M.) was injected locally to reduce opportunity for infection. The analgesic agents bupivacaine (1.5 mg kg^{-1} subcutaneously) and buprenorphine (0.01–0.05 mg kg^{-1} I.M.) were administered subsequently and anaesthesia and mechanical ventilation discontinued. All rats were monitored closely for ≥6 h for arrhythmia development and signs of undue distress (i.e. laboured breathing, etc.) with care administered as appropriate. Rats were also monitored daily (i.e. appetite, weight loss/gain, gait/posture, etc.) according to an intensive 10 day post-operative plan conducted in conjunction with the university veterinary staff.

The final experimental protocol was initiated ≥21 days after surgery. Healthy rats were not subjected to a sham surgical procedure given there are no differences in cardiovascular responses between sham operated and non-sham operated healthy animals (Symons *et al.* 1999). Rats were anaesthetized initially with a 5% isoflurane–O_2 mixture and maintained at 2–3% isoflurane. The carotid artery was cannulated and a two-French-catheter-tipped pressure transducer (Millar Instruments, Houston, TX, USA) was advanced into the LV for measurement of systolic and diastolic pressures and left ventricular delta pressure/delta time (LV dp/dt). 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, Benton, Dickson and Company, Sparks, MD, USA) for measurement of MAP, infusion of the phosphorescent probe, and arterial blood sampling. A catheter (PE-50) was also placed in the caudal (tail) artery. Rats were then transitioned progressively to pentobarbital sodium (administered I.A. to effect), monitored continuously via the toe-pinch and blink reflexes with anaesthesia supplemented as necessary, and placed on a heating pad to maintain core temperature at 38°C (measured via rectal probe). Overlying skin and fascia were then reflected carefully from the mid-dorsal–caudal region of each rat and the right spinotrapezius muscle was exposed in a manner which ensured the integrity of the vascular and neural supply to the muscle (Bailey *et al.* 2000). Silver wire electrodes were then sutured (6–0 silk) to the rostral (cathode) and caudal (anode) regions of the muscle. The exposed spinotrapezius muscle was continuously superfused with a warmed (38°C) Krebs–Henseleit bicarbonate buffered solution equilibrated with 5% CO_2–95% N_2 and surrounding exposed tissue was covered with Saran wrap (Dow Brands, Indianapolis, IN, USA).

Experimental protocol

In 14 (461 ± 25 g) healthy and 12 (528 ± 17 g, $P < 0.05$) CHF rats, the phosphorescent probe palladium meso-tetra(4-carboxyphenyl)porphyrin dendrimer (R2, Oxygen Enterprises, Philadelphia, PA, USA: 15–20 mg kg^{-1} dissolved in saline) was infused via the carotid artery catheter. After a ~15 min stabilization period, the carotid artery catheter was connected to a pressure transducer for continuous monitoring of HR and MAP and P_{O_2mv} measurements (see below) were initiated. Subsequently, 1.2 ml of heparinized saline was infused at a rate of 0.2 ml min^{-1} into the caudal artery catheter to serve as a time and volume control infusion. Following the infusion the caudal artery catheter was connected to a 1 ml plastic syringe and placed in a withdrawl pump (model 907, Harvard Apparatus, Cambridge, MA, USA). Twitch contractions of 1 Hz (~6–8 V, 2 ms pulse duration) were then initiated via a stimulator (model S88, Grass Instrument Co., Quincy, MA, USA). At 180 s of contractions blood withdrawal from the tail catheter was initiated at a rate of 0.25 ml min^{-1}, the carotid catheter was disconnected from the pressure transducer, and ~0.5–0.6 × 10^6 radiolabelled microspheres (15 μm in

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diameter, ^{46}Sc or ^{85}Sr in random order, Perkin Elmer Life and Analytical Sciences, Boston, MA, USA) were infused rapidly into the aortic arch for determination of blood flow in accordance with the reference sample method (Ishise *et al.* 1980, see below for details), and contractions were then terminated. Following a minimum 20 min recovery period, 0.56 mg kg^{-1} (2.1 μmol kg^{-1}) of SMTC was dissolved in 1.2 ml of heparinized saline and infused as described above. Following the infusion and once it was observed that P_{O_2mv} was stable for at least 30 s, contraction and microsphere injection protocols were performed as described for control. Blood samples were taken after each contraction protocol to determine arterial [O_2] and blood [lactate]. Following the experimental protocol rats were killed via an intra-arterial pentobarbital overdose (~50 mg kg^{-1}). For each rat, the lungs and heart were carefully removed and dissected. The lungs and right ventricle (RV) were weighed and normalized to body mass. For CHF rats, infarct size was determined via planimetry as described previously (Ferreira *et al.* 2006*a*).

Measurement of P_{O_2mv} and curve-fitting

The Stern–Volmer relationship allows the calculation of P_{O_2mv} through the direct measurement of a phosphorescence lifetime via the following equation (Rumsey *et al.* 1988):

$$P_{O_2mv} = [(\tau^\circ/\tau) - 1]/(k_Q \times \tau^\circ)$$

where k_Q is the quenching constant (expressed in mmHg^{-1} s^{-1}) and τ° and τ are the phosphorescence lifetimes in the absence of O_2 and the ambient O_2 concentration, respectively. For R2, k_Q is 409 mmHg^{-1} s^{-1} and τ° is 601 μs (Lo *et al.* 1997) and these characteristics do not change appreciably over the physiological range of pH and temperature in the rat *in vivo* herein and, therefore, the phosphorescence lifetime is determined directly by the O_2 pressure (Rumsey *et al.* 1988; Lo *et al.* 1997).

The R2 phosphorescent probe binds to albumin, and consequently is uniformly distributed throughout the plasma. A previous study from our laboratory investigated systematically the compartmentalization of R2 and confirmed that it remains within the microvasculature of exposed muscle over the duration considered in the present experiments, thereby ensuring a valid measurement of P_{O_2mv} (Poole *et al.* 2004). The P_{O_2mv} was determined with a PMOD 5000 Frequency Domain Phosphorometer (Oxygen Enterprises, Philadelphia, PA, USA). The common end of the light guide was placed ~2–4 mm superficial to the dorsal surface of the exposed right spinotrapezius muscle. The randomly selected muscle field was composed principally of capillary blood and P_{O_2mv} was measured continuously and recorded at 2 s intervals throughout the duration of the contraction periods.

For the measured P_{O_2mv} responses, curve-fitting was performed with commercially available software (SigmaPlot 11.2, Systat Software, San Jose, CA, USA) and the data were fit with either a one- or two-component model as described below:

$$\text{One component}: P_{O_2mv(t)} = P_{O_2mv(BL)} - \Delta P_{O_2mv}(1 - e^{-(t-TD)/\tau})$$

$$\text{Two component}: P_{O_2mv(t)} = P_{O_2mv(BL)} - \Delta_1 P_{O_2mv}(1 - e^{-(t-TD_1)/\tau_1}) + \Delta_2 P_{O_2mv}(1 - e^{-(t-TD_2)/\tau_2})$$

where $P_{O_2mv(t)}$ represents the P_{O_2mv} at any given time t, $P_{O_2mv(BL)}$ corresponds to the pre-contracting resting P_{O_2mv}, Δ_1 and Δ_2 are the amplitudes for the first and second component, respectively, TD_1 and TD_2 are the time delays for each component, and τ_1 and τ_2 are the time constants (i.e. time to 63% of the final response value) for each component. Goodness of fit was determined using the following criteria: (1) the coefficient of determination, (2) sum of the squared residuals, and (3) visual inspection and analysis of the model fits to the data and the residuals. The MRT of the kinetics response was calculated for the first component in order to provide an index of the overall principal kinetics response according to the following equation:

$$MRT = TD_1 + \tau^1$$

where TD_1 and τ^1 are as described above. The delta of the initial P_{O_2mv} fall following contractions onset was normalized to τ_1 ($\Delta_1 P_{O_2mv}/\tau_1$) to provide an index of the relative rate of fall.

Determination of muscle blood flow and calculation of $\dot{V}_{O_2}$

Following killing, the right and left spinotrapezius muscles and kidneys were carefully dissected, removed from each rat and weighed. The radioactivity of the spinotrapezius muscles and reference blood samples taken from the tail artery catheter during microsphere infusions were determined via a gamma scintillation counter (Auto Gamma Spectrometer, Cobra model 5003, Hewlett-Packard, Downers Grove, IL, USA) and individual tissue blood flows were expressed in ml min^{-1} (100 g tissue)$^{-1}$ (Ishise *et al.* 1980). In each condition (control and SMTC) the stimulated right, and non-stimulated left, spinotrapezius muscles represented the contracting and resting blood flow measurements, respectively. Vascular conductance (VC) was determined as the ratio of blood flow to MAP using the MAP measured

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immediately prior to each microsphere injection. All rats exhibited <15% difference in blood flow between the right and left kidneys which indicated an adequate mixing of microspheres during the blood flow determinations.

$\dot{V}_{O_2}$ was calculated from blood flow and P_{O_2mv} measurements. Arterial O_2 concentration (C_{a,O_2}) was calculated directly from arterial blood samples and venous muscle effluent blood O_2 concentration (C_{v,O_2}) was approximated from either the baseline (rest) or the contracting steady-state P_{O_2mv} using the rat dissociation curve (Hill's coefficient of 2.6), the measured haemoglobin concentration ([Hb]), a P_{50} of 38 mmHg, and an O_2 carrying capacity of 1.34 ml O_2 (g Hb)$^{-1}$ (Altman & Dittmer, 1974). The measures of the resting and contracting spinotrapezius blood flows were then used to determine $\dot{V}_{O_2}$ via the direct Fick calculation (i.e. $\dot{V}_{O_2}$ = blood flow × ($C_{a,O_2} - C_{v,O_2}$)).

Measurement of muscle force production

Due to differences in surgical preparation required for muscle force and P_{O_2mv} measurement, additional groups of rats (healthy: $n = 7$, 469 ± 10 g; CHF: $n = 7$, 475 ± 26 g, $P > 0.05$) were utilized to determine the effects of CHF on nNOS-mediated control of skeletal muscle force production. In these animals, the caudal end of the exposed spinotrapezius muscle was exteriorized and sutured to a thin, wire horseshoe manifold attached to a swivel apparatus and a non-distensible light-weight (0.4 g) cable which linked the muscle to a force transducer (model FTO3, Grass Instrument Co., Quincy, MA, USA). Rats were placed on a custom-made stabilization platform and the pre-load muscle tension was set to ∼4 g which elicited the muscle's optimum length for twitch force production. Muscle force production was measured throughout control and SMTC contraction bouts as described for the measurement of P_{O_2mv}. Rats were then killed as described above. A previous report from our laboratory indicates that P_{O_2mv} and blood flow do not differ between exposed and exteriorized spinotrapezius muscle (Bailey *et al.* 2000).

Acetylcholine infusions

In order to examine the efficacy of selective nNOS inhibition in the presence of SMTC, rapid ACh infusions (10 μg kg^{-1} in 0.2 ml of saline) were performed in subsets of CHF ($n = 6$) and healthy rats ($n = 7$) under control and SMTC conditions as well as following non-selective NOS inhibition with 10 mg kg^{-1} of L-NAME administered into the caudal artery. ACh was infused rapidly into the caudal artery catheter while MAP was simultaneously recorded via the carotid artery catheter. The time to 50% of the recovery from the hypotensive MAP response to ACh was measured and recorded. Analysis of haemodynamic responses to ACh infusion has been employed previously to examine the efficacy of selective nNOS inhibition with SMTC in both animals (Wakefield *et al.* 2003; Copp *et al.* 2010*b*, 2011) and humans (Seddon *et al.* 2008, 2009).

Table 1. Morphological and haemodynamic characteristics of healthy and CHF rats

	Healthy	CHF
LVEDP (mmHg)	6 ± 1	14 ± 1*
RV/body mass (mg g^{-1})	0.61 ± 0.03	0.68 ± 0.03*
Lung/body mass (mg g^{-1})	3.56 ± 0.14	4.38 ± 0.30
LV d*p*/d*t* (mmHg s^{-1})	8109 ± 168	7113 ± 163*
Infarct size (%)	—	27.9 ± 1.6%

LVEDP, left ventricular end diastolic pressure; RV, right ventricle; LV d*p*/d*t*, left ventricular dpressure/dtime. *$P < 0.05$ *versus* healthy rats.

Statistical analyses

Data for primary study endpoints were compared within (control *vs.* SMTC) and among (healthy *vs.* CHF) groups by mixed ANOVAs with the Student–Newman–Keuls *post hoc* test when significant interactions were indicated. Other comparisons were performed via paired or unpaired Student's *t* tests where appropriate. The *z* test was performed to test when variable changes following SMTC were different from zero. Significance was accepted at $P < 0.05$.

Results

Efficacy of CHF induction

The haemodynamic and morphological indices of CHF are presented in Table 1 and indicate that CHF rats had moderate compensated CHF (Diederich *et al.* 2002).

Effects of CHF on nNOS-mediated blood [lactate] and central haemodynamic regulation

SMTC did not change blood [lactate] in healthy (control: 1.0 ± 0.1, SMTC: 1.0 ± 0.1 mmol l^{-1}, $P > 0.05$) or CHF rats (control: 1.0 ± 0.2, SMTC: 1.1 ± 0.1 mmol l^{-1}, $P > 0.05$). In healthy and CHF rats SMTC elevated MAP and reduced HR compared to control at rest and during contractions steady-state (Table 2). The SMTC-induced MAP elevations (ΔMAP) were significantly attenuated in CHF *versus* healthy rats at rest and during contractions ($P < 0.05$ for both).

In healthy and CHF rats the time to 50% recovery from the hypotensive response to rapid ACh infusions was not

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Table 2. Effects of selective nNOS inhibition with SMTC on HR and MAP in healthy and CHF rats

	Healthy			CHF		
	Control	SMTC	Δ	Control	SMTC	Δ
Rest						
HR (bpm)	351 ± 9	319 ± 8*	−32 ± 8	371 ± 8	353 ± 8*†	−18 ± 9
MAP (mmHg)	111 ± 4	131 ± 5*	19 ± 3	124 ± 4†	134 ± 5*	11 ± 3†
Contractions (steady-state)						
HR (bpm)	352 ± 9	323 ± 9*	−30 ± 7	372 ± 9	354 ± 7*†	−18 ± 9
MAP (mmHg)	115 ± 4	133 ± 5*	18 ± 2	124 ± 4	132 ± 6*	10 ± 2†

Values are means ± SEM, *$P < 0.05$ *versus* control, †$P < 0.05$ *versus* healthy.

different between control and SMTC conditions whereas it was reduced following L-NAME (Fig. 1).

Effects CHF on nNOS-mediated P_{O_2mv}, blood flow and $\dot{V}_{O_2}$ regulation

In healthy rats, SMTC increased baseline P_{O_2mv} (Fig. 2, Table 3) consequent to a 20% reduction in resting $\dot{V}_{O_2}$ (Fig. 3) with no effect on resting blood flow (Fig. 4). In CHF rats, SMTC did not alter resting baseline P_{O_2mv}, blood flow, or $\dot{V}_{O_2}$ ($P > 0.05$ for all). Following onset of contractions, SMTC resulted in a shorter TD_1 and faster MRT and $\Delta P_{O_2mv}/\tau_1$ in healthy rats whereas only a shorter TD_1 following SMTC was found in CHF rats. During the contracting steady-state, SMTC reduced blood flow (↓15%) and $\dot{V}_{O_2}$ (↓15%) in healthy rats such that P_{O_2mv} and Δ blood flow/$\dot{V}_{O_2}$ ratio were unaltered (Fig. 5). SMTC did not change contracting steady-state blood flow, $\dot{V}_{O_2}$, Δ blood flow/$\dot{V}_{O_2}$, or P_{O_2mv} in CHF rats.

In healthy rats, SMTC did not change spinotrapezius muscle VC at rest (control: 0.13 ± 0.03, SMTC: 0.09 ± 0.01 ml min^{-1} (100 g)$^{-1}$ mmHg^{-1}, $P > 0.05$) whereas it was reduced significantly following SMTC during contractions (control: 0.84 ± 0.13, SMTC: 0.59 ± 0.07 ml min^{-1} (100 g)$^{-1}$ mmHg^{-1}, $P < 0.05$). In contrast in CHF rats, SMTC did not alter VC at rest (control: 0.06 ± 0.01, SMTC: 0.06 ± 0.01 ml min^{-1} (100 g)$^{-1}$ mmHg^{-1}, $P > 0.05$) or during contractions (control: 0.48 ± 0.06, SMTC: 0.44 ± 0.05 ml min^{-1} (100 g)$^{-1}$ mmHg^{-1}, $P > 0.05$).

Kidney blood flow was reduced significantly following SMTC in both healthy (control: 498 ± 38, SMTC: 315 ± 30 ml min^{-1} (100 g)$^{-1}$, $P < 0.05$) and CHF (control: 437 ± 23, SMTC: 343 ± 23 ml min^{-1} (100 g)$^{-1}$, $P < 0.05$) rats. Similarly, SMTC reduced significantly kidney VC in healthy (control: 4.57 ± 0.40, SMTC: 2.45 ± 0.24 ml min^{-1} (100 g)$^{-1}$ mmHg^{-1}, $P < 0.05$) and CHF (control: 3.54 ± 0.19, SMTC: 2.57 ± 0.17 ml min^{-1} (100 g)$^{-1}$ mmHg^{-1}, $P < 0.05$) rats. However, the ΔVC following SMTC was significantly greater in healthy (-2.12 ± 0.43 ml min^{-1} (100 g)$^{-1}$ mmHg^{-1}) compared to CHF rats (-0.97 ± 0.29 ml min^{-1} (100 g)$^{-1}$ mmHg^{-1}, $P < 0.05$ *versus* healthy).

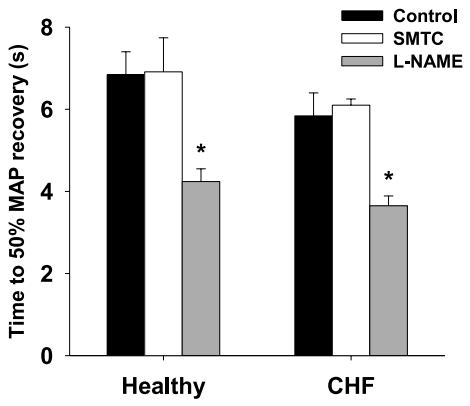

Figure 1. Effects of SMTC and L-NAME on the 50% recovery time from the hypotensive effects of rapid ACH infusions
Data are means ± SEM. *$P < 0.05$ *versus* control and SMTC for healthy ($n = 7$) and CHF ($n = 6$) rats.

Effects of CHF on nNOS-mediated regulation of muscle contractile function

SMTC had no effect on muscle force production at any individual time point in healthy or CHF animals ($P > 0.05$ for all). However, in healthy rats, SMTC elevated significantly the muscle force-time integral by $11 \pm 4\%$ ($P < 0.05$ *versus* zero) whereas there was no effect of SMTC in CHF rats ($1 \pm 4\%$, $P > 0.05$ *versus* zero).

Discussion

The principal novel finding of the present investigation is that nNOS-mediated control of skeletal muscle microvascular function and P_{O_2mv} are impaired, and may even be abolished, in CHF rats. Specifically, the nNOS inhibition-induced changes in P_{O_2mv}, blood flow and $\dot{V}_{O_2}$ evident in healthy rats were essentially absent in CHF rats. These results suggest that improvements in nNOS function

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Table 3. Microvascular partial pressure of O_2 (P_{O_2mv}) at baseline and P_{O_2mv} kinetics parameters during contractions before (control) and after SMTC in healthy and CHF rats

	Healthy		CHF	
	Control	SMTC	Control	SMTC
$P_{O_2mv(BL)}$ (mmHg)	29.7 ± 1.4	34.4 ± 1.9*	25.7 ± 1.6	28.6 ± 2.1†
$\Delta_1 P_{O_2mv}$ (mmHg)	11.9 ± 0.9	14.2 ± 1.2	11.3 ± 1.1	12.1 ± 1.0
$\Delta_2 P_{O_2mv}$ (mmHg)	3.4 ± 0.8	2.1 ± 0.7	3.2 ± 0.7	3.5 ± 1.0
$\Delta_{total} P_{O_2mv}$ (mmHg)	9.9 ± 0.9	13.7 ± 1.1*	8.7 ± 1.1	10.9 ± 1.1
$P_{O_2mv(steady-state)}$ (mmHg)	19.8 ± 1.7	20.7 ± 1.8	17.0 ± 1.4	17.7 ± 1.8
TD_1 (s)	8.7 ± 0.8	5.5 ± 0.5*	9.6 ± 0.7	6.8 ± 1.2*
TD_2 (s)	40.2 ± 6.8	38.8 ± 5.4	63.2 ± 13.2	99.5 ± 22.6†
τ_1 (s)	15.5 ± 1.5	12.9 ± 1.2	13.2 ± 2.6	14.5 ± 2.6
τ_2 (s)	35.4 ± 3.2	25.9 ± 11.7	50.6 ± 12.6	22.4 ± 15.2
MRT (s)	24.2 ± 2.0	18.5 ± 1.3*	22.8 ± 2.6	21.3 ± 3.0
$\Delta P_{O_2mv}/\tau_1$ (mmHg s^{-1})	0.9 ± 0.1	1.4 ± 0.2*	1.2 ± 0.2	1.1 ± 0.2

Values are mean ± SEM. Where two-component models were indicated the values shown reflect data from only those animals (healthy control: $n = 11$, healthy SMTC: $n = 4$, CHF control: $n = 10$, CHF SMTC: $n = 4$). $P_{O_2mv(BL)}$, pre-contracting P_{O_2mv}; $\Delta_1 P_{O_2mv}$, amplitude of the first component; $\Delta_2 P_{O_2mv}$, amplitude of the second component; $\Delta_{total} P_{O_2mv}$; overall amplitude regardless of one- or two-component model fit; $P_{O_2mv(steady-state)}$, contracting steady-state P_{O_2mv}; TD_1, time delay for the first component; TD_2, time delay for the second component; τ_1, time constant for the first component; τ_2, time constant for the second component; MRT, $TD_1 + \tau_1$; $\Delta P_{O_2mv}/\tau_1$, the relative rate of P_{O_2mv} fall. *$P < 0.05$ *versus* control, †$P < 0.05$ *versus* healthy.

specifically may help restore, at least in part, the exercise and functional decrements evidenced by CHF patients.

Effects of CHF on nNOS-mediated regulation of P_{O_2mv}

In healthy rats, nNOS inhibition elevated resting baseline P_{O_2mv} consequent to reductions in resting $\dot{V}_{O_2}$. In this sense, the absence of P_{O_2mv} elevation in CHF rats following SMTC represents the loss of nNOS-mediated control of resting mitochondrial respiration. In healthy rats, nNOS-derived NO slowed the P_{O_2mv} kinetics fall following contractions onset as evidenced by the nNOS inhibition-induced reduction of the TD_1 and MRT and the greater $\Delta P_{O_2mv}/\tau_1$. In CHF rats, only the shorter TD_1 following nNOS inhibition was evident, which denotes that at least a relatively small nNOS-derived NO contribution to the initial $\dot{V}_{O_2}$ and/or blood flow adjustment is preserved during contractions. Interestingly though, this nNOS contribution to P_{O_2mv} regulation immediately following contractions onset appears to diminish as contractions persist. Specifically, the contracting steady-state P_{O_2mv} was not different between control and SMTC conditions in healthy and CHF rats. However, in healthy rats this resulted from similar 15% reductions in blood flow and $\dot{V}_{O_2}$ whereas in CHF there was no effect of SMTC on steady-state blood flow or $\dot{V}_{O_2}$. While this

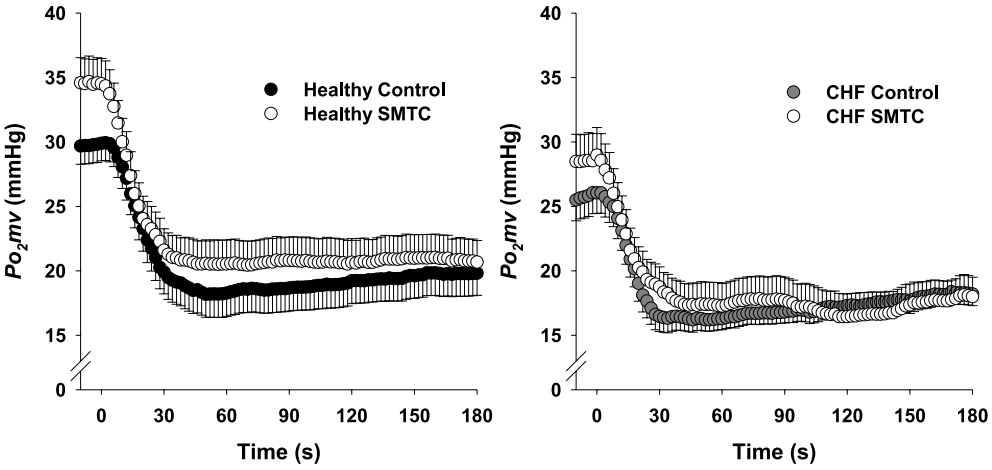

Figure 2. Average raw P_{O_2mv} profiles for healthy (left panel, $n = 14$) and CHF (right panel, $n = 12$) rats during control and SMTC conditions
Uni-directional SEM bars are shown for clarity. Time '0' represents the onset of contractions.

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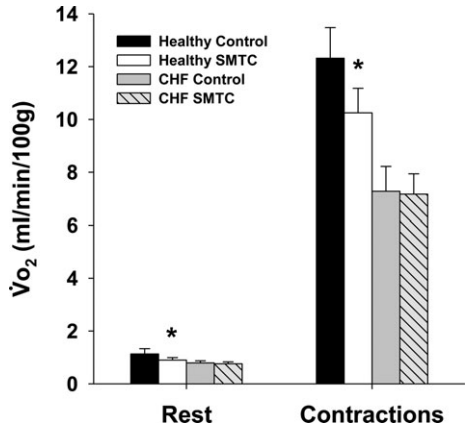

Figure 3. Effects of nNOS inhibition with SMTC on resting and contracting steady-state spinotrapezius muscle $\dot{V}_{O_2}$ in healthy ($n = 14$) and CHF ($n = 12$) rats
Data are means ± SEM. *$P < 0.05$ *versus* control.

may reflect the highly regulated nature of skeletal muscle P_{O_2mv} during contractions, such regulation is achieved very differently (i.e. similar reductions in blood flow and $\dot{V}_{O_2}$ for healthy rats *versus* unchanged values for CHF).

Effects of CHF on nNOS-mediated regulation of O_2 delivery, $\dot{V}_{O_2}$ and contractile function

The potential for nNOS-derived NO specifically to modulate skeletal muscle blood flow in healthy subjects is well-recognized (Thomas *et al.* 1998; Seddon *et al.* 2008; Copp *et al.* 2010*b*, 2011). While nNOS-derived NO may not be obligatory for the hyperaemic response to treadmill exercise in healthy rats due to multiple redundant blood flow pathways (Copp *et al.* 2010*b*), our recent investigation revealed novel roles for nNOS-mediated

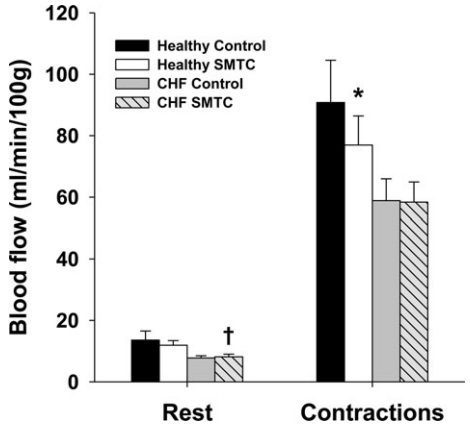

Figure 4. Effects of nNOS inhibition with SMTC on resting and contracting steady-state spinotrapezius muscle blood flow in healthy ($n = 14$) and CHF ($n = 12$) rats
Data are means ± SEM. *$P < 0.05$ *versus* control, †$P < 0.05$ *versus* healthy.

regulation of O_2 delivery/utilization in healthy rat spinotrapezius muscle during electrically induced contractions (Copp *et al.* 2011). The present investigation is the first to find that CHF impairs this modulation as indicated by nNOS inhibition-induced reductions in contracting blood flow and VC in healthy but not CHF rats. Our present finding is consistent with the observation that contracting skeletal muscle functional sympatholysis (an nNOS-driven phenomenon, Thomas *et al.* 1998) is attenuated in CHF rats (Thomas *et al.* 2001). It is noteworthy that compromised nNOS function is likely to account for only a portion of the blood flow decrements observed in CHF and, therefore, dysfunction of other mechanisms of vascular control (i.e. eNOS function, autoregulation, muscle-pump, etc.) must also contribute to the impairment. Interestingly, SMTC reduced kidney blood flow and VC in healthy and CHF rats although the VC reduction in CHF was less than that in healthy rats. Therefore, the degree to which CHF alters nNOS-mediated vascular control evidences heterogeneity among varying tissues and, therefore, possibly among muscles of different fibre-type composition given reports of muscle-fibre type specific CHF-related alterations (Delp *et al.* 1997; Behnke *et al.* 2004; Bertaglia *et al.* 2011). Whether the nNOS-mediated vascular control impairments evident currently are manifest in CHF rats during locomotory exercise remains to be determined.

The SMTC-induced $\dot{V}_{O_2}$ reductions in healthy rat skeletal muscle at rest and during contractions was surprising given the potential for NO to inhibit mitochondrial respiration via competitive inhibition at cyctochrome c oxidase (Brown, 1995), but are consistent with previous studies reporting reductions in rat hindlimb $\dot{V}_{O_2}$ following non-selective NOS inhibition (Krause *et al.* 2005; Baker *et al.* 2006). The findings in healthy rats indicate that nNOS-derived NO may actually stimulate oxidative phosphorylation. The present study demonstrates that CHF impairs, and may abolish, the nNOS-mediated effects on $\dot{V}_{O_2}$. However, nNOS inhibition improved muscle contractile performance (i.e. modestly elevated force-time integral) in healthy rats, which is consistent with the notion that NO exerts an inhibitory influence on muscle contractile function (Reid *et al.* 1998). Taken together, the elevated force–time integral and reduced $\dot{V}_{O_2}$ during contractions in healthy rats suggests an elevated contractile economy. Therefore, the data indicate that the nNOS-mediated effects on contractile function and economy are curtailed in CHF rats. By extension, the muscle contractile dysfunction evident in CHF subjects (Perreault *et al.* 1993; Lunde *et al.* 2002; Ertunc *et al.* 2009) must occur via dysregulation/dysfunction of other mechanisms occurring simultaneous with nNOS downregulation. For example, CHF induces elevated intracellular iNOS, which correlates with exercise intolerance (Hambrecht *et al.* 1999), and

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impaired sarcoplasmic reticulum function and calcium regulation (Perreault *et al.* 1993; Reiken *et al.* 2003), which likely contribute to contractile dysfunction in CHF.

Mechanisms of nNOS dysfunction in CHF

Previous investigations have reported increased (Rush *et al.* 2005) or unchanged (Thomas *et al.* 2001; Rush *et al.* 2005) nNOS expression in skeletal muscle from CHF rats suggesting that the impaired nNOS-mediated microvascular function reported presently reflects reduced nNOS-derived NO bioavailability. This may occur consequent to reduced NO production resulting from nNOS uncoupling following the loss of tetrahydrobiopterin (BH_4) and other essential NOS cofactors (Munzel *et al.* 2005) and/or enhanced nNOS-derived NO inactivation by reactive O_2 species (i.e. superoxide). Specifically, elevated reactive O_2 species may inactivate NO and are the result of CHF-induced increases in muscle oxidoreductases such as, for example, xanthine oxidase (Landmesser *et al.* 2002) which may occur concurrent with reductions in superoxide dismutase and catalase antioxidant protein levels (Rush *et al.* 2005). These effects are likely to contribute to the elevated biomarkers of oxidative stress found in CHF patients (Seddon *et al.* 2007). In addition, elevated levels of the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) have been reported in the circulation of CHF patients (Usui *et al.* 1998) and animals (Feng *et al.* 1998) and it is possible that this substance impairs nNOS function simultaneously with its better-known impediments to eNOS function.

Methodological considerations

Several key lines of evidence support that 0.56 mg kg^{-1} of SMTC inhibited nNOS without impacting eNOS-mediated function. First, Furfine *et al.* (1994) demonstrated that SMTC possesses a 10-fold selectivity for nNOS *versus* eNOS *in vitro* and a 17-fold selectivity for nNOS *versus* eNOS in rat tissue *in vivo*. Second, SMTC did not alter the recovery of the hypotensive response to ACh (mediated largely by eNOS-derived NO) whereas L-NAME significantly blunted the response. However, when the SMTC dose is increased to 5.6 mg kg^{-1} (i.e. 10-fold) a clear impact is seen on the ACh response (authors' unpublished data). Analysis of hemodynamic responses to ACh has constituted the gold-standard assessment of selective nNOS inhibition when SMTC is administered either locally or systemically in intact animal and human models (Wakefield *et al.* 2003; Seddon *et al.* 2008, 2009; Copp *et al.* 2010*b*, 2011). Third, 0.56 mg kg^{-1} SMTC did not impact rat hindlimb skeletal muscle blood flow during treadmill exercise (Copp *et al.* 2010*b*). If SMTC inhibited eNOS significant reductions in blood flow would be expected as seen following L-NAME (Hirai *et al.* 1995). Fourth, the present SMTC dose is similar to that used in investigations from other laboratories that have also reported selective nNOS inhibition with SMTC in rats *in vivo* (0.3 mg kg^{-1}, Ichihara *et al.* 1998; 0.5 mg kg^{-1}, Komers *et al.* 2000).

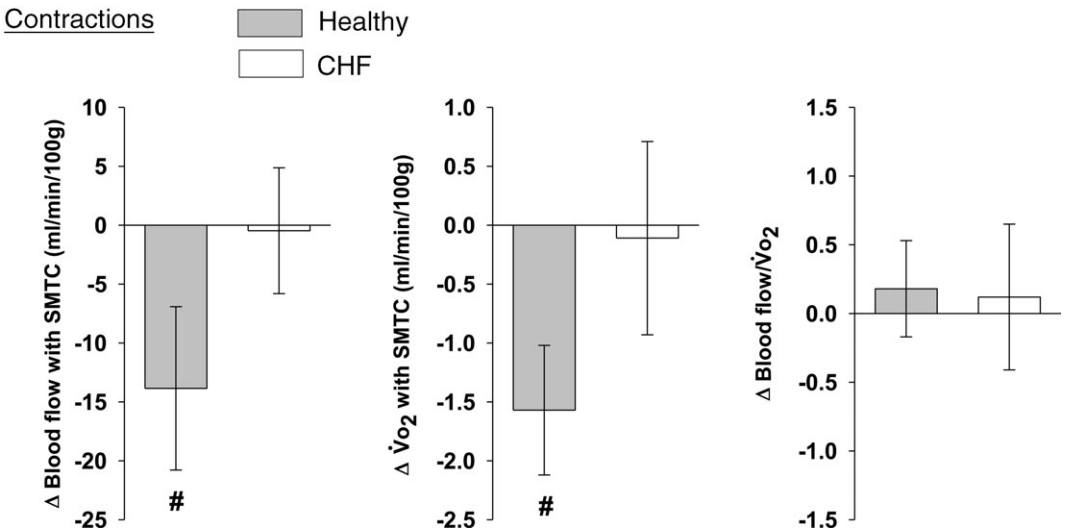

Figure 5. Effects of nNOS inhibition with SMTC on contracting steady-state spinotrapezius muscle blood flow (Δ blood flow), $\dot{V}_{O_2}$ ($\Delta\dot{V}_{O_2}$) and the blood flow/$\dot{V}_{O_2}$ relationship (Δ blood flow/$\dot{V}_{O_2}$, which dictates the steady-state ΔP_{O_2mv})

Note how nNOS inhibition did not alter contracting Δ blood flow/$\dot{V}_{O_2}$ in healthy ($n = 14$) or CHF ($n = 12$) rats; but these values were achieved very differently (i.e. blood flow and $\dot{V}_{O_2}$ reductions in healthy rats compared to no changes in CHF rats). Data are means ± SEM. #$P < 0.05$ *versus* zero.

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Other methodological considerations including time control studies, the validity of the intact spinotrapezius muscle preparation, isolated electrical-contractions paradigm, and systemic *versus* local SMTC administration have been discussed in detail previously (Copp *et al.* 2011).

Conclusions

The nNOS-mediated regulation of P_{O_2mv}, muscle blood flow and $\dot{V}_{O_2}$ is dramatically impaired, if not abolished, in CHF rats. Additionally, nNOS-derived NO inhibits force production in healthy rats and this nNOS-mediated inhibition is suppressed in CHF rats. Investigation of the mechanisms underlying impaired microvascular and muscle function in CHF is critical for the development of treatments designed to mitigate CHF-induced skeletal muscle pathology. Given the impairments of nNOS-mediated P_{O_2mv} regulation in CHF rats reported currently, restoration of nNOS function may constitute a valuable mechanistic target for therapeutic treatments of CHF.

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Conception and design of the experiments: S.W.C., D.M.H., T.I.M., D.C.P. Data collection and analysis: S.W.C., D.M.H., S.K.F., C.T.H., T.I.M. Manuscript preparation and revision:

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