Influence of nitric oxide on vascular resistance and muscle mechanics during tetanic contractions in situ

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Ameredes, Bill T., and Mark A. Provenzano. Influence of nitric oxide on vascular resistance and muscle mechanics during tetanic contractions in situ. J. Appl. Physiol. 87(1): 142–151, 1999.—Studies of the effect of nitric oxide (NO) synthesis inhibition were performed in the isometrically contracting blood-perfused canine gastrocnemius-plantaris muscle group. Muscle blood flow (Q) was controlled with a pump during continuous NO blockade produced with either 1 mM L-argininosuccinic acid (L-ArgSA) or Nω-nitro-L-arginine methyl ester (L-NAME) during repetitive tetanic contractions (50-Hz trains, 200-ms duration, 1/6). Pump Q was set to match maximal spontaneous Q (P<1.4 ml/min·g−1) measured in prior, brief (3–5 min) control contraction trials in each muscle. Active tension and oxygen uptake were 500–600 g/g and 200–230 µl·min−1·g−1, respectively, under these conditions. Within 3 min of L-ArgSA infusion, vascular resistance across the muscle (Rv) increased significantly (from ~100 to 300 peripheral resistance units; P<0.05), whereas Rv increased to a lesser extent with L-NAME (from ~100 to 175 peripheral resistance units; P<0.05). The increase in Rv with L-ArgSA was unchanged by simultaneous infusion of 0.5–10 mM L-arginine but was reduced with 1–3 µg/ml sodium nitroprusside (41–54%). The increase in Rv with L-NAME was reversed with 1 mM of L-arginine. Increased fatigue occurred with infusion of L-ArgSA; active tension and intramuscular pressure decreased by 62 and 66%, whereas passive tension and baseline intramuscular pressure increased by 80 and 30%, respectively. These data indicate a possible role for NO in the control of Rv and contractility within the canine gastrocnemius-plantaris muscle during repetitive tetanic contractions.

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THE MECHANICAL PERFORMANCE of skeletal muscle during repetitive contractions can be indicative of the match between the metabolic demands of the muscle fibers and the supply of substrate (2) and/or metabolite washout (7). The active hyperemic response of contracting muscle is one mechanism that enables high levels of mechanical output over prolonged periods of time, through provision of this supply and washout, during repetitive muscle activation such as exercise. One agent that may play a role in the hyperemic process is the endothelium-derived relaxing factor, presently identified as nitric oxide (NO) (29). NO has been shown to be synthesized in the endothelial cells lining the vasculature, through the activity of a constitutive nitric oxide synthase (NOS) enzyme that acts to produce NO from the precursor L-arginine (28). Once produced, NO acts directly on the vascular smooth muscle to cause relaxation, and there is an attendant drop in resistance as the vessel luminal diameter increases. With respect to skeletal muscle vasculature, it has been shown that the effects of the NO pathway are significant in muscles with a high percentage of oxidative fibers (17), specifically those characterized as slow-oxidative and fast-oxidative glycolytic.

The stimuli that may initiate this response within the muscle vasculature include elevated intravascular shear forces produced by increased flow of blood within the vessels (29) and extravascular shear forces produced by repeated compression of the vessels within the contracting muscle (21), perhaps as a product of intramuscular pressure (PIM) development (4). Evidence for this latter possibility has been demonstrated in rhythmically squeezed rabbit arterial segments that produced elevated cGMP levels in platelets circulating through the segments as an index of NO production (21). Furthermore, a recent study in the canine gastrocnemius-plantaris (GP) muscle has shown production of high intramuscular stress (=1,600 mmHg) during brief tetanic muscle contractions in situ (4), suggesting the propensity for development of large extravascular shear forces acting on the endothelium of the muscle vasculature in this muscle group.

However, studies in several different species and models have produced varied results and a lack of consensus on whether NO plays a significant role in the active hyperemic response (8, 11, 12, 14, 17–19, 21, 27, 30, 34). We believe that three major factors have been responsible for this discrepancy. One is that twitch or low-demand contractions have been utilized in many studies (8, 12, 19, 34), which may not adequately test the capacity of the response (2, 14). Another has been pretreatment with a systemic bolus of competitive NOS inhibitors (19, 30), which may complicate interpretation of results in the contracting muscle vascular bed because of unquantifiable metabolism and sequestration in nonmuscle tissue beds throughout the body. Finally, in some studies, the administered NOS inhibitor effective dose obtained in the resting state was not increased proportionately to account for the increased blood flow during muscle contractions (12, 34). Consideration of these factors suggests that the minimal role reported for NO requires further examination, based on the facts that shear stress is a known strong potential agent of NO production by endothelial cells (29) and endothelialization of the GP muscle vessels results in significantly attenuated hyperemic responses (27). Furthermore, the fiber type composition of the canine GP...
muscle is highly oxidative (45% fast-twitch fatigue resistant, 55% slow-twitch fatigue resistant; Ref. 22), which might predispose it toward significant NO-dependent responses (17).

Thus the purpose of this study was to determine whether NO plays a significant role in control of vascular resistance ($R_v$) during repetitive tetanic contractions in the canine GP muscle in situ with the use of a previously published contraction paradigm that reliably results in achievement of high $P_{IM}$ development, blood flow, and oxygen uptake ($\dot{V}O_2$) (2–4, 10). We hypothesized that inhibition of NO production through local administration of blockers would result in significantly increased $R_v$ if NO played a significant role in this response under these conditions. Rejection of the hypothesis would indicate that even these high-intensity contractions have no effect on the NO system within the canine GP muscle vasculature, suggesting regulation of the resistance response by other, non-NO-related factors.

**METHODS**

General methods. Mongrel dogs of both sexes (10–15 kg), obtained and housed in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee, were utilized in these studies. Initial anesthesia was induced with pentobarbital sodium (30 mg/kg iv), followed by maintenance doses of 60 mg. The animals were ventilated through an endotracheal tube with a Harvard respirator, and endtidal CO$_2$ was maintained at 4.5–5%. Body and muscle temperatures were maintained at 37–39°C. The muscle group studied was the GP with isolated circulation surgically prepared as described previously (2–4, 10). Anticoagulation of the blood was achieved with heparin (2,600 U/kg). The sciatic nerve was dissected and cut, and the distal stump placed in an electrode holder. The calcaneus tendon was freed, cut, and clamped, and the muscle was anchored as described previously (2–4, 10).

Muscle blood flow ($\dot{Q}$) was measured as the rate of venous outflow from the popliteal vein with the use of a 4-mm cannulating-type electromagnetic flow probe. A pressure transducer connected to the venous outflow line provided measurements of mean venous pressure (MVP). A roller pump, fed by the contralateral femoral artery, provided control of Q during the infusion experiments, and a pump bypass line allowed spontaneous perfusion of the muscle directly from the artery when necessary. A pressure transducer connected to the popliteal artery catheter (between the pump and the muscle) provided measurement of the mean arterial pressure (MAP) and allowed calculation of resistance across the muscle ($R_v$) as

$$R_v = \frac{(MAP - MVP)}{\dot{Q}}$$

in peripheral resistance units (PRU = mmHg/g•Q), with $\dot{Q}$ expressed in ml·min$^{-1}$·g wet wt$^{-1}$. $\dot{V}O_2$ was determined in 10 GP muscles. Blood samples (1 ml) for gas analysis were withdrawn simultaneously into glass tuberculin syringes from a side port of the arterial supply tubing and the venous effluent tubing. Oxygen concentration of arterial ([O$_2$]$_a$) and venous effluent ([O$_2$]$_v$) samples was measured by the manometric method of Van Slyke and Neill (33). Samples were taken with the muscle at rest and during repetitive contractions. Additional samples (≤1 ml) were withdrawn for blood-gas analyses by using a microana-

lyzer at 37°C (ABL-3, Radiometer) to determine $P_{O_2}$, $P_{CO_2}$, and pH. A small portion of this arterial sample was also used to measure hematocrit. $\dot{V}O_2$ was calculated by the Fick method as

$$\dot{V}O_2 = \dot{Q}(\text{[O}_2\text{]}_a - \text{[O}_2\text{]}_v)$$

(2)

Because of the complexity of these experiments and the necessity for small timing windows of drug infusion described in Contracting muscle protocol and experiments, samples were not drawn for every series performed. However, as shown in Table 2, these $\dot{V}O_2$ determinations provided evidence that high metabolic rates were attained during the repetitive isometric tetanic contractions at optimal muscle length ($L_o$).

Muscle mechanics. Muscle force development during contractions was monitored with a pneumatic lever specifically designed for the canine GP muscle (13). The force transducer on the lever was calibrated by hanging known weights onto the tendon clamp attachment hook, with the lever perpendicular to the floor. During setup of the lever, precautions were taken to minimize flexing and movement of the apparatus with subsequent tetanic contractions (3, 4). Repetitive isometric tetanic contractions (50 imp/s, 0.2-ms pulse width × 4-V pulse amplitude; 200-ms train duration, 1 contraction/s) were produced at $L_o$, which was determined as the length at which tension of prior twitch contractions (0.2-ms pulse, 4 V) was maximal (102 ± 9 g/l, n = 8 muscles). The activation stimulus train resulted in peak tetanic active tension ($T_{act}$) development of 540 ± 59 g (n = 8) and a twitch/tetanic ratio of 0.19 ± 0.01. The tetanic contraction repetition rate was chosen as one that reliably results in high Q and $\dot{V}O_2$ in this preparation (2, 3, 10). Passive tension ($T_{pass}$) at $L_o$ was 55 ± 10 g/l. During contraction trials, the $T_{pass}$ baseline was monitored as an index of incomplete relaxation to determine whether manipulations of NO produced measurable alterations under these conditions. In some experiments, $P_{IM}$ was measured during contractions by using a solid-state needle-type transducer (model SPR-477, Millar) inserted directly into the muscle. The approach was the same as described previously (4), with $P_{IM}$ being measured in the origin, central, and insertion portions of the medial head of the GP muscle group (zones I, II, and III). The $P_{IM}$ transducer was calibrated with a mercury column (4). As above, alterations in the passive $P_{IM}$ baseline were monitored as an index of significant pressure remaining within the muscle between contractions.

Resting muscle experiments. Just before the tests of resting muscle responses, muscles were stimulated to produce repetitive isometric tetanic contractions (1/s), over a period of 3–5 min, to allow determination of the contraction-induced level of hyperemia and decrement in $R_v$ with no drugs. A systemic indomethacin bolus (0.5 mg/kg iv) was given immediately after the brief contraction trial to block contribution of prostaglandins in the Q response during the subsequent NO synthesis blockade (18, 23). This dose was chosen based on pilot experiments (n = 2) that indicated that a 5 mg/kg systemic dose resulted in elevation of MAP to a level that was considered nonphysiological (>200 mmHg), whereas 0.5 mg/kg produced only mild elevation of MAP to 120–140 mmHg. This treatment may have produced less than a total blockade of prostaglandin synthesis but was considered reasonable with regard to the normal range of systemic MAP.

Toward the end of a subsequent 30-min rest period, the pump-perfusion setup was installed, and Q was monitored in the pump-bypass mode to verify that spontaneous resting values were reestablished. This rest period has been shown to allow Q and $\dot{V}O_2$ to reestablish precontraction values after a
brief repetitive contraction trial (3). Muscle perfusion was then switched to pump control, matching the Q at rest previously measured, and NO agonists ACh and sodium nitroprusside (SNP) were infused at constant rates with the use of the syringe pumps connected to side ports. These agonists provided the ability to test the endothelial-dependent and -independent responses, respectively (18). The syringe pump rate was always set at 10% of the roller pump rate; these rates were typically 1–2 and 10–12 ml/min, respectively. In separate experiments, NO antagonists were also infused at a constant rate, as above. All concentrations of drug in syringes and pump flows were chosen to achieve a desired concentration of drug within the blood delivered to the muscle, typically expressed in millimoles of drug per liter of blood. Two NO synthesis blockers were chosen for these experiments. N^o-nitro-L-arginine methyl ester (L-NAME; Sigma Chemical), a synthetic, competitive, reversible blocker of NO synthesis, was chosen for its ease of solubility in normal isotonic saline and because it has been used in prior twitch contraction studies in this (19) and other in situ preparations (18). L-Argininosuccinic acid (L-ArgSA; Sigma Chemical), a naturally occurring compound, was chosen as a noncompetitive, irreversible inhibitor of NO synthesis (15, 18). In all of the above resting muscle experiments, agonists or antagonists were each infused continuously for 10–20 min (time defined by achievement of a sustained plateau), interspersed with 20–30 min recovery periods.

Contracting muscle protocol and experiments. L-NAME and L-ArgSA dose–response experiments performed during repetitive tetanic contractions followed the same approach outlined in resting muscle experiments, except that the Q achieved during the brief preinfusion contraction trial was used as the perfusion pump set point for Q during the contractions. As above, the syringe pump flow rate was 10% of the roller pump rate, typically 5–6 and 50–60 ml/min, respectively, and drug doses and flows were chosen to achieve concentrations of millimoles of drug per liter of blood within the blood delivered to the muscle. In all experiments, during the preinfusion trial with spontaneous Q, Rv in the resting muscle and during subsequent contractions averaged ~500 and 100 PRU (P < 0.05), respectively, demonstrating a significant drop in resistance with induction of the active hyperemic response. The high resting Rv level was always reestablished during the rest period between the preinfusion and infusion trials, suggesting a robust autoregulatory response. Beginning at minute 3 of contractions under subsequent pump perfusion control, L-NAME or L-ArgSA was constantly infused from the syringe pumps. This time was chosen because prior tetanic contraction experiments in this preparation have shown that maximal Q and VO2 occur from 3 to 5 min of repetitive contractions with the use of this stimulus paradigm (2, 3, 10). Respective NOS inhibitor dosages were increased at 3- to 5-min intervals during the remainder of the contraction trials.

A separate series of competitive experiments was performed in which agonists were infused simultaneously with antagonists during muscle contractions. Muscles were infused continuously with blocker, beginning at minute 3 of contractions, under pump perfusion control with NO agonists being added sequentially over time as contractions continued. The noncompetitive blockade property of L-ArgSA was tested against 0.5, 1.0, 10, and 120 mM L-arginine (Sigma Chemical), the precursor for NO produced by endothelial NOS (28, 29). ACh was not used as an agonist for a complete series, because preliminary experiments indicated that, although effective at lowering Rv, even low doses reduced contraction force by two-thirds to three-fourths of the ongoing level, which we judged as unacceptable for comparison with the other experiments. Thus a series was performed with 1.0 mM L-ArgSA vs. 0.5, 1.0, 2.0, and 3.0 µg/ml SNP. In these experiments, an additional null agonist dosage of saline alone was infused to determine the effect of the agonist vehicle during these experiments. Accordingly, in a separate set of GP muscles, a series of time-control L-ArgSA infusion experiments was performed during repetitive contractions to determine time-based effects of continuous blockade with 1 mM L-ArgSA. Finally, a series of L-NAME vs. L-arginine experiments was conducted to test Rv responses with this competitive antagonist-agonist pair with the use of similar dosages.

After the trials were finished, the animals were killed with an overdose of pentobarbital sodium. The nonstimulated GP muscle was excised, trimmed of connective tissue and fat, and weighed (2–4, 10) for normalization of all measured values, expressed per whole muscle wet weight (40–60 g). Repeated-measures ANOVAs (SigmaStat) were performed on Rv values to determine whether significant differences (P < 0.05) were present. Post hoc analyses of specific values were performed using Duncan’s test. Independent sample t-tests were performed to compare the L-ArgSA time-control, L-ArgSA vs. L-arginine, and L-ArgSA vs. SNP Rv data, also with P < 0.05 considered significant.

RESULTS

Cardiovascular characteristics of the dogs were similar to those obtained in prior experiments that used this preparation (systemic arterial pH = 7.38 ± 0.11, arterial Po2 = 104 ± 16 Torr, arterial Pco2 = 35 ± 4 Torr) (2, 3). No significant changes were noted in any of these arterial variables throughout the experiments; therefore, all animals were considered to be similar with regard to systemic oxygenation and acid-base status.

Responses in resting muscle. Results from ACh-infusion experiments in resting muscles are shown in Fig. 1. Rv displayed a dose-dependent reduction with increasing ACh levels, demonstrating a significant endothelium-dependent response (18) under these conditions. SNP (0.2–1.0 µg/ml) produced similar results,
L-ArgSA reproducibly increased $R_v$ at rest in these experiments. These values reflect a 10-fold increase in $V_{O2}$ from rest, indicating achievement of a high metabolic rate in conjunction with the active hyperemic response resultant of the tetanic contraction regime. $Q$ for all other experiments described below averaged 1.2–1.5 ml·min$^{-1}$·g$^{-1}$ during contractions.

A dose-response relationship showing changes in $R_v$ with sequential L-ArgSA dosages during contractions ($n = 4$ muscles) is shown in Fig. 2. As in the muscles at rest, 1.0 mM was most effective, although significant increases were produced at lesser dosages. Both in the studies shown here and in pilot experiments, 1.0 mM L-ArgSA was tested in a total of 24 muscles, reliably

Table 1. Resistance (PRU) changes with NO-antagonists administered to resting muscle

<table>
<thead>
<tr>
<th></th>
<th>Concentration, mM</th>
<th>n</th>
<th>0.25</th>
<th>1</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-ArgSA</td>
<td></td>
<td>5</td>
<td>+87±48</td>
<td>+278±122*</td>
<td></td>
</tr>
<tr>
<td>L-NAME</td>
<td></td>
<td>3</td>
<td>+2±6</td>
<td>+7±12</td>
<td>+54±38</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of muscles. All agonists continuously delivered for 10–20 min with 20- to 30-min recovery periods between. PRU, peripheral resistance units; NO, nitric oxide; L-ArgSA, L-argininosuccinic acid; L-NAME, N$^G$-nitro-L-arginine methyl ester. *P < 0.05 PRU change.

Table 2. $Q$, $V_{O2}$, and MAP at rest and during contractions

<table>
<thead>
<tr>
<th></th>
<th>Spontaneous $Q$, ml·min$^{-1}$·g$^{-1}$</th>
<th>Pump-controlled $Q$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rest</td>
<td>Contractions</td>
</tr>
<tr>
<td>L-ArgSA</td>
<td>0.34±0.05</td>
<td>1.34±0.13*</td>
</tr>
<tr>
<td>L-NAME</td>
<td>0.38±0.05</td>
<td>1.46±0.13*</td>
</tr>
<tr>
<td>L-ArgSA</td>
<td>21±3</td>
<td>206±13*</td>
</tr>
<tr>
<td>L-NAME</td>
<td>27±6</td>
<td>228±25*</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of muscles. $Q$, muscle blood flow; $V_{O2}$, oxygen uptake; MAP, mean arterial pressure. Respective blocker dose is 1 mM, begun at minute 3 of repetitive contractions; value obtained at minutes 6–7. $V_{O2}$ with blockers was not significant compared with contractions with pump and no blocker. *P < 0.05, compared with prior resting value within $Q$ group; †P < 0.05, blocker compared with contractions with pump and blocker.
arginine competitive experiments, as described below. Inhibition-induced $R_v$ was considered to infer that contractions with no drug ($P < 0.05$). Further administration of 1.0 mM L-arginine reduced $R_v$ by 23 PRU to a level not statistically different from the initial contraction-induced $R_v$. We considered this result to infer that this dose of L-arginine was effective in reversing the effects of 2.0 mM L-NAME. Infusion of the highest dose of L-NAME (10 mM) was not attempted in these experiments, because it represented delivery of a hyperosmotic solution (>310 mosM), which might have opposing vasodilatory effects (32) complicating interpretation of the results. This hyperosmotic vasodilatory mechanism was found to be inducible when 120 mM L-arginine, D-arginine, L-lysine, and sucrose solutions (1,000–1,500 mosM/kg) were administered sequentially during continuous L-ArgSA infusion (Fig. 3) and, therefore, was avoided except in the L-ArgSA vs. L-arginine competitive experiments, as described below.

As shown in Fig. 4, 1.0 mM L-ArgSA during contractions (second bar set) produced a significant increase of $R_v$ by two to four times over its initial low value with no drug (first bar set) in all trials. The time control trial (open bars) indicated the continuous increase in $R_v$ with continued infusion of L-ArgSA over time. The noncompetitive irreversibility of NO synthesis blockade by L-ArgSA was indicated by this same behavior during simultaneous L-arginine infusions (hatched bars), with significant reduction only with 120 mM L-arginine. This same pattern of $R_v$ increase was seen during the initial portion of the SNP infusion trial (solid bars) but was subsequently reduced in a stepwise fashion with increasing doses of SNP. $R_v$ with 1.0 µg/ml SNP was also significantly lower than the matched time control with L-ArgSA alone. With continued infusion of L-ArgSA during this trial, 3.0 µg/ml SNP reduced $R_v$ to a level that was not statistically different from that with contractions alone.

Alterations in muscle mechanics were observed with L-ArgSA administration, such that both $T_{act}$ and $P_{IM}$ loss was exacerbated during infusion and was not reversed with simultaneous L-arginine administration (Fig. 5). With L-ArgSA infusion beginning at minute 3, both $T_{act}$ and peak $P_{IM}$ were observed to decline at a greater rate during minutes 4–12 than during the initial 4 min, and than that of control muscles over the same time period. $T_{pass}$ and passive $P_{IM}$ in these same muscles (Fig. 6) increased significantly during L-ArgSA infusion, possibly through production of incomplete relaxation between activations. No effects on $T_{act}$, $T_{pass}$ or $P_{IM}$ were observed with 1.0–2.0 mM L-NAME.

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The main finding of this study was that NO appears to play a role in control of $R_v$ at rest and during repetitive tetanic contractions within the canine GP muscle in situ. NO synthesis inhibitors, delivered directly into the muscle vasculature, resulted in a significant increase in $R_v$ during electrically stimulated contractions of high force and metabolic rates. The competitive, reversible NO inhibitor L-NAME produced statistically significant increments of $R_v$ during contractions that were reversible with L-arginine. The noncompetitive, irreversible NO inhibitor L-ArgSA produced large increments in $R_v$ that were not reversible with L-arginine. This increase in $R_v$ with L-ArgSA was reversed by administration of SNP, presumably through direct donation of NO to vascular smooth muscle, thus bypassing the endothelial NO blockade. During the administration of L-ArgSA, muscle performance was measurably affected, demonstrating a significant decline in $T_{active}$ and $P_{IM}$ production and a significant increase in $T_{pass}$ and baseline $P_{IM}$ that was not reversible with L-arginine.

Comparisons with other studies of hyperemia. The results of this study agree with those of Hussain et al. (18), in which L-ArgSA was used as a NO synthesis blocker, and NO was reported to play a significant role (22–41%) in the alteration of $R_v$ in the canine diaphragm in situ during twitch contractions (2/s). Our results are also consistent with those of Sagach et al. (27), in which loss of the “functional” hyperemic response was observed with endothelial blockade in the contracting canine GP muscle in situ. Finally, our data are consistent with studies in which L-NAME decreased the diameter of arterioles mainly in contracting, as opposed to resting, hamster cremaster muscles (16).

However, our results appear to be inconsistent with two other studies that used the canine GP muscle, both of which utilized repetitive isometric twitch contractions (8, 19). One study reported no effect of NO on functional hyperemia (8) with N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA, another competitive reversible inhibitor of NOS) infusion during twitch contractions of 4/s.
Another study (19) utilized twitch rates of 1, 2, 4, and 6/s, with blockade produced by iv bolus pretreatment with L-NAME (20 mg/kg). Assuming a normal canine systemic blood volume and uniform distribution, that systemic L-NAME load would peak at 0.99 mM, similar to the dose directly delivered to the muscles in the present study. However, no significant alteration of $R_v$ was observed in any but one case (19). In both studies (8, 19), developed forces (50–150 g/g) and Q $(0.25–0.85 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$) were lower than values achieved in the present study, suggesting a lesser mechanical effect (4) and metabolic response (3).

Thus, one possible explanation for this seeming inconsistency may be the significantly greater muscle mechanical performance and metabolic rates produced with rhythmic tetanic contractions in the present study. The twitch forces measured in the present study (102 g/g) are indicative of the difference between twitch and tetanic contractions, being only $\sim 19\%$ of that developed by tetanic contractions (540 g/g). This would theoretically result in much less contraction-induced vascular deformation with twitch contractions (21), in lower extravascular forces produced (4), and, therefore, in much less potentiation of NO production by this mechanism. Perhaps most importantly, twitch rates above 2/s do not allow for full relaxation between contractions, thus producing an elevation in $T_{\text{pass}}$ and an impediment of the Q which normally occur during the relaxation phase (2), possibly limiting the large hyperemic response that is characteristic of rhythmic high-intensity muscle contractions. Support for these possibilities has been suggested previously by Gilligan et al. (14), who stated that greater effects of NO might be seen with intense rhythmic exercise, thus producing high blood flows. However, it may be possible that NOS inhibition is compensated by other vasodilatory mechanisms during relatively low-demand contractions like twitches, such that NO blockade results in little measurable effect under those conditions (19). Subsequently, these alternative mechanisms may be unable to compensate for inhibition of NOS under high-demand situations, such as tetanic contractions investigated in the present study.

Interpretation of $R_v$ at rest. The resting muscle experiments indicate that NO production is significant even when no contractions are occurring. Furthermore, the results indicate that the competitive, reversible inhibitor L-NAME was less effective than the noncompetitive, irreversible blocker L-ArgSA in blocking this production (Table 1). One possible explanation for this difference is that the concentration gradient for L-arginine favors release from skeletal muscle into the circulation by a factor of 6.5-fold (9), thus presenting a competitive outward flux that would have to be overcome by L-NAME for it to be most effective. However, it should be noted that, although the magnitude of increment of $R_v$ in resting muscle with 1 mM L-ArgSA was significant and eventually similar to that observed with contractions, it occurred over an extended time range (10–20 vs. 3 min). Figure 7 graphically shows the difference in the evolution of the $R_v$ response with L-ArgSA-induced NO blockade during the critical timing window of initial L-ArgSA administration. Taken together, the above data suggest that the rate of NO production was slower in resting muscle compared with contracting muscle. However, we cannot rule out the possibility that these differences might be due to the fact that more L-ArgSA was delivered to more vascular beds within the contracting muscle.

Interpretation of $R_v$ during contractions. The results of the present study suggest several possible interpretations with respect to the influence of NO on $R_v$. One interpretation is that the rate of NO production by the endothelium within this muscle group during rhythmic high-force contractions is so great that an irreversible blocker of NO production (L-ArgSA) given directly over time is necessary to counter these effects. This interpretation is suggested by Figs. 3 and 4. L-Arginine, given in low, matching, and higher doses, did not reverse this effect; its reversal required direct donation of NO to the smooth muscle or induction of a hyperosmotic vasodilatory effect through a separate pathway. The data of Hirai et al. (17), which show a significant role of the NO pathway in determination of $Q$ in exercising muscles with high-oxidative fiber type composition, support this interpretation. Also, similar to the present study, a two- to threefold difference in magnitude of effect between locally administered L-ArgSA and the competitive inhibitor L-NAME has been reported previously for the perfused, repetitively contracting diaphragm muscle (18). By the above interpretation, the significant but less robust results with L-NAME might be expected, because higher doses of a competitive inhibitor would be required to dampen a strongly potentiated NO signal resulting from intense muscular contractions (16). Therefore, there are precedents, to which we are presently inclined, for the above interpretation of the present data.
However, another interpretation of these data is that the significant effects of the blockers are not necessarily due to NOS blockade qualities but may be due to other NO-independent endothelial effects on vascular reactivity. Within this scheme, the L-ArgSA and L-NAME experiments suggest that NO has little, if any, role in the resistance response during contractions. This interpretation also has precedent but only from experiments in which 1) the tension (from twitch contractions) was low and only competitive, reversible blockers were administered (8, 19), or 2) L-NMMA was administered at resting dosages in exercising human forearms, with no increase in drug delivery to account for the dilution that occurs with active hyperemia (12, 34). Thus absent other definitive data on rhythmic tetanic contractions in this preparation, we do not favor this interpretation at present.

A third interpretation is that L-ArgSA produced increases in $R_v$ indirectly, through effects within the contracting muscle fibers, as opposed to a direct effect on the vascular endothelium. For instance, some cross bridges might have persisted after activation, thus prolonging tension during relaxation, elevating baseline $P_{IM}$ between activations, and increasing $R_v$ because of a mechanical restriction or occlusion of the muscle vasculature (4). This interpretation is supported by 1) the fact that $Q$ was decreased significantly despite pump control of perfusion during infusion of L-ArgSA (Table 2), 2) the development of significant $T_{pass}$ and the $P_{IM}$ baseline elevation during infusion of L-ArgSA (Fig. 6), and 3) previous studies showing the Q-limiting and fatigue-enhancing effect of elevated preload (2), possibly resulting in the accentuated decline of $T_{act}$ and peak $P_{IM}$ during L-ArgSA infusion (Fig. 5). Therefore, this indirect mechanism of alteration of $R_v$ by L-ArgSA would be due to a mechanical effect, produced by alterations in muscle-fiber contractile activity and its effect on vessel occlusion during contractions (3, 4).

Finally, it is also possible that large amounts of NO are produced by the myofibers themselves during high-intensity contractions (5), which may augment the shear-induced NO production by the endothelium. Within this interpretation, the $R_v$ effects of both L-ArgSA and L-NAME are again encompassed. The fact that NOS has been reported to exist in skeletal muscle fibers (20) is further consistent with this possibility. If NO production by contracting muscle fibers in situ is significant, it stands to reason that either a noncompetitive, irreversible NOS inhibitor or elevated doses of a competitive, reversible NOS inhibitor would be necessary to counter this NO "load." Furthermore, based on the fact that increased NO production from contracting muscle has been reported to occur (5), it is possible that the concentration gradient for L-arginine release from skeletal muscle into the circulation may become $>6.5$-fold (9). This would present an additional competitive flux that would have to be overcome by higher dosing during contractions for L-NAME to be most effective. Although presently speculative, this interpretation offers an interesting possibility of additional sources of vasoregulatory signals that may be induced with high-intensity muscular activity.

Comparisons with other studies of muscle performance. Prior studies of effects of NO on in situ muscle performance have documented a variety of results. One study of the GP muscle showed that L-NAME increased force development of twitches at 1 and 2 twitches/s, but not at 4 or 6 twitches/s (19), whereas another found no effects of L-NMMA on force development of twitches at 4 twitches/s (8), suggesting some consistency of effects as a function of stimulation frequency. The addition of NO, through administration of S-nitroso-N-acetyl-penicillamine, produced, on average, 3–6% greater fatigue in force than did N-acetyl-penicillamine alone during repetitive twitches of 1.5 and 4/s and tetanic contractions of 40/min, but no effects with 0.5 twitch/s and 12/min tetanic contractions (25). Again, this heterogeneity of response was attributed to stimulation frequency differences but is difficult to compare with the present results because we utilized 60/min tetanic contractions and did not add SNP alone. The absence of these alterations in muscular performance with L-NAME in the present study may have been due to either dosing insufficient to overcome gradients for entry into the skeletal muscle fibers (9) or to intracellular quantities insufficient to compete with native L-arginine (9, 26). Studies have shown that a plasma concentration of 3.0 mM is necessary to saturate the entry mechanism for L-arginine into skeletal muscle (6). Assuming that L-NAME follows this pathway and must compete with intracellular L-arginine for NOS, a reduced or absent response with 1.0 mM L-NAME might be expected in muscle in situ.

The present study has indicated a significant effect of the noncompetitive, irreversible NO blocker L-ArgSA on $T_{act}$, $T_{pass}$, and $P_{IM}$ produced within the GP muscle in situ. This suggests some role for NO in the contraction process. Balon and Nadler (5) have shown significant NO production by contracting skeletal muscles subsequently incubated in vitro, thus suggesting that some mechanism might be present for its use within the myocytes, perhaps in the excitation-contraction coupling process (20). For example, if L-ArgSA blocked NO production and altered reuptake of calcium into the sarcoplasmic reticulum, it might be that incomplete relaxation occurred (evident as elevated $T_{pass}$ in Fig. 6) with persistence of calcium in the sarcoplasm. A possible lack of resequestration of this calcium might also lead to a decrement in its release with subsequent activations (1), leading to the significant decrement of $T_{act}$ observed in Fig. 5.

However, similar to the varied results reported in muscle in situ, studies of muscles in vitro have displayed a heterogeneity of results, which may be dependent on species, fiber type distribution, and NOS enzyme activity (20). For instance, in the rat diaphragm, nitro-L-arginine (a blocker of NO synthesis) produced an ~7% increase in tetanic force, whereas SNP produced 3–7% reductions in tetanic force (20). In contrast, S-nitroso-N-acetyl-penicillamine produced
4–6% increments in tetanic force in both mouse soleus and extensor digitorum longus muscles in vitro (24). Overall, the above results are difficult to compare with those of the present study, but all suggest that NO has some role in the muscle contraction process.

Critique of methods. The design of the experiments in the present study included local administration of NO antagonists and agonists directly into the GP muscle vasculature during contractions with controlled Q. Under these conditions, the time response of the increase in Rv to L-ArgSA was rapid (≤3 min) and ever increasing with continued administration (Fig. 4). This rapid and sensitive behavior of the vasculature necessitated the use of brief timing windows (3–5 min) during drug administration. However, we found that strict regulation of muscle arterial Q was not simple to achieve under these conditions of manual pump control because of the capacity of the muscle vasculature to autoregulate under changing conditions of pressure and metabolic demand (31). For instance, when the arterial supply pump was set to maintain Q and allow arterial pressure to fluctuate as a function of Rv, we observed that the response with L-ArgSA was strong enough to cause a small but significant drop in Q, even though the roller pump speed setting was unchanged (Table 2). Figure 8 shows this effect, which was not significant for the case of L-NAME. The drop in Q probably was the result of the intense vasoconstriction produced by L-ArgSA, also evidenced as the significant increase of MAP shown in Table 2. Attempts to rectify this drop in Q through minor manual adjustments of the pump speed control were abandoned because of the delay between the adjustment and response, typically resulting in repeated overshoot of the target Q and a miss of the timing window. Thus the approach we employed likely resulted in smaller Rv changes than might have otherwise been observed, because more strict rectifications and maintenance of Q would have produced even greater MAP elevations and subsequently greater increases of Rv during NO blockade.

Although these results show a significant role for NO in the control of Rv during tetanic contractions of the GP muscle group, they likely underestimate this response because of the limitations of our experimental technique.

Although not the only NO synthesis inhibitor available, L-ArgSA was utilized as a blocker of endothelial NO synthesis in these studies for several reasons. First, it had a rapid onset of action, as evidenced by the increase of Rv during contractions within 3 min. This property was considered essential to the study of the role of NO by blockade during a time period in which the Q and VO2 response are typically maximal in this preparation (3, 4, 10). Second, L-ArgSA has been shown to be a noncompetitive, irreversible inhibitor of NO synthesis (15, 18), which we have verified functionally in this muscle preparation (Fig. 4). Because we hypothesized originally that NO production was a function of contraction-induced shear forces acting on the endothelium, this blocker was considered ideal for these experiments because it ensured cancellation of endothelial NO effects on regulation of Rv. Third, the use of L-ArgSA provided the possibility of a NO synthesis blockade within the vascular endothelium that could be bypassed by direct donation of NO to the smooth muscle, thereby allowing the observation of a direct NO-dependent alteration of smooth muscle activity on Rv during contractions (Fig. 4). The results suggest that (1) the endothelium plays a significant role in the determination of the resistance response during repetitive contractions of this muscle group, as evidenced by the rise in Rv with its blockade, and (2) the source of this response may be extravascular shear forces on the endothelium during intense contractions. This was concluded because direct supply of NO to the smooth muscle decreased Rv during a simultaneous, irreversible blockade that removed the endothelial response while the shear stimulus of the contractions was still present. The results are suggestive of a mechanism of NO-mediated relaxation of the vascular smooth muscle that lowers Rv and enables the achievement of high flow rates during intense muscular activity.

In summary, given the differences in muscle mechanics and experimental preparations, it is possible that many of the NO/hyperemia studies represent points on a continuum of NO effects on the vascular activity within skeletal muscle. For instance, other metabolic and non-shear-related mechanisms may predominate in the control of vascular activity at rest and low intensities of contractile activity (19), with progressive contribution from the extravascular shear-related mechanisms as the contraction intensity approaches maximum. Also, the differences across studies may be related to differing mechanisms of action between the NO synthesis blockers utilized in each study and the blockade treatment paradigm used, such as systemic bolus pretreatment vs. direct administration during contractions. These possibilities suggest that the present understanding of these differences and mechanisms is incomplete and requires further study.
REFERENCES


