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Neuronal nitric oxide synthase regulation of skeletal muscle functional hyperemia: exercise training and moderate compensated heart failure

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Abstract

Nitric oxide (NO) modulates oxygen delivery-utilization matching in resting and contracting skeletal muscle. Recent reports indicate that neuronal NO synthase (nNOS)-mediated vasoregulation during contractions is enhanced with exercise training and impaired with chronic heart failure (HF). Consequently, we tested the hypothesis that selective nNOS inhibition (*S*-methyl-L-thiocitrulline; SMTC, 2.1 $\mu\text{mol/kg}$) would produce attenuated reductions in muscle blood flow during moderate/heavy submaximal exercise in sedentary HF rats compared to their healthy counterparts. In addition, SMTC was expected to evoke greater reductions in exercising muscle blood flow in trained compared to sedentary healthy and HF rats. Blood flow during submaximal treadmill running (20 min/m, 5% grade) was determined via radiolabeled microspheres pre- and post-SMTC administration in healthy sedentary (Healthy + Sed, $n = 8$), healthy exercise trained (Healthy + ExT, $n = 8$), HF sedentary (HF + Sed, left ventricular end-diastolic pressure (LVEDP) = 12 ± 1 mmHg, $n = 8$), and HF exercise trained (HF + ExT, LVEDP = 16 ± 2 mmHg, $n = 7$) rats. nNOS contribution to exercising total hindlimb blood flow (ml/min/100 g) was not increased by training in either healthy or HF groups (Healthy + Sed: 105 ± 11 vs. 108 ± 16 ; Healthy + ExT: 96 ± 9 vs. 91 ± 7 ; HF + Sed: 124 ± 6 vs. 110 ± 12 ; HF + ExT: 107 ± 13 vs. 101 ± 8 ; control vs. SMTC, respectively; $p > .05$ for all). Similarly, SMTC did not reduce exercising blood flow in the majority of individual hindlimb muscles in any group ($p > .05$ for all, except for the semitendinosus and adductor longus in HF + Sed and the adductor longus in HF + ExT; $p < .05$). Contrary to our hypothesis, we find no support for either upregulation of nNOS function contributing to exercise hyperemia after training or its dysregulation with chronic HF.

Keywords

Blood flow; HFrEF; Myocardial infarction; nNOS; SMTC; Vasodilation

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1. Introduction

The complex regulation of skeletal muscle vasomotor tone and thus blood flow is mediated by multiple central (neurohumoral) and local (including mechanical, metabolic, endothelial and paracrine) mechanisms [31,38]. This synergistic and redundant regulation is crucial to match muscle O₂ delivery to utilization during transitions in metabolic demand and sustain contractile performance. Accordingly, vascular adaptations to exercise training promote increased exercise tolerance whereas vasomotor dysregulation in disease states such as chronic heart failure (HF) predispose to early fatigue [18,23,45].

Nitric oxide (NO) is an important signaling molecule that participates in the exercise hyperemic response [31,38]. Accumulating evidence has implicated NO derived from neuronal NO synthase (nNOS) in this process. nNOS-mediated vasoregulation is exerted via cGMP formation [17,36] and/or functional sympatholysis [13,29,34,51,57] and displays fiber type and contraction intensity dependency [6,8,56]. Consistent with these observations, genetically engineered mice lacking nNOS experience lower hindlimb muscle blood flow during treadmill exercise compared to their wild-type counterparts [34]. Moreover, pharmacologically selective nNOS blockade in healthy rats reduces blood flow particularly to glycolytic fast-twitch muscle fibers during high speed treadmill running (i.e., within the severe domain of exercise; [6,8]). During electrical stimulation of the *in situ* mixed-fiber type spinotrapezius muscle, blood flow reductions with nNOS blockade seen in healthy rats are not observed in those with HF [4,5]. The latter findings are consistent with impaired nNOS-mediated vasoregulation in HF during small muscle mass electrical stimulation. Whether the same holds for conscious, dynamic exercise of large muscle mass which is associated with preserved motor unit recruitment order and competition between active muscle blood flow distribution and systemic blood pressure regulation is unknown. In this context, evaluation of the effects of HF on nNOS-mediated vasoregulation during more physiological conditions such as treadmill running is warranted.

Evidence from human and animal research indicates that nNOS protein expression (and purportedly function) can be modulated by numerous factors, including muscle activity (e.g., exercise training, bed rest; [48,49,59]), hypoxia [28] and inflammatory cytokines [12]. However, inconsistent reports of alterations in skeletal muscle nNOS expression with exercise training [2,14,19,30,40,53,55,60] and HF [50,58] challenge our understanding of nNOS-mediated vasoregulation in these conditions. In healthy rats, increased nNOS expression with exercise training has been found to augment sympatholysis but not to impact hindlimb blood flow during electrically-induced contractions [30]. In HF, nNOS protein level/activity could be dissociated from vascular regulation due to NOS uncoupling and/or NO scavenging secondary to disease-induced oxidative stress [47,54,58]. Thus, whether exercise training modulates nNOS-mediated regulation of exercise hyperemia in healthy and/or HF skeletal muscle remains to be elucidated. This is of clinical relevance given that impaired nNOS function in HF is associated with blunted sympatholysis during contractions [58] potentially contributing to exercise intolerance via compromised muscle blood flow [45].

The purpose of the present study was to determine the effects of exercise training and HF on nNOS-mediated control of skeletal muscle blood flow during large muscle mass dynamic exercise. Specifically, we examined whether nNOS-mediated vasoregulation is altered with exercise training and/or HF; and whether nNOS-derived NO plays an obligatory role in exercise hyperemia in these conditions. Isoformspecific inhibition with *S*-methyl-L-thiocitrulline (SMTC, which has a 17-fold selectivity for nNOS compared to endothelial NOS in rat tissue *in vivo*; [16,61]) was performed during submaximal moderate/heavy intensity treadmill running in sedentary and trained healthy and HF rats. These exercise intensities are highly recommended for most HF patients and thus widely adopted in cardiac rehabilitation programs for their perceived efficacy and safety [44]. Based on the impaired nNOS-mediated vasoregulation during small muscle mass stimulation in HF [4,5], we hypothesized that SMTC would evoke attenuated reductions in exercising muscle blood flow in sedentary HF rats compared to their healthy counterparts. Furthermore, given the potential upregulation of nNOS function with exercise training as discussed above, greater reductions in exercising muscle blood flow with SMTC were expected in trained compared to sedentary healthy and HF rats.

2. Methods

A total of 43 male Sprague-Dawley rats (4–5 months old; Charles River Laboratories; Boston, MA) were used in the present investigation. Rats were housed in accredited facilities and maintained on a 12:12 h light/dark cycle with food and water provided *ad libitum* for the duration of the experimental protocol. All experimental procedures followed guidelines established by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Kansas State University.

Rats were assigned randomly to four groups: healthy sedentary (Healthy + Sed), healthy exercise trained (Healthy + ExT), heart failure sedentary (HF + Sed) and heart failure exercise trained (HF + Ext). Myocardial infarction was induced in HF rats via ligation of the left main coronary artery as described in detail previously [41–43]. Briefly, rats were anesthetized with a 5% isoflurane-O₂ mixture (Butler Animal Health Supply; Dublin, OH) and intubated for mechanical ventilation with a rodent respirator (model 680, Harvard Instruments; Holliston, MA) for the duration of the surgical procedure. The heart was accessed through a left thoracotomy in the fifth intercostal space and the left main coronary artery was ligated (6-0 silk suture) at ~1–2mm distal to the edge of the left atrium. The incision was then closed, and ampicillin (50 mg/kg im) was injected locally to reduce the opportunity for infection. The analgesic agents bupivacaine (1.5 mg/kg sc) and buprenorphine (0.01–0.05 mg/kg im) were administered subsequently and anesthesia and mechanical ventilation discontinued. All rats were monitored closely for 6 h for the development of arrhythmias and signs of undue stress (e.g., labored breathing). Rats were also monitored daily (e.g., appetite, weight loss/gain, gait and posture) according to an intensive 10-day post-operative plan conducted in association with the university veterinary staff. Each animal was allowed 3 weeks of recovery before initiation of any experimental procedure. The survival rate for the rats receiving the myocardial infarction operation was 88% (38 of 43). Previous reports support that this rat myocardial infarction model induces

central and peripheral vascular and microvascular dysfunction consonant with heart failure with reduced ejection fraction (HFrEF; [9,41–43]).

All rats were then familiarized with running on a custom-built motor-driven treadmill over the course of a 1-week period (5–10 min/day at a speed of 20 m/min and 5% grade). This familiarization protocol has been demonstrated previously not to induce exercise training adaptations, such as increases in maximal oxygen uptake ($\dot{V}O_{2\max}$), endurance capacity, skeletal muscle citrate synthase, and/or succinate dehydrogenase activities [1,42]. Following completion of the familiarization sessions, Healthy + Sed and HF + Sed rats were confined to cage activities, whereas Healthy + ExT and HF + ExT rats ran 5 days/week over 6–8 weeks on the treadmill (5% grade). Running duration and speed were increased progressively from 10 min at 25 m/min to 60 min at 35 m/min. This final workload was maintained for the last 3–4 weeks.

Sedentary and exercise trained rats underwent subsequent experimental procedures (described below) after ~5 and 10 weeks from group assignment, respectively. This experimental design was used herein to match central cardiac function in an attempt to isolate peripheral vascular adaptations [21].

2.1. Maximal oxygen uptake measurements

Whole-body $\dot{V}O_{2\max}$ was measured in all rats during a treadmill running test performed in a metabolic chamber placed on the treadmill. As described previously [3,21], the speed was set initially to 25 m/min for 2–3 min and then increased progressively in a ramp-like fashion by ~5–10 m/min until the rat was unable to keep pace with the treadmill belt or no further elevations in $\dot{V}O_2$ were observed despite continued increases in treadmill speed. At this point of the test the $\dot{V}O_{2\max}$ was measured and recorded. Alterations in gait (e.g., lowering of the hindlimbs, dropping of the tail and/or elevation of the snout) normally occurred immediately before termination of the test. Gas measurements were performed in real time via an inline O_2 analyzer (model S-3A/I; AEI Technologies; Pittsburgh, PA). The analyzer was calibrated with precision-mixed gases that spanned the expected range of gas concentrations based on previous studies. We have reported previously highly reproducible $\dot{V}O_{2\max}$ measurements using the abovementioned techniques and protocol [3].

2.2. Measurement of blood flow

On the final experimental day, anesthesia was induced with a 5% isoflurane- O_2 mixture with maintenance on 2–3% isoflurane- O_2 . Subsequently, one catheter (PE-10 connected to PE-50; Clay Adams Brand; Sparks, MD) was placed in the ascending aorta via the right carotid artery and a second catheter was placed in the caudal (tail) artery as described previously [41–43]. Both catheters were tunneled subcutaneously to the dorsal aspect of the cervical region and exteriorized through a puncture wound in the skin. After closure of the incisions, anesthesia was terminated and the animal was given 1 h to recover prior to blood flow measurements under control and selective nNOS inhibition conditions (see Fig. 1 for a schematic representation of the experimental protocol).

Following recovery, each rat was placed on the treadmill and the carotid artery catheter was connected to a pressure transducer (Gould Statham P23ID; Valley View, OH) and chart

recorder for continuous monitoring of mean arterial pressure (MAP) and heart rate (HR). Subsequently, 1.2 ml of heparinized saline was infused at a rate of 0.2 ml/min into the caudal artery catheter to serve as a time and volume control infusion for the nNOS inhibition condition (described below). Following infusion, the tail artery catheter was connected to a 1 ml plastic syringe and a Harvard infusion/withdrawal pump (model 907; Holliston, MA). The speed of the treadmill was then increased progressively during ~30 s to a speed of 20 m/min up a 5% grade [41–43]. The rat was then exercised steadily and after ~3 min of total exercise time, blood withdrawal from the tail artery catheter was initiated at a rate of 0.25 ml/min while MAP and HR were measured and recorded simultaneously via the carotid artery catheter. Subsequently, the carotid artery catheter was disconnected from the pressure transducer and $0.5\text{--}0.6 \times 10^6$ 15- μm diameter microspheres (^{46}Sc or ^{85}Sr in random order; Perkin Elmer; Waltham, MA) were injected into the aortic arch to determine regional blood flows. Following microsphere injection and while the rat was still running a 0.3 ml blood sample was taken from the carotid artery catheter for determination of arterial O_2 saturation, pH and lactate concentration. Exercise was then terminated and blood withdrawal from the caudal artery catheter (which was occurring simultaneously with microsphere injection) was stopped. The reference sample was collected and rats were given a minimum of 30 min to recover.

After recovery from the control exercise bout, 0.56 mg/kg (2.1 $\mu\text{mol/kg}$) of the nNOS-selective inhibitor SMTC (Sigma-Aldrich; St. Louis, MO) dissolved in 1.2 ml of heparinized saline was infused at a rate of 0.2 ml/min into the caudal artery catheter as described above. This SMTC dose was chosen based on previous investigations designed to inhibit selectively nNOS in both human and rodent models [16,52,61] and our analysis of the highest possible SMTC dose that could be administered without impacting the hypotensive response to acetylcholine (ACh; see below), indicative of non-specific eNOS blockade [4–6,22]. Due to its relatively long half-life (~40 min; [16,61]), SMTC was always the second condition to prevent residual effects on systemic and/or regional vascular function. The tail artery catheter was then re-connected to a syringe and the Harvard pump for blood withdrawal. The second bout of exercise, microsphere injection (differently labeled from the first run) and arterial blood sample procedures were performed exactly as described above for the control (i.e., non-SMTC infusion) condition.

2.3. Assessment of the selectivity of nNOS inhibition with SMTC

In order to reconfirm the efficacy of the current SMTC dose to block nNOS without affecting eNOS-mediated function [5,6,16,52,61], rapid ACh infusions (5 $\mu\text{g/kg}$ in 0.2 ml of saline) were performed in subsets of animals (Healthy + Sed, $n = 5$; Healthy + ExT, $n = 6$; HF + Sed, $n = 7$; HF + ExT, $n = 6$) under control and SMTC conditions as well as after non-selective NOS inhibition with N^G -nitro-L-arginine methyl ester (L-NAME; 10 mg/kg) administered in the caudal artery (Fig. 1). ACh was infused rapidly into the caudal artery catheter while the rats sat quietly on the treadmill belt and the hypotensive response to these infusions was measured and recorded simultaneously via the carotid artery catheter. The magnitude of the hypotensive response to ACh was used as an index of eNOS-mediated function. Absence of any blunting of the hypotensive response to ACh following SMTC was

considered consistent with preserved eNOS function. Conversely, the hypotensive response to Ach and thus eNOS function was anticipated to be attenuated after L-NAME.

2.4. Determination of blood flow

Following completion of the experimental protocol each animal was anesthetized with pentobarbital sodium administered via the carotid artery catheter. The thorax was opened, and placement of the carotid artery catheter into the aortic arch was confirmed by anatomical dissection. The animal was then euthanized by removal of the heart. The right and left kidneys, organs of the splanchnic region (adrenals, large intestine, small intestine, stomach, pancreas, spleen and liver) and the 28 individual muscles or muscle parts of the rat hindlimb were identified and removed. The tissues were blotted, weighed and placed immediately into counting vials.

The radioactivity of each tissue was determined on a gamma scintillation counter (Packard Auto Gamma Spectrometer, model 5230; Downers Grove, IL). Taking into account the cross-talk between isotopes, blood flows to each tissue were determined using the reference sample method [37,43] and expressed as ml/min/100 g tissue. Adequate mixing of the microspheres was verified for each injection by demonstrating a < 15% difference between blood flow to the right and left kidneys and/or to the right and left hindlimb musculature.

2.5. Statistical analyses

Data comparisons were performed using unpaired Student's *t*-test, one-way ANOVA or two-way repeated measures ANOVA where appropriate. *Post hoc* analyses were performed with the Student-Newman-Keuls test when a significant *F* ratio was detected. A one-tailed test was performed when *a priori* directional hypotheses were tested. A one sample *t*-test was used to determine differences from zero. Data are presented as means $\pm$ SE. Significance was accepted at $p < .05$.

3. Results

Data from 31 of 38 rats that survived the myocardial infarction surgery and completed successfully the experimental protocol were included for analysis. Seven animals were excluded from the current investigation due to: hemodynamic complications from surgical instrumentation (2 rats), inability/unwillingness to run steadily on the treadmill during blood flow measurements (1 rat) and inadequate mixing of microspheres (4 rats). Results are thus given below for the following animal numbers: Healthy + Sed ($n = 8$), Healthy + ExT ($n = 8$), HF + Sed ($n = 8$) and HF + ExT ($n = 7$).

3.1. Efficacy of chronic HF induction

Morphological and hemodynamic characteristics of sedentary and trained healthy and HF rats are presented in Table 1 and indicate that HF rats had moderate compensated heart failure [9,43]. HF + Sed and HF + ExT rats displayed central indices of disease severity that were not different between groups (including infarct size and LVEDP; $p > .05$ for both) except for LV dp/dt which was lower in HF + ExT ($p < .05$). There were no differences in body mass or hindlimb mass among groups ($p > .05$ for all).

3.2. Maximal exercise tests: efficacy of training

HF + Sed rats had lower $\dot{V}O_{2\max}$ than Healthy + Sed ($p < .05$; Fig. 2). Exercise training elevated $\dot{V}O_{2\max}$ in both Healthy + ExT and HF + ExT rats compared to their sedentary counterparts ($p < .05$ for both; Fig. 2).

3.3. Central hemodynamics

At rest, SMTC increased MAP only in Healthy + Sed and HF + Sed when compared to the control condition ($p < .05$ for both; Table 2). During exercise, there were no differences in MAP measured immediately prior to microsphere injection between control and SMTC in any group ($p > .05$ for all; Table 2) and, therefore, exercising blood flow (as opposed to vascular conductance) data are reported herein.

3.4. Arterial blood samples

There were no differences in O_2 saturation or pH among groups or conditions ($p > .05$ for all; Table 3). SMTC increased lactate concentration only in Healthy + Sed and HF + Sed compared to the control condition ($p < .05$ for both; Table 3). Healthy + ExT and HF + ExT rats displayed lower lactate concentration under both control and SMTC conditions when compared to their sedentary counterparts ($p < .05$ for all; Table 3).

3.5. Skeletal muscle blood flow

Total hindlimb muscle blood flow during exercise was not different between control and SMTC conditions or among groups (Healthy + Sed: 105 ± 11 vs. 108 ± 16 ; Healthy + ExT: 96 ± 9 vs. 91 ± 7 ; HF + Sed: 124 ± 6 vs. 110 ± 12 ; HF + ExT: 107 ± 13 vs. 101 ± 8 ml/min/100 g; respectively; $p > .05$ for all). Accordingly, the change in total hindlimb blood flow elicited by SMTC (blood flow) during exercise was not different among groups or from zero ($p > .05$ for all; Fig. 3). Moreover, SMTC did not change exercising blood flow in the majority of individual hindlimb muscles or muscle parts in any group ($p > .05$ for all, except for the semitendinosus and adductor longus in HF + Sed and the adductor longus in HF + ExT; $p < .05$; Table 4).

3.6. Abdominal organ blood flow

Compared to control, SMTC reduced blood flow under the following conditions: Healthy + Sed (kidneys, adrenals, small intestine, liver), Healthy + ExT (kidneys, adrenals, liver), HF + Sed (adrenals, large intestine, liver) and HF + ExT (kidneys, adrenals, liver) ($p < .05$ for all; Fig. 4). Healthy + ExT rats evidenced greater blood flow than Healthy + Sed to the following vascular beds: kidneys (both control and SMTC), small intestine (SMTC), stomach (SMTC) and pancreas (both control and SMTC) ($p < .05$ for all; Fig. 4 *top panels*). HF + ExT rats had greater blood flow than HF + Sed to the kidneys under the control condition ($p < .05$; Fig. 4 *bottom panels*).

3.7. Acetylcholine infusions

SMTC did not attenuate the relative change in MAP with Ach when compared to control in any group ($p > .05$ for all), whereas L-NAME blunted the magnitude of this hypotensive response in all groups relative to both the control and SMTC conditions ($p < .05$ for all).

(Fig. 5). Taken together, these data support that SMTC selectively inhibited nNOS but did not impair eNOS function in the present study.

4. Discussion

The present investigation is the first to evaluate the impact of exercise training and chronic HF on the contribution of nNOS to systemic and regional hemodynamic control. Contrary to our hypothesis, selective nNOS inhibition with SMTC had essentially no effect on contracting muscle blood flow in sedentary healthy or HF rats. Moreover, exercise training failed to evoke any detectable influence of nNOS in either the healthy or HF muscle blood flow response during exercise. By contrast, exercise training restored renal blood flow responses during exercise in HF + ExT rats largely via nNOS adaptations. These data indicate that, although nNOS-derived NO does not play an obligatory role in the regulation of exercise hyperemia (at least in the ~55–65% $\dot{V}O_{2\max}$ range) in sedentary or trained healthy or HF muscles, therapeutic interventions targeting nNOS such as exercise training could ameliorate renal circulatory dysfunction in HF patient populations.

4.1. Myocardial infarction

In the present study, chronic HF was induced by surgically ligating the left main coronary artery thereby producing myocardial infarction (as described in detail in [41–43]). As expected, this well-established model of heart failure resulted in higher left ventricular end-diastolic pressure (LVEDP) and lower left ventricular developed pressure (LV dp/dt) compared to healthy rats (Table 1). Together with the lack of differences in markers of lung congestion (lung/body mass ratio; Table 1) among groups, these functional and structural features indicate that HF + Sed and HF + ExT rats were in a state of moderate compensated heart failure [9,43].

As reviewed recently [23,45], conflicting results have been found regarding the effects of HF on skeletal muscle bulk blood flow during submaximal exercise. Reports of reduced or unaltered exercising muscle blood flow in either human patients or animal models of HF may reflect differences in disease severity, exercise intensity/mode, measurement techniques, and/or pharmacological therapy. Moreover, compensatory mechanisms (e.g., endothelium-derived hyperpolarizing factors, prostaglandins; [25,32,35]) could prevent or minimize decrements in bulk muscle blood flow and potentially mask disease-induced impairments in specific vasodilatory pathways during submaximal work. Consistent with our previous findings in awake animals [24,41], moderate HF rats showed no impairments in total hindlimb blood flow during submaximal treadmill exercise compared to their healthy counterparts. On the other hand, impaired vasoregulation in moderate HF is likely to be apparent near maximal treadmill exercise [43] and thus contribute directly to reductions in $\dot{V}O_{2\max}$ under those conditions (Fig. 2 and [41]).

In rats with more severe heart failure (i.e., myocardial infarction size approximately 35–40% vs. ~30% herein), impairments in total hindlimb blood flow are evident during both submaximal and near-maximal treadmill exercise [11,24]. Larger myocardial infarctions and severe left ventricular dysfunction are also associated with considerable redistribution of

blood flow from slow-twitch oxidative to fast-twitch glycolytic muscle fibers during exercise [10,39,43].

Interestingly, while moderate HF was found previously to impair nNOS-mediated vasoregulation during electrically-induced small muscle mass contractions [4], the current findings during submaximal treadmill running indicate that nNOS function is not obligatory for exercise hyperemia in sedentary or trained rats with moderate disease (Fig. 3 and Table 4). This discrepancy could result from experimental differences and the fiber type-dependent control exerted by nNOS [6,8] as discussed below.

4.2. Exercise training

The current exercise training protocol was effective in producing several metabolic and cardiocirculatory adaptations in both healthy and HF rats [1,42]. Compared to their sedentary counterparts, Healthy + ExT and HF + ExT rats evidenced a) higher whole-body $\dot{V}O_{2\max}$ (Fig. 2); b) lower blood lactate concentration during exercise performed at the same absolute workload under both control and SMTC conditions (Table 3); and c) sustained greater blood flow to multiple abdominal organs during submaximal exercise (which occurred despite the lack of significant changes in total hindlimb blood flow between sedentary and trained rats; Figs. 3 and 4). Furthermore, the elevations in resting MAP with SMTC observed in sedentary but not exercise trained rats (Table 2) are not only consistent with the obligatory nNOS-mediated regulation of basal hindlimb skeletal muscle, renal and splanchnic blood flow in sedentary humans and animals [6,26,27,52], but also suggest improved systemic vascular control (i.e., greater redundancy) at rest following training. Although not evaluated herein (see *Experimental considerations* below), previous studies using similar exercise training protocols in healthy and HF rats have also produced significant improvements in skeletal muscle oxidative capacity, O_2 delivery to utilization matching (i.e., enhanced muscle microvascular oxygenation assessed via phosphorescence quenching) and exercise tolerance [1,20,21,41,42].

As reviewed recently [23,38], exercise training does not substantially impact skeletal muscle bulk blood flow during submaximal exercise performed at the same absolute intensity in either healthy or HF individuals (unless a slow component behavior is allowed to develop; rev., [46]). Consistent with this notion and previous blood flow data in healthy and HF rats (also acquired via radiolabeled microspheres; [1,42]), no differences in total hindlimb blood flow during submaximal treadmill running were observed herein following training. In the healthy rat subjected to exercise training, Armstrong and Laughlin [1] found that there may be a blood flow redistribution within and among individual muscles with time during submaximal exercise concomitant with no changes in total hindlimb blood flow. More specifically, trained rats exhibited greater blood flow to slow-twitch oxidative fibers whereas sedentary rats had greater blood flow to fast-twitch oxidative/glycolytic fibers after 15 min of treadmill running. However, as discussed by the authors, this spatial and temporal redistribution could be related to enhanced endurance capacity of trained rats, such that progressive recruitment of relatively more glycolytic fibers is delayed with prolonged submaximal exercise compared to sedentary rats [1]. In moderate HF rats undergoing exercise training, Musch and colleagues [42] reported a similar redistribution of blood flow

within and among individual hindlimb muscles after 6 min of submaximal treadmill running. In the current study, blood flow measurements performed at 3 min of submaximal treadmill running revealed no redistribution within or among individual muscles in any group (Table 4). Although at first glance contradictory with the evidence summarized above, our results are consistent with another study by Musch and colleagues [41] investigating exercise training in healthy and moderate HF rats. Interestingly, the latter study also showed no blood flow redistribution in select forelimb or hindlimb muscles following 3 min of submaximal treadmill running in any of the groups examined. Collectively, these data support that fatigue-induced alterations in muscle recruitment that develop with sufficient exercise time may drive the bulk of the redistribution phenomenon as hypothesized initially by Armstrong and Laughlin [1]. In this context, fiber-type specific impairments in vasodilatory regulation with HF and/or adaptations to training [23,24,45] could modulate individual muscle blood flow redistribution under some, but not all, circumstances (e.g., depending on exercise time and/or disease severity as mentioned above).

4.3. Selective nNOS inhibition with SMTC: impact of heart failure

Pharmacologically selective blockade herein evaluated whether impairments in nNOS-mediated vascular control with HF evident during small muscle mass stimulation [4] are manifest during conscious, locomotory exercise engaging a large proportion of muscle mass. Compromised nNOS vasoregulatory function in contracting HF muscle results, at least in part, from impaired functional sympatholysis (which is driven mostly by nNOS-derived NO in glycolytic fibers; [29,56,57]) found in this disease [58]. As shown in Table 2, the lack of difference in the elevation of resting MAP with SMTC between Healthy + Sed and HF + Sed indicate that nNOS-derived NO contribution to resting systemic pressure regulation is not impaired by the disease. However, in conflict with our previous report in the anesthetized sedentary HF rat (notably of the same strain and similar age as herein; [4]), no changes in MAP were observed with SMTC during treadmill exercise in HF + Sed (Table 2). That SMTC had virtually no effects on contracting skeletal muscle blood flow in either Healthy + Sed or HF + Sed (Fig. 3 and Table 4) also contrasts markedly with our previous findings using a small muscle mass model (i.e., electrical stimulation of the spinotrapezius muscle; [4]). It must be noted that electrical stimulation overrides nNOS fiber type-specific vascular control via artificial recruitment of the entire motor unit pool thereby maximizing its role in functional hyperemia [6,8]. The lack of SMTC effects on contracting hindlimb muscle blood flow in sedentary animals herein may also reflect the contraction intensity dependency of nNOS-mediated circulatory control. Accordingly, we demonstrated previously that nNOS blockade with SMTC in healthy sedentary rats reduces blood flow preferentially in glycolytic fast-twitch muscle fibers during near maximal exercise performed at supra-critical treadmill running speeds (i.e., approximately > 50 m/min; [8]). Despite these differences in experimental design, that blood lactate accumulation with SMTC during submaximal exercise herein was not different between Healthy + Sed and HF + Sed (Table 3) suggests that nNOS-mediated modulation of tissue metabolic control (possibly via its influence on the dynamic O₂ delivery-utilization matching; [5]) is not impacted significantly by HF. Taken together, results from the current intact conscious exercise model indicate that the biological role of nNOS-derived NO in the control of resting MAP and contracting muscle metabolism (as implied by blood lactate accumulation) is not distorted by moderate HF.

4.4. Selective nNOS inhibition with SMTC: impact of exercise training

Exercise training has been reported to enhance nNOS-mediated inhibition of sympathetic vasoconstriction (but intriguingly with no attendant increases in hindlimb blood flow) during electrically-induced muscle contractions in healthy rats [30]. Together with potential increases in muscle nNOS protein expression [50, cf. 58], training adaptations could ameliorate nNOS-related vascular dysfunction found previously in contracting HF muscle (albeit during artificial stimulation as mentioned above; [4]) and carry important clinical implications for this patient population. Those data thus served as the basis for our hypothesis for greater participation of nNOS-derived NO in the control of skeletal muscle blood flow during submaximal treadmill running after exercise training. Interestingly, the SMTC-induced changes in resting MAP and exercising blood lactate observed in Healthy + Sed and HF + Sed (Tables 2 and 3, respectively) were absent in Healthy + ExT and HF + ExT presumably denoting improved systemic vasoregulation (i.e., greater participation of alternate vasodilatory mechanisms) following exercise training in both health and disease. However, contrary to our hypothesis, SMTC had essentially no significant effects on exercising muscle blood flow in Healthy + ExT or HF + ExT (Fig. 3 and Table 4). As such, nNOS-derived NO does not appear to play an obligatory role in muscle hyperemia during submaximal locomotory exercise in any of the conditions evaluated presently. By subtraction, this suggests that training-induced increases in NO-induced vasodilation within contracting skeletal muscle likely emanate from enhanced eNOS function and/or decreased eNOS-derived NO scavenging.

During treadmill exercise, the SMTC-induced reductions in blood flow to the kidneys evident in Healthy + Sed rats were absent in the HF + Sed group (Fig. 4). This impaired nNOS function in the renal circulation is similar to that reported in our previous study in the anesthetized sedentary HF rat [4]. It is noteworthy that exercise training restored renal blood flow responses during exercise via nNOS adaptations in HF + ExT (Fig. 4) thereby offering potential therapeutic implications for the HF patient population.

4.5. Experimental considerations

Utilization of acute pharmacological nNOS inhibition as opposed to genetically modified models minimizes the potential for chronic alteration of other isoforms and/or vasodilatory pathways [cf. 34]. The efficacy and selectivity of nNOS inhibition with SMTC has been demonstrated previously in both humans and animals [4–8,16,22,52,61] and was reconfirmed presently in sedentary and trained healthy and HF rats. Accordingly, SMTC did not attenuate the hypotensive response to Ach (which is mediated largely by eNOS-derived NO) in any group whereas L-NAME blunted significantly that response when compared to both control and SMTC conditions (Fig. 5).

Previous studies from our laboratory indicate that nNOS inhibition with systemic SMTC administration in healthy sedentary rats evokes global hemodynamic effects (i.e., increases in MAP) without alterations in renal or lumbar sympathetic nerve discharge [7]. These results support that systemically administered SMTC modulates regional nNOS-mediated vascular control rather than centrally-derived sympathetic vasoconstriction.

Technical limitations related to the presence of radiolabeled microspheres precluded direct assessment of tissue/vascular nNOS enzyme expression herein. While the fiber type selective vasoregulation promoted by nNOS-derived NO is consistent with greater nNOS expression in predominantly glycolytic compared to oxidative rodent muscle [33,36], related data in humans are equivocal [15,40]. Nevertheless, considering that protein expression levels may not necessarily correspond to functional activity [47,54,58], one strength of the current study is evaluation of central and peripheral hemodynamic control *in vivo* during whole-body exercise in sedentary and trained healthy and diseased (HF) states.

4.6. Summary and conclusions

Pharmacological isoform-specific nNOS inhibition with SMTC had essentially no effects on hindlimb muscle blood flow during treadmill running in sedentary or trained healthy or HF rats. In marked contrast to the responses obtained previously during electrically-evoked small muscle mass contractions [4,5], SMTC administration during whole-body submaximal exercise revealed that nNOS-derived NO is not obligatory for the hyperemic response in any of the conditions evaluated herein. On the other hand, exercise training restored renal blood flow responses during exercise in HF + ExT largely via nNOS adaptations thereby presenting potential clinical implications for the chronic HF patient. These novel findings demonstrate the heterogeneous effects of exercise training and disease on nNOS function within various vascular beds. That contracting muscle blood flow adaptation with training or dysregulation with HF are not mediated via modulation of nNOS function provide important mechanistic insights into exercise (in)tolerance and the development/refinement of therapeutic strategies.

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Fig. 1. Schematic representation of the current experimental protocol. Ach, acetylcholine; L-NAME, N^G -nitro-L-arginine methyl ester; SMTC, S -methyl-L-thiocitrulline. See text for further details.

Fig. 2. Whole-body $\dot{V}O_{2\max}$ normalized to body mass in sedentary and trained healthy and HF rats. Healthy + Sed, healthy sedentary; Healthy + ExT, healthy exercise trained; HF + Sed, heart failure sedentary; HF + ExT, heart failure exercise trained. $p < .05$ vs. ^aHealthy + Sed; ^bHealthy + ExT; ^cHF + Sed.

Fig. 3. Absolute change in total hindlimb muscle blood flow (blood flow) elicited by selective nNOS inhibition with SMTC during submaximal treadmill running in sedentary and trained healthy and HF rats. Healthy + Sed, healthy sedentary; Healthy + ExT, healthy exercise trained; HF + Sed, heart failure sedentary; HF + ExT, heart failure exercise trained.

Fig. 4.

Effects of selective nNOS inhibition with SMTC on renal and splanchnic organ blood flow during submaximal treadmill running in sedentary and trained healthy (*top panels*) and HF (*bottom panels*) rats. Note the different scales used for left and right panels. Liver blood flow represents arterial, not portal, values. $p < .05$ vs. ^acontrol; ^bsedentary.

Fig. 5.

Effects of selective nNOS inhibition with SMTC and non-selective NOS inhibition with L-NAME on the relative hypotensive responses to acetylcholine (Δ MAP with Ach) in a subset of sedentary and trained healthy and HF rats. Healthy + Sed, healthy sedentary (n = 5); Healthy + ExT, healthy exercise trained (n = 6); HF + Sed, heart failure sedentary (n = 7); HF + ExT, heart failure exercise trained (n = 6). $p < .05$ vs. ^acontrol; ^bSMTC.

Table 1

Morphological and hemodynamic characteristics of sedentary and trained healthy and HF rats.

	Healthy + Sed	Healthy + ExT	HF + Sed	HF + ExT
Body mass, g	449 ± 4	462 ± 8	452 ± 15	483 ± 14
Hindlimb mass, g	19 ± 1	20 ± 1	20 ± 1	21 ± 1
Infarct size, %	–	–	31.8 ± 1.0	27.3 ± 1.9
LVEDP, mmHg	7 ± 1	7 ± 1	12 ± 1 ^{ab}	16 ± 2 ^{ab}
LV dp/dt, mmHg/s	7375 ± 286	7150 ± 135	6500 ± 157 ^{ab}	5750 ± 278 ^{abc}
LV/body mass, mg/g	2.24 ± 0.10	2.21 ± 0.06	2.38 ± 0.07	2.35 ± 0.04
RV/body mass, mg/g	0.63 ± 0.03	0.62 ± 0.03	0.65 ± 0.03	0.70 ± 0.05
Lung/body mass, mg/g	5.60 ± 0.93	5.44 ± 0.74	5.23 ± 0.94	5.12 ± 0.75

LVEDP, left ventricular end-diastolic pressure; LV dp/dt, left ventricle pressure/ time; RV, right ventricle. Healthy + Sed, healthy sedentary; Healthy + ExT, healthy exercise trained; HF + Sed, heart failure sedentary; HF + ExT, heart failure exercise trained. $P < .05$ vs.

^aHealthy + Sed;

^bHealthy + ExT;

^cHF + Sed.

Effects of selective nNOS inhibition with SMTC on mean arterial pressure (MAP) and heart rate (HR) at rest and during submaximal treadmill running in sedentary and trained healthy and HF rats.

Table 2

	Healthy + Sed		Healthy + ExT		HF + Sed		HF + ExT	
	Control	SMTC	Control	SMTC	Control	SMTC	Control	SMTC
Rest								
MAP, mmHg	132 ± 5	142 ± 5 ^a	125 ± 3	133 ± 4	121 ± 5	129 ± 8 ^a	127 ± 3	128 ± 6
HR, beats/min	447 ± 11	405 ± 17	431 ± 10	393 ± 10	426 ± 17	394 ± 14	472 ± 30	481 ± 48 ^c
Exercise								
MAP, mmHg	145 ± 6 ^b	144 ± 5	136 ± 3 ^b	143 ± 4 ^b	134 ± 2 ^b	137 ± 7	133 ± 1	140 ± 3 ^b
HR, beats/min	533 ± 14 ^b	530 ± 12 ^b	514 ± 9 ^b	500 ± 13 ^b	539 ± 13 ^b	544 ± 13 ^b	529 ± 19	500 ± 12

Healthy + Sed, healthy sedentary; Healthy + ExT, healthy exercise trained; HF + Sed, heart failure exercise trained. $P < .05$ vs.

^a control;

^b rest;

^c Healthy + Sed, Healthy + ExT, HF + Sed.

Effects of selective nNOS inhibition with SMTC on arterial blood O₂ saturation, pH and lactate concentration during submaximal treadmill running in sedentary and trained healthy and HF rats.

Table 3

	Healthy + Sed		Healthy + ExT		HF + Sed		HF + ExT	
	Control	SMTC	Control	SMTC	Control	SMTC	Control	SMTC
O₂ saturation, %	98.0 ± 0.5	97.8 ± 0.8	98.6 ± 0.2	98.8 ± 0.2	97.9 ± 0.1	97.9 ± 0.2	98.2 ± 0.2	98.4 ± 0.2
pH	7.47 ± 0.01	7.46 ± 0.01	7.51 ± 0.01	7.50 ± 0.01	7.46 ± 0.01	7.44 ± 0.01	7.49 ± 0.01	7.48 ± 0.02
Lactate, mmol/l	4.4 ± 0.4	5.7 ± 0.5 ^a	1.4 ± 0.2 ^b	2.0 ± 0.3 ^b	3.6 ± 0.5	5.2 ± 0.8 ^a	1.7 ± 0.3 ^b	2.8 ± 0.7 ^b

Healthy + Sed, healthy sedentary; Healthy + ExT, healthy exercise trained; HF + Sed, heart failure sedentary; HF + ExT, heart failure exercise trained. $P < .05$ vs.

^a control;

^b sedentary.

Effects of selective nNOS inhibition with SMTC on individual hindlimb muscle or muscle part blood flow (ml/min/100 g) during submaximal treadmill running in sedentary and trained healthy and HF rats.

Table 4

	Healthy + Sed		Healthy + ExT		HF + Sed		HF + ExT	
	Control	SMTC	Control	SMTC	Control	SMTC	Control	SMTC
Ankle extensors								
Soleus	256 ± 18	227 ± 36	305 ± 23	275 ± 15	261 ± 22	229 ± 26	308 ± 30	236 ± 39
Plantaris	220 ± 23	213 ± 29	225 ± 29	228 ± 27	236 ± 10	219 ± 25	242 ± 24	209 ± 37
Gastrocnemius, red	393 ± 34	399 ± 52	438 ± 52	394 ± 61	407 ± 30	405 ± 60	415 ± 39	363 ± 59
Gastrocnemius, white	43 ± 6	47 ± 14	23 ± 4	19 ± 4	44 ± 6	47 ± 9	36 ± 7	33 ± 5
Gastrocnemius, mixed	136 ± 16	138 ± 20	143 ± 16	137 ± 15	154 ± 6	159 ± 22	141 ± 12	140 ± 23
Tibialis posterior	172 ± 22	169 ± 23	134 ± 24	130 ± 15	164 ± 20	127 ± 24	129 ± 20	120 ± 25
Flexor digitorum longus	111 ± 19	134 ± 49	66 ± 19	41 ± 6	89 ± 18	80 ± 11	91 ± 19	70 ± 15
Flexor hallucis longus	95 ± 18	94 ± 20	53 ± 12	69 ± 13	89 ± 7	89 ± 10	56 ± 12	60 ± 9
Ankle flexors								
Tibialis anterior, red	370 ± 24	353 ± 48	385 ± 33	338 ± 24	428 ± 16	344 ± 44	358 ± 53	301 ± 19
Tibialis anterior, white	135 ± 13	134 ± 19	121 ± 13	100 ± 13	154 ± 8	130 ± 20	114 ± 24	98 ± 11
Extensor digitorum longus	63 ± 5	90 ± 24	69 ± 15	68 ± 12	87 ± 16	59 ± 6	52 ± 6	64 ± 9
Peroneals	135 ± 13	127 ± 20	124 ± 21	98 ± 16	149 ± 14	118 ± 12	117 ± 18	96 ± 11
Knee extensors								
Vastus intermedius	327 ± 19	312 ± 30	380 ± 31	350 ± 35	352 ± 33	284 ± 30	409 ± 33	388 ± 42
Vastus medialis	145 ± 20	133 ± 17	132 ± 24	128 ± 15	157 ± 16	138 ± 26	156 ± 41	182 ± 65
Vastus lateralis, red	298 ± 28	285 ± 26	413 ± 36	375 ± 40	357 ± 8	327 ± 37	381 ± 53	377 ± 53
Vastus lateralis, white	51 ± 13	52 ± 14	20 ± 4	33 ± 13	47 ± 7	42 ± 8	29 ± 7	34 ± 6
Vastus lateralis, mixed	153 ± 15	144 ± 16	184 ± 16	179 ± 19	194 ± 14	172 ± 24	182 ± 23	183 ± 20
Rectus femoris, red	246 ± 24	215 ± 17	252 ± 39	218 ± 34	282 ± 28	239 ± 35	302 ± 39	282 ± 52
Rectus femoris, white	120 ± 12	118 ± 13	96 ± 14	88 ± 9	142 ± 6	119 ± 15	112 ± 17	113 ± 19
Knee flexors								
Biceps femoris, anterior	39 ± 9	51 ± 13	39 ± 7	42 ± 7	46 ± 8	53 ± 10	57 ± 11	49 ± 8
Biceps femoris, posterior	60 ± 8	69 ± 12	37 ± 5	35 ± 5 ^b	86 ± 8	77 ± 13	58 ± 7	50 ± 6
Semitendinosus	54 ± 6	54 ± 8	42 ± 9	39 ± 7	74 ± 8 ^c	52 ± 6 ^a	37 ± 5 ^b	25 ± 3 ^b

	Healthy + Sed		Healthy + ExT		HF + Sed		HF + ExT	
	Control	SMTC	Control	SMTC	Control	SMTC	Control	SMTC
Semimembranosus, red	96 ± 19	105 ± 22	58 ± 8	60 ± 7	127 ± 28	124 ± 19	87 ± 16	86 ± 16
Semimembranosus, white	28 ± 8	35 ± 10	14 ± 3	16 ± 3	41 ± 12	37 ± 7	18 ± 4	20 ± 3
Thigh adductors								
Adductor longus	317 ± 48	278 ± 38	208 ± 10	170 ± 21	283 ± 35	205 ± 28 ^a	231 ± 34	162 ± 24 ^a
Adductor magnus & brevis	79 ± 9	89 ± 22	53 ± 8	52 ± 5	94 ± 12	66 ± 7	82 ± 30	79 ± 32
Gracilis	33 ± 8	41 ± 11	20 ± 4	18 ± 4	38 ± 8	25 ± 2	24 ± 6	21 ± 6
Pectineus	52 ± 12	83 ± 25	30 ± 6	20 ± 3	59 ± 18	49 ± 8	41 ± 10	39 ± 16

Healthy + Sed, healthy sedentary; Healthy + ExT, healthy exercise trained; HF + Sed, heart failure sedentary; HF + ExT, heart failure exercise trained. *P* < .05 vs.

^a control;

^b sedentary;

^c healthy.