Local L-NAME decreases blood flow and increases leukocyte adhesion via CD18

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Mitchell, Debra J., Jingcheng Yu, and Karel Tyml. Local l-NAME decreases blood flow and increases leukocyte adhesion via CD18. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H1264–H1268, 1998.—Local inhibition of nitric oxide (NO) synthesis with l-arginine analogs such as N^G-nitro-l-arginine methyl ester (l-NAME) decreased red blood cell velocity (V_{RBC}) in capillaries and increased leukocyte adhesion in postcapillary venules in rat skeletal muscle. The goal of the present study was to determine the mechanism of this response to l-NAME. Using intravitral videomicroscopy, we examined blood flow in the surface microvasculature of rat extensor digitorum longus muscle. l-NAME (30 mM in the pipette) locally applied to capillaries (300 µm from feeding arteriole) reduced V_{RBC} (control V_{RBC} = 244 ± 53 (SE) µm/s; ΔV_{RBC} = −52 ± 8%) and increased leukocyte adhesion (from 0.2 ± 0.01 to 1.3 ± 0.3 cells/100 µm) in control animals. Systemic pretreatment with fucoidan (selectin binder), superoxide dismutase and catalase (extracellular antioxidants), and, in separate animals, at high magnification (for leukocyte adhesion). The responses in individual microvessels were averaged for each animal.

Methods
Animal preparation. Experiments were approved by the University Council on Animal Care at the University of Western Ontario (London, ON, Canada). Male Wistar rats [225 ± 8 (SE) g; n = 67; Charles River, St. Constant, PQ, Canada] were anesthetized with pentobarbital sodium (65 mg/kg ip; Somnotol, MTC Pharmaceuticals). Anesthesia was maintained by supplemental pentobarbital (22 mg/kg ip). The rat was placed on the stage of an intravital microscope (Leitz, ELA). Temperature was maintained at 36.5–37.5°C by means of a heating pad (American Medical Systems) and a heating lamp. The left carotid artery and right jugular vein were cannulated to measure blood pressure and to deliver agents, respectively. The animal was placed on its side with one leg held by a clamp attached to the microscope stage. The EDL muscle was exposed as described (13, 18). The exposed muscle was covered by a plastic coverslip (18 × 18 mm) with a hole (3 mm in diameter) surrounded by a silicon ring. The hole was filled with degassed heavy paraffin oil to prevent tissue drying and to allow micropipette access. The muscle was then epi-illuminated. The muscle surface microcirculation was visualized on a closed-circuit video system via a ×10/0.22 NA objective and ×6.3 eyepiece (low magnification) or a ×32/0.40 NA objective and ×10 eyepiece (high magnification). These magnifications yielded fields of view that were 780 × 1050 µm or 154 × 207 µm on the monitor, respectively. Before experiments, we allowed the blood flow to stabilize for 30 min. The experiments lasted up to 60 min.

Local application of materials. Experiments were carried out on randomly chosen surface capillaries in the EDL muscle (1 muscle per rat, up to 4 microvessels per muscle). The capillaries ran parallel to the skeletal muscle fibers and were at least 500 µm in length. Capillaries were locally stimulated 300 µm downstream from the feeding arteriole via pressure injection (3, 13).

Glass pipettes (tip <3 µm in diameter) were backfilled with l-NAME (Sigma Chemical, St. Louis, MO). l-NAME was dissolved in phosphate buffer solution (pH 6.8; composition in mM: 138.9 NaCl, 2.25 KCl, 1.75 KH_{2}PO_{4}, and 1.4 Na_{2}HPO_{4}). Each pipette (mounted on a micromanipulator) was connected to a Picospritzer II (General Valve) that ejected the drug from the pipette using pressurized air (30 psi; 5–10 ms). A small spherical droplet of drug (~65 pl) was formed in the oil layer and then lowered onto the capillary using the technique of Dietrich (3).

Evaluation of microvascular response. The microvascular response was measured both at low magnification (for V_{RBC}) and, in separate animals, at high magnification (for leukocyte adhesion). The responses in individual microvessels were averaged for each animal.

Nitric oxide (NO) is a potent vasodilator synthesized and released from endothelial cells (EC). In the microcirculation, NO has another role, that of an antiadhesive agent. Inhibition of NO synthesis results in decreased red blood cell velocity (V_{RBC}), increased leukocyte adhesion, and increased permeability within mesenteric postcapillary venules (7, 10, 17). Kubes et al. (8) proposed that a decrease in NO production leads to reduced scavenging of extracellular reactive oxygen species (ROS). The increased levels of ROS cause mast cells to degranulate and release proinflammatory mediators. These proinflammatory mediators promote adhesive interactions between the endothelium and leukocytes (8).

When capillary NO synthesis was inhibited with N^G-nitro-l-arginine methyl ester (l-NAME) in rat skeletal muscle, capillary V_{RBC} decreased and leukocyte adhesion increased with no change in arteriolar or venular diameter (13). This response to l-NAME was unique to the capillary inasmuch as application of l-NAME to terminal arterioles or to postcapillary venules had no effect. Because the stereoisomer d-NAME did not cause the same response (13) and there is a significant amount of NO synthesis present in microvascular EC from rat extensor digitorum longus (EDL) muscle (6), l-NAME was presumed to act by decreasing NO production. The objective of this study was to address the mechanism by which l-NAME mediated these vascular effects because l-arginine analogs are being considered for therapy. Our data show that l-NAME decreased V_{RBC} and increased leukocyte adhesion in a CD18-dependent manner.
...V$_{RBC}$ was measured using the flying spot technique (19). We determined the control V$_{RBC}$ (V$_{RBC\text{con}}$) as the average V$_{RBC}$ 1–2 min before L-NAME application. After L-NAME application, the microcirculatory view was recorded until the velocity returned to V$_{RBC\text{con}}$ or to a level within ±15% of V$_{RBC\text{con}}$. If V$_{RBC}$ did not return to ±15% V$_{RBC\text{con}}$, then the experiment was discarded. The lag time of a response was defined as the time between agent application and V$_{RBC}$ reaching a level ±10% from V$_{RBC\text{con}}$. The duration of a response was defined as the time from the onset of the response until the time when V$_{RBC}$ reached V$_{RBC\text{con}}$ or reached a stable level within ±15% of V$_{RBC\text{con}}$. V$_{RBC\text{test}}$ was defined as the velocity at its greatest change from V$_{RBC\text{con}}$, unless stated otherwise. Data were expressed as percent change from V$_{RBC\text{con}}$, as follows: ΔV$_{RBC}$ (%) = 100% × (V$_{RBC\text{test}}$ - V$_{RBC\text{con}}$)/V$_{RBC\text{con}}$.

Leukocyte adhesion was measured off-line in small postcapillary venules of 5–10 µm in diameter. We used this size vessel because this is where leukocytes visibly adhere in this preparation (13). The observed leukocytes were 7–8 µm in diameter, but we could not determine which leukocytes were interacting. We measured the number of adherent leukocytes per 100 µm of postcapillary venule length both before and after the first 3 min after drug application. Leukocytes were considered to be adherent if they remained stationary for ≥30 s.

Experimental protocol. All intravenous agents were dissolved in sterile saline. Rats were randomized into six groups. The first group of animals (n = 9) was used as controls. These animals received the nonbinding mouse immunoglobulin G (IgG) antibody (200 µg/kg iv; Sigma). To determine the role of CD18 in the L-NAME response, a second group of animals (n = 9) was pretreated with the anti-CD18 monoclonal antibody CL26 (200 µg/kg iv; gift from Dr. D. C. Anderson, Upjohn, Kalamazoo, MI) (14). The dose of CL26 does not cause neutropenia in rats (14). To determine whether leukocyte rolling is involved in the L-NAME response, a third group of animals (n = 8) was pretreated with the selectin-binding carbohydrate fucoidan (25 mg/kg iv, Sigma). To determine the role of extracellular superoxide and hydrogen peroxide, a fourth group of animals (n = 10) received the antioxidant superoxide dismutase (SOD; 8 mg/kg iv; Boehringer-Mannheim) and catalase (150,000 U/kg iv; Sigma). To test for the effect of intracellular ROS, a fifth group of animals (n = 6) received dimethylthiourea (DMTU; 500 mg/kg iv; Sigma). For SOD/catalase, leukocyte sticking was measured in venules (10–26 µm in diameter) after the EDL muscle surface was superfused with a mixture of hypoxanthine/xanthine oxidase (5 mM hypoxanthine, 500 U/ml xanthine oxidase, 750 µM EDTA, 500 µM FeCl$_3$), with (n = 3) or without (n = 3) the SOD/catalase pretreatment. To determine the effectiveness of ketotifen, the mesentery was exposed and the percentage of degranulated mast cells per field (0.6 mm$^2$) was measured after treatment with the mast cell activator compound 48/80 (1 µg/ml), with (n = 3) or without (n = 3) ketotifen treatment as described above.

Statistics. All data are reported as means ± SE. Analysis was done using a nested-design analysis of variance (ANOVA) to determine whether there were any differences among treatment groups. When the computed F ratio of the ANOVA reached the critical F ratio (P = 0.05), Student’s t-test with a Bonferroni correction for multiple comparisons was used to identify treatments that were significantly different. Student’s t-test was used to determine whether poststimulation levels were different from prestimulation levels. Values of P < 0.05 were considered significant.

RESULTS

The blood pressure was 99 ± 1 mmHg and was not different among the treatment groups. Baseline V$_{RBC}$ (overall: 231 ± 17 µm/s) and postcapillary venular diameter (overall: 8.2 ± 0.2 µm) also did not differ among the groups. Local drug application did not affect systemic blood pressure. Thus systemic hemodynamics were unlikely to account for the observed local microvascular responses.

Figure 1 shows the effect of local L-NAME application on capillary V$_{RBC}$. In control animals (IgG pretreated), L-NAME application resulted in a significant decrease in capillary V$_{RBC}$ (Fig. 1). Decreases in V$_{RBC}$ occurred within 13 ± 6 s of L-NAME application and lasted for 262 ± 58 s. This response was similar to that seen in naïve animals (13), indicating that systemic administration of a nonbinding antibody did not alter microvascular responsiveness to L-NAME. Similar to Mitchell and Tyml (13), it was observed that other capillaries fed by the same arteriole were largely unaffected by L-NAME. Occasionally, a capillary that drained into the same collecting venule as the stimulated capillary showed decreased V$_{RBC}$ after L-NAME. Because the feeding arteriole was not affected (13), the L-NAME effect was not due to a propagated vasoconstriction.

The L-NAME V$_{RBC}$ response was abolished in CL26-pretreated animals (Fig. 1). This indicates that CD18 was necessary for the V$_{RBC}$ reduction after local L-NAME application. In fucoidan-, SOD/catalase-, DMTU-, or ketotifen-pretreated animals, local L-NAME caused V$_{RBC}$ decreases that were not different from control animals (Fig. 1). These data indicate that selectins, ROS, and mast cells were not involved in the V$_{RBC}$ reductions after L-NAME.

Figure 2 shows the effect of local L-NAME (i.e., applied on the capillary) on leukocyte adhesion in small...
postcapillary venules of 5–10 µm in diameter. In control animals, there was a very low baseline adhesion (Fig. 2). Low baseline adhesion would be expected if there were minimal manipulation and damage to the preparation during surgery (4). After L-NAME, there was a significant increase in leukocyte adhesion (Fig. 2). This is consistent with the response seen in naive animals (13). The observed adhesion was leukocyte-endothelial in nature and not leukocyte-leukocyte (i.e., homotypic). Leukocyte rolling was not observed before or after L-NAME. Rolling is generally defined as the leukocyte velocity ($V_{WBC}$) being less than $V_{RBC}$. Because these vessels are very small and allow for the passage of single blood cells, it was not possible to measure $V_{WBC}$ relative to $V_{RBC}$ at the same time.

Baseline adhesion was nonexistent in CL26 animals but was not different from control animals. Low or no basal adhesion would be expected if CD18 were primarily responsible for firm adhesive interactions (for review, see Ref. 16). CL26-treated animals did not show an increase in adhesion after local L-NAME application (Fig. 2). The response in CL26 animals was different from that in control animals. The baseline adhesion in fucoidan-, SOD/catalase-, and ketotifen-treated animals was low and not different from zero or from that in control animals (Fig. 2). After local L-NAME application, there was a significant increase in adhesion that was similar to that seen in control animals after L-NAME. These data indicate that selectins, extracellular ROS, and mast cells were not necessary for L-NAME to mediate the increase in adhesion.

In the safranine-stained EDL muscle, there were very few mast cells present. Less than five mast cells were seen in the entire exposed muscle surface (55 mm²). This finding is consistent with the lack of effect of ketotifen and indicates that mast cells were not responsible for the response to L-NAME.

In positive control experiments, fucoidan treatment reduced rolling in ischemia-reperfusion animals from $12.7 \pm 1.9$ to $1.0 \pm 0.5$ cells/min ($P < 0.05$). SOD/catalase blocked the increase in leukocyte adhesion after hypoxanthine/xanthine oxidase treatment ($5.3 \pm 1.0$ cells/100 µm in hypoxanthine/xanthine oxidase group vs. $1.33 \pm 0.33$ cells/100 µm in SOD/catalase plus hypoxanthine/xanthine oxidase group; $P < 0.05$). Ketotifen blocked the mast cell degranulation after compound 48/80 ($83 \pm 3.1\%$ after 48/80 vs. $28.4 \pm 1.2\%$ after ketotifen plus 48/80; $P < 0.05$).

**DISCUSSION**

The present study demonstrates that L-NAME required CD18 to decrease capillary $V_{RBC}$ and to increase leukocyte adhesion in rat skeletal muscle capillary in vivo. In control animals, L-NAME locally applied to the capillary decreased $V_{RBC}$ and increased leukocyte adhesion. This L-NAME response was not blocked by SOD/

![Fig. 1. Reversal of N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME; 30 mM)-induced red blood cell velocity ($V_{RBC}$) reductions with CL26 pretreatment (anti-CD18 monoclonal antibody). Animals pretreated with selectin inhibitor (fucoidan), extracellular antioxidants [superoxide dismutase (SOD)/catalase], intracellular antioxidant (dimethylthiourea (DMTU)), or mast cell stabilizer (ketotifen) were without effect. Overall control $V_{RBC}$ was $231 \pm 17$ µm/s. Duration of response to L-NAME in each group (from left to right) was $262 \pm 58$, $157 \pm 31$, $230 \pm 61$, $839 \pm 374$, $160 \pm 32$, and $683 \pm 267$ s, respectively. Responses were different as tested by nested-design analysis of variance (ANOVA). Nos. in parentheses are no. of animals with 1–4 stimulated capillaries per animal. *$P < 0.05$ relative to no $V_{RBC}$ change ($\Delta V_{RBC}$; 0%). **$P < 0.05$ relative to control animals.

![Fig. 2. Reversal of L-NAME (30 mM)-induced leukocyte adhesion with CL26 pretreatment. Animals pretreated with selectin inhibitor (fucoidan), extracellular antioxidants (SOD/catalase), or mast cell stabilizer (ketotifen) were without effect. Overall diameter of postcapillary venules where leukocyte adhesion was measured was $8.2 \pm 0.2$ µm. Responses were different as tested by nested-design ANOVA. Nos. in parentheses are no. of animals with 1–4 stimulated capillaries per animal. *$P < 0.05$ relative to no diameter change (0%). **$P < 0.05$ relative to control animals.](http://alphaheart.physiology.org/)

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catalase or by DMTU, indicating that ROS (extracellular or intracellular) were not involved in the response. ROS do not appear to be involved in the L-NAME response in the capillary. Therefore, capillary NO may not act to scavenge ROS. In contrast, SOD, catalase, and DMTU all attenuated leukocyte adhesion and permeability changes after L-NAME in mesenteric venules (8, 11).

Mast cells were also not involved in the L-NAME response in the capillary. There are very few mast cells at the EDL surface, and mast cell stabilization was unable to block the L-NAME response. The present data indicate that the mechanism of the L-NAME response in skeletal muscle capillaries differs from that described for mesenteric postcapillary venules, which requires both mast cells and ROS (7, 10, 17).

Leukocyte adhesion appears to be responsible for the V_RBC decrease. The time course of the V_RBC decrease correlated with an increased leukocyte adhesion as we reported previously (13). This adhesion occurred in the smallest postcapillary venules and was seen to physically block these vessels. This increase in adhesion could be due to either increased rolling or increased firm adhesion.

In venules, leukocyte rolling (selectin mediated) is a prerequisite for leukocyte adhesion (reviewed in Ref. 16). Rolling allows the leukocyte to come in intimate contact with EC and to sample the local environment for proadhesive signals (reviewed in Ref. 16). Selectin interactions with their ligands can be blocked by the carbohydrate fucoidan (12). Previous studies showed that leukocyte rolling played a role in the L-NAME response (8, 11).

In the present study, fucoidan pretreatment did not block the L-NAME response, indicating that rolling was not a mediator of the L-NAME response. Although larger microvessels require rolling for firm adhesion, it is not surprising that leukocyte adhesion occurred in small postcapillary venules in the apparent absence of rolling. In small blood vessels (e.g., capillaries, small postcapillary venules, liver sinusoids), the vessel is sufficiently small to allow for intimate contact between the leukocyte and endothelium (20).

The β2-integrin family (CD11/CD18) on leukocytes, and their ligands (intercellular adhesion molecules 1 and 2 (ICAM-1 and -2)) on EC, mediate leukocyte adhesion in acute inflammation (16). In mesenteric venules, CD18 was demonstrated to be responsible for leukocyte adhesion in venules after L-NAME (10, 11). In the present study, the anti-CD18 antibody CL26 completely blocked the L-NAME response. Therefore, the L-NAME response was due to an adhesive interaction between the endothelium and leukocytes.

L-NAME may have an effect on the β2-integrins or on the ICAMs. NO has been shown to have an effect on β2-integrin-mediated adhesion in vitro. Physiological levels of NO (nM range) inhibit β2-integrin function to prevent neutrophil adhesion to an EC line (1). It was postulated that NO prevents the conformational change in β2-integrin structure that allows for increased affinity for its ligands (1). NO can also have effects on ICAM expression in vitro (2, 5, 9). These studies examined ICAM levels several hours after exogenous NO was added and, therefore, may not be relevant to the present observations. We do not know which of these adhesion molecules was affected by L-NAME or by which mechanism. To further explore this possible interaction, in vitro studies examining adhesion molecule expression and functional states in response to L-NAME are needed.

In conclusion, we used intravital microscopy to examine blood flow in a rat hindlimb muscle before and after inhibition of NO synthesis. Our data suggest that L-NAME-mediated decrease in V_RBC and increase in leukocyte adhesion was CD18 dependent.

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