

Nitric oxide: An endogenous modulator of leukocyte adhesion

(inflammation/shear rate/ N^G -monomethyl-L-arginine/ N^G -nitro-L-arginine methyl ester/arginine)

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ABSTRACT The objective of this study was to determine whether endogenous nitric oxide (NO) inhibits leukocyte adhesion to vascular endothelium. This was accomplished by superfusing a cat mesenteric preparation with inhibitors of NO production, N^G -monomethyl-L-arginine (L-NMMA) or N^G -nitro-L-arginine methyl ester (L-NAME), and observing single (30- μ m diameter) venules by intravital video microscopy. Thirty minutes into the superfusion period the number of adherent and emigrated leukocytes, the erythrocyte velocity, and the venular diameter were measured; venular blood flow and shear rate were calculated from the measured parameters. The contribution of the leukocyte adhesion glycoprotein CD11/CD18 was determined using the CD18-specific monoclonal antibody IB₄. Both inhibitors of NO production increased leukocyte adherence more than 15-fold. Leukocyte emigration was also enhanced, whereas venular shear rate was reduced by nearly half. Antibody IB₄ abolished the leukocyte adhesion induced by L-NMMA and L-NAME. Incubation of isolated cat neutrophils with L-NMMA, but not L-NAME, resulted in direct upregulation of CD11/CD18 as assessed by flow cytometry. Decrements in venular shear rate induced by partial occlusion of the superior mesenteric artery in untreated animals revealed that only a minor component of L-NAME-induced leukocyte adhesion was shear rate-dependent. The L-NAME-induced adhesion was inhibited by L-arginine but not D-arginine. These data suggest that endothelium-derived NO may be an important endogenous modulator of leukocyte adherence and that impairment of NO production results in a pattern of leukocyte adhesion and emigration that is characteristic of acute inflammation.

Adhesion of polymorphonuclear leukocytes (PMNs, or neutrophils) to vascular endothelial cells is a hallmark of inflammation. A number of factors govern the adhesive interaction between PMNs and endothelial cells in postcapillary venules. These include (i) the expression of adhesion molecules on the surface of activated PMNs and/or endothelial cells; (ii) hydrodynamic dispersal forces (e.g., wall shear rate) that tend to sweep PMNs away from the vascular wall; and (iii) electrostatic charge interaction between the two cell types. In recent years, several published reports have indicated that superoxide, which is produced by both PMNs and endothelial cells, is also an important modulator of leukocyte adherence. Several lines of evidence support this contention: (a) hypoxanthine/xanthine oxidase produces an increased adherence of PMNs and reduces leukocyte rolling velocity in postcapillary venules, an effect that is attenuated by superoxide dismutase (SOD) but not catalase (1), (b) SOD reverses the leukocyte adherence in mesenteric venules elicited by either ischemia-reperfusion (2) or local intraarterial infusion of platelet-activating factor (PAF; ref. 3), and (c) SOD, but not peroxide-inactivated SOD, decreases the adhesion of PMNs to endothelial cell monolayers exposed to anoxia-reoxygen-

ation (2). The observation that SOD does not affect the adhesion of PMNs to biologically inert surfaces (glass or plastic) suggests that superoxide-mediated PMN adhesion is an endothelium-dependent process (2, 3).

The mechanism by which superoxide mediates endothelium-dependent leukocyte adhesion has not been defined; however, one possibility is that superoxide may interact with an endothelial cell-derived antiadhesive substance and render it inactive. Nitric oxide (NO) is a biologically active compound produced by vascular endothelium and is rapidly inactivated by superoxide (4, 5). There is circumstantial evidence in the literature that NO may interfere with the ability of PMNs to adhere to microvascular endothelium. It is well established that NO prevents the adhesion of platelets to endothelial monolayers (6). Additionally, NO inhibits neutrophil aggregation *in vitro*, an effect that is potentiated by SOD (7). The primary objective of this study was to test the hypothesis that endogenous production of NO plays an important role in the modulation of PMN adhesion to endothelial cells in postcapillary venules. This was accomplished by quantifying leukocyte adhesion in cat mesenteric venules that were superfused with N^G -monomethyl-L-arginine (L-NMMA) or N^G -nitro-L-arginine methyl ester (L-NAME), analogues of L-arginine that inhibit NO production. A second objective of this study was to determine whether the pro-adhesive actions of L-arginine analogues could be attributed to up-regulation of the leukocyte adhesion molecule CD11/CD18 and/or a reduction in shear rate within postcapillary venules.

MATERIALS AND METHODS

Intravital Microscopic Studies. The experimental preparation used in this study was the same as that described previously (8). In brief, 21 cats (1.2–2.4 kg) were fasted for 24 hr and initially anesthetized with ketamine hydrochloride (50 mg/kg). The jugular vein was cannulated and anesthesia was maintained by administration of pentobarbital sodium. A tracheotomy was performed to support breathing by artificial ventilation. Systemic arterial pressure was monitored by a Satham P23 A pressure transducer connected to a catheter in the left carotid artery.

A midline abdominal incision was made and a segment of small intestine was isolated from the ligament of Treitz to the ileocecal valve. The remainder of the small and large intestine was extirpated. Body temperature was maintained at 37°C with a thermistor-controlled heating pad (Cole-Parmer) placed beneath the animal. All exposed tissues were moistened with saline-soaked gauze to prevent evaporation.

Abbreviations: mAb, monoclonal antibody; L-NAME, N^G -nitro-L-arginine methyl ester; L-NMMA, N^G -monomethyl-L-arginine; PAF, platelet-activating factor; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leukocyte; SMA, superior mesenteric artery; SOD, superoxide dismutase.

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Heparin sodium (10,000 units, Elkins-Sinn, Cherry Hill, NJ) was administered, and then an arterial circuit was established between the superior mesenteric artery (SMA) and left femoral artery. SMA blood flow was continuously monitored with an electromagnetic flowmeter (Carolina Medical Electronics, Kings, NC) and SMA pressure was measured via a T-tube that was interposed within the arterial circuit and connected to a pressure transducer (Cobe Laboratory, Lakewood, CO). Blood pressures and SMA blood flow were continuously recorded with a Grass physiological recorder (Grass).

Cats were placed in a supine position on an adjustable Plexiglas microscope stage, a segment of mid jejunum was exteriorized through the abdominal incision, and the mesentery was prepared for *in vivo* microscopic observation as described (8, 9). The mesentery was draped over an optically clear viewing pedestal that allowed for transillumination of a 3-cm segment of tissue. The temperature of the pedestal was maintained at 37°C with a constant temperature circulator (Fisher Scientific, model 80). The exposed bowel was draped with saline-soaked gauze while the remainder of the mesentery was covered with SaranWrap (Dow Corning). The exposed mesentery was suffused with warmed bicarbonate-buffered saline (pH 7.4) that was bubbled with a mixture of 5% CO₂ and 95% N₂.

The mesenteric preparation was observed through an intravital microscope (Ernst Leitz Wetzlar) with a ×20 objective lens (Leitz Wetzlar L20/0.32) and a ×10 eyepiece. The image of the microcirculatory bed (×1400 magnification) was recorded using a video camera (Javelin JE 3362) and a video recorder (Panasonic NV8950).

Single unbranched mesenteric venules (30–45 μm in diameter, 250 μm long) were selected for study. Venular diameter was measured either on- or off-line by using a video image-shearing monitor (IPM, LaMesa, CA). The number of adherent leukocytes was determined off-line during playback of videotaped images by two individuals (images shown in ref. 3). A leukocyte was defined as adherent to venular endothelium if it remained stationary for longer than 30 sec. Adherent cells were expressed as the number per 100-μm length of venule. Emigrated leukocytes were quantitated by videotape playback and expressed as number per microscopic field (4.3 × 10⁻² mm²). Red blood cell velocity (V_{RBC}) was measured using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A & M University, College Station, TX) and mean red cell velocity (V_{mean}) was determined as $V_{RBC}/1.6$ (10). Wall shear rate was calculated based on the Newtonian definition: shear rate = (V_{mean}/D_v) × 8 (sec⁻¹).

Experimental Protocol. After a 1-hr stabilization period, baseline measurements of blood pressure, SMA blood flow, V_{RBC} , and leukocyte adherence and emigration were obtained. If V_{RBC} was unstable after the equilibration period, the preparation was not used for the experiment. In the first group of animals ($n = 10$), L-NMMA (100 μM) or L-NAME (100 μM) (Sigma) was superfused on the mesentery for a period of 30 min. Then, repeat measurements were made after which each animal received IB₄ (1 mg/kg, i.v.), a monoclonal antibody (mAb) directed against the common β subunit (CD18) of the leukocyte adhesion glycoprotein (CD11/CD18). Ten minutes later, repeat measurements were made and compared with pretreatment values.

The influence of venular wall shear rate on leukocyte adherence was assessed in four mesenteric preparations. After baseline measurements were obtained, the arterial perfusion circuit was partially occluded so that venular blood flow was reduced in a graded fashion. After each reduction in blood flow, the partial occlusion was released until leukocyte adherence and venular blood flow returned to control values. The mesentery was then superfused with L-NAME (50 μM) and a second series of mesenteric artery compres-

sions was performed. In one experiment, mAb IB₄ was administered and then a second series of venular blood flow reductions was studied in the presence of L-NAME. In another series of experiments, the same protocol was repeated as described above; however, the mesentery was superfused with various concentrations of L-arginine (50–250 μM).

In Vitro Studies. These experiments were designed to assess the direct effects of L-NAME and L-NMMA on PMN adherence. Prior to an experiment, 48-well plates were coated with heat-inactivated fetal bovine serum for 2 hr and then washed three times with phosphate-buffered saline. Cat PMNs were isolated from venous blood by standard dextran sedimentation and gradient separation on Histopaque-1077 (Sigma). This procedure yields a population that is 95–100% viable (trypan blue exclusion) and 98% pure (acetic acid/crystal violet staining). The PMN adherence assay was a modification of the method of Fehr and Dahinden (11). In brief, PMNs were radiolabeled by incubating the purified cells with Na⁵¹CrO₄ (30 μCi/ml of PMN suspension; 1 μCi = 37 kBq) at 37°C for 60 min. The cells were washed three times with cold phosphate-buffered saline to remove unincorporated radioactivity and then resuspended at 2 × 10⁶ cells per ml in Dulbecco's phosphate-buffered saline. Aliquots (500 μl) of the PMN suspension were allowed to adhere in the protein-coated wells for 30 min (37°C) in the presence of either L-NAME or L-NMMA (0, 50, or 100 μM) or 1 μM phorbol 12-myristate 13-acetate (PMA, positive control). The supernatant of each well was then aspirated and the well was gently washed once with phosphate-buffered saline (500 μl). Cells that remained adherent were then lysed by an overnight incubation with 2 M NaOH (500 μl). The cell lysate was collected to assay for ⁵¹Cr activity and PMN adherence was estimated as the ratio of counts in the lysate to counts in the lysate plus supernatant.

In some experiments PMNs were isolated and incubated with either L-NAME or L-NMMA (0, 50, or 100 μM) for 30 min. Flow cytometric analysis was performed on an EPICS 753 flow cytometer/sorter (Coulter) for the simultaneous accumulation of immunofluorescence in addition to forward-angle and 90° light scatter signals. Dead cells and debris were excluded by forward-angle and 90° light scatter gating or, in some experiments, by the exclusion of dead cells, which incorporate propidium iodide (12). Cell preparations were stained as described (13) using anti-CD18 mAb IB₄ as a primary reagent (14) and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Southern Biotechnology Associates, Birmingham, AL) as the secondary reagent. Controls included cells stained with the secondary reagent alone and cells stained with an irrelevant isotype-matched control mAb. Twenty-five thousand cells were analyzed in each experiment and each experiment was conducted three times.

Statistics. Data were analyzed using an analysis of variance and the Sheffe's post-hoc test. The paired Student's *t* test was used to determine statistical difference in the *in vitro* studies. All values are expressed as means ± SE, and statistical significance was set at $P < 0.05$.

RESULTS

Fig. 1 illustrates the effects of the L-arginine analogues L-NMMA (100 μM) and L-NAME (100 μM) on PMN adhesion in postcapillary venules. Both L-NMMA and L-NAME elicited a dramatic increase in PMN adhesion (16- and 21-fold, respectively) 30 min into the superfusion period. Following mAb IB₄ administration, the number of adherent PMNs returned to pretreatment values within 10 min. PMN emigration from venules into the adjacent extravascular compartment increased from 8.5 ± 2.5 to 29.5 ± 1.5 per field.

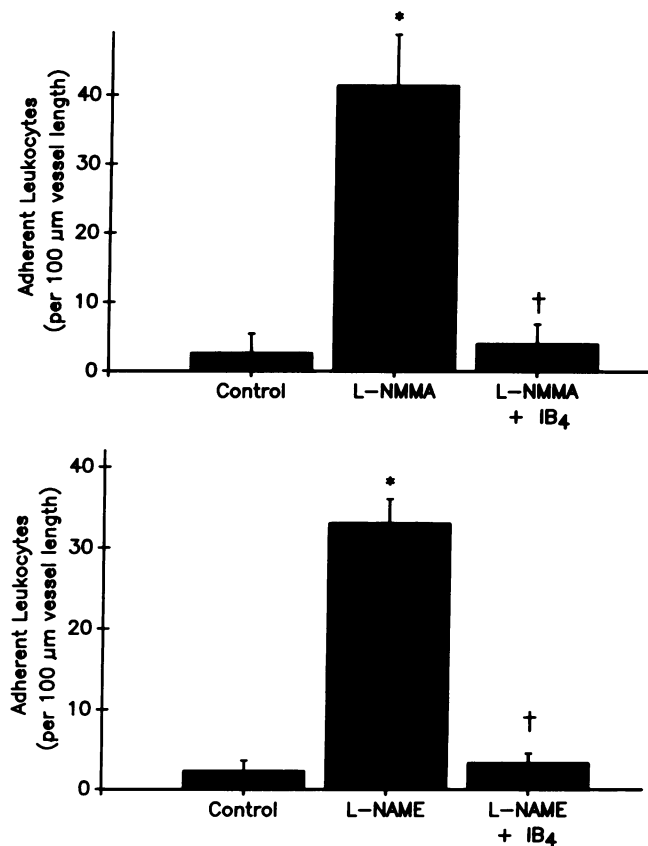


FIG. 1. L-NMMA and L-NAME elicit significant leukocyte adherence that can be completely reversed by the CD18-specific mAb IB₄. *, $P < 0.05$ compared with the pretreatment value (control); †, $P < 0.05$ relative to the L-NMMA- or L-NAME-induced leukocyte adhesion value.

This observation suggests that $\approx 65\%$ of the leukocytes that adhere to postcapillary venules over the 30 min of L-NAME superfusion ultimately emigrate out of the vasculature.

The values for venular diameter, erythrocyte velocity (V_{RBC}), and wall shear rate were $31.2 \pm 3.1 \mu\text{m}$, $4.4 \pm 0.9 \text{ mm/sec}$, and $723 \pm 161 \text{ sec}^{-1}$, respectively. Venular wall shear rate was reduced by $\approx 50\%$ during superfusion with either L-NMMA or L-NAME. The reductions in shear rate resulted entirely from a decrease in erythrocyte velocity; venular diameter remained unchanged from control. The decreased erythrocyte velocity was a result of arteriolar vasoconstriction.

The flow cytometry data (Table 1) revealed that L-NAME had no effect on CD18 up-regulation in isolated feline PMNs, while L-NMMA always led to low-level ($\approx 10\%$, $P < 0.05$) up-regulation of CD18 when compared with PMA ($\approx 65\%$). The L-NMMA-mediated up-regulation of CD18 was reversed by L-arginine. The data from studies of PMN adhesion to

Table 1. Expression of CD11/CD18 on isolated feline neutrophils

Neutrophil treatment	CD11/CD18 expression, fluorescence intensity
None	51.89
L-NAME (100 μM)	50.83
L-NMMA (100 μM)	56.25*
PMA (0.1 μM)	80.07*

In each experiment ($n = 3$), L-NMMA but not L-NAME produced an $\approx 10\%$ increase in CD11/CD18 expression. PMA was used as a positive control.

* $P < 0.05$ relative to control.

protein-coated plastic (not shown) followed a pattern similar to the flow cytometry data; i.e., L-NMMA caused a 10% increase in PMN adhesion whereas L-NAME had no effect. Since L-NMMA appears to exert a direct effect on CD18 expression by PMNs, we used L-NAME for the remainder of the *in vivo* experiments.

The contribution of reduced venular shear rate to increased PMN adherence induced by L-NAME was assessed by comparing the PMN adherence responses of L-NAME to that produced by reductions in venular wall shear rate caused by partial occlusion of the arterial circuit. As illustrated in Fig. 2 *Inset*, reductions in shear rate induced by partial arterial occlusion did lead to a shear rate-dependent increase in PMN adherence as previously reported for cat mesentery (15). However, similar reductions in shear rate induced by L-NAME were associated with much greater increments in PMN adherence. Since superfusion of the mesenteric vasculature with L-NAME (50 μM) initially reduced wall shear rate by $\approx 30\%$, we also evaluated the relationship between PMN adherence and absolute shear rates (Fig. 2 histogram). Clearly, at similar shear rates, PMN adherence was greatly enhanced in the L-NAME group. The responses to L-NAME at various shear rates were abolished by treatment with mAb IB₄.

To determine whether L-arginine could reverse the pro-adhesive effects of L-NAME over the range of shear rates observed in mesenteric venules, we evaluated the effects of L-NAME on leukocyte adherence in the presence of 50 μM , 100 μM , or 250 μM L-arginine (Fig. 3). The L-NAME-induced PMN adhesion was reversed over the entire range of shear rates by the high dose (250 μM) but not the lower doses (50 μM or 100 μM) of L-arginine (data not shown). D-Arginine (250 μM) had no effect on L-NAME-induced leukocyte adherence.

DISCUSSION

It has been recognized for many years that the adhesion of PMNs to microvascular endothelium is a rate-limiting step in the initiation and maintenance of an acute inflammatory response. However, only recently has progress been made in understanding the chemical basis for the adhesive interactions that occur between leukocytes and endothelial cells during inflammation. For example, it is now known that adhesive glycoproteins expressed on the surface of both leukocytes (CD11/CD18) and endothelial cells [intercellular adhesion molecule (ICAM), endothelial-leukocyte adhesion molecule (ELAM)] play an important role in modulating leukocyte adherence (16). A large number of endogenous proinflammatory agents (e.g., PAF, leukotriene B₄, tumor necrosis factor) are known to elicit the expression of these adhesive glycoproteins, thereby providing a mechanism for local tissue regulation of leukocyte adherence and emigration. Less is known about locally produced factors that exert an inhibitory influence on leukocyte adherence. There is circumstantial evidence that NO is a naturally occurring anti-adhesion molecule. NO has been shown to block platelet aggregation and to reduce platelet adhesion to endothelial cell monolayers (6, 17). In addition, it has been reported that NO inhibits PMN aggregation *in vitro* (7). In the present study, we present evidence that inhibitors of NO production greatly enhance PMN adherence and emigration in postcapillary venules, indicating that NO plays an important physiologic role in preventing leukocyte-endothelial cell adhesion in postcapillary venules.

Vascular endothelial cells synthesize NO from the terminal guanidino nitrogen atom of the amino acid L-arginine. Synthesis of NO is inhibited by L-NMMA and L-NAME, two analogues of L-arginine. These reagents reverse the effects of NO; i.e., they promote platelet aggregation and platelet

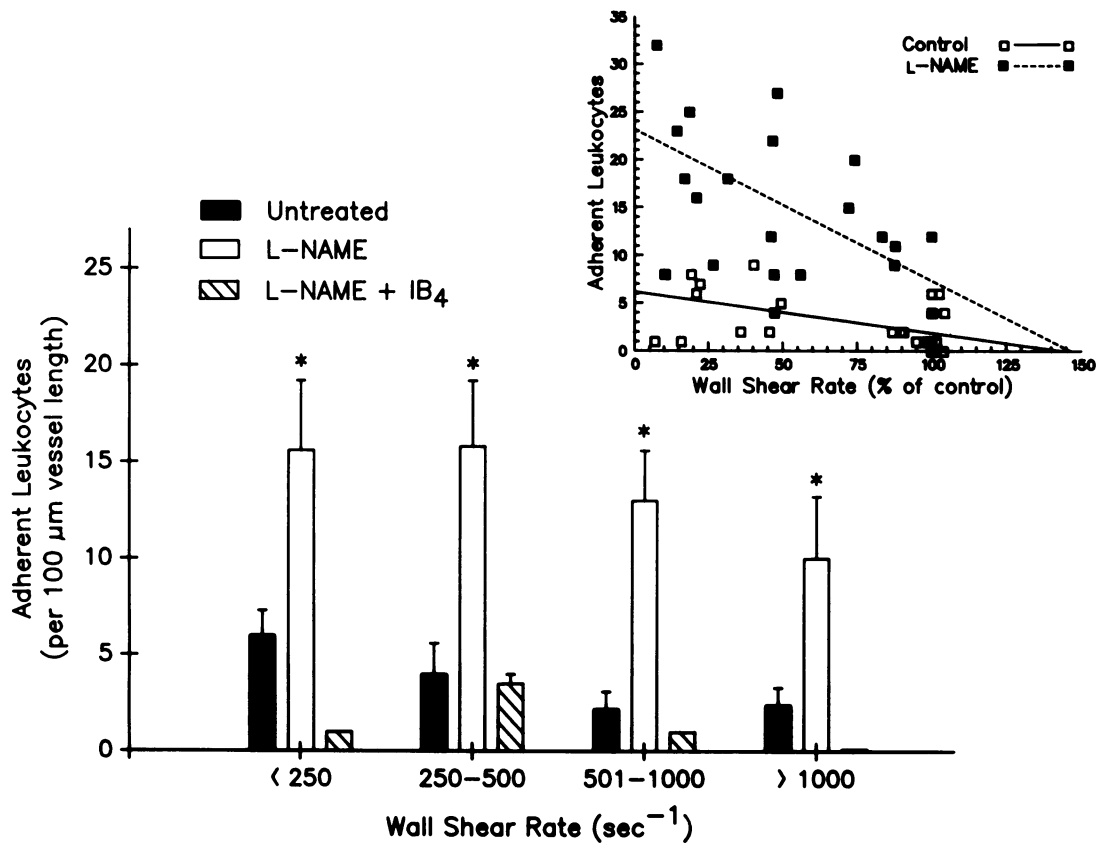


FIG. 2. Relationships between the number of adherent leukocytes and wall shear rate in mesenteric venules under control conditions and during exposure to 50 μM L-NAME. The absolute (histogram) and relative (*Inset*) shear rate data are presented. At all shear rates, L-NAME significantly increased leukocyte adherence. The CD18-specific mAb IB₄ (1 mg/kg) prevented the L-NAME-induced adherence observed at all shear rates. *, $P < 0.05$ relative to untreated controls.

adhesion to endothelium. We have shown that both L-NAME and L-NMMA cause PMNs to adhere to venular endothelium and promote PMN emigration, events that are generally associated with microvascular dysfunction. We were able to reverse the L-NAME-induced PMN adhesion with L-arginine but not with D-arginine. Complete reversal of the effects of L-NAME required a 5-fold excess concentration of exogenous L-arginine. The latter observation is consistent with the view that exogenous L-arginine is unable to compete with

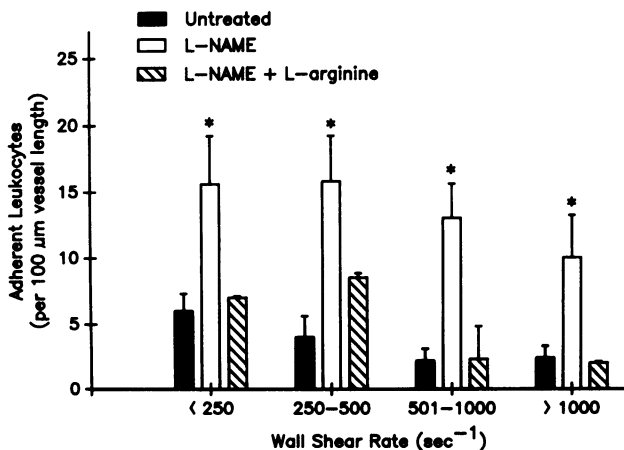


FIG. 3. L-Arginine (250 μM) can prevent the leukocyte adherence in mesenteric venules induced by L-NAME (50 μM). L-NAME increased leukocyte adherence over the entire range of shear rates. The L-NAME-induced leukocyte adherence was largely prevented by L-arginine. *, $P < 0.05$ relative to untreated controls.

endogenous L-arginine, which may be presented more efficiently to the enzyme NO synthase (18, 19).

Although our findings indicate that NO normally functions to limit leukocyte-endothelial cell interactions *in vivo*, the precise mechanism by which NO exerts this effect remains undefined. NO does appear to mediate its antiadhesive effect through the leukocyte adhesion glycoprotein CD11/CD18, inasmuch as the CD18-specific antibody IB₄ largely prevented the leukocyte adherence induced by L-NMMA or L-NAME (Fig. 1). Our results suggest that NO either interferes with the ability of constitutively expressed CD11/CD18 to form an adhesive bond with the endothelial cell surface or acts to suppress CD11/CD18 expression on leukocytes as they roll along venular endothelium. Further studies are needed to address these possibilities as well as the potential involvement of endothelial cell adhesion glycoproteins in NO-mediated inhibition of leukocyte adherence.

Based on our observations, one would predict that decreased NO production and/or increased NO inactivation would promote leukocyte adhesion and ultimately inflammation. Since superoxide is known to inactivate NO (4, 5), conditions associated with enhanced production of superoxide, either by PMNs or endothelial cells, would also be expected to cause leukocyte adherence. Similarly, SOD should prove to be an effective antiadhesive agent in these conditions, since the enzyme would prevent superoxide-mediated inactivation of NO. Indeed, studies from our laboratory have demonstrated that SOD reverses the PMN adherence in mesenteric venules induced by either ischemia-reperfusion (2) or local intraarterial infusion of PAF (3), conditions that should elicit the production of superoxide by neutrophils and/or endothelial cells (20, 21). These observations, coupled with reports that intravascular administration

of either NO or SOD affords protection against intestinal ischemia-reperfusion injury (22, 23), are consistent with the hypothesis that the beneficial actions of SOD can be attributed, at least in part, to its ability to prevent NO inactivation.

Inhibition of NO production causes vasoconstriction and a reduction in the shear forces that tend to push PMNs along venular endothelium. The results of a recent study indicate that significant reductions in shear rate elicit a reversible, CD18-dependent adherence of PMNs in mesenteric venules (15). This observation raises the possibility that inhibitors of NO production (L-NAME and L-NMMA) cause PMN adherence as a consequence of the reduction in venular shear rate. To assess this possibility, we compared the adherence responses elicited by partial arterial occlusion to those observed during superfusion with L-NAME (Fig. 2). The results of this analysis reveal that the PMN adherence induced by L-NAME is about an order of magnitude greater than that caused by passive reductions in venular shear rate. This observation indicates that shear rate-dependent PMN adherence accounts for only a small fraction of the adhesion response associated with inhibition of NO production.

In conclusion, the results of this study indicate that NO may be an important endogenous inhibitor of leukocyte adhesion in postcapillary venules. Inactivation of NO by superoxide may contribute to the leukocyte adherence observed in conditions characterized by enhanced production of superoxide by endothelial cells and/or neutrophils. Such a mechanism would explain the ability of superoxide dismutase to reduce ischemia-reperfusion- and PAF-induced leukocyte adherence in postcapillary venules.

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