Protective Role of Nitric Oxide in a Model of Thrombotic Microangiopathy in Rats

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Abstract. A new model of thrombotic microangiopathy (TMA) was previously developed, and it was demonstrated that endothelial nitric oxide (NO) synthase (NOS) is upregulated in glomeruli in this model. It was hypothesized that the synthesis of NO, a potent vasodilator and platelet inhibitory factor, is induced as a defense mechanism. The goal of this study was to clarify the role of NO in this model. Ex vivo experiments using Western blotting and functional assays demonstrated upregulation of endothelial NOS in isolated glomeruli from TMA rats. In in vivo experiments, five groups of rats were studied, including rats with TMA treated with vehicle, \( N^6 \)-nitro-L-arginine methyl ester (L-NAME) (a NOS inhibitor), or L-\( N^6 \)-(1-iminoethyl)lysine (L-NIL) (a specific inducible NOS inhibitor) and normal control rats treated with vehicle or L-NAME. Blood urea nitrogen levels, BP, urinary nitrate/nitrite excretion, and proteinuria were measured. Histologic assessments using periodic acid-Schiff staining and immunohistologic studies with markers for endothelium, platelets, fibrin, cell proliferation, and apoptosis were also performed. L-NAME inhibition of NO synthesis in rats with TMA resulted in more severe glomerular and tubulointerstitial injury, which was accompanied by thrombus formation and a marked loss of endothelial cells, with more apoptotic cells. These changes were associated with severe renal function deterioration. In contrast, these features were less pronounced in the vehicle- or L-NIL-treated rats with TMA and were absent in the control animals. In conclusion, inhibition of NO production in this model of TMA markedly exacerbated renal injury. The absence of effects with L-NIL treatment suggests a minor role for inducible NOS in this model. These results suggest that production of NO, most likely by endothelial cells, is an important protective mechanism in TMA.

Injury to the vascular endothelium, followed by consequent activation, is critical in inflammation and the promotion of a procoagulant state and is likely to be of major importance in the pathogenesis of various kidney disorders, such as glomerulonephritis, vasculitis, allograft rejection, ischemia-reperfusion injury, and thrombotic microangiopathy (TMA) (1,2). Endothelial injury is also involved in the initiation and propagation of glomerulosclerosis and end-stage renal disease (3–7).

The hemolytic uremic syndrome and related TMA are the most prominent diseases in which injury to the renal microvascular endothelium is apparent. Although animal models of TMA have classically been difficult to develop, we recently reported a model induced by the renal artery perfusion of anti-glomerular endothelial cell antibody (1,8). Similar to hemolytic uremic syndrome in human subjects, this model was associated with glomerular and peritubular capillary endothelial cell injury, with platelet accumulation and fibrin deposition, microangiopathic hemolysis, mild thrombocytopenia, and renal failure (1,9).

We previously reported that endothelial cell injury in this model is followed by a transient increase in glomerular expression of endothelial nitric oxide (NO) synthase (eNOS) (1), which would be expected to result in an increase in local NO generation. Although NO can be injurious, particularly if it reacts with oxidants to generate peroxynitrites and other reactive nitrogen species (10), NO may also function as a protective factor for the renal microvasculature. NO may help maintain vascular patency, because of its potent vasodilatory and antiplatelet effects (11,12).

We thus hypothesized that the local increase in eNOS expression in this model of TMA could represent a protective mechanism of the host to reduce microvascular injury in this model. To test this hypothesis, we examined the effects of \( N^6 \)-nitro-L-arginine methyl ester (L-NAME) [a NO synthase (NOS) inhibitor] and L-\( N^6 \)-(1-iminoethyl)lysine (L-NIL) [a selective inducible NOS (iNOS) inhibitor] (13,14) in this model.
We report that blockade of endogenous NO with l-NAME in this model dramatically exacerbated renal microvascular injury and worsened renal function. l-NIL treatment to block iNOS did not affect the disease manifestations, suggesting that the effects of l-NAME are attributable to blockade of eNOS. These studies document an important protective role of NO, most likely produced by endothelium, in TMA.

Materials and Methods

Animals

Male Wistar rats weighing 200 g were purchased from Nippon Seibutsu Zairyo Center Co. (Saitama, Japan). All rats were housed in individual cages in a temperature- and light-controlled environment in an accredited animal care facility, with free access to water or l-NAME solution (5 mg/dl; total amount ingested per rat, approximately 12.5 mg/d). Either l-NIL (3 mg/kg; total amount injected per rat, 1.2 mg/d) or vehicle was injected intraperitoneally every 12 h throughout the experimental course (13). When urine was to be collected, rats were moved to metabolic cages under the same conditions. All studies conformed to the principles of the Guide for Animal Experimentation at the University of Tokyo.

Induction of the Renal TMA Model

The model was induced by selective perfusion of the right kidney, through the superior mesenteric artery, with the purified IgG fraction of a goat anti-glomerular endothelial cell antibody, as reported previously (1). Kidneys were perfused with 0.2 ml of phosphate-buffered saline (pH 7.2), followed by 50 mg/kg body wt anti-glomerular endothelial cell IgG or phosphate-buffered saline (control). Ischemia time was <6 min. The nonperfused left kidney was removed 4 d before the induction of disease.

Purification of Goat Anti-Glomerular Endothelial Cell IgG

Goat anti-endothelial cell IgG for renal artery perfusion was purified by using a caprylic acid precipitation method, as described previously (15). The purity of anti-glomerular endothelial cell IgG was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Experimental Protocol

To investigate the role of NO synthesized by the endothelium in this model, five groups of rats were studied. These included rats with TMA (group I, n = 5); rats with TMA with l-NAME (Dojindo Molecular Technologies, Gaithersburg, MD) in the drinking water (group II, n = 5); normal control rats with (group III, n = 6) or without (group IV, n = 6) l-NAME in the drinking water; and rats with TMA that were treated with l-NIL (Dojindo Molecular Technologies) by intraperitoneal injection (group V, n = 5). On day −4, rats underwent a left nephrectomy and began treatment with l-NAME (groups II and III) or l-NIL (group V). Baseline measurements before nephrectomy included blood urea nitrogen (BUN), systolic BP, urinary nitrate/nitrite excretion, and proteinuria assessments. On day 0, the TMA model was induced with the right renal artery perfusion of 50 mg/kg body wt anti-glomerular endothelial cell IgG. Twenty-four hours after disease induction, BP was measured, a blood sample was obtained via the tail vein for determination of the BUN level, and a survival biopsy was performed. Rats were housed in metabolic cages for overnight collection of urine on days 1 and 3. On day 3, BP was tested again, a blood sample was obtained via cardiac puncture, and all rats underwent sacrificial biopsies for histologic studies.

Measurement of NOS Activity

To measure NOS activity in glomeruli, we used cultured glomeruli. For this purpose, we measured nitrite generation in normal glomeruli and glomeruli with TMA. One group of glomeruli with TMA were treated with l-NAME in vitro, to confirm the inhibitory effects of this reagent. Another group of glomeruli with TMA were incubated with l-NIL in vitro. We also measured nitrite generation in normal glomeruli treated with lipopolysaccharide (LPS), as a positive control.

Briefly, 24 h after the induction of TMA, glomeruli from diseased or normal kidneys were isolated by conventional sieving methods (16,17). Isolated glomeruli were washed twice in α-minimal essential medium with l-glutamine but without phenol red (Life Technologies, Rockville, MD), supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS), penicillin and streptomycin (Wako Pure Chemical Industries, Osaka, Japan), and 15 mM HEPES, and glomeruli were centrifuged at 1000 rpm for 4 min. Glomeruli were then plated at 4000/ml in 24-well culture clusters (Costar Corp., Cambridge, MA) and incubated at 37°C in 5% CO₂. Some glomeruli were incubated with 1 μg/ml LPS (Wako Pure Chemical Industries), 1 mM l-NAM, or 100 μM l-NIL. Medium without glomeruli was used as a negative control sample. After 48 h of incubation, supernatants were collected for NO₂⁻/NO₃⁻ assays, using nitrate/nitrite colorimetric assay kits (Cayman Chemical, Ann Arbor, MI). Values were derived by subtracting background NO₂⁻/NO₃⁻ values measured in medium incubated without glomeruli (18).

Western Blot Analyses

Twenty-four hours after TMA induction, glomeruli from diseased or normal kidneys were isolated by conventional sieving methods (16,17). The isolated glomeruli were lysed by sonication in 20 mM Tris-HCl, 1 mM ethylenediaminetetraacetate (pH 7.6). The protein concentrations of the lysates were measured with a detergent-compatible protein assay (Bio-Rad, Hercules, CA). Six micrograms of protein were loaded in each lane, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed under reducing conditions. For detection of eNOS, 0.25 μg/ml anti-eNOS monoclonal antibody (clone 3; Transduction Laboratory, Lexington, KY) was used. For detection of iNOS, 0.25 μg/ml anti-iNOS monoclonal antibody (clone 54; Transduction Laboratory) was used. The bound antibodies were detected with alkaline phosphatase-conjugated anti-mouse IgG (Promega, Madison, WI). 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Sigma Fast; Sigma Chemical Co., St. Louis, MO) were used as a substrate. The gels were stained with Coomassie brilliant blue, and all lanes of the stained gels were confirmed to be equivalent by visual inspection. Three independent experiments were performed with a total of six experimental animals, using one TMA rat and one control rat in each set.

BP, BUN, Nitrate/Nitrite Concentration, and Proteinuria Measurements

BP was measured with a tail cuff, using a volume-oscillometric method (UR-5000; UEDA Co., Tokyo, Japan). Mean systolic BP was recorded in the morning on days −4, 1, and 3. BUN levels were determined colorimetrically with a commercial kit that used the urease-indophenol method to measure urea nitrogen (Wako Pure Chemical Industries). Urinary nitrate/nitrite excretion was measured by using the lactate dehydrogenase method, with a nitrate/nitrite colorimetric assay kit (Cayman Chemical). Plasma nitrate/nitrite concentra-
tions were measured by using the same kit, after ultrafiltration of the samples with Ultrafree-MC filter membranes (molecular weight cutoff, 10,000; Millipore, Bedford, MA), according to the protocol provided by the manufacturer. Proteinuria was measured by using a Bio-Rad protein assay kit.

Renal Histologic Assessments

Tissue fixed in methyl Carnoy’s solution was processed and paraffin-embedded. Four-micrometer sections were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. An indirect immunoperoxidase method was used, as described previously (4), to identify the following cell types: renal microvascular endothelial cells with monoclonal antibody JG-12 (a generous gift from Dr. Dontcho Kerjaschki, Institute of Clinical Pathology, School of Medicine, Vienna, Austria) (19); proliferating cells with PC10, an antibody to the proliferating cell nuclear antigen (PCNA) (Dako A/S, Glostrup, Denmark); and platelets with monoclonal antibody PL-1 (generously provided by W. W. Baker, University of Groningen, Groningen, The Netherlands). Formalin-fixed and paraffin-embedded tissue was sectioned at 6 μm, for assessment of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining, using a commercial apoptosis detection kit (TACS2 TdT-Blue Label in situ apoptosis detection kit; Trevigen, Gaithersburg, MD).

Tissue for immunofluorescence assays was embedded in OCT compound (Miles, Inc., Elkhart, IN) and snap-frozen in isopentane. Fibrin was detected by staining with FITC-conjugated goat anti-fibrinogen IgG (Cappel, Durham, NC). Deposition of the pathogenic compound (Miles, Inc., Elkhart, IN) and snap-frozen in isopentane. Glomerular thrombi and platelet infiltration, a semiquantitative scoring system was used, as follows: 0, normal; 1, 0 to 25% of glomerular area involved; 2, 25 to 50% of glomerular tuft area involved; 3, 50 to 75% of tuft area involved; 4, >75% of tuft area involved. A glomerular endothelial score was graded as follows: 0, no positive glomerular tuft staining for endothelium; 1, 0 to 25% of glomerular tuft positive for endothelium; 2, 25 to 50% positive; 3, 50 to 75% positive; 4, 75 to 100% positive. The numbers of proliferating (PCNA-positive) glomerular cells and apoptotic (TUNEL-positive) cells in each glomerular cross-section were also enumerated. Glomerular cross-sections containing only a minor portion of the glomerular tuft (<20 discrete capillary segments/cross-section) were not used. Tubulointerstitial injury was defined as tubular dilation and/or tubular atrophy, tubular cast formation, sloughing of tubular epithelial cells, or thickening of the tubular basement membrane and was quantified on a scale of 0 to 4, as described previously (20).

Statistical Analyses

Data are reported as mean ± SD. Statistical comparisons were analyzed with the program StatView (Abacus Concepts, Berkeley, CA) using ANOVA, followed by the Bonferroni/Dunn method for multiple-group comparisons. Nonparametric data were analyzed with the Kruskal-Wallis test, when appropriate. Differences were considered significant when the P value was <0.05.

Results

Western Blot Analyses

To confirm the upregulation of eNOS expression in the glomeruli of rats with TMA, we performed Western blot analyses of glomeruli isolated by conventional sieving from rats with TMA and normal rats. Expression levels of eNOS in glomeruli from rats with TMA were increased, compared with those from control animals (Figure 1). iNOS was not detectable in any animals in either groups (data not shown).

Inhibition of NOS Activity by l-NAME Treatment

To confirm the functional upregulation of NOS activity in the glomeruli of rats with TMA, glomeruli were isolated by conventional sieving from rats with TMA and normal rats and were cultured for 48 h, and the amount of NO2/NO3 generated in the culture medium (representing products of NOS metabolism) was measured. After 48 h of incubation, NO production (NO2/NO3) was increased in the diseased glomeruli (TMA, 6.5 ± 1.8 μM; control, 1.4 ± 0.8 μM; P < 0.0005). As a comparison, normal glomeruli incubated with LPS also generated increased NO2/NO3 (control plus LPS, 13.4 ± 3.3 μM; control, 1.4 ± 0.8 μM; P < 0.0001). Glomeruli isolated from TMA rats that were treated with l-NAME demonstrated 50 to 90% reductions in NO2/NO3 generation (TMA plus l-NAME, 2.3 ± 0.8 μM; TMA, 6.5 ± 1.8 μM; P < 0.05). Although NO production by glomeruli from TMA rats that were treated with l-NIL was also decreased, the difference was not statistically significant (5.6 ± 2.7 μM, P = 0.6) (Figure 2).

Figure 1. Increased endothelial nitric oxide (NO) synthase (eNOS) expression in isolated glomeruli. Western blot analysis of isolated glomeruli demonstrated increased expression of eNOS in rats with thrombotic microangiopathy (TMA), compared with control animals (arrow). Molecular mass markers are shown on the left (in kilodaltons). A photograph from three independent experiments is shown.
Reduction of Urinary Nitrate/Nitrite Excretion by L-NAME Treatment In Vivo

To test whether the inhibition of NO by L-NAME was successful in reducing NO production, we measured urinary excretion of nitrate/nitrite (NO$_2$/NO$_3$), i.e., end products of NO metabolism. L-NAME-treated animals with TMA demonstrated a significant decrease in urinary nitrate/nitrite excretion on day 1 (group II, 0.4 ± 0.1 mol/24 h; group I, 1.4 ± 0.4 μmol/24 h; P < 0.01). L-NIL treatment resulted in only a marginal decrease in urinary nitrate/nitrite excretion (1.1 ± 0.6 μmol/24 h, P = 0.06). Urinary nitrate/nitrite excretion increased in groups I, II, and V on day 3. Rats in groups III and IV did not exhibit any changes in urinary nitrate/nitrite excretion levels (Figure 3).

We also measured plasma nitrate/nitrite levels for the experimental animals. Plasma nitrate/nitrite concentrations for rats in group III were 25 ± 17 μM on day 1 and 35 ± 23 μM on day 3. Concentrations for animals in group IV were 56 ± 63 μM on day 1 and 46 ± 47 μM on day 3. TMA rats demonstrated increases in plasma nitrate/nitrite levels. Rats treated with L-NAME demonstrated lower plasma nitrate/nitrite concentrations (64 ± 43 μM on day 1 and 69 ± 51 μM on day 3) than did control TMA rats and TMA rats treated with L-NIL (114 ± 35 μM and 108 ± 61 μM on day 1 and 111 ± 68 μM and 108 ± 61 μM on day 3, respectively), but the differences did not reach statistical significance.

Renal Function and BP

Renal function (assessed on the basis of BUN levels) was decreased in rats with TMA (groups I, II, and V), compared with control animals (groups III and IV), on both days 1 and 3. L-NAME treatment for rats with TMA (group II) was associated with worse renal function than that in diseased control rats (group I) and diseased L-NIL-treated rats on day 3 (BUN levels: group II, 208 ± 100 mg/dl; group I, 81 ± 40 mg/dl; group V, 78.1 ± 37.5 mg/dl; P < 0.05) (Figure 4). No significant proteinuria was observed in any group throughout the experimental course (Table 1), as we reported previously (1).

Rats with TMA that were treated with L-NAME demonstrated a slight increase in systolic BP levels on day 1 (group II, 120 ± 6.0 mmHg; group I, 97.4 ± 5.4 mmHg; P < 0.001). Rats treated with L-NIL demonstrated no change in BP levels. On day 3, BP levels for L-NAME-treated rats remained high,
although the difference did not reach statistical significance (group II, 108.7 ± 11.5 mmHg; group I, 95.7 ± 11.0 mmHg; group V, 93.8 ± 17.9 mmHg; P = NS). BP levels for the other groups did not change throughout the experimental course (Table 1).

**L-NAME Exacerbation of Histologic Injury in Rats with TMA**

Light microscopy (with PAS staining) revealed classic changes of TMA in groups I, II, and V. Glomeruli demonstrated generalized hypcellularity, with indistinct capillary walls, often accompanied by intraluminal thrombi. Tubulointerstitial injury was also widespread, with many dilated tubules and often with sloughing of epithelial cells into the tubule lumen, with cast formation, and with patchy areas of necrosis. Infiltrating polymorphonuclear and mononuclear leukocytes were both observed in the tubulointerstitium.

Semiquantitative scoring revealed that both glomerular and tubulointerstitial injuries were significantly worse in the L-NAME-treated rats, compared with vehicle-treated rats or L-NIL-treated rats with TMA, at all times studied (Figure 5 and Table 2). No abnormalities were noted in control rats by light-microscopic assessment.

**Greater Severity of Endothelial Injury in L-NAME-Treated Rats with TMA**

The renal microvascular endothelium was assessed by staining with the endothelial cell-specific mouse monoclonal antibody JG-12. Most glomeruli demonstrated a decrease in JG-12 staining 1 d after the induction of disease in rats with TMA (groups I, II, and V), whereas control rats (groups III and IV) maintained intense staining of the capillary loops. The loss of JG-12 staining 1 d after the induction of disease in rats with TMA that were treated with L-NAME (average score of group II, 0.9 ± 0.7; group I, 2.7 ± 0.5; group V, 2.9 ± 0.4; P < 0.0001) (Figure 6). By day 3, there was a recovery of endothelium in all vehicle-, L-NAME-, and L-NIL-treated rats with TMA and the differences between the groups were no longer significant (average score of group II, 2.7 ± 1.3; group I, 3.1 ± 0.7; group V, 3.4 ± 0.3; P = NS).

**Increases in Renal Thrombi in L-NAME-Treated Rats with TMA**

Fibrin deposition in glomeruli was assessed by immunofluorescence studies with samples obtained after perfusion with anti-glomerular endothelial cell antibody. Glomerular thrombi were common and significantly more severe in rats treated with l-NAME on both days 1 and 3, compared with vehicle-treated and L-NIL-treated rats with TMA (Figure 7 and Table 3). Fibrin deposition was absent in normal and L-NAME-treated control animals.

Marked platelet aggregation was also observed in the glomeruli of rats with TMA. Intraglomerular platelet aggregation exhibited a tendency to be greater in L-NAME-treated rats, but this difference did not reach statistical significance (Table 2). No platelet aggregation was observed in control rats (groups III and IV).

**Glomerular Cell Proliferation in TMA**

The total number of proliferating cells within glomeruli was assessed by immunostaining of tissue sections for PCNA. On day 3, there was a significant increase in proliferating endothelial cells in rats with TMA (groups I, II, and V), compared with normal control animals (groups III and IV) (Table 2). However, there was no significant difference in glomerular cell proliferation in rats with TMA in the presence or absence of L-NAME (group II, 4.8 ± 1.7 cells/glomerulus; group I, 3.9 ± 2.5 cells/glomerulus; P = NS).

**Increase in Endothelial Cell Apoptosis in L-NAME-Treated Rats with TMA**

To investigate the causes of the loss of endothelium demonstrated by JG-12 staining in TMA rats, we assessed apoptosis by TUNEL staining. Almost all TUNEL-positive cells were in intraluminal locations, suggesting that these cells were of endothelial cell origin, consistent with previous studies (21). TUNEL-positive apoptotic cells were significantly evident within the capillary lumen in L-NAME-treated rats with TMA on both days 1 and 3 (on day 1, 0.17 ± 0.07 versus 0.05 ± 0.03 and 0.05 ± 0.03 cells/glomerulus, group II versus groups I and V, P < 0.0001; on day 3, 0.16 ± 0.05 versus 0.008 ± 0.017

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### Table 1. Summary of biological data

<table>
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<tr>
<th>Characteristic</th>
<th>Day</th>
<th>Group I (Control TMA)</th>
<th>Group II (TMA + L-NAME)</th>
<th>Group III (Vehicle + L-NAME)</th>
<th>Group IV (Vehicle Alone)</th>
<th>Group V (TMA + L-NIL)</th>
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<td>2.4 ± 0.6</td>
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a L-NAME, N⁶-nitro-L-arginine methyl ester; L-NIL, L-N⁶-(1-iminoethyl)lysine; TMA, thrombotic microangiopathy.

b P < 0.05 versus Group I.

c P < 0.05 versus Group III.

d P < 0.05 versus Group IV.

e P < 0.05 versus Group V.
Figure 5. Evidence that 1-NAME-treated rats developed more severe renal damage. Three days after disease induction, rats in group II demonstrated indistinct glomerular capillary walls, with formation of intraluminal thrombi in most capillary loops (A). Tubulointerstitial injury was also apparent; tubular cast formation, sloughing of tubular epithelial cells, tubular dilation, and atrophy were observed (B). Damage was less severe for rats in group I (C and D) or group V (G and H) (with a few thrombi in capillary loops) and was absent in control rats in group III or group IV (E and F). Magnification, ×400.
and 0.013 ± 0.018 cells/glomerulus, group II versus groups I and V, \( P < 0.0001 \). Endothelial cell apoptosis was not observed in rats without TMA (Figure 8).

Lack of Effect of Treatment with NOS Inhibitors on Glomerular Deposition of Anti-Glomerular Endothelial Cell IgG

To test whether the difference in the severity of the disease in groups I, II, and V was attributable to differential binding of pathogenic anti-endothelial cell IgG to the kidney, the biopsies obtained 24 h after disease induction were stained with anti-goat IgG. All rats in groups I, II, and V demonstrated bright linear staining in a capillary pattern, with the same intensities, with anti-goat IgG. Semiquantitative analysis of goat IgG deposition in these three groups confirmed this observation (Table 3). The peritubular capillaries and vessels also demonstrated faint staining in these three groups, without any difference. Staining for goat IgG was negative in kidney tissues from groups III and IV (Figure 9).

Discussion

Endothelial cell production of NO may have a protective role for the vasculature. NO may maintain vascular patency because of its vasodilatory and antiplatelet actions (11,22–25). NO is also an important angiogenic and survival factor for endothelial cells (26,27). Vascular endothelial cell growth factor, which is an important endothelial growth factor, requires NO for its angiogenic action (19), and mice lacking eNOS exhibit reduced angiogenic activity (28). The beneficial actions of NO may be particularly important in diseases characterized by primary injury to the microvascular endothelium, such as that observed in TMA. Indeed, increased plasma nitrite levels (a reflection of increased NO production) have been demonstrated in recurrent hemolytic uremic syndrome and chemotherapy-induced TMA (10,29). We also previously observed a transient increase in eNOS expression in glomeruli at 4 and 24 h in the TMA model (1). To test the hypothesis that the transient increase in NO production by the endothelium in this model may have a protective role for the endothelium, we evaluated the effect of blocking the increase in NO production with an inhibitor of NO synthesis (L-NAME). Because L-NAME is a nonspecific inhibitor of NOS, we also used L-NIL to evaluate potential effects of iNOS in this model.

The injection of anti-glomerular endothelial cell antibody resulted in classic features of TMA, with significant renal microvascular endothelial cell injury and loss in association with platelet infiltration, fibrin deposition, tubulointerstitial injury, and renal failure (30). Our Western blot analysis of isolated glomeruli from TMA animals confirmed the upregulation of eNOS in glomeruli, which was previously demonstrated by immunohistochemical methods (1). To examine whether the increase in eNOS expression in glomeruli was associated with its functional activity, we measured the NOS activity of isolated glomeruli from animals with TMA. Our results demonstrated that NOS activity was indeed increased in glomeruli from animals with TMA. When NO synthesis was
inhibited in these animals (as documented by a decrease in glomerular NOS activity and a reduction in urinary nitrate/nitrite excretion), the renal injury became much worse. Both the glomerular and tubulointerstitial injury scores and renal function were significantly worse in L-NAME-treated rats. Therefore, these results document a critical role for endogenous NO production as a host mechanism to counter endothelial cell injury in this model of TMA.

Although eNOS expression was upregulated in our model of TMA, endothelial injury was associated with decreased eNOS expression and upregulation of iNOS in a model of endotoxin-induced TMA (31). This discrepancy can be explained by different pathogenic mechanisms in the two models. Zhou et al. (31) speculated that the decrease in eNOS expression in their model could be attributable to endothelial damage, increased production of cytokines and/or iNOS that downregulate eNOS protein expression, or a direct effect of endotoxin on eNOS gene transcription and translation. Noris et al. (10) also emphasized a cytopathic role for NO interacting with neutrophil-derived superoxide anion in their patients with recurrent TMA. In contrast to the model described by Zhou et al. (31), our model is endotoxin-free and is induced by complement-dependent injury to the glomerular endothelium (1,30). Furthermore, we did not observe neutrophil infiltration of glomeruli in our model (1). The lack of effects of l-NIL treatment in our studies confirmed that our model was independent of iNOS activity. Recent studies using eNOS-knockout mice demonstrated a protective role of NO generated by eNOS in a thrombotic glomerulonephritis model induced with an anti-glomerular basement membrane antibody (32). Therefore, we speculate that upregulation of eNOS in the immunologically damaged glomerular endothelium, in a neutrophil-independent manner, in our model serves as a defensive mechanism against the hypoxic state induced by local thrombus formation.

NO could have provided protection in this model via several different mechanisms. NO causes smooth muscle relaxation and consequent vasodilation, and l-NAME works as a vasococontractor by inhibiting eNOS (11,23). The vasodilatory action may have helped maintain vascular patency and helped minimize ischemia in the tubules (33,34). We also observed an increase in systolic BP for L-NAME-treated TMA rats on days 1 and 3. Although the BP achieved in these rats (120 ± 6.0 mmHg on day 1 and 108.7 ± 11.5 mmHg on day 3) was at a level that is not generally thought to induce endothelial cell injury in normal animals, the observed BP increase may have produced an effect on renal disease manifestations in the presence of previously injured endothelium. The blockade of NO with l-NAME also increases glomerular hydrostatic pressures (35–37), and it is possible that rats treated with l-NAME lost some protection by local NO via its ability to counteract efferent arteriolar vasocostriction.

NO may limit thrombosis via its inhibitory effect on platelet aggregation (24,25). Inhibition of NO production in an endotoxin-induced model promotes glomerular and interstitial thrombosis (38). Our results demonstrating more fibrin depo-

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**Table 2. Summary of histologic and immunochemical studies**

<table>
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<th>Characteristic</th>
<th>Day</th>
<th>Group I (Control TMA)</th>
<th>Group II (TMA + l-NAME)</th>
<th>Group III (Vehicle + l-NAME)</th>
<th>Group IV (Vehicle Alone)</th>
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<td>3</td>
<td>1.6 ± 0.5</td>
<td>3.9 ± 0.4</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>3.2 ± 0.8</td>
</tr>
<tr>
<td>PCNA-positive endothelial</td>
<td>1</td>
<td>0.8 ± 0.3</td>
<td>1.2 ± 1.1</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>cells (no./glomerulus)</td>
<td>3</td>
<td>3.9 ± 2.5</td>
<td>4.8 ± 1.7</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>PL-1 platelet aggregation</td>
<td>1</td>
<td>1.4 ± 1.3</td>
<td>2.0 ± 1.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>1.2 ± 1.2</td>
</tr>
<tr>
<td>score</td>
<td>3</td>
<td>1.0 ± 0.9</td>
<td>1.6 ± 0.8</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>1.0 ± 0.9</td>
</tr>
</tbody>
</table>

*PAS, periodic acid-Schiff; PCNA, proliferating cell nuclear antigen. 
*P < 0.05 versus Group III. 
*P < 0.05 versus Group IV. 
*P < 0.05 versus Group I. 
*P < 0.05 versus Group V. 

**Figure 8.** Detection of glomerular endothelial cell apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining. TUNEL-positive apoptotic glomerular cells were observed for rats in group II on both day 1 and day 3 (A). The intracapillary location suggested that these cells were endothelial in origin. TUNEL-positive cells were not observed for group I, III, IV, or V rats (B). Magnification, ×400.
One of the most striking findings was that there was greater endothelial loss at 24 h in the NO-inhibited rats. This endothelial loss was attributable to a greater number of apoptotic cells in L-NAME-treated rats, which was revealed by our TUNEL analysis. Furthermore, we failed to observe a significant difference in the number of proliferating cells in glomeruli between L-NAME-treated and control rats with TMA on days 1 and 3. NO is known to inhibit endothelial cell apoptosis in response to a variety of stimuli, including shear stress, hypoxia, endotoxin, and tumor necrosis factor-α (26,39,40). We previously reported that the initial endothelial cell injury in this model is mediated by antibody- and complement-dependent apoptosis (21). We therefore speculate that the reduction of NO production by L-NAME resulted in more endothelial cell apoptosis in our study.

In conclusion, NO has an important protective role in experimental TMA. These effects are likely mediated by the vasodilatory, antiplatelet, and endothelial survival effects of NO. Stimulation of endogenous NO may represent a new approach for treatment of this important disorder.

**Acknowledgments**

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