Changes in Inducible Nitric Oxide Synthase Expression in Experimental Glomerulonephritis (44151)

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Abstract. **We assessed changes in transcriptional activation of the inducible isoform of nitric oxide synthase (iNOS) in a model of macrophage-dependent proliferative glomerulonephritis in the rat resembling human forms of rapidly progressive nephritis. By the use of a cDNA probe derived from rat glomerular RNA and an RNase protection assay, iNOS expression was assessed at early and late stages of the disease and was correlated with the extent of macrophage infiltration. Prominent iNOS expression occurred in isolated glomeruli 24 hr after onset of immune injury when marked glomerular infiltration by macrophages also occurred. Treatment of animals with immune injury with the arachidonic acid cyclooxygenase inhibitor, indomethacin, potentiated iNOS expression. iNOS expression was short-lived; it was markedly reduced on Day 2 of injury and undetectable on Days 4 and 10, despite sustained infiltration of glomeruli by macrophages. These observations suggest that in glomerular immune injury the enhanced expression of iNOS is not sustainable possibly due to downregulatory factors generated in the course of injury.** [P.S.E.B.M. 1997, Vol 215]

Nitric oxide (NO) is a reactive radical gas formed from the terminal guanidino atom of L-arginine. Its generation is catalyzed by nitric oxide synthase (NOS). At least three NOS isoenzymes have been described; the endothelial and neuronal isoforms are constitutively expressed and are activated by increased calcium levels in a calmodulin-dependent manner. The inducible NOS (iNOS) is expressed after transcriptional activation by cytokines such as interleukin-1 and tumor necrosis factor, and lipopolysaccharide (1). While production of NO from constitutive forms of NOS is short-lived and regulates a number of homeostatic functions, generation of NO following activation of iNOS is sustained. This can result in high output generation of NO that can bind to superoxide and form the oxidant peroxynitrite (2) thereby causing oxidative cell injury.

The topography of NOS isoenzymes in the kidney has recently become elucidated. In the rat kidney, glomeruli express both constitutive and inducible NOS (3). In addition, isolated normal glomeruli can generate NO under *ex vivo* conditions. Glomerular NO generation becomes enhanced in various forms of glomerular immune injury and this was attributed to activation of the inducible NOS (4). The mechanisms whereby immune injury activates iNOS are unknown. Also unknown are regulators of iNOS expression following injury. Possible regulators are the prostanoids whose intraglomerular synthesis is enhanced following onset of injury. Thus, prostaglandin E_2 downregulates iNOS expression in cultured glomerular mesangial cells stimulated with cytokines (5). Understanding the timing of activation and changes in expression of this NOS isoform in the course of glomerular immune injury is important, as it may allow therapeutic interventions aiming at iNOS inhibition. The observation that glomeruli express inducible iNOS allows development of a cDNA probe for this NOS isoform in order to explore changes in its expression in the course of glomerular immune injury. This study describes use of this approach to assess changes in glomerular iNOS expression in the course of experimental proliferative glomerulonephritis in the rat.

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Materials and Methods

Development of Experimental Proliferative Glomerulonephritis. Male Sprague-Dawley rats weighing 180–200 g were immunized interperitoneally with 1 mg rabbit IgG emulsified in complete Freund's adjuvant and given as a total volume of 0.5 ml. Five days after this immunization, animals were injected in the tail vein with a subnephritogenic dose of rabbit immune serum raised against rat particulate glomerular basement membrane (GBM). This dose of anti-rat GBM serum (0.15 ml/100g body wt) was insufficient by itself to cause significant proteinuria when given to rats not preimmunized with rabbit IgG (subnephritogenic). The intravenous injection of the anti-rat GBM serum was repeated 24 hr following the first injection. This protocol resulted in heavy proteinuria and rapid onset of glomerular cell proliferation and crescent formation. The rabbit anti-rat GBM immune serum was heat inactivated and filtered through 1.2 μM filters prior to each intravenous injection. Control rats were preimmunized with rabbit IgG in complete Freund's adjuvant and subsequently given two intravenous infections of nonimmune rabbit serum. Studies (see below) were performed on Days 1, 2, 4, and 10 following the second injection of anti-rat GBM serum or of nonimmune rabbit serum. At each time point, three experimental and three control animals were studied in parallel. The day prior to sacrifice animals were placed in metabolic cages for urine collection to assess urinary protein excretion. This was measured by a colorimetric assay and expressed as milligrams of protein per milligram of urine creatinine, which was also measured colorimetrically. Upon completion of this collection, animals were sacrificed and nephrectomized. A renal cortical section was obtained for light and immunofluorescence microscopy using routine methods of fixation and processing. In tissue processed for immunofluorescence microscopy, the presence of rabbit IgG bound to GBM was confirmed using a fluorescein-conjugated affinity purified goat anti-rabbit IgG. We also assessed the extent of macrophage infiltration in glomeruli by staining for the presence of cells positive for the rat macrophage determinant ED_1. Fluorescein-conjugated mouse monoclonal antibody against the ED_1 antigen and a direct immunofluorescence method was employed. The remaining cortical tissue was subjected to differential sieving to isolate glomeruli and isolate glomerular total RNA. Glomerular RNA from three experimental animals with comparable levels of proteinuria or from three parallel controls was pooled in order to assess iNOS expression using an RNase protection assay (see below).

Isolation of Glomerular Total RNA. After cortical tissue was obtained for histopathology studies as described above, kidney cortices were separated from medullae, minced, and processed for glomerular isolation using a differential seiving technique that yields a final glomerular preparation of greater than 95% purity as we described in previous studies (6). In this preparation (0.5 ml), 3 ml of a mixture containing guanidium isothiocyanate/phenol-chloroform (RNA Stat-60; Tel-Test Inc., Friendswood, TX) was added under vigorous and prolonged vortexing to inactivate RNases and extract total RNA. The suspension was subsequently allowed to stand at room temperature for 5 min, and 0.2 ml of 100% chloroform was added and mixed thoroughly. After another 5 min at room temperature, the suspension was centrifuged at 4°C for 20 min. Two phases were observed and separated. The upper RNA containing aqueous phase was carefully removed into a microcentrifuge tube and mixed with an equal volume of 100% isopropanol. Glomerular RNA was subsequently precipitated by incubating the isopropanol mixture at −20°C overnight. The isopropanol-precipitated RNA was then centrifuged at 12,000g at 4°C for 20 min. The pellet was redissolved in 300 μl of RNase-free water and extracted once with each of 300 μl of 100% water-saturated phenol, 50% water-saturated phenol/48% chloroform/2% isoamyl alcohol (25:24:1), 96% chloroform/4% isoamyl alcohol (24:1). RNA was subsequently precipitated with 75% ethanol and 0.3 M sodium acetate at −80°C. RNA concentration and quality was assessed spectrophotometrically at wavelengths 260 and 280 nm.

Generation of iNOS and Ribosomal Protein L19 cDNA Probes. *Reverse transcription.* First-strand cDNA was synthesized by using oligo-dT. One microgram of glomerular total RNA was reverse-transcribed in a total volume of 10 μl at 37°C for 2 hr in 1× reaction buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT; USB, Cleveland, OH) containing 10 units/μl MMLV–reverse transcriptase (USB), 0.02 ug/μl oligo-dT (Promega, Madison, WI), 2 units/μl RNasin (Promega), and 0.5 mM of each dNTP. Reactions were stopped by heating the mixtures at 68°C for 5 min.

*Polymerase chain reaction procedures. **Construction of polymerase chain reaction primers.*** Primers for rat iNOS were selected based on a published sequence (7). Studies on characterization of iNOS isoforms expressed in the rat kidney demonstrated the presence of two structurally distinct isoforms: one homologous to murine macrophage iNOS (maciNOS), and another homologous to rat vascular smooth muscle iNOS (vsmiNOS) (3). macNOS was the principal iNOS isoform expressed in glomeruli. However, vsmiNOS was the isoform whose expression markedly increases following injury (i.e., endotoxemia) (3). The particular iNOS isoform amplified by the primers chosen in our studies was identified by sequencing (see below) and was found to be identical to the vsmiNOS. Primers for rat ribosomal protein L19 were designed by using the PRIMER2 Software System (Scientific and Educational Software, Stateline, PA) and on the basis of a published sequence (J02650) obtained from GENEMBL-BANK. L19 is a rat ribosomal protein. Assessment of changes in its expression in isolated glomeruli was used as an indicator of changes in the level of glomerular transcriptional activity in the course of glomerular immune injury (Days 1, 2, 4, and

10 following administration of anti-GBM serum). Primers were synthesized by Operon (Operon Technologies, Inc., Alameda, CA), and were as follows: iNOS—sense, 5′-GCATGGAACAGTATAAGGCAAACA-3′; antisense, 5′-GTTTCTGGTCGATGTCATGAGCAA-3′. L19—sense, 5′-AGCCTGTGACTGTCCATTCC-3′; antisense, 5′-TTGGTCTTAGACCTGCGAGC-3′

The polymerase chain reaction (PCR) products generated from iNOS and L19 mRNAs were 222 and 345 bp, respectively.

Polymerase Chain Reaction. One microliter of undiluted reverse transcription products was used in PCR in a total volume of 50 μl. The 1× reaction buffer contained 50 m*M* KCl, 10 m*M* Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 m*M* $MgCl_2$, 0.2 m*M* of each dNTP, 1 μ*M* of both sense and antisense primers, and 0.02 units/μl Taq-polymerase (Promega). The PCR cycling profile was 1.5 min at 94°C, 2 min at 57°C, and 3 min at 72°C. Thirty-five cycles were carried out and followed by final extention at 72°C for an additional 10 min.

Cloning of PCR products of iNOS and L19. ***Ligations.*** The Strategene pCRscript Amp SK (+) kit was used with minor modification to clone PCR products of iNOS and L19. Five micrograms of Phenol-chloroform purified PCR product was end-in-filled in by *Pfu* DNA plymerase (Stratagene, La Jolla, CA) in a reaction volume of 13 μl. The 1× buffer contained 0.04 unit/μl of *Pfu,* 10 m*M* dNTP mix (2.5 m*M* each), 2 μg of PCR product, 20 m*M* Tris-HCl, pH 8.75, 10 m*M* KCl, 10 m*M* $(NH4)_2SO_4$, 2 m*M* $MgSO_4$, 0.1% Triton X-100, 1 mg/ml BSA. Four hundred nanograms of end-in-filled PCR product were ligated with 10 ng of pCR-Script SK(+) vector (Stratagene) in a final volume of 10 μl at room temperature for 1 hr. The 1× ligation buffer contained 25 m*M* Tris-HCL, pH 7.5, 10 m*M* $MgCl_2$, 10 m*M* DTT, 0.2 mg/ml BSA, 0.5 M*M* ATP, 0.5 units/μl *Srf* I, and 0.4 units/μl T4 DNA Ligase.

Transformation of Escherichia coli. Two microliters of the above ligation product was added to 50 μl of XL-1 Blue MFR′ Kan supercompetent cells (Stratagene). These cells were sequentially incubated on ice for 25 min at 42°C for 30 sec, and on ice again for 2 min. Four hundred and fifty microliters of SOS medium (2% Tryptone, 0.5% yeast extract, 10 m*M* NaCl, 2.5 m*M* KCl, 10 m*M* $MgCl_2$, 10 m*M* $MgSO_4$, 20 m*M* glucose) (Invitrogen, San Diego, CA) brought to 37°C were added to the cells and incubated on an orbital shaker (200 × 1000 rpm) at 37°C for 1 hr. After incubation, 150 μl of the transformed cells were innoculated on a regular LB agar plate in the presence of ampicillin (50 ng/ml), kanamycin (25 ng/ml), and X-gal, and incubated at 37°C for 18 hr. Forty milligrams of X-gal were evenly spread onto the top of the agar plate 1 hr before the bacterial innoculation.

Isolation and Identification of Plasmids Containing iNOS and L19. *Small-scale preparation.* A white colony containing plasmid of either iNOS or L19 grown on the McConkey agar plate was picked and grown in LB broth in the presence of 50 ng/ml of both ampicillin and Kanamycin. One milliliter of the bacterial culture was used for a mini-preparation of plasmids by using the QIA prep Spin Plasmid Kit (Qiagen, Chatsworth, CA). *Hin*dIII (Gibco-BRL) and *Xba*I (Gibco-BRL) were used to digest purified DNA containing the iNOS insert, and *Hin*dIII (Gibco-BRL) and *Sac* I (Pharmacia, Piscataway, NJ) for the L19 insert. Positive colonies were identified by the presence of inserts of predicted sizes released after restriction digestions. Further identification of the exact sequences for each clone was done by DNA sequencing.

Large-scale preparation. A colony containing either insert was selected and let grow in 200 ml of LB broth with 50 μl of kanamycin and ampicillin. Plasmid DNA was purified by a Plasmid Maxi Kit Tip-500 column (Qiagen).

Sequencing of PCR products. The identity of each insert in pCRscripts was identified by dideoxy-sequencing from one direction using established methods. Based on comparison of published sequence of rat vascular smooth muscle (vsm) iNOS (7) with the sequence of the cloned PCR product identified by the dideoxysequencing method, the PCR product had perfect homology with the rat vsmiNOS.

RNAse Protection Assay. *Probe synthesis.* iNOS and L19 plasmids were linearized with *Xba*I and *Hin*dIII, respectively. The linearized DNA was separated by 1% agarose gel electrophoresis. The fragment containing the linear DNA was removed from the gel and purified by GeneClene (Bio 101, Vista, CA). The concentration and purity of each linearized plasmid were determined by O.D. 260/280.

All probes were synthesized using an RNA transcription kit (Strategen). Antisense probe for iNOS was generated by using T7 RNA polymerase and the iNOS plasmid was linearized by *Xba*I. Sense probe for iNOS was generated using T3 RNA polymerase and the iNOS plasmid was linearized by *Hin*dIII. Antisense probe for L19 was generated using T3 RNA polymerase and the L19 plasmid was linearized by *Hin*dIII. Sense probe for PL19 was generated by T7 polymerase and the L19 plasmid was linearized by *Sac* I. The 1× reaction mixture in a final volume of 25 μl contained 1 μg linearized DNA, 1 unit/μl of RNAsin (Promega), CTP ATP GTP each 40 μ*M*, UTP 12 μ*M*, 5 μl of ^{32}P UTP (800 Ci/mmol, 10 μCi/μl), 30 m*M* DTT, and 10 units of polymerase. Buffer (1×) was 40 m*M* Tris-HCl, pH 8.0, 8 m*M* $MgCl_2$, 2 m*M* spermidine, 50 m*M* NaCl. Probes were purified on 8 *M* urea 5% polyacrylamide gel and eluted using a probe elution buffer (Ambion, Austin, TX). Specific activity was determined by liquid scintillation counting.

Hybridization. Twenty-five micrograms of total glomerular RNA was mixed with 10 μg yeast transfer RNA and 100,000 cpm of probe, and precipitated in 0.3 *M* ammonium acetate by 2.5 volumes of absolute ethanol for 30 min at −20°C. The pellet was dissolved in 20 μl hybridization buffer (Ambion) and incubated in a 45°C water bath overnight.

RNase digestion. RNase A (0.5 units) and 20 units of RNase T1 were added to each hybridization tube and the digestion mixture was incubated at 37°C for 35 min. The reaction was stopped, and the RNA was precipitated by RNase Inactivation/Precipitation Buffer (Ambion) at 20°C for 30 min. The RNA was centrifuged at 12,000*g* at 4°C for 20 min. The pellet was dissolved in 8 μl of gel loading buffer (Ambion) and loaded on a 8 *M* 5% polyacrylamide gel. Electrophoresis was performed at 250 V for 3 hr. The gel was exposed to X-ray film overnight at 20°C.

Summary of Methods Described Above. The methods described above can be summarized as follows:

- Isolated glomeruli
- Isolation of total RNA
- Reverse transcription to generate first-strand cDNA
- PCR using primers for rat vascular smooth muscle iNOS (r-vsmiNOS) and for rat ribosomal protein L19
- Identification of PCR products be dideoxy sequencing, and cloning of products using p CRscript Amp SK (+)
- Generation of sense and anti-sense probes from cloned iNOS and L19 cDNAs
- Use of probes in RNase protection assay

Glomerular iNOS expression using the RNase protection assay described above was assessed in the following groups:

1. Rats immunized with rabbit IgG mixed in complete Fruend's adjuvant, and injected intravenously with rabbit anti-rat GBM serum 5 days later. Glomeruli were isolated for assessment of iNOS expression 24 hr, 48 hr, day 4, and day 10 following administration of the anti-GBM serum.
2. Controls: same as in Group 1, above, except the animals were injected with nonimmune rabbit serum instead of rabbit anti-rat GBM serum.
3. Cyclooxygenase inhibition: Same as in Group 1, except these animals received indomethacin 3 mg/kg intravenously 1 hr prior to injection of the anti-GBM serum. Glomeruli were isolated for iNOS expression 24 hr following the anti-GBM serum injection.
4. Cyclooxygenase inhibition control: Same as in Group 3 above, except these animals received indomethacin 3 mg/kg 1 hr prior to injection of nonimmune rabbit serum.

Measurement of NOS Enzyme Activity. This was determined in order to assess whether changes in glomerular iNOS expression were accompanied by changes in nitric oxide synthase (NOS) enzyme activity.

Glomeruli isolated from experimental ($n = 4$) and control ($n = 3$) animals as defined above were quickly frozen in a dry ice–ethanol mixture in a buffered proteinase inhibitor cocktail containing 100 μ*M* phenyl methyl sulphonyl fluoride, 5 μg/ml aprotinin, 1 mg/ml chymostatin, and 5 μg/ml pepstatin. Glomeruli were subsequently disrupted with a pestel and repeated freeze-thaw cycles were performed. Cytosols were collected after centrifugation and assayed for NOS enzyme activity using previously described methods (8). The assay buffer contained 15 mmol HEPES, pH 7.5, 100 mmol NADPH, 1 mmol dithiothreitol, 10 μ*M* FAD, 100 μ*M* tetrahydrobiopterin, and 10 μ*M* L-arginine. [^{3}H]L-arginine (New England Nuclear), labeled in the guanidino position, was used in the reaction mixture. The total reaction mixture consisted of 3 μl of sample in a total reaction volume of 50 μl, and measurements were performed in duplicate or triplicate. The assay is based on conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline by nitric oxide synthase. Reactions were allowed to proceed for a 20-min period and were stopped with 1 ml of ice-cold stop buffer consisting of 20 m*M* HEPES, 2 m*M* EDTA, 2 m*M* EDGA, pH 5.5. To separate the [^{3}H]L-citrulline formed from [^{3}H]L-arginine, the entire reaction mixture was passed over a plastic column containing 1 ml of equilibrated Dowex exchange resin. The eluate containing [^{3}H]L-citrulline was collected into liquid scintillation vials to measure radioactivity by liquid scintillation counting. The Dowex exchange resin binds the [^{3}H]L-arginine allowing the [^{3}H]L-citrulline to flow through. After substraction of background counts, enzyme activity was calculated and expressed as picomoles of citrulline generated based on the specific activity of the [^{3}H]arginine added in the reaction mixture. As iNOS activity is Ca^{++} independent, $CaCl_2$ was omitted from the assay buffer so that the L-arginine to L-citrulline conversion would proceed in the absence of calcium thereby reflecting iNOS activity. Data was expressed as micromoles L-citrulline per microgram glomerular protein per minute (mean ± SE, $n = 3$). Comparisons between control and experimental groups employed *t* test statistics for unpaired observations.

RESULTS

The histologic appearance of glomerular lesions on Day 4 following administration of the anti-GBM antibody is shown in Figure 1. Linear deposition of the anti-GBM antibody (left panel) and increased cellularity and crescent formation (middle panel) were prominent features. A normal glomerulus is shown in the right-hand panel.

Infiltration of glomeruli by macrophages was also a prominent feature (Fig. 2). Macrophage infiltration was prominent on Day 1 (Fig. 2B) and persisted on day 10 (Fig. 2C). Heavy proteinuria developed in all animals.

In Figure 3, changes in glomerular iNOS expression using the RNase protection assay are shown in the course of glomerulonephritis. Detectable levels of iNOS mRNA were found in glomeruli isolated from control rats studied on Day 1 following injection of nonimmune rabbit serum (Lane 1), but not in glomeruli from control animals studied at other time points (Lanes 3, 5, and 7). iNOS expression in glomeruli isolated from rats following injection of anti-GBM serum was prominent on Day 1 of the disease (Lane 2), became markedly reduced on Day 2 (Lane 4) and was undetectable on Days 4 and 10 (Lanes 6 and 8).

In Figure 4, changes in expression of ribosomal protein L19 using the RNase protection assay are shown. In contrast

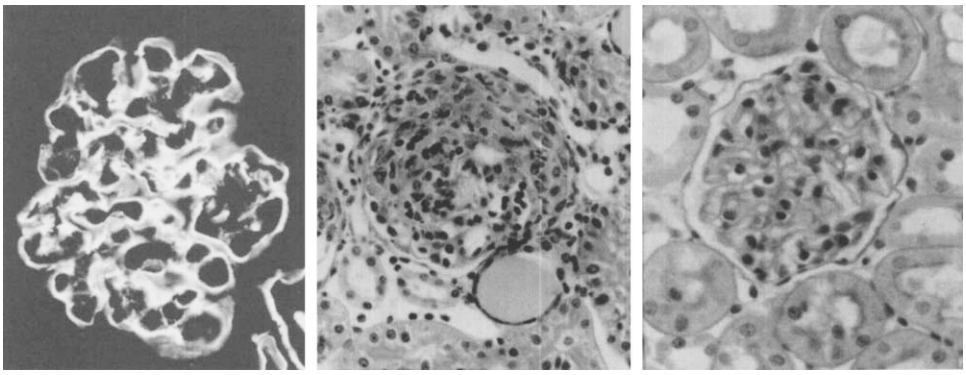

Figure 1. Histologic appearance of glomerular lesions of proliferative glomerulonephritis. Linear deposition of anti-GBM antibody (left panel) and increased glomerular cellularity and crescent formation (middle panel) are shown. A control glomerulus is shown in the right-hand panel.

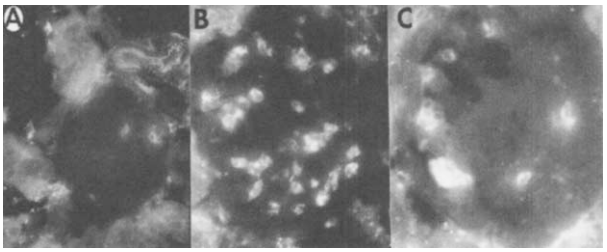

Figure 2. Glomerular infiltration by macrophages identified by staining for the antigenic marker ED_1. (A) Control glomerulus. (B) Prominent infiltration of glomerulus by ED_1 (+) cells (macrophages) 24 hr following onset of immune injury. (C) Macrophage infiltration on Day 10 of injury.

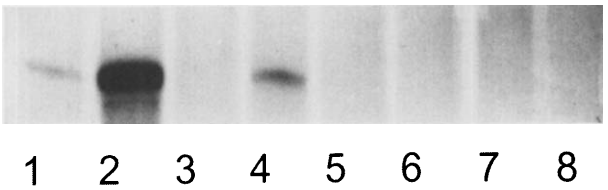

Figure 3. Changes in iNOS gene expression in the course of glomerular immune injury assessed in glomerular total RNA by RNase protection assay. Lane 1, Control day 1; Lane 2, Experimental day 1; Lane 3, Control day 2; Lane 4, Experimental day 2; Lane 5, Control day 4; Lane 6, Experimental day 4; Lane 7, Control day 10; Lane 8, Experimental day 10.

to iNOS expression, L19 expression was present in glomeruli isolated from control animals at all time points of study (Lanes 1, 3, 5, and 7). Moreover, L19 expression was persistently increased in immunologically injured glomeruli at all time points (Lanes 2, 4, 6, and 8).

Figure 5 demonstrates the effect of cyclooxygenase inhibition using indomethacin on iNOS expression assessed 24 hr following administration of anti-GBM serum. iNOS expression was enhanced in glomeruli isolated from indomethacin-treated animals with immune injury compared with expression in glomeruli from animals with immune injury not treated with indomethacin.

In Figure 6, the level of glomerular NOS enzyme activity on Day 1 of immune injury is shown. In glomeruli isolated from rats given anti-GBM serum (experimental) there was enhanced enzyme activity, compared with the level of activity detected in glomeruli isolated from rats given nonimmune rabbit serum (control).

Discussion

Immune injury of the renal glomerular capillary frequently results in an inflammatory reaction. In the course of this reaction, generation and release of various proinflammatory and vasoactive molecules can occur. Among the biochemical pathways activated in response to glomerular immune injury, activation of the L-arginine:NO pathway has recently been described (4). A key enzyme in this pathway is nitric oxide synthase. In glomerular immune injury, we have demonstrated that activation of the inducible NOS isoform occurs and attributed this activation to infiltrating macrophages (4, 9). NO derived from iNOS can be sustained and of high output. As NO is a vasodilator, this sustained output may have a beneficial effect on the glomerular vasculature by preventing ischemia resulting from immune injury. Indeed, work from other investigators in the model of anit-GBM antibody-induced injury has shown that inhibition of NOS activity reduces glomerular plasma flow at early stages of injury (10). On the other hand, sustained high output generation of NO may cause oxidative injury of glomerular capillary cells. Putative agonists that can stimulate transcription of the iNOS gene in the course of glomerular immune injury include the cytokines interleukin-1 and tumor necrosis factor and the interferons (1). Knowledge of the time course of transcriptional activation of iNOS following injury is useful as it can indicate when in the course of injury pharmacologic inhibition of iNOS can be attempted in order to abbrogate sustained generation of NO. As selective iNOS inhibitors are now becoming available, iNOS inhibition as an anti-inflammatory strategy in glomerulonephritis may soon come under consideration. Therefore, evaluation of changes in iNOS expression in the course of the disease becomes an important issue.

Our present studies were prompted by the observation that normal rat glomeruli express inducible NOS (3). Using the method of polymerase chain reaction and iNOS gene–specific primers amplifying a 222-bp fragment of a pub-

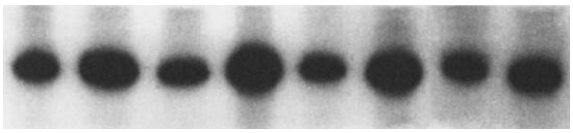

Figure 4. Changes in expression of the rat ribosomal protein L19 gene assessed in glomerular total RNA in the course of glomerular immune injury by RNase protection assay. Designation of lanes is same as shown in the legend of Figure 4.

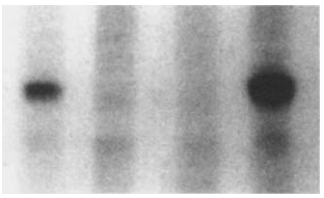

Figure 5. iNOS expression in RNA of glomeruli isolated from: rats that received anti-GBM serum (Lane 1), rats that received nonimmune serum (Lane 2), rats that received indomethacin followed by nonimmune serum (Lane 3), and rats that received indomethacin followed by anti-GBM serum (Lane 4).

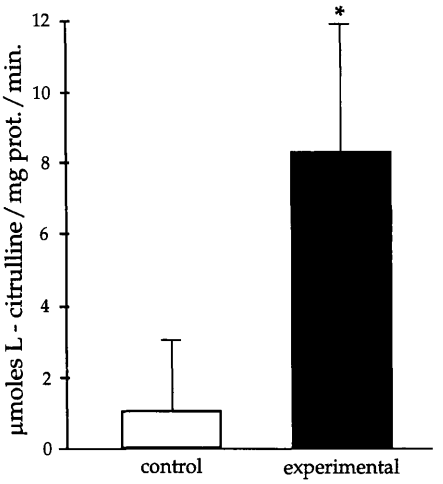

Figure 6. NOS enzyme activity measured in isolated glomeruli on Day 1 of glomerulonephritis in control ($n = 3$) and experimental ($n = 3$) animals. $^*P < 0.05$ compared with control (unpaired t test).

lished sequence, we generated a 222-bp cDNA probe from RNA of normal glomeruli. We then employed this probe as a tool to assess changes in glomerular iNOS expression in the course of proliferative glomerulonephritis using an RNase protection assay. Prominent iNOS expression was observed at 24 hr following onset of glomerular immune injury (Fig. 3), when marked glomerular infiltration by macrophages occurred (Fig. 2B). At the same time point, increased glomerular NOS enzyme activity was also present (Fig. 6). The enhancement in iNOS expression was short-lived; following peak expression at 24 hr, it was markedly reduced on Day 2 of injury and was undetectable on Days 4 and 10 (Fig. 3). The short-lived enhancement of glomerular iNOS expression occurred despite persistent infiltration of glomeruli by macrophages (Fig. 2), and despite a persistently enhanced glomerular transcriptional activity assessed by changes in expression of the ribosomal protein L19 (Fig. 4).

Previous immunohistochemical studies to identify glomerular cells expressing iNOS protein in glomeruli of rats with this form of glomerulonephritis have localized the iNOS protein in the infiltrating macrophage (4). In addition to being a major site of iNOS expression, the infiltrating macrophage also accounts, to a significant extent, for the generation of NO by glomeruli isolated from rats with macrophage-dependent forms of glomerular immune injury. This was shown by performing macrophage depletion studies. To this extent, we previously reported that macrophage depletion using x-irradiation to deplete bone marrow–drived macrophages, markedly reduced NO generation by glomeruli isolated from rats with anti–Thy 1 antibody induced glomerular injury (9). These observations indicate that the infiltrating macrophage is a major contributor of iNOS expression and NO generation in glomerular immune injury.

The short-lived nature of transcriptional activation of iNOS in glomeruli may indicate that factors released in the glomerular microenvironment in the course of glomerulonephritis downregulate iNOS expression. Factors that may downregulate iNOS expression in the course of glomerulonephritis are unknown. One potential factor is the arachidonic acid metabolite, prostaglandin (PG) E_2. PGE_2 downregulates iNOS expression in cultured glomerular cells stimulated with interleukin-1 (5). In various forms of glomerular immune injury, including the one used in the present studies, glomerular PGE_2 synthesis is enhanced. This occurs early (24 hr) following onset of injury and remains sustained over at least a 14-day period (11). To explore the role of PGE_2 as a downregulator of iNOS expression, we treated animals with immune injury with the cyclooxygenase inhibitor, indomethacin, given 1 hr prior to injection of the anti-GBM antibody. In glomeruli isolated from these rats, iNOS expression was accentuated (Fig. 5). This implicates arachidonic acid cyclooxygenation products such as PGE_2 as downregulators of iNOS expression in glomerular immune injury. Another potential downregulator of iNOS expression is transforming growth factor–β1 (TGFβ1). This cytokine reduces NOS mRNA induction and NOS protein mass in murine macrophages stimulated with γ-interferon (12). Enhanced expression and synthesis of TGFβ1 occurs in the course of anti-GBM antibody-induced nephritis, and this is localized in macrophages infiltrating glomeruli (5, 13).

In summary, we have assessed changes in transcriptional activation of iNOS in glomeruli in the course of a macrophage-dependent form of glomerulonephritis. Prominent iNOS expression occurred at early stages of immune injury (24 hr) and temporally correlated with marked macrophage infiltration. However, iNOS expression was short-lived, despite the sustained presence of macrophages in glo-

meruli. This indicates downregulation of transcriptional activation of iNOS by factors generated in the course of injury. These factors include arachidonate cyclooxygenation eicosanoids.

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1. Moncada S, Palmer RJ, Higgs EA. Nitric oxide: Physiology, pathophysiology and pharmacology. Pharmacol Rev **43:**109–142, 1991.
2. Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. J Biol Chem **266:**4244–4250, 1991.
3. Mohaupt MG, Elzie LJ, Ahn KY, Clapp WL, Wilcox CS, Kone BC. Differential expression and induction of mRNAs encoding two inducible nitric oxide synthases in rat kidney. Kidney Int **46:**653–665, 1994.
4. Cook HT, Ebraham H, Jensen AS, Foster ER, Larigan P, Cattell V. Expression of the gene for inducible nitric oxide synthase in experimental glomerulonephritis in the rat. Clin Exp Immunol **97:**315–320, 1994.
5. Tetsuka T, Daphna-Iken D, Srivastava SK, Baier LD, DuMaine J, Morrison A. Cross-talk between cyclooxygenase and nitric oxide pathways: Prostaglandin E_2 negatively modulates induction of nitric oxide synthase by interleukin-1. Proc Natl Acad Sci U S A **91:**12168–12172, 1994.
6. Lianos EA, Orphanos V, Cattell V, Cook T, Anagnou N. Glomerular expression and cell origion of transforming growth factor–β1 in anti-GBM disease. Am J Med Sci **307:**1–6, 1994.
7. Nunokawa Y, Ishida N, Tanaka S. Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells. Biochem Biophys Res Commun **191:**89–94, 1993.
8. Sherman PA, Laeubach B, Reep BR, Wood ER. Purification and cloning of a cytokine-induced nitric oxide synthase from a human tumor cell line. Biochemistry **32:**11600, 1993.
9. Cattell V, Lianos EA, Largen P, Cook T. Glomerular iNOS synthase activity in mesangial cell immune injury. Exp Nephrol **1:**36–40, 1993.
10. Ferrario R, Takahashi K, Fogo A, Badr KF, Munger KA. Consequences of acute nitric oxide synthesis inhibition in experimental glomerulonephritis. J Am Soc Nephrol **4:**1847–1854, 1994.
11. Lianos EA, Andres G, Dunn MJ. Glomerular prostaglandin and thromboxane synthesis in rat nephrotoxic serum nephritis. Effects on renal hemodynamics. J Clin Invest **74:**1439–1448, 1983.
12. Vodovotz Y, Bogdan C, Paik J. Mechanisms of suppression of macrophage nitric oxide release by transforming factor β. J Exp Med **178:**605–613, 1993.
13. Lianos EA, Liu J, Guglielmi K. Quantitation of changes in transforming growth factor (TGF)–β_1 gene expression in experimental crescentic glomerulonephritis. Proc Soc Exp Biol Med **214:**180–186, 1997.