Am J Physiol Heart Circ Physiol 284: H133–H140, 2003.
First published September 5, 2002; 10.1152/ajpheart.00957.2001.

Vascular protection by estrogen in ischemia-reperfusion injury requires endothelial nitric oxide synthase

ALYSON J. PROROCK,[1] ALI HAFEZI-MOGHADAM,[4]
VICTOR E. LAUBACH,[2] JAMES K. LIAO,[5] AND KLAUS LEY[1,3]
Departments of [1]Biomedical Engineering and [2]Surgery and [3]Cardiovascular Research Center, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908; [4]The Center for Blood Research and Department of Pathology, Harvard Medical School, Boston 02115; and [5]Division of Cardiovascular Medicine, Brigham & Women's Hospital and Harvard Medical School, Boston, Massachuesetts 02115

Submitted 2 November 2001; accepted in final form 16 August 2002

Prorock, Alyson J., Ali Hafezi-Moghadam, Victor E. Laubach, James K. Liao, and Klaus Ley. Vascular protection by estrogen in ischemia-reperfusion injury requires endothelial nitric oxide synthase. *Am J Physiol Heart Circ Physiol* 284: H133–H140, 2003. First published September 5, 2002; 10.1152/ajpheart.00957.2001.—Estrogen increases nitric oxide (NO) production by inducing the activity of endothelial NO synthase (eNOS) (Simoncini et al. *Nature* 407: 538, 2000). Ischemia (30 min) and reperfusion (I/R) increased the number of adherent leukocytes and decreased their rolling velocities in mouse cremaster muscle venules with a strong dependence on wall shear rate. Minimum rolling velocity at ~5 min after the onset of reperfusion was accompanied by increased P-selectin expression. This preceded the peak in leukocyte adhesion (at 10–15 min). In untreated wild-type mice, I/R caused a decrease of leukocyte rolling velocity from 37 to 26 μm/s and a 2.0-fold increase in leukocyte adhesion. Both were completely abolished by 0.25 mg ip estrogen 1 h before surgery. In $eNOS^{-/-}$ mice, the decrease of leukocyte rolling velocity and increase in adhesion were similar but were only marginally improved by estrogen. We conclude that the protective effect of estrogen, as measured by leukocyte rolling and adhesion, is significantly reduced in $eNOS^{-/-}$ mice, suggesting that induction of eNOS activity is the major mechanism of vasoprotection by estrogen in this model.

leukocyte adhesion; rolling; P-selectin; microcirculation; inflammation

INFLAMMATORY LEUKOCYTE RECRUITMENT generally fulfills a crucial function for host defense; however, unrestrained it can cause considerable damage (49). For example, ischemia-reperfusion (I/R) injuries occur in myocardial infarction and stroke and cause inappropriate inflammatory leukocyte recruitment (9, 19, 24, 29, 42).

Nitric oxide (NO) is produced by three different NO synthase (NOS) enzymes and has a variety of functions. Neuronal NOS (nNOS) is primarily localized in nervous tissue, where it generates NO for neurotransmission (35, 36, 38). Inducible NOS (iNOS) can be found in macrophages and other tissues and is thought to generate large amounts of NO in response to tissue stimulation with various cytokines and proinflammatory agents (35, 36, 38). Little is known about the effects of nNOS- or iNOS-generated NO on leukocyte-endothelium interactions (20). NO released by endothelial NOS (eNOS) is a potent vasodilator that regulates mean arterial blood pressure and inhibits platelet and leukocyte adhesion and aggregation (7, 13–15, 31, 36). In I/R injury, the mechanism of inhibition of leukocyte-endothelium interactions by eNOS is not well understood. Our study focused on eNOS-deficient ($eNOS^{-/-}$) mice generated by gene targeting and homologous recombination (45). These mice show significant increases in blood pressure and plasma renin concentration and a significant decrease in heart rate. Leukocyte-endothelium interactions have not been investigated in these mice; however, experiments conducted in a different strain of $eNOS^{-/-}$ mice show slightly increased interactions under baseline conditions (23, 33).

Recently, we reported that estrogen can reduce I/R-mediated injury through stimulation of eNOS activity (46). The activation of eNOS by estrogen involves the interaction of the estrogen receptor with the regulatory subunit of phosphatidylinositol 3-kinase (PI3K). PI3K catalyzes the synthesis of second messenger lipid mediators (2, 39), which recruit proteins containing specific phosphatidylinositol (3,4,5)-trisphosphate-binding or Pleckstrin homology domains, such as phosphatidyl-dependent kinases (PDK)-1 and -2 (8, 48). The PDKs phosphorylate the serine-threonin protein kinase Akt (5). Akt mediates many of the downstream cellular effects of PI3K (11, 12), one of which is the phosphorylation and activation of eNOS (48). Interestingly, this effect does not involve gene transcription or translation.

To test whether the protective effect of estrogen depends on eNOS, we investigated a standardized I/R

Address for reprint requests and other correspondence: K. Ley, Health System Dept. of Biomedical Engineering, PO Box 800759, Charlottesville, VA 22908-0759 (E-mail: klausley@virginia.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

injury in untreated and estrogen-treated wild-type (WT) and eNOS$^{-/-}$ mice and quantified the leukocyte-endothelium interactions, namely leukocyte rolling velocity, flux, and adhesion, in the venules of the mouse cremaster muscle. Leukocyte rolling velocity is an important parameter regulating successful transition to firm adhesion, because slower rolling leukocytes have longer contact with inflamed endothelium (27) and hence a higher likelihood for arrest (32). The leukocyte recruitment observed after I/R has been shown to be primarily dependent on P-selectin because P-selectin-deficient mice showed no leukocyte-endothelium interactions before or after I/R, and the increase in leukocyte rolling observed in untreated WT mice after I/R could be abolished by administration of an anti-P-selectin antibody (28). Furthermore, several studies have suggested that the P-selectin upregulation caused by I/R in this model and other models of microvascular I/R is regulated by eNOS (10, 16, 22, 25). Therefore, we also investigated P-selectin expression at baseline conditions and after I/R.

MATERIALS AND METHODS

Animals. Intravital experiments were performed on a total of 29 male mice 6–14 wk old and weighing 20–40 g. Mice included WT C57BL/6 (Hilltop; Scottsdale, PA) and gene-targeted mice deficient in eNOS (45). eNOS knockout mice were backcrossed into the C57BL/6 background for at least seven generations. Data (red blood cell velocity and leukocyte rolling velocity and adhesion) from five of the eNOS$^{-/-}$ mice used in the control group also appear in a study by Hafezi-Moghadam et al. (18).

Reagents. Estrogen-treated animals were injected with conjugated estrogen (0.25 mg ip, Ayerst Laboratories; Philadelphia, PA) 1 h before the start of surgery. The cremasters of two estrogen-treated eNOS$^{-/-}$ mice were superfused with N^G-nitro-L-arginine methyl ester (L-NAME; 0.1 mM, Sigma; Louis, MO), a nonspecific NOS inhibitor (40), during the ischemia period. FITC-conjugated mAb RB40.34 (rat IgG$_1$, 30 μg/mouse iv, PharMingen; San Diego, CA), which binds to murine P-selectin, was used for P-selectin histology. Sodium azide was removed using an Amicon Centricon 30 Microconcentrator (Amicon; Danvers, MA).

Intravital microscopy. Mice were anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg, Ketalar, Parke-Davis; Morris Plains, NJ), xylazine (0.05 mg/kg), and atropine (0.1 mg/kg, Elkins-Sinn; Cherry Hill, NJ). Animals were kept at 37°C throughout the experiment with a heating pad. The cremaster muscle was externalized over a Plexiglas observation platform and pinned in place as previously described (3). The preparation was superfused with a thermocontrolled 35°C bicarbonate-buffered saline saturated with 95% N_2-5% CO_2 throughout the experiment. Microscopic observations were made with the use of an Axioskope intravital microscope (Zeiss; Thornwood, NY) with a saline immersion objective (SW 40/0.75 numerical aperture). Between one and four venules per animal with diameters ranging from 20 to 35 μm were observed before the 30-min ischemia period and for 60 min of reperfusion after ischemia. Ischemia was achieved by tying off the supplying arteries with polyethylene-10 tubing and visually confirming that blood flow had ceased. Video recordings were made using a charge-coupled device camera system (model VE-1000CD, Dage-MTI; Michigan City, IN) on a Panasonic S-VHS recorder. Venules were recorded for ~1.5-min segments before ischemia and at 5-min intervals throughout the reperfusion period. Red blood cell centerline velocity was measured using an optical Doppler and cross-correlation system (Circusoft Instrumentation; Hockessin, DE). Centerline velocities were converted to mean blood flow velocities by multiplying by an empirical factor of 0.625 (34). Shear rates were calculated using the equation $\gamma_w = 2.12 \times 8V_b/d$, where γ_w is the wall shear rate, V_b is the mean blood flow velocity, d is the in vivo diameter of the vessel, and 2.12 is an empirical correction factor for the shape of the velocity profile (41). All vessels had calculated wall shear rates between 400 and 3,700 s^{-1}. Leukocyte rolling velocity was measured using Scion Image (Scion; Frederick, MD) software on a G4 Macintosh computer. Rolling velocity was measured before ischemia and at 10-min intervals starting at 5 min after the end of the

Table 1. *RBC velocity, vessel diameter, and wall shear rate before ischemia and 5 and 15 min into the reperfusion period*

	RBC Velocity, mm/s	Vessel Diameter, μm	Wall Shear Rate, s^{-1}
	WT mice		
No treatment			
Before ischemia	3.8 ± 0.70	24.5 ± 0.58	1,600 ± 320
After 5-min reperfusion	3.4 ± 0.51	26.1 ± 0.91	1,400 ± 210
After 15-min reperfusion	3.5 ± 0.88	25.8 ± 0.94	1,400 ± 320
Estrogen treatment			
Before ischemia	5.2 ± 0.69	25.2 ± 1.3	2,300 ± 390
After 5-min reperfusion	3.7 ± 0.81	26.1 ± 1.3	1,500 ± 360
After 15-min reperfusion	3.4 ± 0.89	25.7 ± 1.1	1,400 ± 390
	eNOS$^{-/-}$ mice		
No treatment			
Before ischemia	5.0 ± 0.95	27.6 ± 2.2	2,100 ± 530
After 5-min reperfusion	3.2 ± 0.67	29.2 ± 1.9	1,400 ± 300
After 15-min reperfusion	3.4 ± 0.74	24.7 ± 1.4	1,800 ± 450
Estrogen treatment			
Before ischemia	3.9 ± 0.84	24.1 ± 1.5	1,700 ± 400
After 5-min reperfusion	4.1 ± 1.7	24.9 ± 2.0	1,800 ± 790
After 15-min reperfusion	3.1 ± 0.90	25.5 ± 2.0	1,300 ± 390

Values are means ± SE. RBC, red blood cell; WT, wild type; eNOS$^{-/-}$, endothelial nitric oxide synthase deficient.

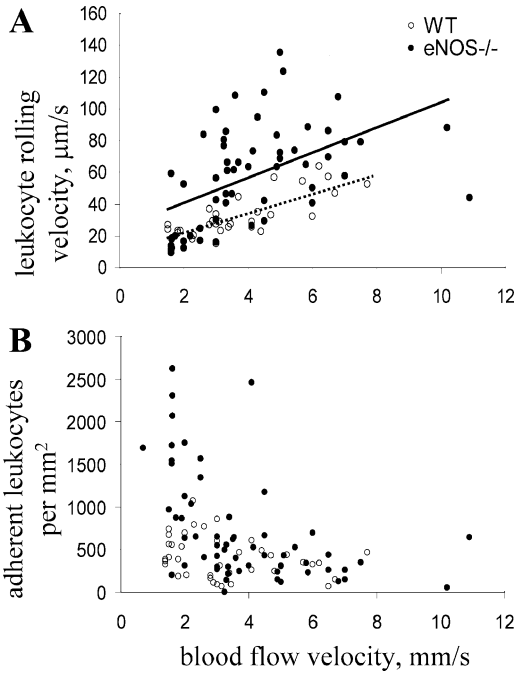

Fig. 1. Untreated endothelial nitric oxide synthase (eNOS)-deficient ($eNOS^{-/-}$; solid line) mice show a higher baseline leukocyte rolling velocity than untreated wild-type (WT; dotted line) mice. *A*: leukocyte rolling velocity versus red blood cell velocity. There was a positive correlation between rolling velocity and red blood cell velocity for both WT and $eNOS^{-/-}$ mice ($P < 0.01$). *B*: leukocyte adhesion versus red blood cell velocity.

ischemic period. Each rolling leukocyte passing a line perpendicular to the vessel wall was followed for 0.5–1 s. Adherent cells and rolling flux were counted before ischemia and at 5-min intervals starting at 2 min after the end of the ischemic period. A cell was counted as adherent if it was stationary for at least 30 s.

Immunostaining for P-selectin. Whole cremaster muscles subjected to either 30 min of ischemia and 10 min of reperfusion or no treatment were harvested from WT and $eNOS^{-/-}$ mice injected intravenously with FITC-conjugated mAb RB40.34 (30 μg/mouse, Pharmingen) 10 min before the end of the ischemic period and perfused with PBS to remove blood and unbound antibody after the 10-min reperfusion period (26). The cremaster muscle was laid flat on a gelatin-coated slide and allowed to air dry for ~5–10 min. Slides were then coverslipped using VectaShield (Vector Laboratories; Burlingame, CA) and viewed and photographed using a Nikon Microphot SA microscope with attached Nikon CoolPix 900 digital camera (Nikon; Melville, NY).

eNOS activity assay. Mouse cremasters were homogenized in ice-cold PBS containing 1 mM EDTA using a polytron. The homogenates were pelleted in a microfuge (2 min, 13,000 rpm, 4°C) to remove the insoluble material. Protein concentration was determined with the micro BCA assay kit (Pierce; Rockford, IL), and 5 μg of protein extracts from each sample were used for the eNOS assay. The eNOS activity was detected by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline at 37°C for 30 min with the eNOS assay kit (Calbiochem; La Jolla, CA) as described. Unlabeled L-arginine was added to L-[^{3}H]arginine (specific activity 60 Ci/mmol) at a ratio of 3:1. Rat cerebellum extracts, containing elevated amounts of nNOS, were used as positive controls, whereas samples incubated in the presence of the competitive NOS inhibitor L-NAME (1 mM) were used to determine nonspecific activity. Nonspecific activity accounted for 20–35% of the total activity.

Statistical analysis. All data are presented as means ± SE. Data from all vessels from each animal were averaged and used as an individual data point. Statistical analyses were performed using each animal rather than each vessel as an entity. Two-group comparisons between animals before and after ischemia were performed using paired Student's *t*-test.

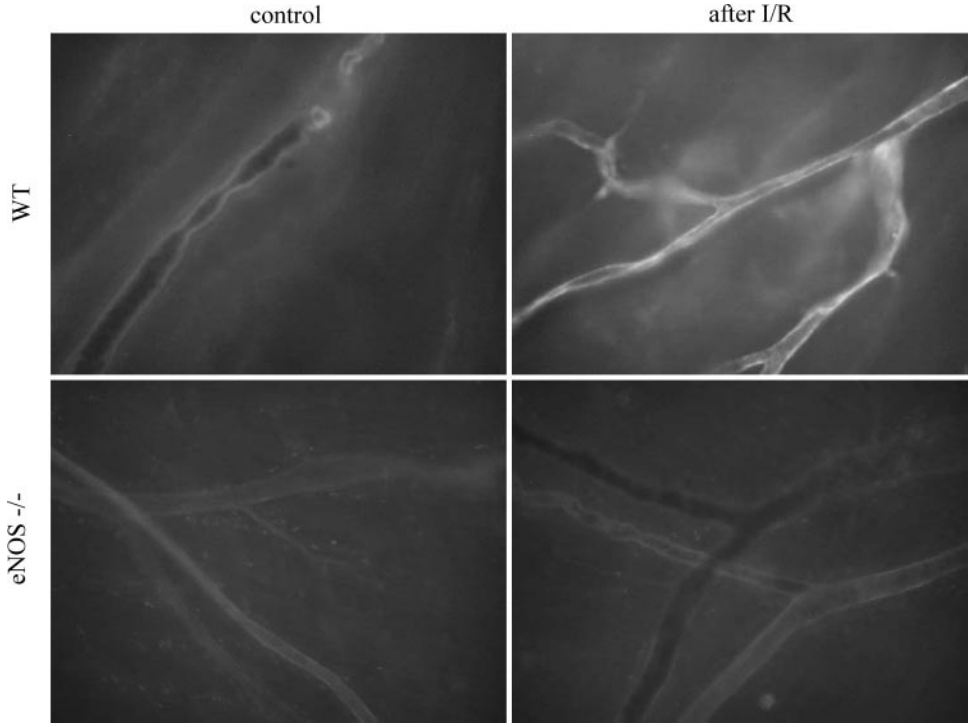

Fig. 2. Expression of endothelial P-selectin (infusion of mAb RB40.34) present on the luminal surface in WT and $eNOS^{-/-}$ mice. Control, no ischemia; after ischemia-reperfusion (I/R), sample taken at 5 min of reperfusion.

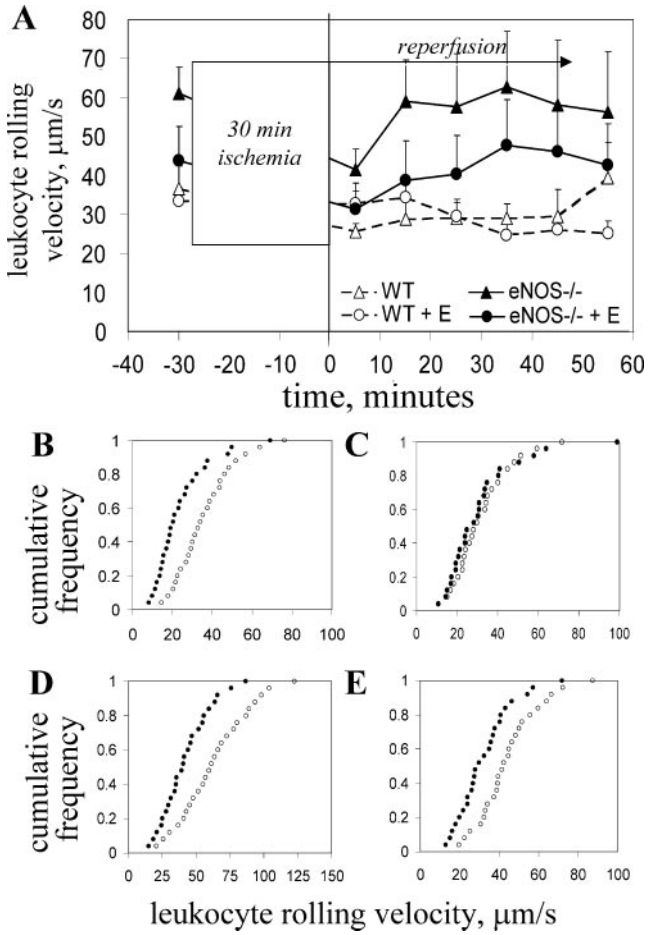

Fig. 3. *A*: average leukocyte rolling velocities (means ± SE). *B–E*: cumulative histograms of leukocyte rolling velocities before and 5 min after reperfusion for untreated WT mice (*B*), estrogen (E)-treated WT mice (*C*), untreated eNOS$^{-/-}$ mice (*D*), and estrogen-treated eNOS$^{-/-}$ mice (*E*).

Probabilities of 0.05 or less were considered statistically significant. All statistical analysis was performed using Microsoft Excel and NCSS (Kaysville, UT).

RESULTS

Hemodynamic data. Red blood cell rolling velocities, diameters and wall shear rates measured before the start of ischemia and 5 and 15 min into the reperfusion period are presented in Table 1. In individual vessels, we observed a positive correlation between leukocyte rolling velocity and red blood cell velocity, as shown in Fig. 1*A* ($P < 0.05$ for all groups), and a negative correlation between the number of adherent cells and red blood cell velocity, as shown in Fig. 1*B*. Rolling velocities were significantly higher ($P < 0.05$, one-tailed *t*-test) in eNOS$^{-/-}$ mice compared with WT mice, consistent with lower P-selectin expression in eNOS$^{-/-}$ than WT mice (Fig. 2). At low blood flow velocities (<3 mm/s), significantly more adherent leukocytes were observed in eNOS$^{-/-}$ mice. Because there were no systematic hemodynamic differences among the groups, all data are presented without adjustments for hemodynamics.

Leukocyte rolling velocity. Leukocyte rolling velocities were measured at 10-min intervals, and a time course is shown in Fig. 3*A*. The numbers of mice and venules represented by each data point are presented in Table 2. The maximal decrease in rolling velocity occurred ~5 min into the reperfusion period except in estrogen-treated WT mice (no decrease). In WT mice, I/R caused a decrease of leukocyte rolling velocity from 37 to 26 μm/s ($P < 0.05$, one-tailed *t*-test) in untreated mice (Fig. 3*B*) but no change (33 μm/s before and after I/R, not significant) in estrogen-treated mice (Fig. 3*C*). In eNOS$^{-/-}$ mice, I/R caused a similar decrease of leukocyte rolling velocity from 61 to 42 μm/s ($P < 0.05$) as in untreated mice (Fig. 3*D*), which was not reversed in estrogen-treated mice (Fig. 3*E*). Application of L-NAME to estrogen-treated eNOS$^{-/-}$ mice produced no further reduction in rolling velocity, suggesting that eNOS indeed accounted for all NO production relevant to leukocyte rolling in this model. There was no significant decrease of rolling velocity at later times in any group, suggesting that the injury induced by 30 min of ischemia and reperfusion was transient and reversible.

P-selectin expression. P-selectin expression was investigated under baseline conditions and after 30 min of ischemia and 10 min of reperfusion in WT and eNOS$^{-/-}$ mice. We observed an increase in P-selectin expression after I/R in WT mice (Fig. 2). In eNOS$^{-/-}$ mice, P-selectin expression was low, but present, and did not increase after I/R. These findings suggest that increased leukocyte adhesion and reduced rolling velocity may be caused by altered P-selectin expression in WT mice. The expression levels of P-selectin in eNOS$^{-/-}$ mice do not account for increased leukocyte adhesion, suggesting a different molecular mechanism.

Leukocyte adhesion. The number of adherent leukocytes per square millimeter of endothelial surface area was measured in each vessel. The time course is shown in Fig. 4. Maximal increase in adherent leukocytes occurred between 10 and 15 min into the reperfusion period except in the estrogen-treated wild type mice (no increase). The number of adherent leukocytes after I/R increased 62% (from 430 to 700 cells/mm^2) in untreated WT mice ($P < 0.01$) but remained unchanged in WT mice treated with estrogen (390 cell/mm^2 before vs. 380 cell/mm^2 after). We observed a 130% increase (from 430 to 980 cells/mm^2, $P < 0.01$) in untreated eNOS$^{-/-}$ mice and a 50% increase (from 430 to 650 cells/mm^2, not significant) in eNOS$^{-/-}$ mice treated with estrogen. Estrogen treatment reduced the dura-

Table 2. *Numbers of mice and venules used in statistical analysis*

Groups	WT		eNOS$^{-/-}$	
	No. of mice	No. of venules	No. of mice	No. of venules
No treatment	8	14	9	12
Estrogen	5	8	5	12
Estrogen + L-NAME	ND	ND	2	6

L-NAME, N^G-nitro-L-arginine methyl ester; ND, not done.

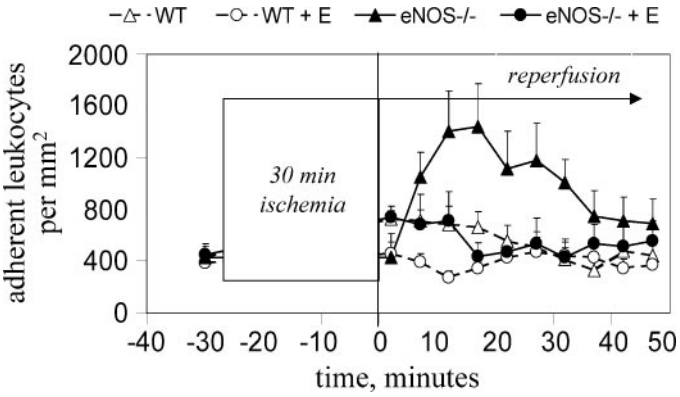

Fig. 4. Leukocyte adhesion before and after I/R (means ± SE).

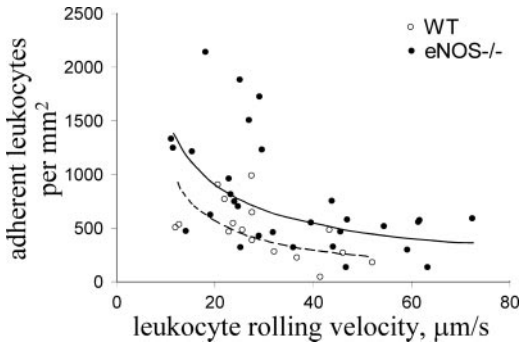

Fig. 6. Leukocyte rolling velocity 5 min after reperfusion versus adherent leukocytes 2–12 min after reperfusion. There was a significant negative correlation [$P < 0.05$ for WT (dashed line); $P < 0.01$ for eNOS$^{-/-}$ (solid line)] between rolling velocity and adhesion.

tion of increased leukocyte adhesion in eNOS$^{-/-}$ mice from 35 to 15 min.

Figure 5 summarizes the peak effects on leukocyte rolling velocity (at 5 min after onset of reperfusion) and leukocyte adhesion (averaged between 2 and 12 min after the onset of reperfusion). Significant differences are indicated by asterisks (Fig. 5). In addition to the groups shown in Figs. 3 and 4, we also treated eNOS$^{-/-}$ mice with L-NAME, an inhibitor of all NOS isoforms. This treatment did not affect rolling velocity or leukocyte adhesion in eNOS$^{-/-}$ mice, suggesting that other NOS isoforms do not significantly contribute to the inflammatory response in this model of I/R. We have previously (46) shown that L-NAME completely abolished the protective effect of estrogen on I/R injury in WT mice.

Finally, we investigated the correlation between reduced rolling velocity as measured at 5 min after onset of reperfusion and increased leukocyte adhesion (Fig. 6). Previous studies (27) have suggested that rolling velocity could be a predictor of adhesion. Indeed, we found a significant negative correlation between rolling velocity and leukocyte adhesion in WT ($P < 0.05$) and eNOS−/− ($P < 0.01$) mice (Fig. 6). This suggests that leukocyte rolling velocity may be a predictor of leukocyte adhesion, not only in cytokine-induced inflammation, but also in I/R.

eNOS activity. eNOS activity was measured in cremaster muscles harvested from untreated and estrogen-treated WT and eNOS$^{-/-}$ mice used for intravital experiments (Fig. 7). eNOS activity in WT mice after ischemia was significantly increased in estrogen-treated cremasters (2.8 vs. 4.4 pmol·mg^{-1}·min^{-1}, $P < 0.05$), whereas there was no difference in eNOS$^{-/-}$

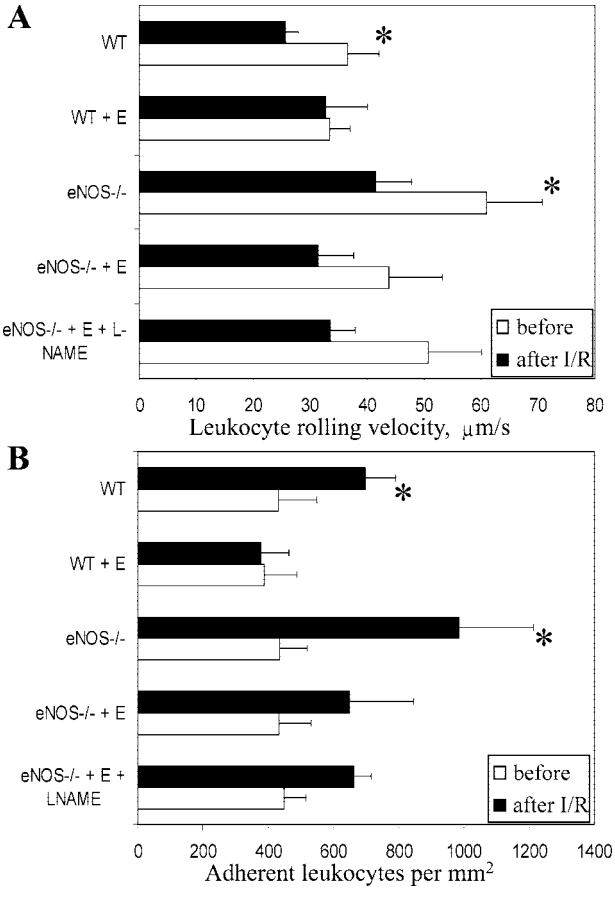

Fig. 5. *A*: rolling velocity for all treatment groups before and 5 min after reperfusion (means ± SE). *B*: leukocyte adhesion for all treatment groups before and 2–12 min after reperfusion (means ± SE). L-NAME, N^G-nitro-L-arginine methyl ester. *Significant differences.

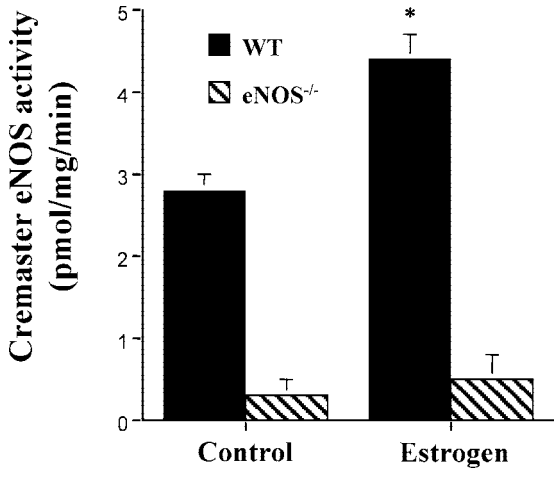

Fig. 7. Cremaster eNOS activity in untreated and estrogen-treated WT and eNOS$^{-/-}$ mice. *Significant difference.

mice (0.3 vs. 0.5 pmol·mg^{-1}·min^{-1}). We found no significant correlation between eNOS activity and adherence or rolling velocities in individual mice.

DISCUSSION

In this study, we examined the effect of pretreatment with estrogen on I/R injury in the microvasculature of WT and eNOS$^{-/-}$ mice. We found that, unlike WT mice, the eNOS$^{-/-}$ mice were not significantly protected by estrogen treatment. In eNOS$^{-/-}$ mice, I/R caused a 32% decrease of leukocyte rolling velocity in untreated mice that was similar in magnitude to that seen in untreated WT mice (30%) and that was not improved in estrogen-treated mice (decrease of 28%). We also observed increases in leukocyte adhesion of 130% and 50% in untreated and estrogen-treated eNOS$^{-/-}$ mice, respectively. The increase in adhesion in the untreated eNOS$^{-/-}$ mice (130%) was much greater than that seen in the untreated WT mice (62%). Also, eNOS activity was significantly increased by 57% in estrogen-treated WT mice compared with untreated WT mice after I/R.

In WT and eNOS$^{-/-}$ mice, the maximum decrease in rolling velocity occurred within 5 min after the onset of reperfusion. Likewise, the peak increase in leukocyte adhesion was observed ~10–15 min into the reperfusion period. This suggests that the injury created in this model of I/R is mild and transient. The lack of severity of the injury is supported by the absence of a significant reactive hyperemia at the onset of reperfusion. The rapid decrease in rolling velocity suggests that the adhesion molecules involved in this phenomenon are not synthesized de novo but are already present in the endothelial cells.

It was suggested by a previous study (46), and established in this study, that the protective effect of estrogen is partially mediated via an increase in production of NO by eNOS; however, the mechanism behind the protective effect of NO remains unknown. P-selectin, an adhesion molecule involved in the early stages of leukocyte-endothelium interactions, is stored in Weibel-Palade bodies and can be rapidly translocated to the endothelial surface upon cellular activation (47). Previous studies have indicated that P-selectin is the primary mediator of leukocyte adhesion and rolling in the cremaster I/R model (28), and the present study confirms a rapid increase in endothelial cell surface expression of P-selectin. Many studies have suggested a link between NO and P-selectin expression (1, 6, 16, 17, 22, 25, 33, 37, 43, 44). However, P-selectin expression was low in cremaster muscle venules of eNOS$^{-/-}$ mice and was not upregulated after I/R, so it is unlikely to be responsible for the increased amount of adhesion seen in this model. Upregulation of P-selectin may not be the only cause of increased adhesion seen in WT mice after I/R. Some evidence indicates that NO may also modulate expression of platelet activating factor (PAF), ICAM-1, and CD18 integrins (30, 31). PAF can activate CD18 integrins on leukocytes (21, 50). Further work will be necessary to elucidate the molecular mechanism of increased adhesion after I/R.

Other investigators have identified roles for iNOS and nNOS in other models of I/R injury (4, 20, 42). Generally, activation of nNOS and iNOS exacerbates the I/R-induced injury by producing toxic levels of NO; however, it is uncertain what role these enzymes may play in the complete absence of eNOS (4, 42). In this study, we superfused several of the cremasters from the estrogen-treated eNOS$^{-/-}$ mice with L-NAME during the ischemic period to discern whether other nNOS and iNOS play a role in this model. We did not observe any additional reperfusion injury in the L-NAME-treated mice, nor was there a protective effect, indicating that NO produced by eNOS is the only NO-producing system involved in this model.

Other observations from this study include elevated baseline leukocyte rolling velocity in eNOS$^{-/-}$ mice compared with WT mice. The reason for this increased rolling velocity could be related to decreased P-selectin expression in these mice. The interpretation of the present data is complicated by the lack of I/R-induced P-selectin expression in eNOS$^{-/-}$ mice. The mechanisms responsible for postischemic leukocyte recruitment are likely different in eNOS$^{-/-}$ and WT mice. Estrogen treatment has little effect on this recruitment mechanism, which may be the result of adaptive changes that occur during the development of eNOS$^{-/-}$ mice. We show that estrogen increases eNOS activity and reduces leukocyte rolling and adhesion, but our data stop short of demonstrating a causal relationship, because the molecular mechanisms underlying the modulation of leukocyte adhesion through eNOS products are essentially unknown.

In conclusion, the protective effect of estrogen as measured by leukocyte rolling and adhesion is abolished in eNOS$^{-/-}$ mice, suggesting that induction of eNOS activity is the major mechanism of vasoprotection by estrogen in this model. Furthermore, experiments conducted with the NOS inhibitor L-NAME suggest that other NOS isoforms do not significantly contribute to the inflammatory response in this model of I/R. However, the estrogen-treated eNOS$^{-/-}$ mice seemed somewhat protected because the number of adherent leukocytes was reduced and the duration of leukocyte accumulation was decreased. Therefore, estrogen may exert additional anti-inflammatory effects unrelated to NO production. Finally, leukocyte rolling velocity may be a predictor of leukocyte adhesion, not only in cytokine-induced inflammation, but also in I/R.

We thank John Sanders for the purified FITC-RB40.34, Dao-Shan Chui for performing the eNOS assay, and Dr. Terry Turner for use of the fluorescence microscope and camera.

This study was supported by National Heart, Lung, and Blood Institute Grants HL-64381 (to K. Ley), HL-48242 (to V. E. Laubach), and HL-52233 and HL-70274 (to J. Liao).

REFERENCES

1. **Armstead VE, Minchenko AG, Schuhl RA, Hayward R, Nossuli TO, and Lefer AM.** Regulation of P-selectin expression in human endothelial cells by nitric oxide. *Am J Physiol Heart Circ Physiol* 273: H740–H746, 1997.

2. **Auger KR, Serunian LA, Soltoff SP, Libby P, and Cantley LC.** PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. *Cell* 57: 167–175, 1989.
3. **Baez S.** An open cremaster muscle preparation for the study of blood vessels by in vivo microscopy. *Microvasc Res* 5: 384–394, 1973.
4. **Bolanos JP and Almeida A.** Roles of nitric oxide in brain hypoxia-ischemia. *Biochim Biophys Acta* 1411: 415–436, 1999.
5. **Burgering BM and Coffer PJ.** Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* 376: 599–602, 1995.
6. **Davenpeck KL, Gauthier TW, and Lefer AM.** Inhibition of endothelial-derived nitric oxide promotes P-selectin expression and actions in the rat microcirculation. *Gastroenterology* 107: 1050–1058, 1994.
7. **De Graaf JC, Banga JD, Moncada S, Palmer RM, de Groot PG, and Sixma JJ.** Nitric oxide functions as an inhibitor of platelet adhesion under flow conditions. *Circulation* 85: 2284–2290, 1992.
8. **Delcommenne M, Tan C, Gray V, Rue L, Woodgett J, and Dedhar S.** Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. *Proc Natl Acad Sci USA* 95: 11211–11216, 1998.
9. **Entman ML and Smith CW.** Postreperfusion inflammation: a model for reaction to injury in cardiovascular disease. *Cardiovasc Res* 28: 1301–1311, 1994.
10. **Eppihimer MJ, Russell J, Anderson DC, Epstein CJ, Laroux S, and Granger DN.** Modulation of P-selectin expression in the postischemic intestinal microvasculature. *Am J Physiol Gastrointest Liver Physiol* 273: G1326–G1332, 1997.
11. **Franke TF, Kaplan DR, and Cantley LC.** PI3K: downstream AKTion blocks apoptosis. *Cell* 88: 435–437, 1997.
12. **Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR, and Tsichlis PN.** The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* 81: 727–736, 1995.
13. **Freedman JE, Sauter R, Battinelli EM, Ault K, Knowles C, Huang PL, and Loscalzo J.** Deficient platelet-derived nitric oxide and enhanced hemostasis in mice lacking the NOSIII gene. *Circ Res* 84: 1416–1421, 1999.
14. **Furchgott RF and Vanhoutte PM.** Endothelium-derived relaxing and contracting factors. *FASEB J* 3: 2007–2018, 1989.
15. **Gaboury J, Woodman RC, Granger DN, Reinhardt P, and Kubes P.** Nitric oxide prevents leukocyte adherence: role of superoxide. *Am J Physiol Heart Circ Physiol* 265: H862–H867, 1993.
16. **Gauthier TW, Davenpeck KL, and Lefer AM.** Nitric oxide attenuates leukocyte-endothelial interaction via P-selectin in splanchnic ischemia-reperfusion. *Am J Physiol Gastrointest Liver Physiol* 267: G562–G568, 1994.
17. **Gauthier TW, Scalia R, Murohara T, Guo JP, and Lefer AM.** Nitric oxide protects against leukocyte-endothelium interactions in the early stages of hypercholesterolemia. *Arterioscler Thromb* 15: 1652–1659, 1995.
18. **Hafezi-Moghadam A, Simoncini T, Yang Limbourg FP, Plumier JC, Rebsamen MC, Hsieh CM, Chui DS, Thomas KL, Prorock AJ, Laubach VE, Moskowitz MA, French BA, Ley K, and Liao JK.** Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. *Nat Med* 8: 473–479, 2002.
19. **Hearse DJ, Maxwell L, Saldanha C, and Gavin JB.** The myocardial vasculature during ischemia and reperfusion: a target for injury and protection. *J Mol Cell Cardiol* 25: 759–800, 1993.
20. **Hickey MJ.** Role of inducible nitric oxide synthase in the regulation of leucocyte recruitment. *Clin Sci (Colch)* 100: 1–12, 2001.
21. **Hogaboam CM, Donigi-Gale D, Shoupe TS, Bissonnette EY, Befus AD, and Wallace JL.** Platelet-activating factor synthesis by peritoneal mast cells and its inhibition by two quinoline-based compounds. *Br J Pharmacol* 105: 87–92, 1992.
22. **Horie Y, Wolf R, Anderson DC, and Granger DN.** Nitric oxide modulates gut ischemia-reperfusion-induced P-selectin expression in murine liver. *Am J Physiol Heart Circ Physiol* 275: H520–H526, 1998.
23. **Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, and Fishman MC.** Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* 377: 239–242, 1995.
24. **Jeroudi MO, Hartley CJ, and Bolli R.** Myocardial reperfusion injury: role of oxygen radicals and potential therapy with antioxidants. *Am J Cardiol* 73: 2B–7B, 1994.
25. **Jones SP, Girod WG, Palazzo AJ, Granger DN, Grisham MB, Jourd'Heuil D, Huang PL, and Lefer DJ.** Myocardial ischemia-reperfusion injury is exacerbated in absence of endothelial cell nitric oxide synthase. *Am J Physiol Heart Circ Physiol* 276: H1567–H1573, 1999.
26. **Jung U and Ley K.** Regulation of E-selectin, P-selectin, and intercellular adhesion molecule 1 expression in mouse cremaster muscle vasculature. *Microcirculation* 4: 311–319, 1997.
27. **Jung U, Norman KE, Scharffetter-Kochanek K, Beaudet AL, and Ley K.** Transit time of leukocytes rolling through venules controls cytokine-induced inflammatory cell recruitment in vivo. *J Clin Invest* 102: 1526–1533, 1998.
28. **Kanwar S, Smith CW, and Kubes P.** An absolute requirement for P-selectin in ischemia/reperfusion-induced leukocyte recruitment in cremaster muscle. *Microcirculation* 5: 281–287, 1998.
29. **Kloner RA, Przyklenk K, and Whittaker P.** Deleterious effects of oxygen radicals in ischemia/reperfusion. Resolved and unresolved issues. *Circulation* 80: 1115–1127, 1989.
30. **Kubes P, Kurose I, and Granger DN.** NO donors prevent integrin-induced leukocyte adhesion but not P-selectin-dependent rolling in postischemic venules. *Am J Physiol Heart Circ Physiol* 267: H931–H937, 1994.
31. **Kubes P, Suzuki M, and Granger DN.** Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci USA* 88: 4651–4655, 1991.
32. **Kunkel EJ, Dunne JL, and Ley K.** Leukocyte arrest during cytokine-dependent inflammation in vivo. *J Immunol* 164: 3301–3308, 2000.
33. **Lefer DJ, Jones SP, Girod WG, Baines A, Grisham MB, Cockrell AS, Huang PL, and Scalia R.** Leukocyte-endothelial cell interactions in nitric oxide synthase-deficient mice. *Am J Physiol Heart Circ Physiol* 276: H1943–H1950, 1999.
34. **Lipowsky HH and Zweifach BW.** Application of the "two-slit" photometric technique to the measurement of microvascular volumetric flow rates. *Microvasc Res* 15: 93–101, 1978.
35. **Michel T and Feron O.** Nitric oxide synthases: which, where, how, and why? *J Clin Invest* 100: 2146–2152, 1997.
36. **Moncada S, Palmer RM, and Higgs EA.** Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 43: 109–142, 1991.
37. **Murohara T, Parkinson SJ, Waldman SA, and Lefer AM.** Inhibition of nitric oxide biosynthesis promotes P-selectin expression in platelets. Role of protein kinase C. *Arterioscler Thromb* 15: 2068–2075, 1995.
38. **Nathan C.** Nitric oxide as a secretory product of mammalian cells. *FASEB J* 6: 3051–3064, 1992.
39. **Rameh LE and Cantley LC.** The role of phosphoinositide 3-kinase lipid products in cell function. *J Biol Chem* 274: 8347–8350, 1999.
40. **Rees DD, Palmer RM, Schulz R, Hodson HF, and Moncada S.** Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br J Pharmacol* 101: 746–752, 1990.
41. **Reneman Woldhuis RB, oude Egbrink M, Slaaf D, and Tangelder G.** Concentration and velocity profiles of blood cells in the microcirculation. In: *Advances in Cardiovascular Engineering*, edited by Hwang N, Turitto V and Yen M. New York: Plenum, 1993, p. 25–40.
42. **Samdani AF, Dawson TM, and Dawson VL.** Nitric oxide synthase in models of focal ischemia. *Stroke* 28: 1283–1288, 1997.
43. **Scalia R, Gauthier TW, Murohara T, and Lefer AM.** Oligotide attenuates leukocyte-endothelial cell interaction via P-

selectin in the rat mesenteric vascular bed. *Eur J Pharmacol* 296: 181–187, 1996.
44. **Scalia R, Kochilas L, Campbell B, and Lefer AM.** Effects of defibrotide on leukocyte-endothelial cell interaction in the rat mesenteric vascular bed: role of P-selectin. *Methods Find Exp Clin Pharmacol* 18: 669–676, 1996.
45. **Shesely EG, Maeda N, Kim HS, Desai KM, Krege JH, Laubach VE, Sherman PA, Sessa WC, and Smithies O.** Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc Natl Acad Sci USA* 93: 13176–13181, 1996.
46. **Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, and Liao JK.** Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* 407: 538–541, 2000.
47. **Smith CW.** Endothelial adhesion molecules and their role in inflammation. *Can J Physiol Pharmacol* 71: 76–87, 1993.
48. **Stephens L, Anderson K, Stokoe D, Erdjument-Bromage H, Painter GF, Holmes AB, Gaffney PR, Reese CB, McCormick F, Tempst P, Coadwell J, and Hawkins PT.** Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science* 279: 710–714, 1998.
49. **Sullivan GW, Sarembock IJ, and Linden J.** The role of inflammation in vascular diseases. *J Leukoc Biol* 67: 591–602, 2000.
50. **Zimmerman GA, McIntyre TM, Mehra M, and Prescott SM.** Endothelial cell-associated platelet-activating factor: a novel mechanism for signaling intercellular adhesion. *J Cell Biol* 110: 529–540, 1990.