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Effects of chronic nitric oxide synthase inhibition on responses to acute exercise in swine

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Abstract

Nitric oxide (NO) is potentially involved in several responses to acute exercise. We tested the hypotheses that inhibition of NO formation reduces maximal O₂ delivery to muscle, but does not affect O₂ utilization by muscle, therefore lowering maximal O₂ consumption. To test these hypotheses, swine (~30 kg) drank either tap water (Con, $n = 25$) or water with N^G-nitro-L-arginine methyl ester ($8.0 \pm 0.4 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for ≥ 4 wk; LN, $n = 24$). Treatment efficacy was reflected by higher mean arterial pressure and lower plasma NO metabolite concentration in LN than Con (both $P < 0.05$). Swine completed two graded treadmill running tests to maximum. In the first test, O₂ consumption was determined at rest through maximal exercise intensity. O₂ consumption did not differ between groups at rest or at most exercise intensities, including maximum (Con, $40.8 \pm 1.8 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$; LN, 40.4 ± 2.9 ; not significant). In the second test, tissue-specific blood flows were determined using the radiolabeled-microsphere technique. At rest, blood flows were lower ($P < 0.05$) in LN compared with Con for a number of tissues, including kidney, adrenal, lung, and several skeletal muscles. During both submaximal and maximal exercise, however, blood flows were similar between Con and LN for all 16 muscles examined; only blood flows to kidney (Con, $99 \pm 16 \text{ ml} \cdot \text{min}^{-1} \cdot 100 \text{ g}$; LN, 55 ± 15 ; $P < 0.05$) and pancreas (Con, 25 ± 7 ; LN, 6 ± 2 ; $P < 0.05$) were lower in LN at maximum. Endothelium-dependent, but not -independent, relaxation of renal arterial segments was reduced ($P < 0.05$) in vitro. These data indicate that exercise-induced increases in muscle blood flows are maintained with chronic inhibition of NO formation and that maximal O₂ consumption is therefore preserved. Redundant vasodilatory pathways and/or upregulation of these pathways may underlie these findings.

Keywords

exercise; blood flow; oxygen consumption; muscle; kidney

Many additional roles have been ascribed to nitric oxide (NO) in physiological function since the discovery of its involvement in endothelium-dependent vasodilation by Furchgott and Zawaski (11) more than 25 yr ago. Among these roles are two that may directly impact performance of exercise, vasodilation and modulation of O₂ consumption by cardiac and skeletal muscle. The former would impact O₂ delivery, whereas the latter could affect O₂ utilization. Effect(s) on either or both of these factors could, in turn, alter whole body O₂ consumption during exercise.

Available data are equivocal concerning the role of endothelium-dependent, NO-mediated dilation in the cardiovascular response to acute exercise; this controversy has been the subject of several recent reviews (e.g., 5). Nonetheless, by virtue of the vasodilatory action of NO, inhibition of its formation could lower maximal O₂ delivery to muscle. Interestingly, for cardiac muscle, the literature uniformly indicates that NO is not required for exercise-induced increases in coronary blood flow (1, 4, 28, 30, 37); in the skeletal muscle circulation, available data are less uniform. Data from studies involving rodents have implicated NO in the hyperemic response to exercise (7, 14, 20). Studies involving larger animals have frequently (8, 26, 30), although not exclusively (31, 33), not supported a role for NO in exercise-induced hyperemia. Studies in humans are also equivocal, with some lending support for (e.g., 13) but others not supporting (e.g., 9) a role for NO in exercise hyperemia within large skeletal muscle masses. A limitation of most of these studies has been that only submaximal exercise intensities were examined; a role for NO may only be revealed at maximal exercise where demand for O₂ delivery is high. Another consideration is that these studies have utilized acute inhibition of NO formation in an attempt to elucidate the role(s) for NO in the response to acute exercise. While acute inhibition is experimentally sound, chronic inhibition may more closely mimic the in vivo condition; for example, in its early stages, the chronic disease atherosclerosis is characterized by reduced NO formation in the vascular endothelium (38). This reduction in endothelium-dependent, NO-mediated vasodilation likely contributes to abnormal responses to acute exercise exhibited by atherosclerotic patients (10). Thus a model in which NO formation is chronically inhibited may more closely resemble human patient populations than one in which NO synthesis is acutely inhibited.

The literature is also equivocal regarding the role of NO in modulating O₂ utilization. Originally reported by Hintze and colleagues (32) for resting cardiac muscle, this hypothesis asserts that NO exerts a tonic inhibition of O₂ utilization by mitochondria. Thus, with inhibition of NO formation, this restraint of mitochondrial function is reduced and O₂ utilization therefore increases. Studies involving whole body exercise are few in number, as well as mixed in their findings. Shen and coworkers reported that canine hindquarter O₂ consumption across a range of treadmill running speeds was increased after acute inhibition of NO synthesis (31). Interestingly, the same group found that myocardial O₂ consumption across the same range of running speeds was unchanged after NO formation was inhibited (4). Duncker and colleagues (8) reported unchanged whole body O₂ consumption after acute inhibition of NO formation in swine running on a treadmill at several different intensities, as did Frandsen and coworkers (9) for leg O₂ consumption in humans performing knee extensor exercise.

Based on the aforementioned literature, we hypothesized that NO has an important role in the vasodilatory response to whole body exercise and, therefore, O₂ delivery to cardiac and skeletal muscle, particularly at maximal exercise intensity. Second, we hypothesized that, at maximal exercise, NO does not modulate tissue O₂ utilization. Thus reduced O₂ delivery and unchanged O₂ utilization produced by inhibition of NO formation was hypothesized to cause a reduction in maximal whole body O₂ consumption. To test these hypotheses, we used a porcine model that we subjected to chronic inhibition of NO synthesis, reasoning that a large animal treated chronically with a NO synthase inhibitor would better model some features of human atherosclerosis. Moreover, we determined tissue blood flow and O₂ consumption responses to a wide range of exercise intensities, including maximum, to more completely examine potential roles for NO in whole body responses to exercise.

METHODS

Animals and treatment

Female Yucatan miniature swine were studied in four series. In each series, swine were randomly assigned to either a control group or a group that was chronically administered *N*^G-nitro-L-arginine methyl ester (L-NAME; Fisher Scientific). Control swine (Con; 30.7 ± 0.9 kg, total $n = 25$) drank tap water, whereas treated swine (LN; 30.2 ± 0.9 kg, total $n = 24$; not significant vs. Con) drank water with 10 mg L-NAME/100 ml water. Treatment duration was ≥ 4 wk. Daily water consumption was measured for 7 days preceding each animal's terminal experiment to determine individual L-NAME dose. These treatments and other procedures (see below) were approved by the Animal Care and Use Committee of the University of Missouri.

Assessment of treatment efficacy

Effectiveness of chronic L-NAME treatment was assessed by determining resting mean arterial pressure and plasma NO metabolite (NO_x) concentration. Mean arterial pressure was measured via an internal thoracic arterial catheter (see below) connected to a pressure transducer and blood pressure analyzer (model BPA, MicroMed). Plasma NO_x concentration was determined via VCl_3 -induced reduction of NO_x to NO and its subsequent reaction with O_3 , producing chemiluminescence (model NOA 280i, Sievers).

Determination of O_2 consumption

Each animal underwent two identical graded exercise tests to maximum on consecutive days. Graded exercise consisted of 5 min each of treadmill running at 2.0, 3.2, 5.0, and 7.1 miles/h (all 0% grade), as well as 7.1 miles/h (5% grade) and 7.1 miles/h (10% grade). After each stage (excluding 2.0 miles/h), 5 min of walking at 2.0 miles/h was allowed.

During the first exercise test, whole body O_2 consumption was determined using a modification of the method of Armstrong and colleagues (3). A plastic mask was fitted over the animal's nose and mouth using straps attached to a neck strap. This mask was carefully inspected for cracks pre- and posttest, and, if found, data from that test were excluded from analysis. Expired air was pulled from the mask using a vacuum (Fuller). Vacuum rate (in l/min) was set to 10 times body weight (i.e., well in excess of porcine expired ventilatory rate) using a variable transformer (model AEEC, Jameco Electronics) and an in-line flowmeter (model FL-400A, Omega). Vacuumed air was sampled for O_2 concentration determination using an O_2 analyzer (model S-3A/1, Applied Electrochemistry). Sampled air was passed through a chamber containing Drierite (Fisher Scientific) and Ascarite (Sigma-Aldrich) to remove H_2O and CO_2 , respectively, before entering the O_2 analyzer. O_2 consumption was calculated as follows:

$$\text{O}_2 \text{ consumption} = \text{vacuum rate} \times ([\text{inspired O}_2] - [\text{expired O}_2])$$

Temperature and barometric pressure were recorded before each test, O_2 consumption was corrected to STPD, and brackets denote concentration.

Determination of tissue blood flows

Surgery to implant catheters in the left atrium and internal thoracic artery was conducted 8–10 days before exercise tests. To implant the left atrial catheter, after cutting the sternabrae on the midline and opening the pericardium, the tip of the left atrium was incised while its base was clamped. The catheter (Micro-Renathane; outside diameter, 0.095 in.; Braintree Scientific) was advanced ~5 cm into the atrium, and then it was secured to the atrial wall

using a purse-string suture. A short loop of catheter was left in the pericardium as the latter was closed. To implant the internal thoracic arterial catheter, the artery was isolated in the cranial thorax. After incising the artery, the catheter (Micro-Renathane; outside diameter, 0.080 in.; Braintree Scientific) was advanced cranially to the junction of the internal thoracic artery and aorta and secured. After closing the sternabrae using stainless steel sutures, the catheters were tunneled subcutaneously using a trocar from the thoracic inlet to a position between the scapulae. At the exit site, the catheters were wrapped, secured to the dorsal surface, and covered with a jacket until the day of exercise testing with blood flow determinations. Routine postoperative care was provided, and each animal was familiarized with treadmill running while wearing the O₂ consumption mask for 4–5 days before its first exercise test.

On the day following O₂ consumption testing, a second identical graded exercise test was administered. Mean arterial pressure was continuously monitored via the internal thoracic arterial catheter, except while reference blood samples were being withdrawn (see below). The radiolabeled-microsphere technique was used to determine tissue blood flows while the animal stood on the treadmill before exercise, as well as during submaximal exercise (5.0 miles/h) and at maximal exercise, as done previously (3, 24). Briefly, for each blood flow determination microspheres labeled with ⁴⁶Sc, ⁵¹Cr, ¹⁰³Ru, or ¹⁴¹Ce (15 µm diameter; Perkin Elmer) in 1.0 ml of saline with Tween 80 were infused via the left atrial catheter. Microspheres were sonicated and vortexed before infusion; their infusion was followed by a 10-ml saline flush. A reference blood sample was obtained simultaneously via the internal thoracic arterial catheter using a syringe pump (Harvard Apparatus). The rate of withdrawal of this reference blood sample was 4.12 ml/min. Efficacy of microsphere-blood mixing in the left atrium was assessed by comparing bilateral blood flows to the kidneys and adrenals. A difference of ≤15% was considered acceptable. After completion of the second exercise test, the animal was anesthetized (Thiopental, 10 mg/kg) and then euthanized by removal of the heart. Tissue samples were then dissected, weighed, and counted in a gamma counter (model CompuGamma CS; LKB Wallac), and tissue blood flows were calculated using standard procedures (3, 24). For each tissue, conductance was calculated as the quotient of blood flow and mean arterial pressure. Viscera sampled included the liver, spleen, pancreas, stomach, jejunum, colon, kidneys, and adrenals. Forelimb skeletal muscles sampled included the triceps brachii lateral head [Tlth; both deep (D) and superficial (S) portions], triceps brachii accessory head (TAH), triceps brachii medial head (TMH), triceps brachii long head (TLH; both D and S portions), biceps brachii (Bic Br), and brachialis (Brach). Hindlimb muscles sampled included the vastus lateralis (VL; both D and S portions), vastus intermedius (VI), vastus medialis (VM), rectus femoris (RF; both D and S portions), biceps femoris (Bic Fem), and semitendinosus (Semit). In addition, the left and right ventricles, diaphragm, and lung were sampled.

Vasomotor responses of renal arteries

Renal arterial segments were prepared for determination of vasomotor responses in vitro as done previously (22). Briefly, the renal artery was cleaned of fat and connective tissue and cut into 4 segments ~3 mm in axial length. Axial length, as well as outside and inside diameters, were determined. Each renal segment was then mounted in a vessel rig (Global-town Microtech) containing 20 ml of standard Krebs bicarbonate buffer solution, with two wires passing through the segment's lumen. One wire was connected to a force transducer and the other to a micrometer microdrive. The former, in conjunction with a data acquisition system (Powerlab), allowed for continuous monitoring of isometric force; the latter permitted stretching of the vascular segment by 10% (of passive outside diameter) increments. Segments were stretched to optima of their individual length (stretch)-developed tension relationships by repeated exposures to KCl (60 mM) at progressively increasing

stretch. Optimal stretch was indicated by an increase of $\leq 5\%$ in developed tension with a 10% increase in stretch. All subsequent vasomotor responses were determined at optimal stretch. Krebs bicarbonate buffer solution bathing each renal segment was maintained at 37°C and pH 7.4, the latter via bubbling with a 95% O_2 -5% CO_2 gas mixture.

Concentration-response (isometric tension) relationships were determined for several vasorelaxing agents, including bradykinin (BK; 1×10^{-11} to 1×10^{-6} M, in half-log increments), acetylcholine (ACh; 1×10^{-10} to 1×10^{-4} M, in half-log increments), and sodium nitroprusside (SNP; 1×10^{-10} to 1×10^{-4} M, in whole-log increments). BK and ACh were tested as endothelium-dependent agents, whereas SNP was tested as an endothelium-independent agent. Before each relationship, renal arterial segments were contracted with 3×10^{-5} M $\text{PGF}_{2\alpha}$. Contractile force development before all concentration-response relationships was similar for Con and LN. For each concentration-response relationship, one segment was not exposed to any endothelial inhibitor, whereas the remaining three segments were incubated with 3×10^{-4} M L-NAME, 5×10^{-6} M indomethacin (a cyclooxygenase inhibitor), and both L-NAME and indomethacin, all for 30 min before $\text{PGF}_{2\alpha}$ administration.

Immunohistochemistry

Immunohistochemical analyses for endothelial NO synthase (eNOS), cyclooxygenase-1 (COX-1), and cyclo-oxygenase-2 (COX-2) were performed as described in detail previously (12). Sections of renal artery (5 μm in thickness) were incubated overnight with primary antibodies against eNOS (1:800 dilution; BD Transduction), COX-1 (1:400 dilution; Santa Cruz Biotechnology), and COX-2 (1:800; Cayman Chemical). Appropriate positive controls were incubated in parallel.

Immunoblotting

Expression of COX-1 and -2 protein was determined for endothelial extracts of contralateral renal arteries to those for which vasomotor responses were determined. Protein expression was determined via immunoblotting, as described in detail previously (12). Primary antibodies for COX-1 (Santa Cruz Biotechnology) and -2 (Cayman Chemical) were diluted 1:1,000 and 1:500, respectively.

Biochemical determinations

Plasma concentrations of total cholesterol, high-density lipoprotein cholesterol, and triglyceride, as well as hemoglobin and hematocrit, were determined using previously described methods (36). Citrate synthase activity was determined using the method of Srere (34) adapted for porcine skeletal muscle (23).

Statistical analysis

All data are presented as means $\pm$ SE. For most variables, Con and LN were compared using independent *t*-tests (35). In addition, O_2 consumption data for each animal within the treadmill running intensity range for which intensity was a continuous variable (i.e., 2.0–7.1 miles/h, all 0% grade) were subjected to two different analyses. First, two-way ANOVA, with repeated measures on running intensity, was used to compare Con and LN (35). Second, linear regression analysis (35) was conducted, and slopes of regression lines for individual Con and LN swine were then compared using the independent *t*-test. Relaxation responses to BK, ACh, and SNP were also analyzed using two-way ANOVA, with repeated measures on vasorelaxing agent concentration. The Scheffé's test was used to compare Con and LN at individual agent concentrations after ANOVA revealed an overall difference between Con and LN (35). For all analyses, $P < 0.05$ was considered significant.

RESULTS

Treatment efficacy

Swine in the LN group consumed $8.0 \pm 0.4 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ ($n = 24$) L-NAME via their drinking water. This was associated with increased mean arterial pressure at rest (Con, $87 \pm 5 \text{ mmHg}$, $n = 9$; LN, $118 \pm 7 \text{ mmHg}$, $n = 8$; $P < 0.05$) and reduced plasma NO_x concentration (Con, $12.5 \pm 1.9 \text{ } \mu\text{M}$, $n = 21$; LN, $8.1 \pm 1.2 \text{ } \mu\text{M}$, $n = 19$; $P < 0.05$) in LN compared with Con. Despite elevated mean arterial pressure, no cardiac hypertrophy was observed (Con, $5.87 \pm 0.23 \text{ g/kg}$, $n = 23$; LN, $5.54 \pm 0.21 \text{ g/kg}$, $n = 22$; not significant). Blood lipids (Table 1), including total cholesterol, high-density lipoprotein cholesterol, and triglyceride, did not differ between Con and LN. Neither hematocrit nor hemoglobin differed between groups (Table 1). LN consumed $2,448 \pm 170 \text{ ml}$ drinking water/day ($n = 24$), while a subset of Con drank a similar daily volume ($3,008 \pm 969 \text{ ml/day}$, $n = 5$; not significant vs. LN).

O₂ consumption

In swine for which O₂ consumption was determined, data were excluded for a total of 7 swine (2 Con, 5 LN) due to technical difficulties (e.g., mask cracking). Resting O₂ consumption was similar between groups (Con, $7.1 \pm 1.1 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, $n = 12$; LN, $6.7 \pm 1.4 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, $n = 11$; not significant). Figure 1A shows O₂ consumption as a function of treadmill running intensity. O₂ consumption across the range of running intensities did not differ between groups. In addition, slope of the O₂ consumption-running intensity relationship over the 2.0–7.1 miles/h (all 0% grade) range did not differ between groups (Con, $2.71 \pm 0.21 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} \cdot \text{miles}^{-1} \cdot \text{h}^{-1}$, $n = 17$; LN, $2.70 \pm 0.43 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} \cdot \text{miles}^{-1} \cdot \text{h}^{-1}$, $n = 13$; not significant). O₂ consumption at 7.1 miles/h, 5% grade also did not differ between groups (Con, $38.5 \pm 1.6 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, $n = 15$; LN, $34.6 \pm 3.1 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, $n = 6$; not significant); at 7.1 miles/h, 10% grade, O₂ consumption was lower in LN (Con, $43.4 \pm 2.1 \text{ ml/min}^{-1} \cdot \text{kg}^{-1}$, $n = 8$; LN, 34.0 ± 3.8 , $n = 5$; $P < 0.05$). This difference at the latter running intensity was primarily due to a difference in power (Con, $100.9 \pm 5.3 \text{ W}$; LN, 85.0 ± 6.3 ; $P < 0.05$), in turn due to lower body weights of LN that achieved this intensity. Maximal O₂ consumption, irrespective of intensity at which it was attained, did not differ between groups (Fig. 1B).

Tissue blood flows at rest

Figures 2A, 3A, and 4A illustrate blood flows to viscera, forelimb skeletal muscles, and hindlimb muscles, respectively, before treadmill exercise. Kidney and adrenal blood flows, as well as those to certain muscles of the fore- and hindlimbs, were lower in LN than Con. Resting left and right ventricular blood flows, as well as blood flow to the diaphragm, did not differ between groups; lung blood flow was less in LN than in Con (Table 2). Because mean arterial pressure was elevated in LN, conductance was reduced in a number of tissues at rest, including liver, pancreas, kidney, and adrenal, as well as left and right ventricles, lung, TAH, TMH, VL/D, VL/S, VI, VM, and RF/D (data not shown).

Tissue blood flows during exercise

Figures 2B, 3B, and 4B illustrate blood flows to viscera, forelimb skeletal muscles, and hindlimb muscles, respectively, during treadmill running at 5.0 miles/h. This intensity was submaximal, requiring 75–80% of maximal O₂ consumption (Fig. 1). Mean arterial pressure was greater in LN (Con, $112 \pm 4 \text{ mmHg}$, $n = 8$; LN, 148 ± 9 , $n = 4$; $P < 0.05$). As expected, visceral blood flows decreased from resting levels (Fig. 2B vs. Fig. 2A), and muscle blood flows increased over those at rest (Figs. 3B and 4B vs. Figs. 3A and 4A, respectively) in both Con and LN. There was, however, only one muscle (Brach) and no viscera for which blood

flow differed between Con and LN. Similarly, diaphragm and left and right ventricular blood flows did not differ between groups (Table 2). Conductance was lower in LN compared with Con for only left ventricle, lung, and VI (data not shown).

Figures 2C, 3C, and 4C illustrate blood flows to viscera, forelimb skeletal muscles, and hindlimb muscles, respectively, during treadmill running at maximum. Mean arterial pressure was greater in LN (Con, 126 ± 11 mmHg, $n = 5$; LN, 160 ± 4 , $n = 3$; $P < 0.05$). Similar to findings for submaximal exercise, blood flows did not differ between Con and LN for most tissues. Blood flows to the left and right ventricles, as well as the diaphragm and lung, were similar between groups (Table 2). Chronic L-NAME treatment only reduced conductance in pancreas and kidney at maximum (data not shown).

Vasomotor responses

Because renal conductance at rest was reduced by ~45% in LN compared with Con, by nearly 25% ($P = 0.06$) during submaximal exercise, and by ~60% at maximal exercise intensity, we conducted follow-up experiments in which relaxation responses of renal arterial segments were determined in vitro. These segments were of similar dimensions for Con and LN (Table 3). Chronic L-NAME treatment reduced dose-dependent relaxation responses to the endothelium-dependent agent BK (Fig. 5A). Acute treatment with L-NAME reduced ($P < 0.05$) responses to BK in Con (all concentrations $\geq 1 \times 10^{-9}$ M; Fig. 5B vs. Fig. 5A). In LN, acute L-NAME administration reduced the response to BK only at the highest concentration tested (1×10^{-6} M). Thus Con and LN did not differ with L-NAME present in vitro. Conversely, acute treatment with indomethacin reduced responses to BK in LN ($P < 0.05$; Fig. 5C vs. Fig. 5A at all concentrations $\geq 3 \times 10^{-8}$ M), but not Con, such that groups differed ($P < 0.05$) across the range of BK concentrations tested. Combined acute treatment with L-NAME and indomethacin resulted in blunted, but similar, relaxation responses to BK in Con and LN (Fig. 5D). Relaxation responses to ACh, another endothelium-dependent agent, did not differ significantly between Con and LN, either without or with endothelial inhibitors present (Fig. 6). There was, however, a tendency for responses to ACh in LN to be less than those of Con with indomethacin present ($P = 0.07$; Fig. 6C), as was the case with BK. Relaxation responses to the endothelium-independent agent SNP did not differ between groups under any condition in vitro (Fig. 7, A–D).

Immunohistochemistry

Figure 8 shows immunohistochemical analysis for COX-1 in renal arteries from Con and LN. COX-1 protein expression was demonstrated in endothelium of renal arteries from both Con and LN (Fig. 8). COX-2 was not detected in any vessels examined; as expected, eNOS expression was demonstrated in endothelium of all renal arteries examined (not shown).

Immunoblotting for COX-1 and -2

Figure 9 shows data for expression of the proteins COX-1 and -2 in renal artery endothelium. COX-1 expression tended ($P = 0.09$) to be greater in LN than Con; COX-2 expression did not differ between groups ($P > 0.10$).

Citrate synthase activity

Activity of citrate synthase, a marker enzyme for mitochondrial content, was determined in several skeletal muscles. Citrate synthase activity did not differ (all $P > 0.10$) between Con ($n = 8$) and LN ($n = 9$) for any muscle examined, including TAH (Con, 14.7 ± 1.1 ; LN, 13.6 ± 0.5), TMH (Con, 14.9 ± 0.7 ; LN, 15.5 ± 0.8), TLH (Con, 18.6 ± 1.1 ; LN, 19.5 ± 1.3), and deltoid (Con, 16.9 ± 0.5 ; LN, 17.6 ± 1.1).

DISCUSSION

Key new findings of this study were that cardiac and skeletal muscle blood flows during maximal exercise, as well as conductance within these tissues, were predominantly normal after chronic inhibition of NO formation. These findings suggest that compensation for inhibition of NO formation occurred, via either redundant, preexisting dilatory pathways in the vasculature of these tissues or an upregulation of one or more of these pathways. The kidney, however, exhibited reduced blood flow and conductance. Another important finding of this study was that whole body O_2 consumption was not altered, either at rest or during exercise, by chronic inhibition of NO synthesis. These findings improve our understanding of the roles for NO in the response to acute exercise by demonstrating that NO is not obligatory for maximal hyperemic responses in cardiac and skeletal muscle. In addition, our data indicate that whereas NO may modulate O_2 consumption in certain tissues, it does not appear to impact whole body O_2 consumption.

Treatment efficacy

We found that chronic administration of L-NAME via drinking water was associated with increased mean arterial pressure and reduced plasma NO_x concentration at rest. The former finding has been reported for studies involving rodents (18, 27); to our knowledge, the present study is the first one to demonstrate hypertension with chronic inhibition of NO formation in a large-animal model. Plasma NO_x concentration, another indicator of effective inhibition of NO synthesis, was lower in LN. Furthermore, superimposing acute L-NAME administration on a background of chronic L-NAME treatment had minimal additional effect on endothelium-dependent relaxation of the renal artery in vitro. Our laboratory has previously reported similar observations for arteries of the coronary (17) and skeletal muscle circulations (25). Our mean daily dose of L-NAME was somewhat less than those used in acute studies (typically $20-30 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) because our swine were intolerant of higher doses, as previously reported for dogs. In that study, anorexia and weight loss (~10%) accompanied inhibition of NO formation for 1 wk (28). Nonetheless, the above lines of evidence indicate that our model provides a useful test of the importance of NO in cardiorespiratory responses to exercise. It is important to note that L-NAME treatment did not alter any blood lipid concentrations, because it is well known that some blood lipids modulate eNOS activity.

NO and muscle blood flow

Because skeletal muscle comprises a significant fraction of body mass and therefore must make an important contribution to systemic blood pressure, we anticipated that L-NAME-induced hypertension would be associated with decreased muscle blood flow. Although some fore- and hindlimb muscles that we sampled indeed exhibited lower blood flows in LN than Con at rest, a majority had normal resting blood flow and conductance values. Furthermore, none of the 16 muscles/muscle sections that we sampled exhibited impaired blood flow during treadmill exercise at either 5.0 miles/h (submaximal) or at maximum. Interestingly, most muscles with lower blood flows at rest after chronic inhibition of NO formation, including the TMH, VI, and VM, are composed of significant numbers of slow-twitch, high-oxidative fibers that are recruited for posture (3). Our determinations of resting blood flows were made while swine stood quietly on the treadmill prior to exercise, presumably recruiting postural muscles. In rats, muscles of this fiber-type composition have been reported to exhibit robust endothelium-dependent, NO-mediated vasodilation (21). Our findings in swine may therefore be indicative of a role for NO in regulation of blood flow to slow oxidative skeletal muscle at rest.

During exercise, however, NO was clearly not obligatory for skeletal muscle perfusion in our swine. This likely contributes to the finding that maximal O₂ consumption was unchanged with L-NAME treatment, because neither blood flow to any muscle examined nor arterial O₂ content differed between Con and LN at maximal exercise. Furthermore, although we did not measure arterial-venous O₂ difference during maximal exercise, citrate synthase activity for several muscles was not different between groups. This variable is an established index of mitochondrial content and, therefore, the potential for O₂ extraction and utilization (15).

Although our findings are at odds with those from some studies that have demonstrated that inhibition of NO synthesis reduces skeletal muscle blood flow during exercise, those studies involved acute administration of L-NAME (13, 14, 20, 31, 33). It may be that our prolonged treatment period permitted vascular adaptations to occur that compensated for inhibition of NO formation. Indeed, we have found that endothelium-dependent dilatory responses of skeletal muscle 2A arterioles from chronically L-NAME-treated swine are normal (25), as has been reported for similarly treated rats (39). These resistance vessels are important determinants of muscle blood flow. Other studies, however, have found no effects of inhibition of NO formation on muscle blood flow, in agreement with the present findings (8, 9, 26, 30). Our study extends those studies by examining muscle blood flow responses to maximal exercise; indeed, the present study is the first to determine muscle blood flow at maximum in a large animal model. Thus an alternate explanation for the findings of the present study is that multiple pathways mediate vasodilation in skeletal muscle such that when one pathway (endothelium dependent, NO mediated) is inhibited, other preexisting pathways are able to compensate (5).

In addition to skeletal muscle, we found that cardiac muscle was normally perfused during exercise in L-NAME-treated swine. This observation is consistent with several previous studies (1, 4, 28, 30, 37) and with the notion that multiple dilatory pathways also exist in the coronary circulation. There was, in fact, a strong tendency ($P = 0.06$) for left ventricular blood flow to be greater in LN at maximal exercise. This may be attributed to greater myocardial O₂ consumption in LN due, in turn, to increased afterload.

NO and renal blood flow

Similar to skeletal and cardiac muscle, visceral blood flows during exercise were largely unaffected by chronic L-NAME treatment. Only blood flows to pancreas and kidney were lower in LN than Con (by ~75 and 45%, respectively) at maximum; no visceral flows were significantly reduced by L-NAME treatment at our submaximal level of exercise. At rest, both renal and adrenal blood flows were lower in LN. Given the effects of L-NAME on blood flow to and conductance in the kidney, we examined potential mechanisms underlying reduced renal blood flow by determining renal artery vasomotor function in vitro. Endothelium-dependent, but not-independent, relaxation was reduced in renal arteries from LN compared with Con. Acute L-NAME treatment eliminated this difference by reducing the relaxation response in Con. Acute indomethacin treatment, on the other hand, reduced endothelium-dependent relaxation in LN only, suggesting that cyclooxygenase products (e.g., PGI₂) were more important for endothelium-dependent relaxation in LN. Consistent with these findings expression of COX-1, the isoform involved in vasodilatory prostaglandin formation, tended to be greater in endothelium from renal arteries of LN. This apparently greater reliance on cyclooxygenase products with chronic inhibition of NO formation has previously been observed in the coronary circulation of dogs (28). Our in vitro data, along with the immunohistochemical demonstration of eNOS and COX-1 in endothelium of the renal artery and our in vivo data, provide evidence of the potential importance of endothelium in regulation of blood flow to the kidney at rest and during exercise in a large animal model. That both NO (18, 27) and cyclooxygenase products (16) are important

regulators of renal blood flow in rodents is well established. Nonetheless, demonstration of protein expression and functional evidence of its participation in vasodilation *in vitro* do not constitute proof of involvement *in vivo*. In addition, it is important to recognize that apparently greater reliance on COX-1-derived vasodilatory prostaglandins in the kidney of LN was insufficient to normalize renal blood flow and conductance, either at rest or during exercise.

NO and O₂ consumption

O₂ consumption, at rest as well as at submaximal and maximal exercise intensities, was similar for Con and LN. Since cardiac and skeletal muscle are primarily responsible for the exercise-induced increase in whole body O₂ consumption, it is instructive to examine findings from previous studies involving these tissues. Increases in myocardial O₂ consumption (32), as well as that of hindlimb skeletal muscle (31), have been observed with inhibition of NO synthesis, although contrary data are available (4, 8, 9). Our data unequivocally indicate that one determinant of maximal O₂ consumption, O₂ delivery, was similar for Con and LN at maximum (see above). We are less certain of the other determinant, O₂ extraction, because we did not determine arterial-venous O₂ difference in our experiments. Nonetheless, given that maximal O₂ consumption did not differ between groups, it is clear that on a whole body basis extraction of O₂ also did not differ between groups at maximum. This does not preclude regional differences in O₂ consumption between groups that were not detectable at the whole body level. It may be, for example, that increased myocardial O₂ consumption with inhibition of NO formation was diluted by unaltered skeletal muscle O₂ consumption, given their relative masses.

Alternatively, skeletal muscle character may underlie differences between the present O₂ consumption data and previous data. Canine skeletal muscle, which comprises a majority of the hindquarter tissue examined by Shen and colleagues (31), is uniformly high oxidative in phenotype (2) and may permit increased O₂ consumption with inhibition of NO synthesis. Similar to human skeletal muscle, porcine muscle is more modest in its oxidative capacity (3, 23). Thus reduced restraint on mitochondrial function may not result in a detectable effect in porcine muscle, which has a lower ceiling for O₂ consumption. This may be particularly germane at maximal exercise, where electron transport chain flux is already high. Importantly, unchanged O₂ consumption with L-NAME treatment is consistent with unaltered blood flows at maximum. Matching of O₂ delivery to O₂ demand is an established feature of the cardiorespiratory response to acute exercise (29).

Physiological perspectives

Our findings may have important implications for human patient populations. Chronic L-NAME administration clearly reduced NO bioavailability, as reflected by reduced plasma NO_x concentration. Reduced NO bioavailability is a hallmark of the early stages of atherosclerosis (38); thus chronically L-NAME-treated animals may model this component of early-stage human atherosclerosis. Interestingly, normal maximal O₂ consumption in a human population with no intima-media thickening, but probable endothelial dysfunction due to the presence of multiple risk factors, has been reported (19). That chronic loss of NO in our swine did not affect cardiac and skeletal muscle blood flows, as well as maximal O₂ consumption, is encouraging since endothelial dysfunction (and eventually frank atherosclerosis) frequently affects these two vascular beds. It also lends support for therapeutic measures designed to halt progression of the disease beyond this stage. Our findings of reduced blood flow to the kidney, however, are of concern because renal insufficiency and failure constitute an increasingly important public health problem (6).

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Fig. 1.

A: O_2 consumption as a function of treadmill running intensity in control swine (Con; $\circ$) and treated swine that drank water with 10 mg N^G -nitro-L-arginine methyl ester (L-NAME)/100 ml water (LN; $\bullet$). Values are means $\pm$ SE; $n = 17$ and 13 for Con and LN, respectively. O_2 consumption did not differ between Con and LN across the range of running intensities. B: maximal O_2 consumption achieved for Con (open bar) and LN (filled bar). Values are means $\pm$ SE; $n = 17$ and 13 for Con and LN, respectively. Maximal O_2 consumption did not differ between Con and LN.

Fig. 2.

Visceral blood flows at rest (A), during treadmill running at 5.0 miles/h (B), and at maximal running intensity (C) in Con (open bars) and LN (filled bars). Values are means $\pm$ SE; $n = 9$ and 8 for Con and LN, respectively, at rest, 8 and 4 during submaximal exercise, and 5 and 3 at maximal exercise. * $P < 0.05$ vs. Con.

Fig. 3. Forelimb muscle blood flows at rest (A), during treadmill running at 5.0 miles/h (B), and at maximal running intensity (C) in Con (open) and LN (filled bars). Values are means $\pm$ SE; $n = 9$ and 8 for Con and LN, respectively, at rest, 8 and 4 during submaximal exercise, and 5 and 3 at maximal exercise. TLtH, triceps brachii lateral head; D, deep portion; S, superficial portion; TAH, triceps brachii accessory head; TMH, triceps brachii medial head; TLH, triceps brachii long head; Bic Br, biceps brachii; Brach, brachialis. Note different scaling of y-axis for B and C vs. A. * $P < 0.05$ vs. Con.

Fig. 4. Hindlimb muscle blood flows at rest (A), during treadmill running at 5.0 miles/h (B), and at maximal running intensity (C) in Con (open bars) and LN (filled bars). Values are means $\pm$ SE; $n = 9$ and 8 for Con and LN, respectively, at rest, 8 and 4 during submaximal exercise, and 5 and 3 at maximal exercise. VL, vastus lateralis; D, deep portion; S, superficial portion; VI, vastus intermedius; VM, vastus medialis; RF, rectus femoris; Bic Fem, biceps femoris; Semit, semitendinosus. Note different scaling of y-axis for B and C vs. A. * $P < 0.05$ vs. Con.

Fig. 5. Dose-dependent relaxation responses of renal arterial segments from Con (○; $n = 6$) and LN (●; $n = 7$) to the endothelium-dependent agent bradykinin. Relaxation responses expressed as percentage of developed tension induced by $\text{PGF}_{2\alpha}$. Brackets denote concentration. **A:** segments not treated with inhibitors. **B** (L-NAME), **C** (Indomethacin), **D** (L-NAME + Indomethacin): segments incubated with L-NAME and/or indomethacin before determination of responses to bradykinin. * $P < 0.05$ vs. Con.

Fig. 6. Dose-dependent relaxation responses of renal arterial segments from Con (○; $n = 6$) and LN (●; $n = 7$) to the endothelium-dependent agent acetylcholine. Relaxation responses expressed as percentage of developed tension induced by $\text{PGF}_{2\alpha}$. *A*: segments not treated with inhibitors. *B* (L-NAME), *C* (Indomethacin), *D* (L-NAME + Indomethacin): segments incubated with L-NAME and/or indomethacin before determination of responses to acetylcholine. There were no significant differences between Con and LN in responses to acetylcholine under any condition.

Fig. 7.

Dose-dependent relaxation responses of renal arterial segments from Con (○; $n = 6$) and LN (●; $n = 7$) to the endothelium-independent agent sodium nitroprusside. Relaxation responses expressed as percentage of developed tension induced by $\text{PGF}_{2\alpha}$. *A*: segments not treated with inhibitors. *B* (L-NAME), *C* (Indomethacin), *D* (L-NAME + Indomethacin): segments incubated with L-NAME and/or indomethacin before determination of responses to sodium nitroprusside. There were no significant differences between Con and LN in responses to sodium nitroprusside under any condition.

Fig. 8. Immunohistochemical demonstration of cyclooxygenase (COX)-1 protein expression in renal arteries from Con (*top left*) and LN (*top right, bottom left*) and in positive control (arcuate artery; *bottom right*). Lumen located on *left* in panels with renal arteries. Note positive staining for COX-1 in endothelium adjacent to lumen in each renal artery.

Fig. 9.

COX-1 and -2 protein expression in endothelial scrapes of renal arteries from Con (unfilled bars) and LN (filled bars). Values are means $\pm$ SE; $n = 6$ and 5 for Con and LN, respectively. *Insets*, immunoblots. Con and LN do not differ significantly in either COX-1 or -2 expression.

Table 1

Blood variables

Group	Total Cholesterol, mg/100 ml	High-Density Lipoprotein Cholesterol, (mg/100 ml)	Triglyceride, mg/100 ml	Hematocrit, %	Hemoglobin, g/100 ml
Con	90±3	33±2	64±4	29±1	10.0±0.3
LN	93±2	37±1	76±4	29±1	10.2±0.3

Values are means ± SE; for total cholesterol, high-density lipoprotein cholesterol, triglyceride, *n* = 10 and 8 for swine that drank tap water (Con) and swine that drank water with *N*⁶-nitro-L-arginine methyl ester (LN) respectively; for hematocrit and hemoglobin, *n* = 14 each. There were no differences between Con and LN.

Table 2

Cardiothoracic blood flows

Tissue	At Rest	Submaximal Exercise	Maximal Exercise
Left ventricle			
Con	135±9	406±29	519±81
LN	124±19	429±52	734±78
Right ventricle			
Con	127±15	422±36	581±39
LN	121±18	477±74	729±142
Lung			
Con	139±46	675±130	383±229
LN	42±9*	285±72*	353±113
Diaphragm			
Con	38±7	135±21	281±26
LN	40±10	152±29	401±160

Values are means $\pm$ SE given in $\text{ml} \cdot \text{min}^{-1} \cdot 100 \text{ g}^{-1}$; for Con and LN, respectively, $n = 9$ and 8 (at rest), 8 and 4 (submaximal exercise), and 5 and 3 (maximal exercise). Submaximal exercise, treadmill running at 5.0 miles/h; maximal exercise, maximal treadmill running intensity.

* $P < 0.05$ vs. Con.

Table 3

Characteristics of renal arterial segments

Group	OD, mm	ID, mm	Axial Length, mm	Stretch, %	Tension (Rest), g
Con	2.92±0.08	1.82±0.12	3.21±0.06	133±2	5.8±0.7
LN	2.98±0.15	1.59±0.09	3.28±0.13	133±3	5.6±0.4

Values are means ± SE; *n* = 6 and 7 for Con and LN, respectively. OD, outside diameter; ID, inside diameter; stretch, increase above passive diameter associated with greatest tension development in response to KCl; tension (rest), tension associated with vessel segment stretched to optimum. There were no differences between Con and LN.