Effect of Nitric Oxide Synthase Inhibition on Proteinuria in Glomerular Immune Injury

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In glomerular immune injury, the inducible isoform of nitric oxide synthase (iNOS) becomes a major catalyst of NO production. Although iNOS-catalyzed NO production is sustained and can be cytotoxic, iNOS inhibition exacerbates the magnitude of proteinuria that accompanies immune injury. To investigate putative mechanisms of this effect, we assessed changes in glomerular permeability to albumin by using the following two approaches: (i) an *in vivo* rat model of glomerular immune injury induced by antibody against the glomerular basement membrane (GBM), in which urine albumin excretion was measured under conditions of iNOS inhibition, and (ii) an *ex vivo* model of isolated rat glomeruli, in which changes in glomerular capillary permeability to albumin were assessed under conditions of NOS inhibition.

In rats with anti-GBM antibody–induced glomerular injury, there was an increase in urine albumin excretion. Treatment with two structurally dissimilar iNOS inhibitors at doses sufficient to decrease urine nitrate and/or nitrite exacerbated proteinuria. In these animals, urine excretion of the isoprostane 8-iso-PGF2α (marker of oxidative stress) was increased. In isolated glomeruli incubated with the NOS inhibitor L-NMMA, the permeability to albumin increased. This effect was reversed by the NO donor DETA NONOate and by the superoxide dismutase mimetic Tempol.

We conclude that NOS-catalyzed NO production is an important mechanism in regulating glomerular permeability to protein. This mechanism involves control of the bioavailability of superoxide. Exp Biol Med 231:576–584, 2006

Key words: NOS; glomerulus; proteinuria; nephritis

Introduction

In glomerular immune injury, the inducible isoform of nitric oxide synthase (iNOS) becomes a major catalyst of NO formation. Thus, although glomerular levels of constitutive isoforms of NOS remain constant (1) or decrease (2), those of iNOS increase (3–5). iNOS-catalyzed NO production is sustained and of high output, and can cause cell injury either on its own or by complexing with superoxide ($O_2^{\bullet -}$) to form the potent and relatively stable radical, peroxynitrite ($ONOO^-$) (6).

Studies assessing the effect of NOS inhibition on the extent of glomerular immune injury have reported conflicting results. In a model of glomerular immune injury induced by administration of antibody against the mesangial cell antigen Thy-1 that resulted in mesangiolysis, it was shown that NOS activity is increased (7) and that its inhibition attenuates the extent of lytic injury (8). Surprisingly, however, in this and other models of glomerular immune injury, pharmacologic NOS inhibition exacerbates the magnitude of proteinuria. This was shown using both nonselective NOS inhibitors (9) and selective iNOS inhibitors (10). The mechanism of this effect is unexplored and raises the possibility that NO production regulates the glomerular capillary permeability to protein. To address this question, we assessed changes in glomerular permeability to protein by use of a rat model of proteinuria secondary to immune injury induced by administration of antibody against the glomerular basement membrane, in which iNOS-catalyzed NO production was inhibited; and use of an *ex vivo* system of isolated glomeruli, in which permeability of the glomerular capillary to protein was assessed under conditions of NOS inhibition.

Materials and Methods

Glomerular Immune Injury. This was induced by administration of immune serum raised in rabbits against rat particulate glomerular basement membrane (GBM), as previously described (11). Briefly, Lewis rats (180–200 g) were immunized with 1 mg rabbit IgG mixed in complete Freund's adjuvant and given as a single intraperitoneal

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injection. Six days later, the animals received two intravenous injections (0.3 ml per animal given 24 hrs apart) of heat-inactivated rabbit anti–rat GBM serum. This protocol results in glomerular inflammation characterized by macrophage infiltration and crescent formation (12). Proteinuria begins as early as 6 hrs after the second injection of anti-GBM antibody (13) and peaks at 24–48 hrs, at which point glomerular iNOS expression also peaks (5).

Histopathologic Analysis. Glomerular lesions were evaluated by light microscopy of cortical sections (4 μm thick) stained by routine methods of hematoxylin-eosin or periodic acid–Schiff. Peroxynitrite modified proteins (protein-bound 3-nitrotyrosine) were detected by immunohistochemistry analysis. This was performed using an immunohistochemistry kit obtained from InnoGenex (San Ramon, CA). The sections were incubated overnight in a humidified chamber at room temperature with monoclonal antibody against 3-nitrotyrosine (dilution, 1 μg/ml; Upstate, Charlottesville, VA) in phosphate buffer solution (PBS) containing 1% bovine serum albumin (BSA). After sections were washed with PBS containing 0.1% Tween-20 (PBS-T), they were incubated with biotinylated anti-mouse secondary antibody for 20 mins at room temperature. After sections were rinsed in PBS-T, they were incubated with streptavidin-horseradish peroxidase conjugate for 20 mins at room temperature and rinsed in PBS-T. The antibody-bound peroxidase was visualized by 3,3′-diaminobenzidine (DAB) chromogen. Sections were counterstained with Mayer's hematoxylin and mounted in Permount. Specificity of staining was verified by preincubation of the antibody with 10 m*M* nitrotyrosine in PBS or by omitting the primary antibody.

iNOS Inhibition. The following two structurally dissimilar inhibitors of iNOS were used: the acetamide-containing analogue of arginine, L-N^6-(1-iminoethyl)-lysine (L-NIL), and the acetamide-containing analogue of arginine, (S)-2-amino-7-acetamidino-5-thioheptanoic acid (also known as "GW274150").

L-NIL is an irreversible inhibitor of iNOS with no effect of COX activity (14). It is 28-fold more selective for iNOS than it is for rat brain constitutive NOS ($IC_{50} = 3.3$ μ*M* for iNOS vs. $IC_{50} = 92$ μ*M* for constitutive (NOS) and 30- to 50-fold more selective for iNOS than eNOS. Daily administration of L-NIL for 7 days in the drinking water of rats with glomerular immune injury has no effect on systemic blood pressure (15), which indicates no systemic inhibitory effect on endothelial NOS (eNOS) and makes this compound appropriate for use in renal disease models in which systemic hypertension could impact the extent of glomerular injury. Additional properties that make L-NIL appropriate for use in models of glomerular immune injury are its lack of an effect on cyclooxygenase activity; its lack of other known pharmacologic actions, apart from competition with L-arginine (iNOS substrate) for cellular uptake; and its lack of an effect on the extent of inflammatory response (i.e., inflammatory cell infiltrates) to glomerular injury induced by anti-GBM antibody (10). Compared with L-NIL, GW274150 has an even greater selectivity for iNOS, which is 333-fold that for eNOS. The IC_{50} value for inhibition of recombinant iNOS is 2.19 ± 0.23 μ*M*, which is comparable to that of L-NIL (1.58 ± 0.10 μ*M*) (16).

L-NIL was given intravenously at 15 mg/kg for 30 mins before each of the two doses of anti-GBM serum. This protocol of administration was shown to prevent activation of iNOS that ensues after onset of anti-GBM antibody–induced glomerular injury (10). GW274150 was given in a similar manner (5 mg/kg for 30 mins before injection of each of the two doses of anti-GBM serum).

Animals were placed into groups (6–8 animals per group) and received either (i) anti-GBM serum only as described above, (ii) anti-GBM serum and L-NIL or GW274150 given intravenously 30 mins before administration of the anti-GBM serum as described above, or (iii) nonimmune rabbit serum (controls), given in a manner identical to that of anti-GBM serum and L-NIL or GW274150 as in the second group. Animals were placed in metabolic cages after the second injection of anti-GBM or nonimmune serum for an 18-hr urine collection. The metabolic cages were placed on an automated refrigerated collection rack that freezes urine to –20°C as it is collected. This prevents bacterial growth and decomposition of the various excreted metabolites.

Urine Albumin and Creatinine Excretion. Excreted concentrations of urine albumin and creatinine were measured using established colorimetric methods and commercially available kits (Bio-Rad, Hercules, CA).

Urine NOx Excretion. The excreted concentration of urine NOx was assessed by measuring urine nitrate (NO_3^-) and nitrite (NO_2^-) levels (collectively referred as NOx). Urine samples were diluted in water, and NO_3^- was converted to NO_2^- by use of vanadium chloride (17). NO_2^- was detected in samples by means of the Griess reagent (Promega, Madison, WI). Results were compared against the standard curve for $NaNO_3^-$, which was treated in an identical manner as the NOx samples.

Urine Isoprostane Excretion. Isoprostanes are formed nonenzymatically by the attack of superoxide ($O_2^{\bullet -}$) or $O_2^{\bullet -}$-derived reactive oxygen species on arachidonic acid. Their levels in biological samples have been shown to reflect the extent of lipid peroxidation and oxidant stress *in vivo* (18). The best studied isoprostane is 8-isoprostane F2α (8-iso-$PGF_{2\alpha}$). The rationale for measuring this isoprostane in the present studies is based on the possible consequences of iNOS inhibition on the bioavailability of $O_2^{\bullet -}$. NO reacts very rapidly with $O_2^{\bullet -}$ at near diffusion-controlled rates with a reaction constant of 4.3×10^9–6.7×10^9 $M^{-1}s^{-1}$ (19). This reaction is twice as fast as the maximum velocity of the reaction between $O_2^{\bullet -}$ and tissue superoxide dismutase (SOD), indicating that NO is an important $O_2^{\bullet -}$ scavenger. By inhibiting NOS, the ensuing NO depletion could increase the bioavailability of $O_2^{\bullet -}$, thereby promoting oxidant injury. Assessment of the

excretion of 8-iso-$PGF_{2\alpha}$ in the urine thus allows inferences to be made about whether iNOS inhibition in animals treated with these iNOS inhibitors intensified oxidant injury.

Urine 8-iso-$PGF_{2\alpha}$ was measured using a commercially available competitive assay (enzyme-linked immunosorbent assay [EIA]; Cayman Chemicals, Ann Arbor, MI). The assay is based on the competition between 8-isoprostane and an 8-isoprostane acetylcholinesterase conjugate (8-isoprostane tracer) for a limited number of 8-isoprostane–specific rabbit antiserum binding sites. The rabbit antiserum–8-isoprostane complex binds to a mouse monoclonal antibody against rabbit IgG that is attached to the wells of the assay plates. After the plates were washed to remove unbound reagents, Ellman's reagent (which contains the substrate to acetylcholine) is added, and the product of this enzymatic reaction (distinct yellow color) absorbs at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of 8-isoprostane tracer bound to the well, which is inversely proportional to the amount of free 8-isoprostane present in the well during the incubation. Because urine may contain contaminants that can interfere with the assay, testing for interference was performed by diluting urine samples to obtain two different dilutions for each sample yielding %B/Bo (% bound/maximum bound; the ratio of the absorbance of a sample or standard well to that of the maximum amount of the tracer that the antiserum can bond in the absence of free analyte) values of 20%–80% (the assay typically yields 50% B/Bo at 30 pg/ml and 80% B/Bo at 50 pg/ml). A difference of less than 20% between the two dilutions was considered to be acceptable for that particular sample. Otherwise, sample purification was performed using 8-isoprostane affinity columns and affinity sorbent (Cayman Chemical). The solid-phase extraction purification method we used has been validated by comparison of results of the EIA and of gas chromatography and/or negative ion chemical ionization–mass spectrometry of samples derivatized as pentafluorobenzyl esters, as described elsewhere (20).

Effect of NOS Inhibition on Glomerular Capillary Permeability to Albumin (P_{alb}) in Isolated Glomeruli. Although changes in urine protein excretion in glomerular injury generally reflect changes in glomerular capillary permeability to protein, it is now recognized that the tubules may significantly contribute to the processing (uptake and degradation) of albumin or other filtered proteins (21). Because the excreted peptide fragments are not detected by conventional urine albumin or total protein assays, use of the albumin level or the total protein level in the urine as indicators of changes in glomerular protein filtration can be seriously flawed. To directly assess the effect of NOS inhibition on glomerular permeability to protein, we used an *ex vivo* system of isolated glomeruli that allows detection of rapid and subtle changes in P_{alb} without the influence of hemodynamic or circulating factors. We and other investigators have used this system to study the physiologic and pathophysiologic characteristics of glomerular capillary permeability to protein (22, 23).

Briefly, glomeruli were isolated from healthy rats anesthetized with halothane. After the kidney capsule was removed, the outer cortex was minced in 1- to 2-mm fragments, passed through consecutive 80- and 120-mesh sieves, and recovered from the 200-mesh sieve. Isolation of glomeruli was performed at room temperature in a medium containing 115 m*M* sodium chloride, 5.0 m*M* potassium chloride, 10.0 m*M* sodium acetate, 1.2 m*M* dibasic sodium phosphate, 25.0 m*M* sodium bicarbonate, 1.2 m*M* magnesium sulfate, 1.0 m*M* calcium chloride, 5.5 m*M* glucose, 6.0 m*M* L-alanine, 1.0 m*M* sodium citrate, and 4.0 m*M* sodium lactate. BSA (5.0 g/dl) was included in the medium as an oncotic agent (i.e., an isolation and/or incubation buffer). The pH of the medium was adjusted to 7.4. The oncotic pressure was measured using a membrane colloid osmometer (Model 4100; Wescor Inc., Logan, UT).

Changes in glomerular P_{alb} were measured by calculating the glomerular volume response to an applied oncotic gradient generated by defined concentrations of albumin (Fig. 1). Glomeruli were incubated in control media or in media containing the NOS inhibitor L-N^G-monomethyl arginine (L-NMMA) in the presence and in the absence of the NO donor diethylenetriamine (DETA) NONOate (Cayman Chemicals) or the superoxide dismutase mimetic Tempol (see below). Incubations were performed after glomeruli were affixed to coverslips coated with poly-L-lysine (1 mg/ml). Individual glomeruli were observed using videomicroscopy before and 1 min after the initial incubation medium was replaced by medium containing 1.0 g/dl BSA. This replacement produced an oncotic gradient across the glomerular capillary wall (5.0 and 1.0 g/dl BSA in the lumen and bathing medium, respectively) and resulted in a net fluid influx and an increase in glomerular volume. This change in volume (DV) was calculated from the average of 4 diameters of the video image (Fig. 1). The increase observed in each glomerulus in response to the oncotic gradient was expressed as $DV = (V_{final} - V_{initial})/V_{initial} \times 100\%$ (24).

Reflection Coefficient of Albumin (σ_{alb}). There is a direct relationship between the increase in glomerular volume (ΔV) and the change in the oncotic gradient ($\Delta\Pi$) across the capillary wall (24). We used this principle to calculate σ_{alb} by using the ratio of the experimental ΔV to the control ΔV in response to identical oncotic gradients (i.e., $\sigma_{alb} = \Delta V_{experimental}/\Delta V_{control}$).

Convectional P_{alb}. Convectional P_{alb} was defined as $1 - \sigma_{alb}$ to describe the movement of albumin consequent to water flow. When σ_{alb} is zero, albumin moves at the same rate as water, and P_{alb} is 1.0. When σ_{alb} is 1.0, albumin cannot cross the membrane with water, and P_{alb} is zero (24).

Stock solutions of reagents were prepared by dissolving each reagent at defined concentrations indicated below in the isolation-incubation buffer containing 5% BSA. In each experiment, control glomeruli were incubated with equiv-

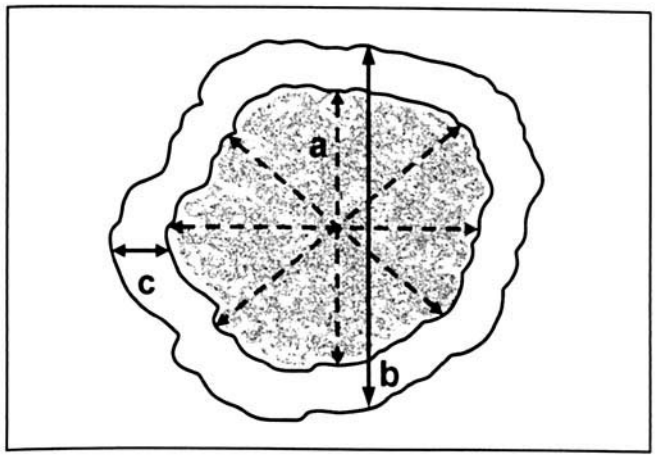

Figure 1. Diagrammatic presentation of changes in the diameter of an isolated glomerulus after application of an oncotic gradient. When the concentration of albumin in the bathing medium is changed from 5% to 1%, water influxes from the bathing medium into the lumen of the glomerular capillaries, where the albumin concentration is still that of the original medium (5%). As a result, the initial glomerular diameter (a) recorded at 5% BSA increases (b). The change in diameter (c) reflects the change in glomerular volume (ΔV).

alent volumes of the isolation-incubation buffer, and P_{alb} was determined. All incubations were performed at 37°C for the indicated periods. The duration of incubation (10–15 mins) and the concentrations used were chosen on the basis of results of pilot experiments.

To assess the effect of NOS inhibition on P_{alb}, isolated glomeruli were incubated with freshly prepared L-NMMA, (0.5–2 m*M*; Cayman Chemical) for 15 mins at 37°C, and P_{alb} was determined (see below). L-NMMA is a prototypical and frequently used L-arginine analogue–type inhibitor of NOS. It has strong inhibitory activity against all NOS isoforms, the inhibition being reversible and competitive with respect to L-arginine (22). Normal glomeruli do not express iNOS (4, 10), whereas the neuronal NOS (nNOS) isoform is expressed mainly at the macula densa, where it participates in regulation of the tubular-glomerular feedback loop (25). For these reasons, L-NMMA was determined to be the most appropriate NOS inhibitor in these experiments.

Given that L-NMMA may alter glomerular protein permeability because of effects other than NOS inhibition, in separate experiments we explored whether the effect of L-NMMA on P_{alb} can be reversed by exogenous NO. In these experiments, glomeruli were incubated with L-NMMA and the NO donor DETA-NONOate. DETA-NONOate belongs to the class of 1-substituted diazen-1-ium-1, 2-diolate compounds containing the $[N(O)NO]^-$ functional group with half lives ranging from 1 min to 1 day in physiological buffers, which makes them suitable for a variety of applications in which controlled generation of NO is required (26). The 20-hr half-life of DETA NONOate provides a relatively constant flux of NO that is required in these experiments, making this compound an ideal NO donor. Isolated glomeruli were incubated with 2 m*M* L-NMMA and 500 μM DETA NONOate for 15 mins, and changes in P_{alb} were determined as described above.

As mentioned earlier, NOS inhibition results in NO depletion, which may increase the bioavailability of $O_2^{\bullet -}$. To determine the role of the interaction between NO and $O_2^{\bullet -}$ on changes in P_{alb}, we performed the following experiment. Isolated glomeruli were incubated with 2 m*M* L-NMMA in the presence or in the absence of the membrane-permeable SOD mimetic Tempol (4-hydroxyl-2, 2, 6, 6-tetramethylpiperidinoxyl, 5 m*M*; Sigma-Aldrich, St. Louis, MO), and P_{alb} was measured. Control glomeruli were incubated with Tempol alone. This low molecular weight, metal-independent SOD mimetic has been used as a spin trap for $O_2^{\bullet -}$ (27). It attenuates $O_2^{\bullet -}$-related injury in models of ischemia reperfusion (28) and inflammation (29).

Statistical Analyses. Levels of urine protein, NOx, and isoprostane excretion were factored by urine creatinine excretion and expressed as means ± SEM. Levels between

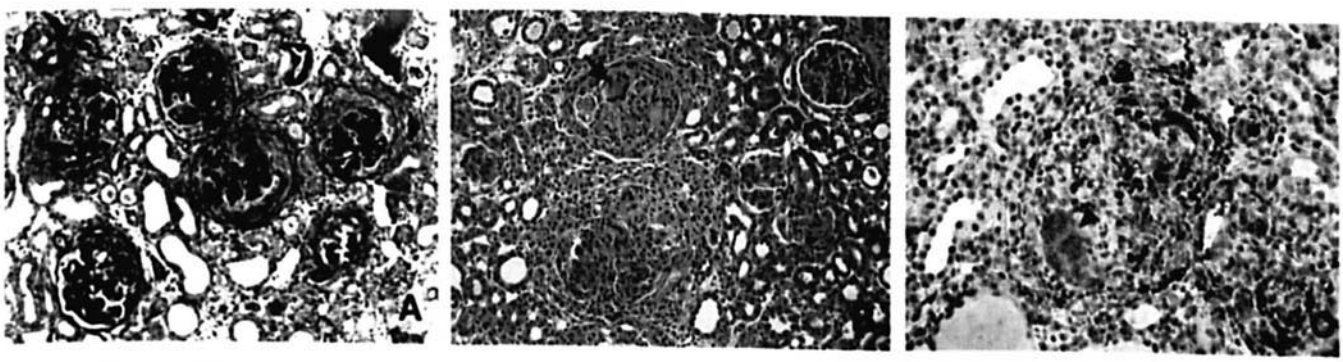

Figure 2. Results of histopathologic and immunohistochemistry analyses showing (A) glomeruli with crescent formation at late stages of glomerular inflammation (Day 21) and (B) glomeruli at early stages (18–24 hrs) of inflammation. Note the presence of multinucleated giant cells (arrow) originating from fusion of infiltrating monocytes. (C) Nitrotyrosine staining localized in multinucleated giant cells (red arrows) and in intrinsic glomerular cells (yellow arrows).

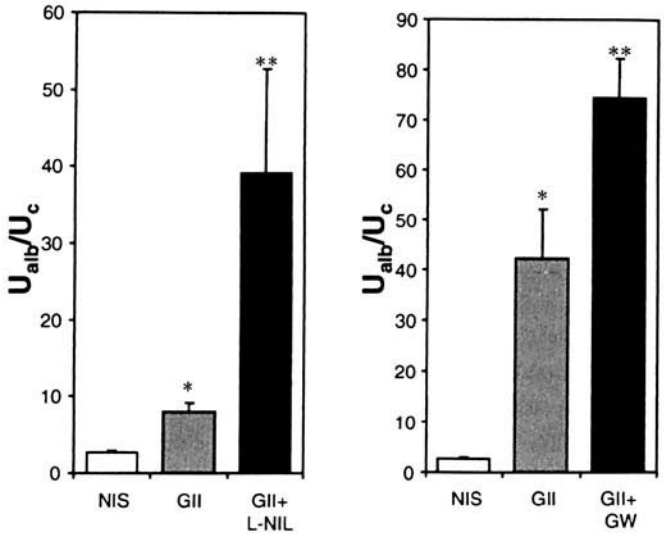

Figure 3. Effect of treatment with the iNOS inhibitors L-NIL and GW274150 on albuminuria, expressed as the ratio of urine albumin (U_{alb}) to urine creatinine (Uc). $^*P < 0.05$, compared with control animals that received nonimmune serum (NIS). $^{**}P < 0.05$, compared with animals with glomerular immune injury (GII) that did not receive the iNOS inhibitors.

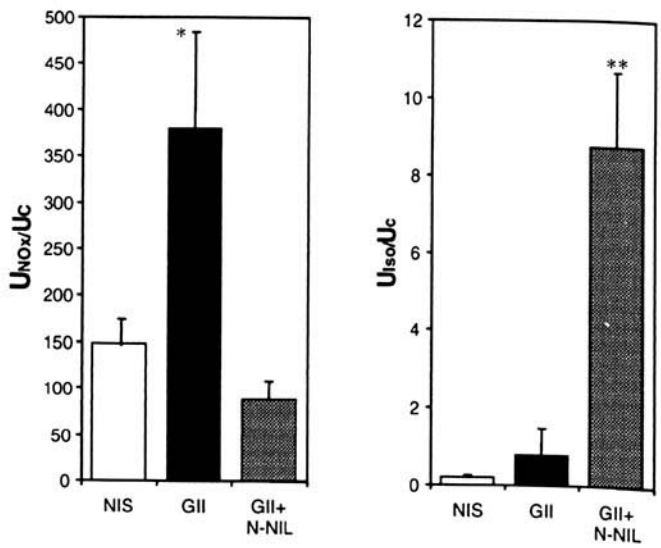

Figure 4. Effect of treatment with the iNOS inhibitor L-NIL on levels of urine nitrate/nitrite (U_{nox}) and 8-iso-$PGF_{2\alpha}$ (U_{iso}) excretion, factored by creatinine excretion (Uc). In animals with glomerular immune injury (GII), levels of NOx and 8-iso-$PGF_{2\alpha}$ excreted in urine increased. In animals with GII that were treated with L-NIL, levels of NOx excretion in urine decreased, whereas levels of 8-iso-$PGF_{2\alpha}$ excreted in urine further increased. $^*P < 0.05$, compared with control animals that received nonimmune serum (NIS). $^{**}P < 0.05$, compared with animals with GII.

groups were compared using unpaired Student's *t* tests. P_{alb} values in isolated glomeruli were expressed as means ± SEM, and values in groups of glomeruli exposed to different treatments were compared using ANOVA.

Results

In Glomerular Immune Injury, iNOS Inhibition Exacerbates Albuminuria and the Magnitude of Oxidative Injury. In Figure 2, histopathologic changes assessed in an animal with anti-GBM antibody–induced glomerular inflammation are shown. The cortical sections were obtained on Day 21 following administration of the antibody, when changes such as crescent formation become established (Fig. 2A), and at early stages of inflammation (18–24 hrs), when infiltration by monocytes-macrophages is prominent (Fig. 2B). Figure 2C also shows the localization pattern of nitrotyrosine staining: nitrotyrosines are localized in intrinsic glomerular cells and in multinucleated giant cells (formed from fusion of infiltrating monocytes-macrophages).

The effect of the iNOS inhibitors, L-NIL and GW 274150 on urine albumin excretion assessed at 18–24 hrs following onset is shown in Figure 3. Treatment with either of the inhibitors exacerbated urine albumin excretion (expressed as the urine albumin to creatinine ratio).

The effect of iNOS inhibition in animals with anti-GBM antibody–induced glomerular injury treated with the iNOS inhibitor L-NIL on NO_x and 8-iso-$PGF_{2\alpha}$ excretion is shown in Figure 4. NO_x excretion in animals with glomerular immune injury increased, compared with that in controls, which received nonimmune serum. In the group with glomerular immune injury that was treated with L-NIL, NO_x excretion was no different than that in controls (Fig. 4). Urine 8-iso-$PGF_{2\alpha}$ excretion was increased in the group with glomerular immune injury, compared with that in controls (Fig. 4). In animals with glomerular immune injury treated with L-NIL, 8-iso-$PGF_{2\alpha}$ excretion was further increased.

NOS Inhibition Increases Glomerular Capillary P_{alb}. Although the observations described above were based on changes in the level of urine albumin excretion, such changes may not accurately reflect effects of NOS inhibition on permeability to protein at the glomerular level because the increase in urine albumin excretion observed following NOS inhibition is an inaccurate reflection of changes in glomerular permeability to protein. This is because, as mentioned earlier, tubules significantly contribute to the processing (i.e., uptake and degradation) of filtered protein. Further compounding the problem is the recognition that, after tubular degradation of filtered protein, peptide fragments excreted in the urine are not detected by conventional urine protein assays, and systemic administration of NOS inihibitors can cause significant changes in the glomerular hemodynamic characteristics (9, 30), which can impact glomerular protein permeability in a manner independent of changes in NO production. Direct measurement of changes in the glomerular capillary P_{alb} under conditions of NOS inhibition (i.e., NO depletion) circumvents these issues and also allows study of underlying mechanisms. We, therefore, used the *ex vivo* system of isolated glomeruli, in which rapid and subtle changes in P_{alb} could be detected without the influence of hemodynamic

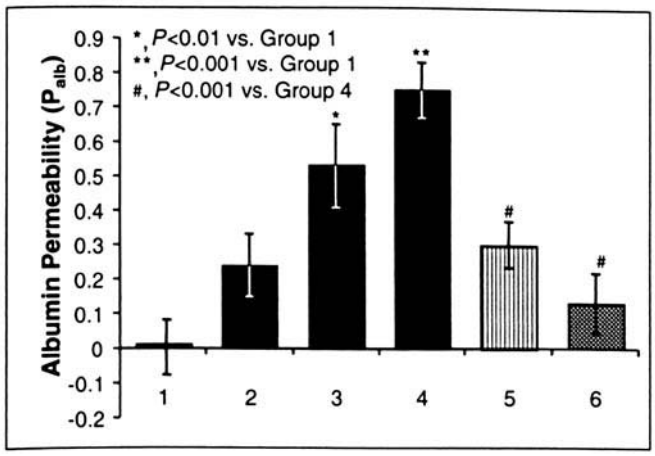

Figure 5. Isolated glomeruli were incubated for 15 mins at 37°C without any treatment and used as control (Group 1). Glomeruli in experimental Groups 2, 3, and 4 were incubated with 0.5, 1.0, and 2.0 m*M* L-NMMA, respectively. Glomeruli in experimental Group 5 were incubated with 2 m*M* L-NMMA and 500 μ*M* DETA-NONOate. Glomeruli in experimental Group 6 were incubated with 2 m*M* L-NMMA and 5 m*M* tempol. L-NMMA at 1- or 2-m*M* concentrations increased P_{alb} significantly ($P < 0.01$ and $P < 0.001$, respectively). Coincubation with DETA-NONOate (Group 5) or tempol (Group 6) reversed the L-NMMA–induced increase in P_{alb} ($P < 0.001$, both groups).

factors (i.e., changes in GFR) or of circulating factors (i.e., cytokines) that could also alter glomerular permeability to protein (31).

The effect of glomerular NOS inhibition using L-NMMA on glomerular P_{alb} is shown in Figure 5. L-NMMA was ineffective at concentration 0.5 m*M* but significantly and dose-dependently increased P_{alb} at 1 and 2 m*M*. These results indicate that NO depletion increases glomerular P_{alb}. To further validate these observations, we explored whether exogenous NO can reverse the increase in P_{alb} observed in the presence of L-NMMA. In these experiments, glomeruli were coincubated with L-NMMA and the NO donor, DETA-NONOate. The 20-hr half-life of DETA NONOate provides a relatively constant flux of NO during the incubation periods used (10–15 mins). In these experiments, isolated glomeruli were coincubated with 2 μ*M* L-NMMA and 500 μ*M* DETA-NONOate. As shown in Figure 5, in glomeruli incubated with L-NMMA and DETA NONOate, the increase in P_{alb} was attenuated. At concentrations less than 500 μ*M*, DETA-NONOate was ineffective.

The apparent inverse relationship between NO_x and 8-iso-$PGF_{2\alpha}$ excretion following iNOS inhibition (Fig. 4) raises the question of whether NO depletion increases the bioavailability of superoxide ($O_2^{\bullet -}$), which can intensify the extent of oxidant injury, as reflected by generation of 8-iso-$PGF_{2\alpha}$. We addressed this question using isolated glomeruli to determine whether the increase in P_{alb} caused by the NOS inhibitor L-NMMA can be attenuated by an $O_2^{\bullet -}$ scavenger. In these experiments, glomeruli were coincubated with L-NMMA (2 m*M*) and the superoxide dismutase (SOD) mimetic Tempol, which is a low–molecular weight (MW; Ref. 172), membrane-permeable, metal-independent SOD mimetic used as a spin trap for $O_2^{\bullet -}$ (27). As shown in Figure 5, in the presence of Tempol at a concentration of 5 m*M*, the L-NMMA–induced increase in P_{alb} was abrogated. At concentrations less than 5 μ*M*, Tempol was ineffective.

Discussion

The demonstration that iNOS-catalyzed NO production is sustained and can cause cytotoxicity (32) prompted investigators to explore whether iNOS inhibition can attenuate the extent of renal injury and hemodynamic dysfunction in experimental models of glomerulonephritis and interstitial nephritis associated with proteinuria. The observations that were reported varied depending on the model used. In a mouse model of systemic lupus erythematous associated with immune complex glomerulonephritis, use of the iNOS inhibitor aminoguanidine significantly reduced proteinuria and the degree of glomerulosclerosis following a 4-month treatment (33). However, interpretation of these results is difficult because aminoguanidine is not a very selective inhibitor of iNOS. Selective inhibition of iNOS-catalyzed NO increases glomerular iNOS protein levels, because of a negative feedback regulatory interaction between iNOS-driven NO production and iNOS protein synthesis (1). In the studies by Yang *et al.* (33), aminoguanidine treatment actually decreased glomerular iNOS protein levels. In another study involving the mouse model of systemic lupus erythematous–associated glomerulonephritis, the effect of the nonselective NOS inhibitor L-NMMA, as well as that of the iNOS inhibitor L-NIL, on glomerular histopathologic characteristics and proteinuria was assessed during a 12-week period. Both inhibitors decreased the level of protein excretion and improved the histopathologic characteristics, and L-NIL was more effective in reducing renal disease scores (34). However, an earlier study that used the same disease model in mice lacking a functional gene encoding iNOS described no differences in glomerular pathologic findings between iNOS (–/–) mice and iNOS (+/–) or iNOS (+/+) mice (35). Collectively, these studies concluded that iNOS-driven NO production has a variable impact on the manifestations of the glomerular inflammation that develops in the murine model of systemic lupus erythematous because of heterogeneity in mechanisms of this disease.

Two independent studies performed in a model of glomerulonephritis induced by administration of anti-GBM antibody and in a model of autoimmune interstitial nephritis reported exacerbation rather than attenuation of proteinuria after iNOS inhibition (5, 36), whereas renal hemodynamic dysfunction deteriorated (9). This raises the question of whether iNOS-catalyzed NO production in these models has beneficial rather than detrimental effects by offsetting factors that can directly injure the glomerular microvasculature, thereby increasing permeability of the glomerular capillary to protein. One such factor is superoxide

($O_2^{\bullet -}$), whose production in the model of anti-GBM antibody–induced glomerular immune injury that was used in the present studies is increased (37, 38). Because NO contains an unpaired electron and is paramagnetic, it rapidly reacts with $O_2^{\bullet -}$ at a near diffusion-controlled rate constant. This makes NO an effective $O_2^{\bullet -}$ scavenger. In this reaction, the availability of NO determines the rate constant, whereas concentrations of $O_2^{\bullet -}$ determine the amount of the reaction product (peroxynitrite, $ONOO^-$) formed (19). Although the $NO/O_2^{\bullet -}$ reaction serves to scavenge $O_2^{\bullet -}$, the reaction product $ONOO^-$ is a potent and relatively stable oxidant for thiols (39) and lipids (40). It follows that NO depletion that occurs after NOS inhibition could increase the bioavailability of $O_2^{\bullet -}$, which can promote oxidative injury either on its own or by giving rise to more potent radicals, including $^{\bullet}OH$, H_2O_2 and $ONOO^-$. Indirect evidence for $ONOO^-$ formation in the present studies is shown in Figure 2, in which presence of nitrotyrosines (considered to be a footprint of protein nitration by $ONOO^-$ or other nitrating species) is shown. The localization of nitrotyrosines in infiltrating macrophages is not surprising in view of evidence that these cells are a major source of iNOS-driven NO production (3) and of $O_2^{\bullet -}$ (3, 13, 37).

These considerations may explain the exacerbation of albuminuria in rats with anti-GBM antibody–induced glomerular immune injury treated with the iNOS inhibitors N-NIL or GW274150 (Fig. 3). These inhibitors of the inducible isoform of NOS were chosen because this isoform is overexpressed in glomeruli after glomerular immune injury (3–5). Of the constitutive NOS isoforms, nNOS is expressed in the macula densa (25), whereas eNOS is the main constitutive isoform in glomeruli. The choice of the L-NIL compound was based not only on its selectivity for iNOS but also on the demonstration that it is currently the best available iNOS inhibitor for *in vivo* use in models of glomerular immune injury that involve the Lewis rat strain (15). Furthermore, inhibition of iNOS activity by means of L-NIL is not associated with increased systemic blood pressure, as could be expected because of NO depletion. This was shown in rats fed a high-salt diet (41) and, of direct relevance to the present studies, in a rat model of mesangioproliferative glomerulonephritis (15). However, although L-NIL is 30- to 50-fold more selective for iNOS than for eNOS, caution is still necessary in attributing the observed effect (exacerbation of proteinuria) purely to iNOS inhibition; this effect could be due to some degree of eNOS inhibition. To further confirm that the exacerbation of proteinuria in L-NIL treated animals with anti-GBM antibody–induced glomerular injury was not due to eNOS inhibition, we used the acetamide amino acid compound GW274150, which has a very high degree of selectively for iNOS (333-fold more selective for iNOS than for eNOS) without an effect on systemic blood pressure in chronically instrumented conscious mice (16).

In rats with glomerular immune injury treated with L-NIL, changes in levels of urine NOx and 8-iso-PGF2α excretion moved in opposite directions; levels of the former decreased, whereas levels of the latter increased (Fig. 4). Although the decrease in NOx excretion was expected because of iNOS inhibition, the ensuing NO depletion may explain the increase in the level of 8-iso-PGF_{α} excretion. As mentioned earlier, NO is an effective $O_2^{\bullet -}$ scavenger. Therefore, NO depletion could increase bioavailability of $O_2^{\bullet -}$ and the extent of ongoing oxidative injury, which is reflected as increased 8-iso-$PGF_{2\alpha}$ excretion.

The *ex vivo* experiments using glomeruli isolated from normal rats were prompted by the limitations of measuring the level of urine protein excretion as a quantitative marker of changes in glomerular permeability to protein, as discussed above. These limitations make it difficult to determine whether the mechanism of increased urine albumin excretion observed in animals treated with the iNOS inhibitors involves NO depletion *per se*. In normal rats treated with the NOS inhibitor L-NMMA (given as a bolus infection of 20 mg followed by a continuous infusion of 0.4 mg/min), marked systemic hypertension, glomerular arteriolar vasoconstriction, and glomerular hypoperfusion were described, whereas the glomerular ultrafiltration coefficient decreased by approximately 40% (30). Because these hemodynamic changes can impact on the glomerular capillary permeability to macromolecules, the question of whether NO depletion *per se* alters this permeability is difficult to answer by means of *in vivo* approaches alone. This highlights the advantages of the *ex vivo* system of isolated glomeruli that was used in the present studies (see Materials and Methods). In this system, the effects of NO depletion on glomerular capillary permeability to protein can be assessed without regard to systemic or glomerular hemodynamic changes. One could argue that the ideal design would be the use of anti-GBM antibody to cause glomerular immune injury both *in vivo* and in isolated glomeruli, such that changes in protein permeability could be measured. However, anti-GBM antibody–induced immune injury of the glomerular capillary cannot be reproduced in preparations of isolated glomeruli because development of injury requires complement activation, the presence of specifically activated macrophages and T lymphocytes, and the presence of specific cytokines (13, 31). Because normal glomeruli do not express detectable levels of iNOS or nNOS protein or enzyme activity, the *ex vivo* experiments involved the prototypical and frequently used L-arginine analogue–type NOS inhibitor L-NMMA. In glomeruli incubated with this inhibitor, P_{alb} increased, and this effect was reversed by NO originating from the NO donor DETA-NONOate, as well as by the $O_2^{\bullet -}$ scavenger Tempol (Fig. 5). These observations indicate that the increase in P_{alb} was mechanistically linked to NO depletion and increased bioavailability of $O_2^{\bullet -}$.

The structural elements of the glomerular capillary permeability barrier altered by NO depletion have to be identified. This barrier consists of a fenestrated endothelial layer, the glomerular basement membrane (GBM), and an

epithelial cell (podocyte) layer. The contribution of each layer to the barrier function differs, with the epithelial layer contributing most to this function. The endothelial layer contributes to a lesser but significant degree, whereas the amount of GBM contribution is debatable (42). A major structural component of the epithelial cell barrier to protein movement is the slit diaphragm (an extracellular structure composed of nephrin strands). Epithelial cell proteins, especially nephrin and podocin, play a key role in the structural integrity of this diaphragm (43). It is unlikely that NO depletion or increased bioavailability of $O_2^{\bullet -}$ had a detectable effect on the synthesis of these proteins, because changes in P_{alb} in glomeruli incubated with L-NMMA in the presence and absence of DETA NONOate or Tempol occurred within 15 mins of incubation with these reagents. (see Materials and Methods). It is conceivable, however, that NO depletion and the associated increase in $O_2^{\bullet -}$ could cause changes in the spatial orientation of these proteins in the slit diaphragm, thereby altering the size of protein–filtration pores of this diaphragm.

In summary, our observations point to NOS-catalyzed NO production as an important mechanism in preserving the glomerular capillary permeability barrier to protein in the immunologically injured glomerulus. This mechanism likely involves control of the bioavailability of superoxide.

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