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The iNOS /-- mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and were generated as described previously (19, 31). In the present studies, we utilized both C57BL/6 and SV129 as wild-type controls for the gene-targeted mice.

Surgical procedures. Mice were anesthetized with pentobarbital sodium (120 mg/kg) injected intraperitoneally. A polyethylene catheter (PE-10) was inserted in the left carotid artery to monitor MABP. MABP was recorded on a Grass model 7 oscillographic recorder using a Statham model P23AC pressure transducer (Gould, Cleveland, OH). All experiments were acute, and the animals were killed with an overdose of pentobarbital (250 mg/kg ia). All experiments were approved by the Louisiana State University Medical Center and Thomas Jefferson University Animal Care Committees (IACUC).

Intravital microscopy of the mouse perintestinal venules. The abdominal cavity was opened via a midline laparotomy as described earlier (29). Briefly, a loop of ileal mesentery was exteriorized through the midline incision and placed in a temperature-controlled fluid-filled Plexiglas chamber for observation of the mesenteric microcirculation via intravital microscopy. The mesentery was placed over a Plexiglas pedestal in the superfusion chamber, and the ileum was secured for stabilization of the viewing field. The ileum and mesentery were superfused throughout the experiment with a modified Krebs-Henseleit solution (containing, in mM, 118 NaCl, 4.74 KCl, 2.5 CaCl2, 1.19 KH2PO4, 1.19 MgSO4, 12.5 NaHCO3, warmed to 37°C, and bubbled with 95% N2-5% CO2). A Microphot microscope (Nikon, Tokyo, Japan) was used to visualize the mesenteric microcirculation and the mesenteric tissue. The image was projected by a high-resolution color video camera (model DC-330; DAGE-MTI, Michigan City, IN) onto a color Sony high-resolution video monitor (Multiscan 200-sf), and the image was recorded with a videocassette recorder. All images were then analyzed using computerized imaging software (Phase 3 Image System, Media Cybernetics) on a Pentium-based IBM-compatible computer (Micron Millenia Mxe; Micron Electronics, Nampa, ID). Red blood cell velocity was determined on-line using an optical Doppler velocimeter (3) obtained from the Microcirculation Research Institute, College Station, TX. This method gives a value for red blood cell velocity, which is digitly displayed on a meter, and allows for the calculation of shear rates. Red blood cell velocity (V) and venular diameter (D) were used to calculate venular wall shear rate (g) employing the formula g = 8(Vmean/D) (Vmean = Vred/L.6), where Vmean is the average red blood cell velocity, and Vred is the measured red blood cell velocity (13).

The mice were allowed to stabilize for 20–30 min following surgery. After stabilization, a 20- to 45-µm diameter postcapillary venule was chosen for observation. A baseline recording was made to establish basal values for leukocyte rolling and adherence. Video recordings were made at 0, 10, 20, and 30 min after initiation of superfusion for quantification of leukocyte rolling and adherence. The number of rolling and adhered leukocytes was determined off-line by playback analysis of the videotape. Leukocytes were considered to be rolling if they were moving at a velocity significantly slower than that of red blood cells. Leukocyte rolling is expressed as the number of cells moving past a designated point per minute (i.e., leukocyte flux). A leukocyte was judged to be adherent if it remained stationary for >30 s. Adherence is expressed as the number of leukocytes adhering to the endothelium per 100 µm of vessel length.

Immunohistochemistry. Immunohistochemical localization of P-selectin was determined after intravital microscopy was completed. Both the superior mesenteric artery and superior mesenteric vein were then rapidly cannulated for perfusion fixation of the small bowel. The ileum was first washed free of blood by perfusion with Krebs-Henseleit buffer warmed to 37°C and bubbled with 95% O2-5% CO2. Once the venous perfusate was free of red blood cells, perfusion was initiated with iced 4% paraformaldehyde mixed in phosphate-buffered 0.9% NaCl (PBS) for 5 min. A 3- to 4-cm-long segment of ileum was isolated from the perfused intestine and fixed in 4.0% paraformaldehyde for 90 min at 4°C. The ileum was then cut into rings, and the tissue was dehydrated using graded acetone washes at 4°C. Tissue sections were embedded in plastic (Innomed; Polysciences, Warrington, PA), and 4-µm thick sections were cut and transferred to Vectabond coated slides (Vector Laboratories, Burlingame, CA).

Immunohistochemical localization of P-selectin was accomplished using the avidin-biotin immunoperoxidase technique (Vectastain ABC Reagent; Vector Laboratories) as previously described (29). Tissue sections were treated with 0.25% trypsin (Sigma Chemical, St. Louis, MO) to improve reagent penetration. Blocking serum (horse) was applied to the tissue for 30 min to reduce nonspecific binding, and then the tissue sections were incubated, with the primary antibody directed against P-selectin (RB40.34, Pharmingen) at a dilution of 1:100, for 24 h. The tissue was then incubated with the biotinylated secondary antibody, and the peroxidase staining was carried out using 3,3'-diaminobenzidine (DAB). Control preparations consisted of omission of the primary antibody or omission of the secondary antibody. Expression of P-selectin was determined by microscopic observation of the brown peroxidase reaction product on the venular endothelium of the tissue sections. Positive staining was defined as a venule displaying brown reaction product on greater than 50% of the circumference of its endothelium. Fifty venules were analyzed per tissue section, 20 sections were examined per group, and the percentage of positive staining venules was tallied.

Peritonitis experiments. Mice (wild type, iNOS /--iNOS /-- or /---; nNOS /--iNOS /--iNOS /----, and ecNOS /--iNOS /--iNOS /----) were injected (intraperitoneally) with 1.0 ml of thioglycollate medium-135C (Becton-Dickinson). Six hours following thioglycollate injection, the mice were killed via cervical dislocation. A midline incision was made over the abdominal cavity, and the skin layers were separated by hand. Normal PBS solution (5 ml) was injected into the peritoneum through the muscle layers with a 10-ml syringe connected to a 20-gauge needle. The abdominal cavity was gently massaged, and the PBS was removed with the 10-ml syringe and transferred into a 15-ml conical test tube. This procedure was repeated, and the PBS solution was then centrifuged at 1,500 rpm for 10 min. The resulting cell pellet was suspended in 1.0 ml of PBS solution, and the cells were stained with 3.0% acetic acid/0.5% crystal violet. The number of polymorphonuclear neutrophils (PMNs) per microliter was determined in each animal using a hemocytometer.

Chemicals and reagents. The murine-specific anti-P-selectin monoclonal antibody, RB40.34, was purchased from Pharmingen (San Diego, CA) and administered to mice intravenously at a dose of 1.0 mg/kg. The recombinant, soluble high-affinity P-selectin ligand, PSGL-1, was generously provided by Michael Eppihimer, Ph.D., and Gray Shaw, Ph.D., at Genetics Institute (Cambridge, MA), and administered to mice at a dose of 1.0 mg/kg intravenously.

Data analysis. All data are presented as means ± SE. Data were compared by ANOVA using post hoc analysis with Fisher’s corrected t-test. All data on leukocyte rolling and adherence, as well as arterial blood pressure and shear rates, were analyzed by ANOVA, incorporating repeated measure-
RESULTS

Hemodynamic data. Baseline MABP and venular shear rate data for all the groups of mice studied are presented in Table 1. MABP values were similar in the two wild-type strains of mice (C57BL/6 and SV129) compared with the iNOS-deficient and nNOS-deficient mice at all time points during the 30-min observation period. However, the MABP was significantly greater (P < 0.05) in the ecNOS /-/- mice compared with all other study groups at all times during the experimental procedures. Furthermore, the venular shear rates (s^-1) were comparable in all of the wild-type and mutant mice throughout the entire experimental protocol.

Circulating blood cells. Data for circulating neutrophil and platelet counts (number of cells x 10^10/µl) are presented in Table 2. There were no differences in circulating neutrophil counts between any of the study groups. However, the circulating platelet count was significantly (P < 0.05) lower in the SV129 compared with the C57BL/6 mice. No other significant differences were observed in circulating platelet counts between any of the study groups.

Leukocyte rolling and adhesion: baseline conditions. Leukocyte rolling was determined in mesenteric venules in wild-type and the NOS gene-targeted mice under control conditions, and these data are depicted in Fig. 1A. Baseline leukocyte rolling was similar in the SV129 mice compared with the C57BL/6 mice, and there was no significant difference in leukocyte rolling between these two wild-type strains. Leukocyte rolling in the iNOS /-/- mice was similar to the wild type. Baseline rolling was significantly augmented in both the nNOS /-/- and ecNOS /-/- mice compared with wild-type animals.

Baseline data for leukocyte adherence are shown in Fig. 1B. Baseline leukocyte adherence (cells/100 µm of vessel length) was relatively low in the SV129, C57BL/6, and in the iNOS /-/- group. In contrast, the adherence of leukocytes was dramatically increased in the nNOS /-/- and ecNOS /-/- mice.
Baseline P-selectin expression in mesenteric vessels. The level of P-selectin expression was measured in the microcirculation of the mesentery of all the mouse strains, and these data are presented in Fig. 2. P-selectin expression in the mesenteric endothelium was very similar in the SV129, C57BL/6, and iNOS \(-/-\), with \(\sim 10\%\) of vessels demonstrating positive staining for P-selectin. The percentage of vessels that stained positive for P-selectin expression was significantly elevated in the nNOS \(-/-\) and ecNOS \(-/-\) mesentery.

Anti-P-selectin therapy effects on leukocyte rolling and adherence. The effects of a murine-specific monoclonal antibody that neutralizes P-selectin (RB40.34) were studied in wild-type, nNOS \(-/-\), and ecNOS \(-/-\) mice. Leukocyte rolling data for RB40.34-treated animals are presented in Fig. 3A, and data for leukocyte adherence in the presence of RB40.34 are presented in Fig. 3B. Leukocyte rolling and adherence data were collected at 30 min following the administration of either an IgG1 control antibody or RB40.34. Treatment with this specific P-selectin antibody did not significantly alter leukocyte rolling in wild-type mice and significantly attenuated the rolling response in both nNOS \(-/-\) and ecNOS \(-/-\) mice. Leukocyte rolling was reduced in nNOS \(-/-\) mice (\(P < 0.001\)). Furthermore, leukocyte rolling was also dramatically attenuated (\(P < 0.001\)) in ecNOS \(-/-\) mice treated with RB40.34 compared with ecNOS \(-/-\) mice that received the control antibody. Following administration of RB40.34, leukocyte adherence was also significantly reduced in the nNOS \(-/-\) mice. In addition, P-selectin antibody therapy also attenuated leukocyte adherence in the ecNOS \(-/-\) group of animals.

We also investigated the effects of P-selectin inhibition in nNOS \(-/-\) and ecNOS \(-/-\) mice with the high-affinity P-selectin ligand, PSGL-1, on leukocyte rolling and adhesion. Leukocyte rolling data are presented in Fig. 4A, and adhesion data are presented in Fig. 4B. Leukocyte rolling and adhesion were determined at 30 min following administration of the PSGL-1 (1 mg/kg). Both leukocyte rolling and firm adhesion were significantly reduced in the nNOS- and ecNOS-deficient mice following treatment with PSGL-1.

Leukocyte rolling and adhesion following thrombin stimulation. The effects of direct application of thrombin (0.25 U/ml) to the mesentery of wild-type, iNOS \(-/-\), nNOS \(-/-\), and ecNOS \(-/-\) mice were also investigated, and these data are presented in Fig. 5, A and B. The relatively low dose of thrombin that was employed failed to induce any significant increase in leukocyte rolling in the wild-type and iNOS \(-/-\) animals (Fig. 5A). In contrast, leukocyte rolling significantly (\(P < 0.05\) vs. baseline) increased in the nNOS \(-/-\) mice at 10–30 min following thrombin. Leukocyte
iNOS −/− mice with positive staining of 49.0 ± 6.5% of venules. P-selectin immunostaining of vessels was 72.0 ± 8.0% in nNOS −/− mice and 80.0 ± 7.4% in ecNOS −/− mice 30 min following thrombin treatment.

Neutrophil extravasation following thioglycollate injection. In additional experiments, neutrophil accumulation in the peritoneum was determined at 6 h following intraperitoneal injection of thioglycollate. Data for wild-type, iNOS −/−, nNOS −/−, and ecNOS −/− are presented in Fig. 7. Neutrophil accumulation (number of cells $\times 10^3/\mu l$) was significantly enhanced in both the nNOS −/− and the ecNOS −/− mice compared with wild-type and iNOS −/− animals.

**Fig. 4.** A: leukocyte rolling under conditions of treatment with the P-selectin ligand, PSGL-1 (1 mg/kg). Leukocyte rolling was significantly attenuated in nNOS −/− and ecNOS −/− mice receiving PSGL-1 compared with their respective control levels. B: leukocyte adherence following treatment with soluble PSGL-1 in wild-type, nNOS −/−, and ecNOS −/− mice. PSGL-1 treatment significantly reduced the degree of adherent leukocytes in nNOS −/− and ecNOS −/− mice compared with baseline conditions. †P < 0.05 vs. control and **P < 0.001 vs. control.

**Fig. 5.** A: leukocyte rolling (cells/min) following superfusion of mesentery with thrombin (0.25 U/ml). Thrombin stimulation resulted in significant increases in leukocyte rolling in wild-type, nNOS −/−, and ecNOS −/− during the 30-min observation period. Leukocyte rolling was greatest in ecNOS −/− and nNOS −/− mesentery. †P < 0.05 vs. control. B: leukocyte adherence (cells/100 $\mu$m) following treatment of mesentery with thrombin. Leukocyte adherence was dramatically enhanced compared with baseline values in all of the study groups following application of thrombin. The greatest leukocyte adherence was observed in ecNOS −/− mice at all time points. †P < 0.05, *P < 0.01, and **P < 0.001 vs. baseline.
The most striking observation of this study is the profound increase in baseline leukocyte rolling and adhesion that occurs in the mesentery of nNOS-deficient and ecNOS-deficient mice. We observed a greater than twofold increase in basal leukocyte rolling in the nNOS−/− mice and a sixfold increase in leukocyte rolling in the ecNOS−/− mice. Furthermore, firm adhesion of leukocytes was increased by greater than twofold in the nNOS−/− mice and by approximately sixfold in mice lacking ecNOS activity. These results serve to further demonstrate the importance of NO released by endothelial cells in the regulation of leukocyte adherence and point to a newly discovered role for NO liberated by nNOS on leukocyte function in the vasculature. Data are also presented in this study demonstrating that the transmigration of neutrophils into the peritoneum following thioglycollate challenge is significantly augmented in both the nNOS−/− and ecNOS−/− mice. One potential limitation of the present study is the use of knockout mice of a mixed genetic background and studying control mice with a single (SV129 or C57) genetic background. However, we do not feel that differences in background strain can account for increased leukocyte rolling in the ecNOS−/− and nNOS−/− animals.

Immunostaining of intestinal tissue revealed that the enhanced basal rolling and adhesion of leukocytes in the nNOS−/− and ecNOS−/− mice may be a result of increased P-selectin expression. Surface expression of P-selectin was increased by twofold in nNOS-deficient mice and by approximately sevenfold in the ecNOS-deficient mice compared with wild-type control mice. Thus decreased basal NO production by either nNOS or ecNOS serves to upregulate endothelial cell surface expression of P-selectin, and tonic release of NO by these enzymes may be an endogenous inhibitor of P-selectin expression. We also determined that inhibition of P-selectin function with either a monoclonal antibody or soluble PSGL-1 markedly attenuated the rolling response and adhesion of leukocytes in ecNOS−/− and nNOS−/− mice. Taken together, these data strongly suggest that the exaggerated leukocyte-endothelial cell interactions observed in the nNOS−/− and ecNOS−/− mice are P-selectin mediated.

Previous studies have demonstrated that NO is a critical modulator of leukocyte-endothelial cell interactions within the circulation (9, 23). Inhibition of NO synthesis has been shown to promote leukocyte rolling and firm adherence in the microcirculation by a number of investigators (2, 9, 11, 23, 26, 39). In addition, it is now well established that NO therapy with NO-donating agents or with L-arginine can reduce the extent of neutrophil-mediated tissue injury in animal models of inflammation (1, 8, 11, 25, 28, 40, 48, 49). The mechanism by which NO protects against the deleterious effects of circulating neutrophils is thought to be a result of NO-mediated downregulation of a number of endothelial cell adhesion molecules. NO has been shown to inhibit the expression of P-selectin within the microcirculation as well as the expression of a number of other endothelial cell adhesion molecules (4, 11). NO can also reduce the formation of cytokines and serves to inhibit NF-κB (4, 43). Previous experimental results have suggested that the acute protective effects of NO are mediated by NO that is released by the vascular endothelium and not by NO liberated by nNOS or by iNOS.

Development of mice that are deficient in one particular isoform of NOS allows for the elucidation of the precise contribution of ecNOS, iNOS, and nNOS to the physiological actions of NO. Previous studies of ecNOS-deficient mice have reported a profound hypertension in these mice and suggest that NO derived from endothelial cells plays a critical role in blood pressure homeostasis (18, 42, 45). Pulmonary hypertension and vascular remodeling are also exacerbated in ecNOS-
deficient mice (45). In the present study, we observed a significantly elevated blood pressure in the ecNOS−/− mice compared with wild-type, nNOS−/−, and iNOS−/− mice. It has also been reported that vascular reactivity to endothelium-dependent vasodilators is impaired, whereas responses to NO-generating compounds are enhanced in ecNOS knockout mice (6). More recently, it has been demonstrated that defective NO synthesis by the endothelium in ecNOS transgenic mice reduces the degree of angiogenesis (37) and that ecNOS deficiency exacerbates intimal proliferation following vascular injury (36). Furthermore, studies of ecNOS mutant mice have reported that cerebral infarct size and exciototoxicity is enhanced in ecNOS−/− mutant mice (16).

Early studies have suggested that NO is produced in large quantities by iNOS in response to a number of inflammatory stimuli and the NO generated by this enzyme contributes to organ injury, decline in blood pressure, and death (35). Mice lacking iNOS have also been developed and studies have been conducted in which the responses of these animals to a variety of insults have been characterized. Interestingly, it has been reported that the lack of iNOS activity does not prevent mortality in mice treated with a lethal dose of lipopolysaccharide (27). In addition, iNOS-deficient mice exhibit enhanced leukocyte-endothelium interactions in endotoxemia (15). Furthermore, it has also been demonstrated that iNOS activity plays a critical protective role in the response to injury in intestinal inflammation (32). Furthermore, arteriosclerosis following cardiac transplantation is significantly enhanced in iNOS-deficient mice (22). In contrast, it has been reported that renal vascular disease is attenuated in iNOS knockout mice (12). In the present study, we failed to demonstrate a role for iNOS in baseline leukocyte-endothelial cell interactions or following treatment with thrombin or thiglycollate. Clearly, further studies utilizing iNOS null mice are required to elucidate the role of this enzyme in disease states.

Perhaps the most novel and interesting observation of the present study is the profound effect of NO generated by nNOS on the adhesion interactions between circulating leukocytes and the venular endothelium. To our knowledge, this is the first report of immunomodulatory actions of nNOS within the vasculature. Data presented in this study clearly indicate that nNOS deficiency promotes leukocyte rolling and adhesion under basal conditions and following inflammatory stimuli such as thrombin or thiglycollate. It has been previously reported that nNOS modulates hypoxic-ischemic injury and that nNOS augments neuronal damage (7, 18). However, at present very little is known regarding the potential role of nNOS synthesized NO and the cardiovascular system. It is unclear how nNOS might regulate leukocyte function, and further studies are required to determine the precise cellular mechanisms involved in this process. However, there is experimental evidence suggesting that NOs are present in cardiac nerve fibers and neurons of both rat and guinea pig hearts (21). In addition, NO released by nerves in the brain has been shown to modulate vascular tone of cerebral blood vessels and thereby regulate blood flow (46, 47). Furthermore, nNOS-derived NO is also thought to regulate ACh-mediated relaxation of pial arterioles in ecNOS-deficient mice (33). Thus it is possible that NO released by nNOS from nerve fibers innervating blood vessels may exert an anti-adhesive effect on circulating leukocytes.

In summary, we have presented clear data that mice deficient in ecNOS and nNOS exhibit enhanced leukocyte adhesion to vascular endothelium in the mesentery. Exaggerated leukocyte adhesion responses are observed under baseline conditions and during acute inflammatory conditions. This hyperadhesive condition is associated with elevated surface expression of P-selectin within the microcirculation. In addition, both the rolling response and firm adhesive interactions can be completely abolished by a P-selectin monoclonal antibody and by soluble PSGL-1.

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