Lack of Endothelial Nitric Oxide Synthase Aggravates Murine Accelerated Anti-Glomerular Basement Membrane Glomerulonephritis

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Nitric oxide (NO) radicals generated by endothelial nitric oxide synthase (eNOS) are involved in the regulation of vascular tone. In addition, NO radicals derived from eNOS inhibit platelet aggregation and leukocyte adhesion to the endothelium and, thus, may have anti-inflammatory effects. To study the role of eNOS in renal inflammation, the development of accelerated anti-glomerular basement membrane (GBM) glomerulonephritis was examined in mice lacking a functional gene for eNOS and compared with wild-type (WT) C57BL/6j mice. WT C57BL/6j mice (n = 12) and eNOS knockout (−/−) mice (n = 12) were immunized intraperitoneally with sheep IgG (0.2 mg in complete Freund’s adjuvant). At day 6.5 after immunization, mice received a single i.v. injection of sheep anti-mouse GBM (1 mg in 200 μl PBS). Mice were sacrificed at day 1 and 10 after induction of the disease. All WT mice survived until day 10, whereas 1 eNOS−/− mouse died and 2 more became moribund, requiring sacrifice. At day 10, eNOS−/− mice had higher levels of blood urea nitrogen than WT mice (P < 0.02), although proteinuria was comparable. Immunofluorescence microscopy documented similar IgG deposition in both WT and eNOS−/− mice, but eNOS−/− mice had more extensive glomerular staining for fibrin at day 10 (P < 0.007). At day 10, light microscopy demonstrated that eNOS−/− mice had more severe glomerular thrombosis (P < 0.003) and influx of neutrophils (P < 0.006), but similar degrees of overall glomerular endocapillary hypercellularity and crescent formation. In conclusion, accelerated anti-GBM glomerulonephritis is severely aggravated in eNOS−/− mice, especially with respect to glomerular capillary thrombosis and neutrophil infiltration.

These results indicate that NO radicals generated by eNOS play a protective role during renal inflammation. (Am J Pathol 2000, 156:879–888)

The production of nitric oxide (NO) radicals has been recognized as an important mediator system in various physiological and pathophysiological processes. NO is produced from the terminal guanidine nitrogen of L-arginine by nitric oxide synthases (NOS). Three isoforms have been identified encoded by distinct genes: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS).1 nNOS is expressed in neurons and plays a role in neurotransmission. It is also found in the macula densa of the kidney where it may be involved in the regulation of the vascular tone of the afferent and efferent arterioles.2 Expression of eNOS is found predominantly in endothelial cells and plays a significant role in the regulation of vascular tone. Additionally, NO generated by eNOS has been shown to inhibit platelet aggregation and platelet and leukocyte adhesion to the endothelium, and thus may have anti-inflammatory effects.3,4 Both nNOS and eNOS are calcium- and calmodulin-dependent, are constitutively expressed, and generate small amounts (measurable in pmol) of NO. In contrast, iNOS is a calcium- and calmodulin-independent enzyme expressed in a variety of cell types, including intrinsic renal cells, only after exposure to inflammatory stimuli. Once induced, iNOS is capable of producing large amounts (measurable in μmol) of NO for prolonged periods of time.5

Increased NO production has been demonstrated in several experimental models of glomerulonephritis, most likely derived from infiltrating inflammatory cells expressing iNOS.6,7 Increased production of NO radicals can be cytotoxic and cytostatic and may contribute to tissue injury. The exact role of NO in glomerulonephritis, however, is controversial. Results from studies attempting to inhibit excessive NO production in experimental models of glomerulonephritis are inconclusive. Whereas some studies have shown a beneficial effect of NOS inhibition

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on renal inflammation, detrimental effects have been reported in others.\(^{10,11}\)

In an experimental model of anti-myeloperoxidase-associated crescentic glomerulonephritis (CGN), we recently investigated the temporal expression of NO synthases in conjunction with platelet aggregation, inflammatory cell influx, generation of reactive oxygen species (ROS), and nitrotyrosine formation. These studies showed that eNOS expression was markedly decreased and, in the early stages, was associated with extensive platelet aggregation. In addition, a marked transient induction of iNOS in polymorphonuclear cells and macrophages was observed coinciding with the generation of ROS and formation of nitrotyrosines.\(^{12}\) These studies suggested that NO generated by eNOS may be protective, whereas NO generated through iNOS may be involved in tissue injury in this experimental model of anti-myeloperoxidase-associated CGN. However, in a recent study, Cattell et al demonstrated that accelerated anti-glomerular basement membrane (GBM) glomerulonephritis in mice lacking iNOS is indistinguishable from that in wild-type (WT) mice, indicating that iNOS does not play an essential role in this form of glomerulonephritis in mice.\(^{13}\)

The present study was designed to determine the role of NO radicals generated by eNOS in the development of murine accelerated anti-GBM glomerulonephritis. To this end, the course of murine anti-GBM glomerulonephritis was examined in mice homozygous for the disruption of the eNOS gene and compared to that in WT mice.

**Materials and Methods**

**Animals**

eNOS-deficient mice were generated by homologous recombination as described previously.\(^{14}\) These mice were backcrossed to C57BL/6j mice for 10 generations to obtain congenic knockout mice. WT C57BL/6j mice (Jackson Laboratories, Bar Harbor, ME) were used as controls. Tail DNA from each mouse was analyzed by PCR to confirm the genotype for eNOS. All mice were bred and maintained in a specific-pathogen-free facility and received standard chow and water ad libitum.

**Induction of Accelerated Anti-GBM Glomerulonephritis**

Mice were immunized by i.p. injection of 0.2 mg of sheep IgG in complete Freund’s adjuvant (1:1 v/v, 200 μl total volume). After 6.5 days, mice were intravenously injected with 1 mg of sheep anti-mouse GBM IgG in phosphate buffered saline (PBS; 200 μl total volume) prepared as described previously.\(^{15}\) The endotoxin concentration in the stock anti-GBM preparation was 45 pg/ml. No endotoxin (<0.5 pg/ml) could be detected in the diluted anti-GBM preparation used for injection.

**Albuminuria**

One day after the sheep anti-GBM IgG injection and before the day of sacrifice, mice were placed in individual metabolic cages for the collection of urine. Urinary albumin content was measured with the mouse albumin enzyme-linked immunosorbent assay (ELISA) quantitation kit (Bethyl Laboratories, Montgomery, TX) using purified mouse albumin as a reference. Briefly, ELISA strips (Costar, Cambridge, MA) were coated with affinity-purified goat anti-mouse albumin in 0.1 mol/L sodium carbonate buffer, pH 9.0. Next, plates were incubated with serial dilutions of mouse urine in 1% goat serum in PBS/0.05% Tween 20 followed by peroxidase-labeled goat anti-mouse albumin. Binding was detected using tetramethyl benzidine (TMB, Sigma, St. Louis, MO) and the reaction was stopped with 2 mol/L H₂SO₄. Plates were analyzed at 450 nm and albumin concentrations were determined from the standard curve and corrected for urine volume.

**Anti-Sheep IgG Response**

Sera collected at the time of sacrifice were tested for the presence of anti-sheep IgG antibodies by ELISA. Briefly, ELISA strips (Costar) were coated with sheep IgG (20 μg/ml) in 0.1 mol/L sodium carbonate buffer, pH 9.0. Next, strips were incubated with serial dilutions of the mouse sera starting at a 1:50 dilution in 1% donkey serum in PBS/0.05% Tween 20. Antibody binding was detected with donkey anti-mouse IgG (Jackson Laboratories) followed by p-nitrophenyl phosphate substrate (Sigma). The optical density was analyzed at 405 nm and a standard curve was prepared from a reference serum. Antibody concentrations were computed from the linearized titration curve after log-logit transformation of the absorbances of the respective dilutions of the reference serum. The antibody concentration of the reference serum was arbitrarily set at 100 U.

**Blood Urea Nitrogen Measurement**

Blood urea nitrogen (BUN) was measured in sera collected at the time of sacrifice by an enzymatic degradation assay on a Vitros 250 automated analyzer (Johnson & Johnson, South Brunswick, NJ).

**Light Microscopy**

Renal tissue was fixed in 10% buffered formalin and embedded in paraffin using standard procedures. Two-micron sections were stained with periodic acid-Schiff (PAS) and hematoxylin and eosin (H&E). Sections were examined for glomerular hypercellularity, necrosis, thrombosis, crescents, sclerosis, interstitial leukocyte infiltrates, and fibrosis. A glomerular crescent score for each animal was determined by evaluating crescent formation in 30 glomeruli per mouse. Only glomeruli that had two or more layers of cells in Bowman’s space were considered crescentic. In addition, the presence of PAS-
positive material within the glomeruli was semiquantitatively scored in 50 glomeruli per mouse according to the following scoring system: 0, absent; 1, up to one-third; 2, one-third to two-thirds; 3, more than two-thirds of the total glomerular area effaced by PAS-positive material. Results were expressed as mean scores per mouse.

Immunohistochemistry

Renal tissue was snap-frozen in isopentane and embedded in Tissue-Tek (Sakura Finetek, Torrance, CA). To determine the absence or presence of eNOS and iNOS protein, renal tissue sections were fixed in acetone and incubated with a rabbit polyclonal antibodies directed against eNOS (Santa Cruz Biotechnology, Santa Cruz, CA) or iNOS (Transduction Laboratories, Lexington, KY) after incubation with the primary antibody, sections were washed in PBS and endogenous peroxidase activity was blocked by incubation with 0.05% H<sub>2</sub>O<sub>2</sub>/PBS for 20 minutes. Next, sections were incubated with peroxidase-labeled goat anti-rabbit IgG followed by peroxidase-labeled rabbit anti-goat IgG (both from Dakopatts, Glostrup, Denmark) both diluted in 5% normal mouse serum in PBS. Binding was visualized using aminothiocyanate-labeled rabbit anti-sheep IgG, rabbit anti-mouse IgG, and rabbit anti-human fibrinogen (cross-reactive with mouse; all from Dakopatts). The glomerular basement membrane and tubulointerstitial eNOS expression was observed, which in most glomeruli was focal and segmental (Figure 1C-E). At day 1 and 10 after induction of anti-GBM glomerulonephritis was found in interstitial vessels and glomerular and tubular capillaries (Figure 1B). At day 1 and 10 after induction of anti-GBM glomerulonephritis was found in interstitial vessels and glomerular and tubular capillaries (Figure 1B).

Experimental Design

Mice between 9 and 14 weeks of age were used in this study. The following groups were studied: (i) C57BL/6J WT mice, 1 day (n = 5) and 10 days (n = 7) after induction of accelerated anti-GBM glomerulonephritis; (ii) eNOS<sup>−/−</sup> mice, 1 day (n = 5) and 10 days (n = 7) after induction of accelerated anti-GBM glomerulonephritis; (from the one mouse that died at day 6 after induction of accelerated anti-GBM glomerulonephritis, only tissue for light microscopy was obtained); and normal WT (n = 4) and eNOS<sup>−/−</sup> (n = 3) mice.

Results

Circulating Mouse Anti-Sheep IgG Antibody

Both WT and eNOS<sup>−/−</sup> mice immunized with sheep IgG in complete Freund’s adjuvant developed antibodies against sheep IgG as measured by ELISA. No significant difference was found in the anti-sheep IgG titer between the two groups (arbitrary units: 252.5 ± 80.4 vs. 227.14 ± 84.9 at day 10 in eNOS<sup>−/−</sup> and WT mice, respectively).

Expression of eNOS and iNOS

Immunohistochemistry for eNOS in untreated eNOS<sup>−/−</sup> mice confirmed the absence of eNOS protein (Figure 1A). In untreated WT mice, strong immunoreactivity for eNOS was found in interstitial vessels and glomerular and tubular capillaries (Figure 1B). At day 1 and 10 after induction of anti-GBM nephritis, a decrease or absence in glomerular eNOS expression was observed, which in most glomeruli was focal and segmental (Figure 1, C-E). At these time points, strong expression of eNOS could still be observed in cortical vessels (Figure 1, C and E). By immunohistochemistry, no difference in iNOS expression was detected between WT and eNOS<sup>−/−</sup> mice. In all mice, weak immunoreactivity for iNOS was found in tubular epithelial cells and vascular smooth muscle cells. In addition, only occasional iNOS positive cells were detected in interstitium and glomeruli of all mice.

Histological Features of Glomerulonephritis in WT and eNOS<sup>−/−</sup> Mice

By light microscopy, renal morphology in normal eNOS<sup>−/−</sup> mice was indistinguishable from that in normal WT mice (Figure 2, A and B).
Preimmunized C57BL/6J WT mice developed a proliferative and crescentic glomerulonephritis after administration of sheep anti-mouse GBM antibody. In the early phase (day 1), renal injury was characterized by a mild influx of PMN and mild focal segmental glomerular thrombus formation (Figure 2, C and E). All mice survived until day 10. At this time point, a proliferative glomerulonephritis had developed with crescent formation in 17 ± 5% of glomeruli and focal interstitial infiltrates of leukocytes (Figure 3, A and B). Deposits of PAS-positive material in the glomerular tufts, consistent with capillary thrombosis, were focal and mild, affecting 6 to 20% (mean, 11.3 ± 5.5%) of the glomeruli (Figure 3, A and B).

In eNOS−/− mice, the development of glomerulonephritis was severely aggravated, especially the development of thrombosis and necrosis. One mouse died at day...
6 after induction of the disease and two additional mice became moribund at day 8 and 9, respectively, and were sacrificed. All these mice had severe ascites. By light microscopy, at day 1, a mild influx of neutrophils was observed. In addition, glomerular thrombus formation was observed that was more extensive than in WT mice, reflected by a significantly higher score for glomerular deposits of PAS-positive material (Figure 2, D and F, PAS stain). In some glomeruli all glomerular capillaries showed thrombus formation. Severe glomerular injury developed by day 10, characterized particularly by deposits of PAS-positive material in 60 to 78% (mean, 70.5 ±

Figure 2. Light microscopy of renal tissue from normal WT (A) and eNOS−/− (B) mice and at day 1 after administration of sheep anti-mouse GBM Ig. Focal and segmental glomerular thrombosis was observed in WT mice (C and E, PAS stain). More extensive glomerular thrombosis was found in eNOS−/− mice (D and F, PAS stain). Original magnifications, ×200 (A-D), ×400 (E and F).
Figure 3. Light microscopy of renal tissue 10 days after administration of sheep anti-mouse GBM Ig in WT (A, B, PAS stain) and eNOS−/− mice (C-F, PAS stain). A: Overview of renal tissue in a WT mouse demonstrating mild glomerular hypercellularity and glomerular crescent formation. B: Glomerulus from a WT mouse showing crescent formation and mild periglomerular inflammatory cell accumulation. C: Overview of renal tissue from an eNOS−/− mouse that became moribund and was sacrificed at day 8 after sheep anti-mouse GBM Ig administration. Glomerular deposits of PAS positive material are prominent in all glomeruli. D: Glomerulus from the same mouse as in C showing extensive capillary thrombosis and necrosis. E: Overview of renal tissue from an eNOS−/− mouse at 10 days after administration of sheep anti-mouse GBM Ig. Glomeruli show extensive deposits of PAS-positive material. F: Glomerulus from the same mouse as in E showing deposits of PAS-positive material throughout the glomerulus, segmental karyorrhexis, and the development of a small crescent (top right). Original magnifications, ×200 (A, C, E) and ×400 (B, D, F).
8.2%) of glomeruli (Figure 3, E and F). In the mice that died or became moribund, deposits of PAS-positive material were even more extensive, affecting more than 90% of glomeruli (mean, 94.7 ± 2.3%; Figure 3, C and D).

Also, as revealed by semiquantitative analysis, the amount of PAS-positive material per glomerulus was significantly higher in the eNOS−/− mice compared with WT mice (Figure 4). In the eNOS−/− mice, small crescents developed in 13 ± 4% of glomeruli, which was not significantly different from that in WT mice.

**Immunofluorescence**

By immunofluorescence, strong linear glomerular deposition for sheep IgG was found in both WT and eNOS−/− mice at days 1 and 10 after administration of sheep anti-mouse GBM IgG (Figure 5, A and B). The median endpoint titer for detection was 1:1600 in both groups. Also, a prominent granular-to-linear staining was observed for mouse IgG at both time points in WT and eNOS−/− mice (median endpoint titer 1:400 in both groups). At day 1 after administration of sheep anti-mouse GBM, glomerular fibrin deposition was mild in both WT and eNOS−/− mice. However, at 10 days, a marked increase in fibrin deposition, indicative of extensive thrombosis, was found in eNOS−/− mice compared
Characterization of Leukocyte Infiltrate

In WT mice, a mild glomerular influx of PMNs and CD11b-positive cells was found at day 1 (Figure 7). At 10 days, the number of glomerular CD11b-positive cells increased and a mild influx of FA/11-positive and T cells was observed (Figure 7). At this time point, the number of intra-glomerular PMNs had slightly decreased. In eNOS−/− mice, similar results were found for CD11b-positive cells, FA/11-positive cells, and T cells. However, glomerular influx of PMNs at day 10 was significantly higher compared to WT mice (1.3 ± 0.12 vs. 0.91 ± 0.26, P < 0.006; normal, 0.18 ± 0.08; Figure 7). In the mice that became moribund, the glomerular PMN infiltrates were even more prominent, averaging 3.2 and 2.0 PMNs per glomerular cross-section.

**BUN and Albuminuria**

BUN levels in normal WT and eNOS−/− mice were <20 mg/dl. In WT mice a slight increase in BUN levels was detected at day 10 (Figure 8A). At this time point, eNOS−/− mice developed severe renal impairment with markedly increased BUN levels (105.0 ± 31.0 mg/dl vs. 47.0 ± 21.0 mg/dl, P < 0.01; Figure 8A). In the two eNOS−/− mice that became moribund, BUN levels were 166.0 mg/dl and 150.0 mg/dl.

Albuminuria was detectable at day 1, increased at day 2, and remained elevated until day 10 (Figure 8B; albuminuria in normal WT (n = 4) and eNOS−/− mice (n = 3) was <50 μg/18 hours). However, no significant differences were found between eNOS−/− and WT mice at any time point.
been shown to initiate lipid peroxidation and induces several experimental models of glomerulonephritis. In production of NO radicals has been demonstrated in reactive compound peroxynitrite. Peroxynitrite has through direct cytotoxic actions. In addition, NO radicals production of NO radicals can contribute to renal injury and iNOS protein by immunohistochemistry. Increased cleavage protection assays and reverse transcription PCR, tis, iNOS mRNA can be detected in glomeruli by ribonu-
al in infiltrating inflammatory cells or intrinsic renal cells such as mesangial cells. In these models of glomerulonephritis, iNOS mRNA can be detected in glomeruli by ribonuclease protection assays and reverse transcription PCR, and iNOS protein by immunohistochemistry. Increased production of NO radicals can contribute to renal injury through direct cytotoxic actions. In addition, NO radicals can react with superoxide radicals generating the highly reactive compound peroxynitrite. Peroxynitrite has been shown to initiate lipid peroxidation and induces nitrination of tyrosine residues, leading to loss of protein structure and function.

To further define the role of increased NO production in renal inflammation, several studies have attempted to decrease excessive NO production either by using inhibitors of NO synthases or by depletion of circulating arginine. In some of these studies, inhibition of NO production has been shown to augment glomerulonephritis, implicating a toxic role for NO radicals. In other studies, however, blockade of NO production aggravated renal injury, pointing to a protective role of NO radicals. Until recently, the major NOS inhibitors lacked NOS isoform specificity, which may explain part of the conflicting data. Overall, these studies have indicated that increased NO production during renal inflammation may have detrimental or protective effects, which probably depends on the NOS isoform generating NO radicals, the amount of NO radicals produced, and the simultaneous production of ROS. The present study clearly demonstrates that inactivation of eNOS by disruption of the eNOS gene exacerbates renal injury in the accelerated anti-GBM glomerulonephritis model. As such, these results provide direct evidence for a protective role of eNOS derived NO radicals in renal inflammation.

The exact mechanisms by which lack of eNOS-derived NO radicals exacerbates renal injury in this model are not clear, but several possibilities may be considered. Mice lacking a functional gene for eNOS were originally generated to study the involvement of NO radicals produced by eNOS in blood pressure regulation. These studies showed that eNOS−/− mice were indistinguishable from WT mice in general appearance and histology, but had significantly increased blood pressures associated with a twofold increase in plasma renin activities. Also, long term treatment with the nonselective NOS inhibitors L-NAME or L-NNA in rats causes hypertension and leads to proteinuria and renal morphological changes, such as capillary collapse and tubulo-interstitial injury. In addition, hypertension is a factor known to exacerbate glomerulonephritis in rats. Thus, the increased blood pressures in eNOS−/− mice may have contributed to the increased renal injury after induction of accelerated anti-GBM glomerulonephritis we observed; this possibility warrants further investigation.

In vitro studies have also demonstrated that endothelial cell-derived NO radicals inhibit platelet aggregation and platelet and leukocyte adhesion to the endothelium. In vivo, administration of the nonselective NOS inhibitor L-NAME in models of endotoxemia exacerbates liver and kidney injury associated with increased thrombosis and leukocyte infiltration, especially PMNs. Part of these effects have been attributed to increased adhesion molecule expression (ICAM-1, P-selectin) by the endothelium after inhibition of endogenous NO production most likely derived from eNOS. Part of the disease in eNOS−/− mice. In addition, a small but significant increase in glomerular PMN influx was observed in the eNOS−/− mice at day 10.

In conclusion, the course of accelerated anti-GBM glomerulonephritis is severely aggravated in mice lacking
NO production by eNOS. These results point to a protective role of eNOS-derived NO production in glomerulonephritis and emphasize its importance in maintaining the antithrombogenic and anti-inflammatory properties of the endothelium.

References