Nitric oxide produced via neuronal NOS may impair vasodilatation in septic rat skeletal muscle

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Gocan, Nigel C., Jeremy A. Scott, and Karel Tyml. Nitric oxide produced via neuronal NOS may impair vasodilatation in septic rat skeletal muscle. Am J Physiol Heart Circ Physiol 278: H1480–H1489, 2000.—Impaired vascular responsiveness in sepsis may lead to maldistribution of blood flow in organs. We hypothesized that increased production of nitric oxide (NO) via inducible nitric oxide synthase (iNOS) mediates the impaired dilation to ACh in sepsis. Using a 24-h cecal ligation and perforation (CLP) model of sepsis, we measured changes in arteriolar diameter and in red blood cell velocity ($V_{\text{RBC}}$) in a capillary fed by the arteriole, following application of ACh to terminal arterioles of rat hindlimb muscle. Sepsis attenuated both ACh-stimulated dilation and $V_{\text{RBC}}$ increase. In control rats, arteriolar pretreatment with the NO donors S-nitroso-N-acetylpenicillamine or sodium nitroprusside reduced diameter and $V_{\text{RBC}}$ responses to a level that mimicked sepsis. In septic rats, arteriolar pretreatment with the “selective” iNOS blockers aminoguanidine (AG) or S-methylisothiourea sulfate (SMT) restored the responses to the control level. The putative neuronal NOS (nNOS) inhibitor 7-nitroindazole also restored the response toward control. At 24-h post-CLP, muscles showed no reduction of endothelial NOS (eNOS), elevation of nNOS, and, surprisingly, no induction of iNOS protein; calcium-dependent constitutive NOS (cNOS; nNOS) enzyme activity was increased whereas calcium-independent iNOS activity was negligible. We conclude that 1) AG and SMT inhibit nNOS activity in septic skeletal muscle, 2) NO could impair vasodilative responses in control and septic rats, and 3) the source of increased endogenous NO in septic muscle is likely upregulated nNOS rather than iNOS. Thus agents released from the blood vessel milieu (e.g., NO produced by skeletal muscle nNOS) could affect vascular responsiveness.

arteriole; acetylcholine; nitric oxide synthase

Nitric oxide (NO) is a potent vasodilator, synthesized by a family of isoenzymes, nitric oxide synthases (NOS; 32). This family is subdivided into two categories: one of constitutively expressed enzymes (cNOS) that produce low (nmol) levels of NO and require an increase in intracellular calcium to stimulate activity; and another that is a high-output, inducible enzyme [inducible nitric oxide synthase (iNOS), type II NOS] for which activity is independent of a calcium trigger. The high levels of NO produced via iNOS subserve pathophysiological events requiring macrophage cytotoxic activity (23). The cNOS enzymes are subdivided into two distinct gene products, which produce an endothelial (eNOS, type III) and a neuronal (nNOS, type I) form, based on the cell types in which these isozymes were first described (9). Furthermore, a splice-variant of the nNOS gene exists, which is expressed primarily within skeletal muscle (µNOS; 37). The constitutive isozymes produce NO to subserve autonomic and central nervous control (3, 5), to subserve vascular tone control (16), and to participate in pathophysiology (8).

Sepsis is often defined as a systemic inflammatory response to a local infectious or noninfectious insult. In terms of cardiovascular effects, the reduced responsiveness to vasoconstrictive (14) and vasodilative stimuli (1, 25, 34, 49) is one of the key features of sepsis. Impaired vascular response may lead to inappropriate distribution of blood flow to vital organs (19), maldistribution of blood flow within an organ (18), abnormal microvascular control of tissue oxygenation (36), and eventual multiple organ dysfunction (18). It has been shown that sepsis-induced production of NO by iNOS could be responsible for the vasoconstriction and vasodilation deficits (1, 14, 25, 38). In terms of the vasodilatation deficit, data from our laboratory (46) underscored the significant role of NO in that the attenuation of arteriolar dilation after local application of ACh in septic skeletal muscle was eliminated by the “selective” iNOS blocker aminoguanidine (AG).

The mechanism of reduced vascular sensitivity to ACh (or to other endothelium-dependent stimuli) in sepsis is not clear. Although the intracellular response to ACh in the endothelium can occur along several pathways, it is likely that sepsis affects the endothelium-derived relaxing factor (EDRF)/NO pathway. Previous reports suggest that downregulation (22, 24, 52, 53) and/or functional inhibition (negative feedback) of eNOS, mediated by the continuous production of NO during sepsis (4, 6, 26, 38), could possibly account for the reduced sensitivity. Our recent study (46) supported the latter possibility, because the restoration of the vasodilatation to ACh occurred within 5–7 min after AG application, not allowing sufficient time for de novo synthesis of eNOS.

The role of NO in functional inhibition of agonist-induced dilation in sepsis is poorly understood. On the basis of our recent work (38, 46), the objective of the
present study was to determine the effect of an increased level of NO, produced either exogenously or via iNOS, on arteriolar dilation to ACh in septic skeletal muscle. To this end, we have examined the NO-mediated attenuation of vasodilation to ACh using both NO donors and “selective” inhibitors of iNOS.

MATERIALS AND METHODS

Animal Preparation

The experimental protocol was approved by the University Council on Animal Care at the University of Western Ontario. Male Sprague-Dawley rats (300–350 g) were acclimatized for 1 wk and then divided into control and septic groups. Rats in the septic group were subjected to the same model of sepsis as described previously (46). We defined sepsis as the outcome of cecal ligation and perforation (CLP), laparotomy, line insertion, and resuscitation with a fluid mixture, to mimic the clinical situation of sepsis involving a surgical intervention (e.g., laparotomy) and fluid resuscitation via an inserted line. Consistent with the previous study (46), control rats did not undergo surgery or fluid resuscitation.

Briefly, rats in the septic group were given a mixture of halothane (1–2%) and oxygen (remainder) throughout the CLP procedure. A right carotid artery catheter (PE-50) was tunnelled through a posterior neck incision and advanced to the ascending aorta to permit infusion of fluids, withdrawal of blood samples [for blood gases, lactate, and NO metabolite (nitrate and nitrite) determinations], and measurement of blood pressure. Subsequently, the carotid catheter was attached to a swivel harness to allow free movement of the rat in its cage. A midline laparotomy was performed, and the cecum was isolated and ligated distal to the ileocecal valve to maintain bowel integrity. The cecum was punctured twice with an 18-gauge needle and returned to the peritoneal cavity. Incisions were sutured, and the carotid line was reconnected to a transilluminated preparation. For this reason, the majority of responses to arteriolar stimuli were evaluated in terms of changes in red blood cell velocity (V$_{RBC}$) in a daughter capillary fed by the stimulated arteriole. The acute arteriolar diameter change is almost entirely responsible for the ensuing acetylcholine (ACh) response (data not shown). Furthermore, V$_{RBC}$ change is a more sensitive index of the response than the diameter change (40). Nevertheless, arteriolar diameters were measured where possible for confirmatory purposes.

Evaluation of Microvascular Response

The epi-illuminated EDL muscle preparation is not optimal for arteriolar diameter measurement because the microscopic image of the vessel wall cannot be seen as sharply as in a transilluminated preparation. For this reason, the majority of responses to arteriolar stimuli were evaluated in terms of changes in red blood cell velocity (V$_{RBC}$) in a daughter capillary fed by the stimulated arteriole. The acute arteriolar diameter change is almost entirely responsible for the ensuing acetylcholine (ACh) response (data not shown). Furthermore, V$_{RBC}$ change is a more sensitive index of the response than the diameter change (40). Nevertheless, arteriolar diameters were measured where possible for confirmatory purposes.

Capillary blood flow was visualized at the final magnification of ×240 using the Leitz microscope [×10/0.22 numerical aperture (NA) objective and ×6.3 eyepiece], connected to a closed-circuit television system (Panasonic MV5410 monitor, MT1 camera, Mitsubishi U82 SVHS videotape recorder). V$_{RBC}$ was measured by the video flying spot technique (45). Arteriolar diameter was measured from the video screen at the final magnification of ×1,220 (×32/0.40 NA objective and ×10 eyepiece) at a resolution of ± 1 µm.

Arterioles selected arbitrarily were stimulated 100–200 µm upstream from the most distal capillary fed by the arteriole. An average of six arterioles were stimulated in one EDL muscle per rat. Diameters were measured at the site of stimulation along a 100-µm segment before and after the stimulation. Measurements along the segment were averaged to obtain control diameters (D$_{CON}$) and peak poststimulus diameters (D$_{PEAK}$). The percent diameter response was calculated as ∆D (%) = 100 × (D$_{PEAK}$ – D$_{CON}$)/D$_{CON}$. V$_{RBC}$ response was measured in a capillary fed by the stimulated arteriole. We required that the 2-min average baseline V$_{RBC}$ (V$_{RBC}$ base) in this capillary was in the range of 100–200 µm/s. Our preliminary experiments in control rats demonstrated that the percent V$_{RBC}$ increase caused by the feeding arteriole stimulation with ACh was inversely related to V$_{RBC}$ base (data not shown). The selection of capillary with velocity within the range was required to ensure that a possible treatment-induced attenuation of the V$_{RBC}$ response to ACh was not due...
to the baseline $V_{RBC}$ effect. In general, $V_{RBC}$ response was calculated as $\Delta V_{RBC} (%) = 100 \times (V_{RBC\;peak} - V_{RBC\;con}) / V_{RBC\;con}$. Responses where poststimulus $V_{RBC}$ did not return to $\pm 15\%$ of $V_{RBC\;con}$ were rejected.

Immunoblotting

Immunoblotting was performed on septice and naive control EDL muscle homogenates. Both septic and naive EDL muscles were homogenized in $5$ volumes (wt/vol) of ice-cold homogenization buffer ($50$ mM Tris·HCl, $1.25$ mM CaCl$_2$, $0.2$ mM phenylmethylsulfonyl fluoride, $1$ mM EDTA, $10$ µM anti-

pain, $10$ µM leupeptin, $10$ µM pepstatin, $10$ µM chymostatin, and $10$ µM soybean trypsin inhibitor; pH $7.4$). A total of $80$ µg of protein for eNOS and iNOS analyses and $100$ µg of protein for nNOS analyses were loaded into each well of the mini-gel apparatus (Mini Gel II; Bio-Rad). Separation of NOS isoforms was performed by electrophoresis on a $7.5\%$ SDS-tris-glycine polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with $5\%$ wt/vol nonfat milk powder and incubated with mouse primary monoclonal antibodies against eNOS ($1:2,500$ dilution), iNOS ($1:500$), and nNOS ($1:500$) proteins (Transduction Laboratories). Lysates of human endothelial cells, cytokine-activated murine macrophages, and human pituitary cells were used as positive controls, respectively. Membranes were then incubated with a horseradish peroxidase-conjugated anti-mouse secondary antibody ($1:1,000$; Amersham) for $1$ h. Blots were developed by enhanced chemiluminescence (ECL; Amersham) and exposed to X-ray film for $45–60$ s. Optical density readings of the blots were obtained and quantified (model GS700; multianalyst version 1.1; Bio-Rad). Control experiments were carried out to ensure that no NOS isozyme cross-reactivity of the employed antibodies occurred.

NOS Activity

NOS enzyme activity in EDL muscle homogenates was measured as described previously by us ($38, 39$). In separate experiments, concentration-dependent AG inhibition of nNOS, eNOS, and iNOS isoforms was also determined. nNOS was isolated from rat cerebellar supernatant. Briefly, rat cerebellum were homogenized in $6$ volumes (wt/vol) of homogenizing buffer ($10$ mM HEPES buffer, $0.1$ mM EDTA, $1$ mM dithiothreitol, $1$ mg/ml phenylmethylsulfonyl fluoride, and $0.32$ M sucrose; pH $7.4$) and centrifuged ($100,000 \times g$) for $60$ min at $4^\circ C$ in a L5–65B Ultracentrifuge, Beckman Instruments, Mississauga, Ontario, Canada). The cytosolic eNOS from the supernatant was used for subsequent assays. Recombinant bovine eNOS ($20$ U/ml) and rat iNOS isoforms ($250$ U/ml) were purchased from Cayman Chemical (Ann Arbor, MI) and diluted to $1:10$ (vol/vol) and $1:50$, respectively, in homogenization buffer. Inhibition curves were constructed by incubating aliquots (25 µl) of each of the isozymes under control conditions (i.e., all cofactors and substrate provided, with no inhibitors), with increasing concentrations of AG ($10^{-10}$ to $10^{-1}$ M), and with $1$ mM N$^+$-nitro-L-arginine methyl ester (to control for background nonspecific radioactivity).

Nitrate and Nitrite Determination

Blood samples from control and septic rats were drawn into heparin sodium vacuum containers and centrifuged for $15$ min at $1,000 \times g$. Plasma ($10–20$ µl) was injected into a purge chamber containing vanadium(III) chloride [to convert NO$_2$ + NO$_3$ (NO$_2^-$) to NO gas]. NO gas was then carried via inert carrier gas (He) to the NO analyzer (model 270B, Sievers). NO measurement was based on a gas-phase reaction between NO and ozone, producing light detectable by a photomultiplier tube. NO concentrations in plasma samples were determined from a standard curve constructed to sodium nitrate.

Experimental Design: In Vivo Studies

Effect of L-NNA on arteriolar response to ACh. The objective was to establish the presence of the EDRF/NO pathway in the arteriolar response to ACh in control rats. A pretreatment of arteriole with the competitive L-arginine inhibitor (L-NNA) was used for this purpose. The experiment consisted of (1) a $2$-min control video recording of blood flow in a capillary, (2) application of a droplet of L-NNA ($15$ mM) on the feeding arteriole, (3) application ($3$ min later) of a droplet of ACh ($10$ mM) on the same arteriolar site, and (4) recording of the ensuing $V_{RBC}$ response in the capillary. In a further experiment, after the $2$-min control flow recording in a capillary fed by a different arteriole, a droplet of ACh ($10$ mM) was applied on this arteriole and the resulting capillary $V_{RBC}$ response recorded. To assess the effect of L-NNA pretreatment, we compared $\Delta V_{RBC}$ values from these two experiments based on $V_{RBC}$ measurements obtained from the video recordings.

Effect of NO donors on arteriolar response to ACh in control rats. To examine whether NO could affect the microvascular response to ACh in skeletal muscle, we pretreated arterioles with either SNP or SNAP ($5$ mM each; two structurally unrelated NO donors). In practice, blood flow in a capillary was recorded for $2$ min and, concurrently, the average $V_{RBC\;con}$ in this capillary was estimated via online $V_{RBC}$ monitoring. SNP or SNAP was then locally ejected on the feeding arteriole, and the ensuing $V_{RBC\;con}$ response was monitored. As soon as $V_{RBC\;con}$ returned to the $V_{RBC\;con}$ level ($\sim 3–4$ min), capillary blood flow was further recorded for $30$ s (concurrent on-line monitoring ensured that $V_{RBC\;con}$ remained stable at the $V_{RBC\;con}$ level). Thirty seconds after the end of this recording (i.e., $4–5$ min after SNP/SNAP), ACh ($10$ mM) was applied on the same arteriolar site and the ensuing $V_{RBC\;con}$ response was recorded. Computed $\Delta V_{RBC}$ was based on off-line $V_{RBC}$ measurements obtained from the $30$-s control and the ACh-stimulated response recordings. Arteriolar diameter response to the sequential application of SNAP + ACh was also determined in separate arterioles. Here the delay between the SNAP and ACh applications was not based on on-line monitoring, but rather on the average delay measured in the SNAP + ACh $V_{RBC\;con}$ experiment. The effect of sequential SNP + ACh or SNAP + ACh application was compared with the effect of sequential vehicle + ACh or ACh alone applications, in separate arterioles. Because NO donors themselves cause both dilation and increase in capillary $V_{RBC\;con}$, the nonspecific effect of dilation and $V_{RBC\;con}$ increase on the subsequent response to ACh was also examined using a different vasodilator (NECA). Arterioles were pretreated with NECA ($10$ µM), and capillary $V_{RBC\;con}$ was monitored. After $V_{RBC\;con}$ returned to the baseline and the $V_{RBC\;con}$ stability ($30$ s) was ensured, ACh was applied (i.e., $4–5$ min after NECA) on the same arteriolar site and the resulting $V_{RBC\;con}$ response was recorded. The effect of sequential NECA + ACh application was compared with the effect of ACh alone application on separate arterioles.

Effect of iNOS blockers and NO donors on arteriolar response in septic rats. To determine whether NO is involved in impaired vasodilation in septic skeletal muscle, we aimed to show that 1) the “selective” iNOS blockers AG and SMT eliminate the deficit in ACh-stimulated arteriolar response in septic rats, and 2) that NO donors added to AG or SMT re-establish the deficit. A droplet of AG ($10$ mM) or SMT ($5$ µM) was applied on the septic arteriole; $5$ min later, ACh ($10$ mM) was applied on the same site and the capillary $V_{RBC\;con}$ response was recorded. A droplet of the mixture ($10$ mM AG +
5 mM SNAP, 10 mM AG + 5 mM SNAP, or 5 mM SMT + 5 mM SNAP) was then applied on separate septic arterioles and the capillary V_{RBC} was measured. When V_{RBC} returned to the baseline and V_{RBC, con} stability was ensured, ACh (10 mM) was applied (i.e., 4–5 min after the mixture) on the same arteriolar site and the V_{RBC} response was recorded. Arti- dopolar diameter responses to AG+ACh and to (AG+SNAP)+ACh were also obtained. Responses to AG+ACh, SMT+ACh, (AG+SNAP)+ACh, (AG+SNAP)+ACh, or (SMT+SNAP)+ACh were compared with responses to vehicle+ACh or to ACh alone applied on separate septic arterioles. In this study we also tested whether the 5-min pretreatment with AG or SMT affected the V_{RBC} response to ACh in control rats. Responses to AG+ACh or to SMT+ACh were compared with responses to ACh alone measured in separate control arterioles.

Effect of NO on endothelium-independent vasorelaxation in control and septic rats. NO stimulates guanylate cyclase to produce smooth muscle relaxation. The overproduction of NO in sepsis could desensitize guanylate cyclase and indirectly result in a deficit in ACh-stimulated vasodilation. The objectives were to test 1) the effect of NO donor on the endothelium-independent arteriolar dilation (caused by SNAP), 2) the presence of endothelium-independent dilatation in septic rats, and 3) the effect of NO on a possible enhancement of endothelium-independent dilatation in septic rats. Control arterioles were locally stimulated with SNAP (5 mM); when capillary V_{RBC} returned to the baseline and V_{RBC, con} stability was ensured, SNAP (5 mM) was applied again (i.e., 4–5 min after the previous SNAP) on the same site. V_{RBC} responses to sequential SNAP+SNAP and responses to SNAP alone were compared. Septic arterioles were then locally stimulated with SNAP (5 mM) and capillary V_{RBC} responses were recorded. Finally, separate septic arterioles were locally pretreated with AG (10 mM); after 5 min they were stimulated with SNAP (5 mM). For septic arterioles, we compared capillary V_{RBC} responses between SNAP and AG+SNAP groups; we also compared responses to SNAP between control and septic arterioles.

Experimental Design: In Vitro Studies

Immunoblotting of NOS isozymes in EDL muscles of control and septic rats. It is possible that the deficit in vasodilatation to ACh in sepsis is due to an increased production of NO via iNOS or due to downregulation of the eNOS protein itself (22, 24, 53). Assessment of the relative amount of the NOS isozymes protein levels in control and septic muscles was carried out to address these possibilities.

cNOS and iNOS enzyme activities. To complement the immunoblotting study, we determined the cNOS (i.e., nNOS and eNOS) and iNOS enzyme activities of control and septic EDL muscles. To complement the in vivo studies, we also tested for the effect of the “selective” iNOS blockers AG (100 µM) and SMT (30 nM). Enzyme activities with AG or SMT treatment were compared with those with potassium phosphate buffer. On the basis of our previous work (28) indicating that drugs in the pipette were diluted ~100× before reaching the arteriolar wall, these blocker concentrations approximated the effective concentrations of our in vivo experiments.

Data Analysis

For each rat, the effect of a particular stimulus was analyzed in one to three arterioles; data from these arterioles were averaged. Data in this study are presented as mean ± SE, based on the number of rats (n). For both control and septic groups, ANOVA was performed among D_{con} in different experiments to ensure that control and septic rats came from the same populations. Data from in vivo experiments were analyzed using Student’s t-test with a Bonferroni correction for multiple comparisons, unless stated otherwise. Systemic parameter measurements (Table 1), immunoblot optical density, and enzyme activity data were compared with the Student’s t-test. P values <0.05 were considered significant. All tests were calculated with Systat 6.01 statistical software.

RESULTS

Baseline measurements of systemic parameters in control and septic rats under the conditions of pentobarbital anesthesia and ventilation are shown in Table 1 (note that not all parameters could be measured in all control and septic animals). Sepsis induced 2.6- and 2.0-fold increases in plasma lactate and NOx levels, respectively. Despite fluid resuscitation, septic rats had a slightly lower arterial pressure than control rats; however, both groups were normotensive. Data of systemic parameters were consistent with our earlier studies (18, 46) of this model of sepsis. The baseline diameter of terminal arterioles was also slightly reduced in septic rats (Table 1). This reduction appears to contradict the reported no change (2) or increase in diameter of terminal arterioles in sepsis (1, 25). The discrepancy could be due to the inherent technical difficulty associated with arteriolar diameter measurement in the model. Because of epi-illumination of the EDL muscle surface, deeper arterioles or proximal segments of arterioles (i.e., >200 µm upstream from the last bifurcation) were not accessible for measurement. It is possible that the selection process excluded deeper or proximal vessels that could be dilated in sepsis. Thus conclusions based on arteriolar diameter measurement in the present study are limited to the population of arterioles examined. Postmortem examination of both the lungs and abdominal cavity revealed necrotic areas of the lungs, necrotic necum, and purulent peritoneal fluid in septic rats. In contrast, control rats had normal lungs and abdominal peritoneal cavity. The mortality rate associated with the CLP procedure was 23%.

Response to ACh in control rats. Similar to our previous study (46), ACh caused peak increases of 100–120% and 25–30% in capillary V_{RBC} and arteriolar diameter, respectively. Responses occurred within sev-

### Table 1. Baseline parameters in control and septic rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Septic</th>
<th>Significance</th>
<th>P</th>
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<tbody>
<tr>
<td>Body wt, g</td>
<td>326 ± 5 (46)</td>
<td>339 ± 3 (52)</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Arterial blood pH</td>
<td>7.51 ± 0.01 (34)</td>
<td>7.51 ± 0.01 (50)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Arterial blood PO_{2}, Torr</td>
<td>107 ± 4 (34)</td>
<td>105 ± 4 (50)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Arterial PCO_{2}, Torr</td>
<td>33 ± 1 (34)</td>
<td>31 ± 1 (50)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Blood lactate, mmol/l</td>
<td>0.73 ± 0.03 (42)</td>
<td>1.9 ± 0.1 (51)</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>114 ± 1 (46)</td>
<td>102 ± 2 (43)</td>
<td>&lt;0.01</td>
<td></td>
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<tr>
<td>Plasma NOx, µM</td>
<td>20.9 ± 3.6 (10)</td>
<td>43.1 ± 5.8 (15)</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Arteriolar diameter, µm</td>
<td>9.0 ± 0.3 (20)</td>
<td>8.0 ± 0.2 (21)</td>
<td>&lt;0.01</td>
<td></td>
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Values are means ± SE. Numbers in parentheses indicate number of rats. NS, nonsignificant difference between groups. P > 0.05. (N.B. from all rats prepared for intravital microscopic examination of skeletal muscle only a subset was used for arteriolar diameter measurement.)
eral seconds after the application of ACh and lasted ~2–3 min. Pretreatment with L-NNA resulted in a significant 67% reduction in the \( V_{RBC} \) response to ACh \([116 \pm 17\% \text{ increase after ACh versus L-NNA+ACH; } V_{RBC \text{ con}}: 140 \pm 7 \text{ µm/s (n = 14)} \) and 158 \pm 9 \text{ µm/s (n = 11)}, respectively], indicating the presence of the EDRF/NO pathway in the arteriolar response to ACh. The residual response to ACh (i.e., 39% increase) could have reflected the fact that the concentration of L-NNA was not sufficiently high to completely block the response to ACh, or that an alternative pathway (e.g., endothelium-derived hyperpolarizing factor, EDHF) was involved in the response. L-NNA itself had no significant effect on \( V_{RBC} \). Arterioles treated with the vehicle PBS did not affect \( V_{RBC} \) (12 \pm 20% change, n = 5) or the \( V_{RBC} \) response to ACh (95 \pm 25%, n = 5).

The effect of NO donors on ACh-stimulated \( V_{RBC} \) increase in control rats is shown in Fig. 1. SNP and SNAP pretreatment caused significant reductions (63 and 59%, respectively) in the response to ACh, respectively, whereas pretreatment with the saline vehicle had no effect. Arteriolar diameter measurements confirmed these results. Diameter increases after ACh alone (30 \pm 3%, \( D_{\text{con}} = 8.6 \pm 0.6 \text{ µm, n = 11} \)) and after saline + ACh (26 \pm 4%, \( D_{\text{con}} = 8.3 \pm 0.6 \text{ µm, n = 6} \)) did not differ, whereas the increase after SNAP + ACh (10 \pm 3%, \( D_{\text{con}} = 9.1 \pm 0.5 \text{ µm, n = 9} \)) was significantly smaller. SNP or SNAP pretreatment caused a temporary ~100% increase in \( V_{RBC} \) that lasted 3–4 min. Arteriolar pretreatment with NECA had no effect on the \( V_{RBC} \) response to ACh; we measured 116 \pm 17% (n = 14) and 123 \pm 18% (n = 7) increases after ACh alone and after NECA + ACh applications, respectively.

Response to ACh in septic rats. Sepsis caused a significant attenuation in the ACh-stimulated increase in capillary \( V_{RBC} \) \([36 \pm 6\% \text{ in controls (Fig. 1)] vs. 116 \pm 17\% in septic arterioles with saline (i.e., the vehicle of AG and SMT), which was not different from the dilution to ACh seen in control rats. Pretreatment of septic arterioles with saline (i.e., the vehicle of AG and SMT) had no effect on the deficit (Fig. 2). Diameter data confirmed this effect, because \((\text{AG+SNAP}) \) caused a 24 \pm 3\% increase \((D_{\text{con}} = 7.5 \pm 0.5 \text{ µm, n = 12} \), which was not different from the dilution to ACh seen in control rats. Pretreatment of septic arterioles with saline (i.e., the vehicle of AG and SMT) had no effect on the deficit (i.e., ACh induced only a 32 \pm 10\% increase \((D_{\text{con}} = 8.6 \pm 0.6 \text{ µm, n = 6} \)) did not affect the \( V_{RBC} \) response to ACh; responses to AG versus ACh (92 \pm 18%, n = 8) and to SMT + ACh (117 \pm 20%, n = 5) did not differ from the response to ACh alone (Fig. 1).

Response to an endothelium-independent stimulus. Figure 3 shows that, in terms of the \( \Delta V_{RBC} \) response to SNAP, there was no difference in the endothelium-independent response between control and septic rats. In control rats, pretreatment with the NO donor SNAP did not affect the response to SNAP (i.e., SNAP + SNAP data in Fig. 3). In septic rats, pretreatment with AG did not affect the response to SNAP either (AG + SNAP data in Fig. 3).

Western blots and enzyme activity. Figure 4 (left and middle immunoblots) shows that sepsis had no effect on the level of iNOS protein (120 kDa) or the level of eNOS protein (135 kDa) in the EDL muscle at the 24-h time point. Figure 5 shows that sepsis significantly elevated the nNOS (i.e., calcium-dependent eNOS + nNOS) activity, whereas it did not affect the iNOS (i.e., calcium-dependent) activity at 24 h. Protein concentrations in the control \((15.9 \pm 1.9 \text{ mg/ml}) \) and septic \((14.7 \pm 1.6 \text{ mg/ml}) \) homogenates were similar. Figure 6 shows that, in septic rats, AG (100 µM) and SMT (30 nM) reduced the nNOS activity toward the level seen in control rats.
In control rats, addition of AG (100 µM) to the incubation buffer resulted in cNOS activity (1.37 ± 0.19 pmol L-[3H]citrulline·min⁻¹·mg protein⁻¹) not significantly different from the cNOS activity measured with the vehicle only (Fig. 5, left open bar).

Involvement of nNOS. The lack of elevation in the iNOS protein and enzyme activity levels at the 24-h time point of sepsis was unexpected. Because the increased cNOS (i.e., eNOS+nNOS) activity in sepsis was reduced by AG to the cNOS level observed in control rats, we pursued the possibility that sepsis elevated nNOS rather than iNOS. Figure 4 (right immunoblots and densitometry readings) demonstrates that the nNOS protein expression in EDL muscles from septic rats was indeed significantly higher than in control rats. The upper band in the septic lane (165 kDa; seen consistently only in nNOS blots from septic muscles) was apparently of a slightly lower molecular mass than the pituitary lysate nNOS protein. This was likely due to the presence of the truncated, splice-variant skeletal muscle µNOS, which still retains the epitope to the antibody (9). Alternatively, the upper band (and the lower band seen in both control and septic lanes) may represent denaturation products of the nNOS in the preparation of the protein sample. Densitometry was performed using both upper and

Fig. 3. Effect of an endothelium-independent stimulus (SNAP) on capillary \( V_{\text{RBC}} \) in control and septic rats. Open bars, effect of a droplet of SNAP (5 mM) applied on arteriole in control rats, with and without 4–5 min arteriolar pretreatment with SNAP (5 mM). Solid bars, effect of SNAP (5 mM) applied on arteriole and of SNAP after 5-min arteriolar pretreatment with AG (10 mM) in septic rats. \( V_{\text{RBC}} \) baselines (from left to right, 133 ± 14, 147 ± 12, 133 ± 12, and 143 ± 15 µm/s) were comparable. Numbers in parentheses denote number of animals.

Fig. 4. Examples of immunoblots of control and septic rat EDL muscle homogenates (A), and densitometry readings (B), using anti-inducible nitric oxide synthase (iNOS), anti-endothelial NOS (eNOS), anti-neuronal NOS (nNOS) monoclonal antibodies, and cytokine-induced murine macrophages (Mac), human endothelial cell (EC) lysate, and human pituitary cell lysate as positive controls, respectively. Molecular mass of iNOS, eNOS, and nNOS (upper band in septic lane) proteins was 120, 135, and 165 kDa, respectively. Densitometry readings for eNOS and nNOS were normalized to average reading in the control lane. Numbers in parentheses denote number of animals. *Significant difference between control and septic groups.

Fig. 5. In vitro constitutive NOS (cNOS) (i.e., eNOS+nNOS) and iNOS enzyme activities measured in control and septic EDL muscle homogenates. Numbers in parentheses denote number of animals. *Significant difference between cNOS activities in control and septic muscles.
lower bands; it consistently exhibited an increased nNOS-positive protein in the septic lanes compared with controls lanes.

To determine whether functional inhibition of nNOS affects the ACh-stimulated vasodilation deficit in septic rats, we used the putative nNOS inhibitor 7-NI (17) in our in vivo experiment. First, we used a cerebellum homogenate from rat (a source of high nNOS content; 10) to confirm that 7-NI (1 µM) is capable of blocking nNOS enzyme activity (data not shown). Next, arterioles in septic rats were pretreated locally for 5 min with 7-NI (100 µM), and the V_{RBC} response to ACh was examined. Figure 7 shows that 7-NI significantly increased this response in septic rats. Finally, concentration-dependent inhibition of nNOS, eNOS, and iNOS isozyme activities by AG was determined in vitro. On the basis of inhibition curves done in quadruplicate for each isozyme (Fig. 8), IC_{50} values for AG were calculated (Fig. 8) using a linear regression of the Hill plot (i.e., intercept values). These IC_{50} values indicate that all of the NOS isozymes are capable of inhibition by AG.

Fig. 6. In vitro cNOS and iNOS activities in septic rat EDL muscle homogenates in the presence or absence (control bars on left) of iNOS inhibitors AG (100 µM) or SMT (30 nM). AG and SMT reduced cNOS activity to level seen in control rats (Fig. 5, left). Numbers in parentheses denote number of animals. *Significant change from cNOS activity in control (untreated) homogenates.

DISCUSSION

The key findings of the present study were 1) the deficit in ACh-stimulated arteriolar dilation in sepsis was "immediately" eliminated by AG and SMT, but restored by NO donors; 2) in the EDL muscle, sepsis was not associated with an induction of iNOS or a change in eNOS, but rather there was an increase in nNOS protein level; and 3) sepsis elevated calcium-dependent (eNOS + nNOS) but not calcium-independent iNOS enzyme activity. These results are consistent with the hypothesis that NO inhibits arteriolar dilation to ACh but indicate that nNOS rather than iNOS could be the source of increased NO in skeletal muscle during sepsis.

Effect of NO donors on arteriolar response to ACh in control rats. The rationale for using NO donors in control rats was to establish that exogenous NO per se can affect the vascular response to ACh. Although the response can be mediated by one of, or a combination of, at least three pathways (i.e., EDRF/NO, EDHF, and prostaglandins), the major component of this response in arterioles of the present EDL muscle model appeared to be mediated by the EDRF/NO pathway. L-NNA caused a substantial attenuation (67%) of the V_{RBC} response to ACh. A similar contribution of the EDRF/NO pathway was reported for the response in terminal arterioles of the rat cremaster muscle (47).

The present data (Fig. 1) demonstrate that exogenous NO can inhibit the vascular response to ACh, but they do not explain the exact mechanism of this inhibition. An indirect inhibitory effect of NO on muscarinic receptor/G protein function via activation of guanylyl cyclase is possible (29), because an NO donor has been shown to modulate the bradykinin (BK)-signaling pathway in endothelial cells in vitro by selectively inhibit-
ing G proteins of the G_{i} and G_{q} family (29). Alternatively, it has been shown in vitro that NO can directly inhibit NOS activity, in particular eNOS (3, 35), likely through interaction with the heme moiety of NOS (50). There is also evidence that NO can participate in the regulation of the EDRF/NO pathway in vivo. Cohen et al. (6) showed that infusion of an NO donor into rabbit hindquarters attenuated the endothelium-dependent (agents: ACh, BK), but not the endothelium-independent, vasodilator response (agent: SNAP). Our data confirmed and extended this observation by determining the effect of NO donors at the level of a single microvessel (Figs. 1 and 3). Furthermore, we have demonstrated that this effect was not due to a non-specific vasodilator effect of the donor since NECA (another vasodilator) failed to attenuate the V_{RBC} response to ACh.

Our protocol of sequential application of NO donors and ACh did not permit an assessment of the maximal inhibition by NO donors. Assuming that the amount of NO given off by the donors at the arteriole dictated the degree of dilation and V_{RBC} increase, the maximum effect of donors would have occurred several seconds after their application, at the peak of dilation. The necessity of waiting for the diameter and V_{RBC} to return to baseline might have resulted in a smaller amount of NO donors (and hence, NO) at the site of the arteriole at the time of ACh application. In terms of the vascular response to ACh, the effect of the smaller amount of NO at the arteriole in control rats could have mimicked the effect of sepsis. This is because 1) the immediate pre-ACh arteriolar diameter was similar to that in septic rats, and 2) the donor-mediated attenuation in response to ACh was similar to the attenuation seen in septic rats (compare SNAP+ACh or SNAP+ACh data in Fig. 1 to ACh data in Fig. 2). Hence, the NO levels in SNP/SNAP-treated controls were likely similar to those in septic EDL muscles, resulting in similar attenuation of the ACh-stimulated vasodilation.

Effect of sepsis on arteriolar response to ACh. The sepsis-induced deficit in dilation (i.e., an 8% instead of 30% diameter increase) of the present study agrees with the reported attenuation of response to ACh in several models of sepsis (1, 25, 34, 46, 48). The present deficit was not due to the limited ability of the arteriole to relax, because there were several stimuli that yielded responses well above the level of deficit (i.e., see responses to AG+ACh, SMT+ACh, and SNAP in Figs. 2 and 3). Our data (Fig. 3, SNAP in sepsis) agree with reports of no attenuation of endothelium-independent dilation (25, 34, 48) and suggest that the deficit was mediated through the loss of endothelium-dependent dilation.

The attenuated responsiveness to ACh could be due to a sepsis-induced change in the muscarinic receptor and/or G protein function. Indeed, sepsis has been shown to alter the expression of G protein regulator proteins (33) and of specific G protein α-subunits (51). However, the immediate and complete restoration of the dilation to ACh, following treatment with AG or SMT (Fig. 2), indicated that an NO-dependent mechanism was more likely responsible for the vasodilation deficit. This deficit was unlikely to be explained by a loss of substrate (l-arginine) for eNOS (49) or insufficient production of the cofactor tetrahydrobiopterin (13) in endothelial cells, because both the l-arginine transporter and GTP cyclohydrolase I, respectively, are upregulated in vitro following treatment with lipopolysaccharides (LPS). Endotoxemia has been reported to reduce both basal eNOS activity (30), ACh-stimulated eNOS activity, and Ach-stimulated NO production in blood vessels (31). Cytokines and/or endotoxin has been shown to downregulate eNOS mRNA (24, 52), whereas the CLP model of sepsis reduced the eNOS protein level in aortic endothelial cells (53). However, downregulation of eNOS in skeletal muscle was an unlikely mechanism of the deficit in the present study, because the level of eNOS protein was not altered by sepsis whereas the cNOS (eNOS+nNOS) enzyme activity was substantially increased. Furthermore, the 5-min period required by AG or SMT to eliminate the deficit (Fig. 2) would seem too short for de novo synthesis of eNOS.

It has been proposed that the continuous production of NO via iNOS during endotoxemia is responsible for the decreased sensitivity to ACh-mediated vasodilation (38, 46). Endotoxemia is known to be associated with elevated iNOS mRNA, protein, and enzyme activity levels in many tissues (8, 21, 38, 43). At first glance, the data in Fig. 1 appear to agree with this proposal, because experiments in control rats indicated that NO could attenuate the arteriolar response to ACh. Furthermore, the data in Fig. 2[(AG+SNP)+ACh, (AG+SNAP)+ACh, and (SMT+SNAP)+ACh groups] confirmed that NO could cause a comparable attenuation in septic rats, and that the “selective” iNOS inhibitors AG and SMT eliminated the deficit in ACh-stimulated response. However, the lack of iNOS protein expression and activity in the septic EDL muscle (Figs. 4 and 5) contradict the literature reports from other organs (8, 21, 38). If increased NO were involved in the observed vasodilation deficit, but iNOS was not present, where did the increased NO come from?

The endotoxin-induced increase in iNOS expression in skeletal muscle (8, 43) has been shown to be much more transient than that observed in large blood vessels following LPS or CLP treatment (38, 39). In mouse skeletal muscle, LPS-stimulated iNOS mRNA expression peaked around 4 h and then subsided at 8 h, whereas the iNOS protein level followed a similar time course resulting in a dramatic reduction by 12 h (43). In the rat diaphragm, iNOS protein and enzyme activity peaked 12 h after endotoxin injection and disappeared by 24 h (8). These earlier findings agree with the present lack of iNOS protein and enzyme activity found 24 h after the CLP procedure. However, the present findings do not exclude a possible, yet undiscovered, earlier effect of elevated iNOS level (e.g., at 12 h) on the vasodilation deficit seen at 24 h.

Contrary to the findings (38, 39) in conduit vessels in CLP rats, cNOS activity in our septic EDL muscle doubled compared with controls (Fig. 5). Recently, it has been shown (8) in septic rat skeletal muscles that cNOS activity rises substantially within 12 h after...
endotoxin injection and remains elevated for 24 h. The increase of cNOS activity has been attributed to an induction of nNOS (or the skeletal muscle splice-variant of nNOS (µNOS)), or to a decrease in the tissue level of an endogenous protein inhibitor of nNOS (11). The present results from septic rats confirm these findings in terms of significant increases in cNOS activity and in protein expression of nNOS in the EDL muscle. Thus an NO-mediated functional inhibition of ACh-stimulated response could involve nNOS rather than iNOS upregulation in septic skeletal muscle. This proposal is consistent with the current observation that the putative nNOS inhibitor (7-NI) improved the \( V_{RBC} \) response to ACh in sepsis (Fig. 7).

In endotoxin-injected rats, the increase in nNOS protein in rat skeletal muscle has previously been shown (8) to be localized in the subsarcolemmal space. On the basis of this report, the present study suggests that, in terms of NOS protein profile at 24 h post-CLP (Fig. 4), the effect of sepsis was precipitated in the parenchymal tissue rather than in the vasculature. Thus sepsis-induced changes in the parenchyma (i.e., increased NO production) appeared to affect the vascular behavior (i.e., arterial response to ACh). Therefore, we propose that studies addressing the effect of sepsis in different segments of the vascular tree should take into account the alteration of the parenchymal cellular milieu.

Effect of “selective” iNOS inhibition with AG and SMT. The lack of detectable change in iNOS protein in septic EDL muscle raises the question of how the “selective” iNOS inhibitors AG and SMT restored the ACh-mediated vasodilation in septic arterioles. Because 1) AG and SMT did not attenuate the \( V_{RBC} \) response to ACh (i.e., indirect measure of eNOS activity) in control rats, 2) eNOS protein expression was not increased in sepsis (Fig. 4), and 3) iNOS was not detectable, it is possible that the inhibitors affected nNOS rather than iNOS in septic skeletal muscle. Thus the nNOS likely produced sufficient NO to attenuate the ACh-mediated vasodilation, which would otherwise have been attributed to the elevated iNOS activity in sepsis. This is corroborated by the current observations that 1) AG did not significantly reduce cNOS activities (i.e., the eNOS plus nNOS activities) in control muscle, 2) AG restored the high eNOS activity in septic muscle to the level seen in control tissues (Fig. 6), and 3) AG inhibited both eNOS and nNOS at higher concentrations (Fig. 8). Although both AG and SMT have been previously shown to be 30–40 times more selective for iNOS than the cNOS isoforms (12, 27, 43), the present data agree with the reports that AG and SMT are capable of inhibiting both isoforms (15, 20, 41). Thus caution must be exercised when these “selective” inhibitors are used in situations that may involve both nNOS and iNOS isoforms.

In conclusion, the present study demonstrated for the first time that NO donors can attenuate the microvascular response to ACh in both control and septic rats and that, surprisingly, the possible increased endogenous NO may come from upregulated nNOS and not iNOS protein at 24 h after the CLP procedure. The study is consistent with the hypothesis that increased production of NO, rather than downregulation of eNOS, is responsible for the deficit in vasodilation to ACh observed in the microvasculature of septic rat skeletal muscle.

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REFERENCES

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