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Lack of a Role for Inducible Nitric Oxide Synthase in an Experimental Model of Nephrotic Syndrome

Lisa M. Walker, Sudhir V. Shah† and Philip R. Mayeux*‡*

Departments of *Pharmacology and Toxicology and †Medicine, Division of Nephrology, University of Arkansas for Medical Sciences, Little Rock, AR 72205, U.S.A.

ABSTRACT. Puromycin aminonucleoside (PAN) administration in rats produces an experimental model of nephrotic syndrome characterized by glomerular epithelial cell injury and proteinuria. The purpose of this study was to examine the role of nitric oxide (NO) in this model of minimal change glomerular disease. Aminoguanidine (AG) was used to inhibit inducible nitric oxide synthase (iNOS). Sprague–Dawley rats were divided into Control (N = 9), PAN (N = 14), AG (N = 2), and PAN + AG (N = 12) treatment groups. Control animals received saline (i.v.), PAN animals received PAN (75 mg/kg, i.v.), and PAN + AG animals received PAN plus AG (50 mg/kg, i.p., twice daily). AG animals received a saline injection (i.v.) on day 0 in the place of PAN and then AG on the same schedule as the PAN + AG group. Animals were kept in metabolic cages, and urinary protein excretion and nitrite (NO_2^-) excretion were measured daily. PAN administration increased urinary NO_2^- excretion by day 2, and levels remained elevated through day 7. AG prevented this PAN-induced increase in urinary NO_2^- excretion. Plasma nitrate (NO_3^-) and NO_2^- (NOx) concentrations were also increased in the PAN and PAN + AG groups. iNOS protein expression was not detected in either the glomeruli or the cortex at day 7. Proteinuria developed in PAN animals on day 4 and increased steadily through day 7. PAN + AG animals showed a pattern similar to that of the PAN group. These results indicated that in contrast to models of proliferative glomerulonephritis, NO formation during PAN-induced nephrotic syndrome is increased but does not participate in the development of glomerular injury as measured by proteinuria. BIOCHEM PHARMACOL **60**;1:137–143, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. puromycin aminonucleoside; nephrotic syndrome; inducible nitric oxide synthase; aminoguanidine

Nephrotic syndrome is characterized by renal glomerular injury resulting in heavy proteinuria and lipiduria and is associated with varying degrees of edema and hypoalbuminemia. Minimal change nephrotic syndrome accounts for more than 75% of the cases of nephrotic syndrome in children and 20–30% of nephrotic syndrome in adults [1–3]. Because the underlying mechanisms of glomerular injury are not known, treatment of minimal change disease is for the most part empirical.

NO§ is an important signaling molecule generated by a five-electron oxidation of the guanidino nitrogen group of the amino acid L-arginine. This reaction is catalyzed by the NOS family of enzymes [4]. Recent studies have shown a complex role for NO in glomerular injury [5–9]. As a vasodilator, NO participates in the regulation of glomerular hemodynamics. In fact, chronic blockade of NO production results in glomerular sclerosis [10]. Yet, in some models of glomerular injury, NO synthesis may be deleterious. In anti-Thy-1 and anti-myeloperoxidase models of proliferative glomerulonephritis, increased NO formation by iNOS is suggested to participate in injury [8, 11, 12].

A single injection of PAN into rats causes injury to the glomerular epithelial cells and results in massive proteinuria and glomerular morphological changes that are similar to minimal change nephrotic syndrome in humans [13]. Thus, it has been used as a model of non-proliferative glomerulonephritides such as minimal change disease and focal segmental glomerulosclerosis. PAN enhances the generation of reactive oxygen species by glomeruli [14], and antioxidants such as SOD, deferoxamine, and dimethylthiourea all decrease or prevent PAN-mediated kidney injury [15–19]. It is becoming increasingly clear that an interaction between NO and reactive oxygen species generates damaging reactive nitrogen species [20, 21]. Because the aforementioned inhibitors all interfere with the generation of or directly scavenge reactive nitrogen species [22–24], we examined the role of NO in this model using the iNOS inhibitor AG [25].

‡ Corresponding author: Philip R. Mayeux, Ph.D., Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, 4301 West Markham Street, Mail Slot 611, Little Rock, AR 72205. Tel. (501) 686-8895; FAX (501) 686-5521; E-mail: mayeuxphilipr@exchange.uams.edu

§ *Abbreviations*: NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible NOS; PAN, puromycin aminonucleoside; SOD, superoxide dismutase; AG, aminoguanidine; LPS, lipopolysaccharide; and NOx, NO_3^- (nitrate) + NO_2^- (nitrite).

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MATERIALS AND METHODS

Materials

PAN and all other chemicals (unless noted otherwise) were purchased from Sigma-Aldrich. Rabbit polyclonal anti-iNOS antibody was purchased from Cayman Chemical. Donkey anti-rabbit IgG peroxidase conjugate was purchased from Amersham Life Sciences. The peroxidase detection system SuperSignal® BLAZE™ chemiluminescent substrate and Coomassie® Plus protein assay reagent were purchased from Pierce. Hanks' Balanced Salt Solution was purchased from Gibco BRL.

Experimental Design

Housing and treatment of the animals were in accordance with the guidelines established by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Resource Council. Male Sprague–Dawley rats were divided into four treatment groups: Control (N = 9), PAN (N = 14), AG (N = 2), and PAN + AG (N = 12). PAN animals received PAN on day 0 (75 mg/kg, i.v.) in saline. PAN + AG animals received PAN (75 mg/kg, i.v.) on day 0 plus AG (50 mg/kg, i.p.) administered twice daily for 7 days following PAN administration. The first dose of AG was given 30 min prior to the PAN injection, and each subsequent dose was given 12 hr apart. Control animals received saline (i.v.) on day 0. The AG group received a saline injection on day 0, and AG on the same schedule as the PAN + AG group. All animals were kept in metabolic cages, and urine was collected daily, at regular intervals, to obtain 24-hr collections of spontaneously voided urine. The urine volume voided, urinary protein excretion, and urinary NO_2^- excretion per 24 hr was measured. On day 7 following PAN administration, animals were anesthetized with pentobarbital sodium (50 mg/kg) and were killed by exsanguination. Blood was collected in heparinized syringes. Kidneys were excised, and glomeruli were isolated or kidney cortex was frozen in liquid nitrogen for western blot analysis.

Urinary Protein Excretion

The protein concentration in urine was determined using the Coomassie® Plus protein assay reagent. Samples were diluted in H_2O and mixed with detection reagent. Absorbance was measured at 590 nm, and the amount of protein per sample was determined by comparing sample absorbance to a BSA standard curve. Results were normalized to urine volume and were expressed as milligrams protein per day.

Urinary NO_2^- Excretion

The NO_2^- concentration in the urine was determined using Griess reagent. Urine samples (50 μL) were diluted in H_2O, and $ZnSO_4$ was added (final concentration = 1.5%) to precipitate protein. Samples were incubated for 15 min at room temperature and were centrifuged at 16,000 *g* for 5 min to separate particulate matter. The supernatant was collected. An aliquot was added to a microtiter plate, and the volume was brought to 100 μL with H_2O. Griess reagent (100 μL) was added to start the reaction. Griess reagent consisted of equal volumes of 1% sulfanilamide in 2.5% H_3PO_4 and 0.1% *N*-(1-naphthyl)-ethylenediamine in H_2O. Plates were incubated for 5 min at room temperature, and the absorbance was read at 550 nm. Results were compared against a standard curve of $NaNO_2$, and the amount of NO_2^- (in nanomoles) was determined for each sample. The results were normalized against the volume of urine voided in 24 hr and were expressed as nanomoles NO_2^- per day.

Plasma NOx

NOx concentrations were determined in the plasma (day 7) using a colorimetric non-enzymatic NO assay kit from Oxford Biomedical Research. Samples (50 μL) were diluted in H_2O, and $ZnSO_4$ was added (final concentration = 1.5%) to precipitate protein. Samples were incubated for 15 min at room temperature and were centrifuged at 16,000 *g* for 5 min to separate particulate matter. The supernatants were collected and added to a microcentrifuge tube containing 6–7 cadmium beads. The samples were mixed on a tube shaker overnight. The following day, samples were centrifuged, and the resulting supernatant was tested for NO_2^- using Griess reagent. The samples were diluted in H_2O and mixed with an equal volume of Griess reagent. Plates were incubated for 5 min at room temperature, and the absorbance was read at 550 nm. Results were compared against a standard curve of $NaNO_2$, and the concentration of NO_2^- was determined for each sample. Data were expressed as micromolar.

Urine NOx

NOx concentrations were determined in the urine (day 7) using a colorimetric NO assay kit from Oxford Biomedical Research. Because cadmium did not convert NO_3^- to NO_2^- in the urine samples, nitrate reductase was used to determine NOx concentration. Samples were diluted in H_2O, and NO_3^- was converted to NO_2^- using nitrate reductase according to the manufacturer's directions. NO_2^- was detected in samples using Griess reagent. Results were compared against a standard curve of $NaNO_3$ treated in an identical manner as the samples. The NOx was determined for each sample and expressed as micromoles per day.

Creatinine Clearance

Plasma creatinine and urinary creatinine concentrations were determined using a Beckman Synchron CX7 Analyzer. Creatinine clearance for day 7 was determined for all

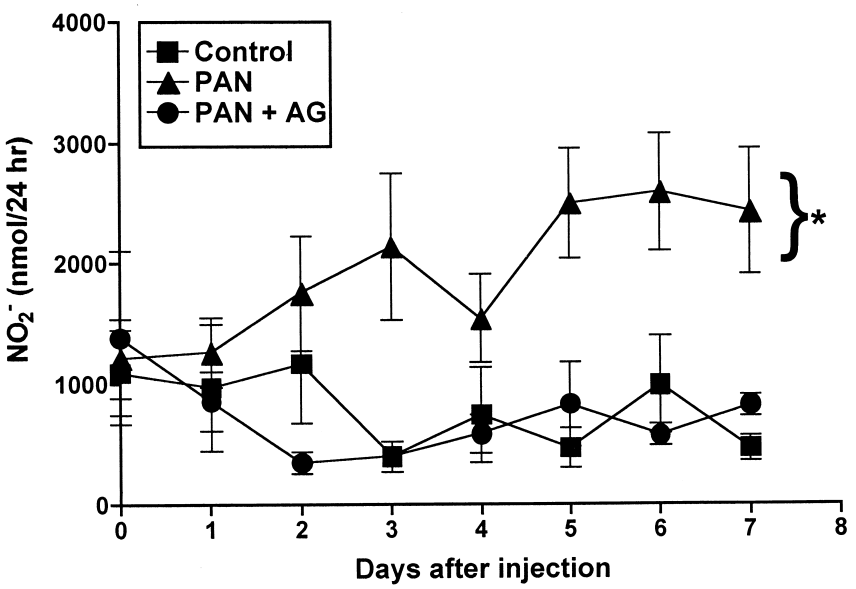

FIG. 1. Urinary NO_2^- excretion following PAN administration. Urine was collected daily from all animals, and NO_2^- excretion rates were measured by the Griess reagent method. Data are expressed as means ± SEM. N = 9, 14, and 12 for the Control, PAN, and PAN + AG groups, respectively. Statistical significance was determined using a two-way ANOVA followed by Tukey's test. Key: (*) $P < 0.05$ for PAN vs Control and the PAN + AG groups.

animals, and was expressed as microliters per day per 100 g body weight.

Isolation of Glomeruli from Kidney Cortex

Rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and the abdominal cavity was opened. A polyethylene catheter (PE-100) was inserted into the lower abdominal aorta immediately above the bifurcation. The aorta was clamped above the renal arteries, the renal veins were cut, and the kidneys were perfused until blanched with 30 mL (10 mL/min) of ice-cold Hanks' Balanced Salt Solution (pH 7.4). Then kidneys were excised, and the kidney cortex was dissected from the medulla. Purified glomeruli (<5% contaminating tubular fragments) were isolated by differential centrifugation and sieving as previously described [26].

Tissue Preparation for Western Blot Analysis

Kidney cortices were homogenized in homogenization buffer (125 mM sucrose, 10 mM HEPES, 1 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 1.5 μM pepstatin) using a Dounce glass homogenizer. Isolated glomeruli were sonicated in homogenization buffer. Then homogenates were centrifuged at 14,000 *g* for 5 min. The supernatants from glomeruli were concentrated by filtration (10,000 molecular weight cutoff filters), and final protein concentrations were determined using Coomassie® Plus protein assay reagent.

Samples (25 or 100 μg protein/lane) were separated by SDS–PAGE on 4–20% polyacrylamide gels, and were transferred to nitrocellulose membranes. Nonspecific protein binding was blocked by incubation with blocking buffer (10 mM Tris, 100 mM NaCl, 0.1% Tween 20, and 5% non-fat milk) overnight at 4°. To detect iNOS protein, the membranes were incubated for 3 hr at room temperature with rabbit polyclonal anti-iNOS antibody diluted 1:500 in blocking buffer. The membranes were washed and then incubated with donkey anti-rabbit IgG peroxidase conjugate, diluted 1:50,000 in blocking buffer, for 60 min at room temperature. The membranes were washed and developed using the SuperSignal® substrate according to the manufacturer's directions. Between each step the membranes were washed for 40 min with four changes of washing buffer every 10 min (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20).

Statistical Analysis

Data are expressed as means ± SEM. Statistical differences were detected by ANOVA. A two-way ANOVA followed by a Tukey post-hoc test was performed on the urinary NO_2^- and protein excretion rates. For all other comparisons, a one-way ANOVA was performed followed by a Newman–Keuls post-hoc test. $P < 0.05$ was considered significantly different for all tests performed.

RESULTS

In a preliminary study, we observed an increase in urinary NO_2^- excretion in rats 7 days after PAN administration. The following studies were performed to examine the role of NO in PAN-induced proteinuria. Urinary NO_2^- levels were monitored over a 7-day period in rats following PAN administration (Fig. 1). Control rats maintained a consis-

tent level of NO_2^- excretion throughout the time course of the experiment. PAN-treated rats showed a significant increase in urinary NO_2^- excretion beginning at day 2. Levels remained elevated through day 7 and were significantly different from Control ($P < 0.05$). The PAN + AG group showed levels of NO_2^- excretion not different from the Control, indicating that the iNOS inhibitor AG prevented the increase in PAN-induced NO_2^- excretion. Urinary NO_2^- levels in the AG group were similar to Control (data not shown). These results suggest that PAN administration causes an increase in NO production by iNOS.

NOx species are stable metabolites (NO_2^- and NO_3^-) of NO and often are used to monitor NO formation in biological fluids. On day 7 of the experiment, NOx levels were measured in plasma from all of the animals. Control animals had levels of 12 ± 1.0 μM (Fig. 2A). Both the PAN and the PAN + AG treatment groups showed a significant increase in plasma NOx levels to 22 ± 2.2 and 18 ± 2.0 μM, respectively, compared with the Control, but were not significantly different from each other. The total amount of NOx filtered (filtered load) during a 24-hr period was determined at day 7 for each animal by multiplying the plasma concentration of NOx by each animal's creatinine clearance. Control, PAN, and PAN + AG groups had creatinine clearance of 549 ± 41.3, 461 ± 35.7, and 472 ± 37.1 (μL/min/100 g body weight), respectively, and these values were not significantly different from one another. The filtered load of NOx is shown in Fig. 2B. Control animals filtered 26 ± 3.0 μmol/day. PAN treatment caused a significant increase in NOx filtered to 38 ± 2.4 μmol/day ($P < 0.05$ vs Control). In the PAN + AG group, the NOx filtered load decreased slightly to a value (33 ± 4.0 μmol/day) not different from PAN or Control groups. NOx excretion for day 7 is shown in Fig. 2C. Values were 10.1 ± 1.8, 4.6 ± 1.1, and 9.1 ± 1.5 μmol/day, for Control, PAN, and PAN + AG, respectively. Values for the PAN group were significantly different from the Control and PAN + AG groups ($P < 0.05$). The NOx value for the PAN + AG group was not significantly different from the Control group.

NO_2^- excretion was normalized to creatinine excretion for day 7. Values were 6.2 ± 1.5, 47.6 ± 11.8, and 10.9 ± 1.5 nmol/mg creatinine/day, for Control, PAN, and PAN + AG, respectively. NO_2^- excretion in the PAN group was significantly different from the other groups ($P < 0.05$). NOx excretion was also normalized to creatinine excretion for day 7. Values were 126 ± 18, 71 ± 14, and 136 ± 20 nmol/mg creatinine/day for Control, PAN, and PAN + AG, respectively. NOx excretion in the PAN group was significantly different from the other groups ($P < 0.05$).

iNOS expression in the kidney was evaluated on day 7 because the excretion of NO_2^- was increased following PAN administration and AG prevented this increase. Western blot analysis for iNOS was performed in isolated glomeruli and kidney cortex. No evidence of iNOS expression was observed in either the glomeruli (Fig. 3A) or

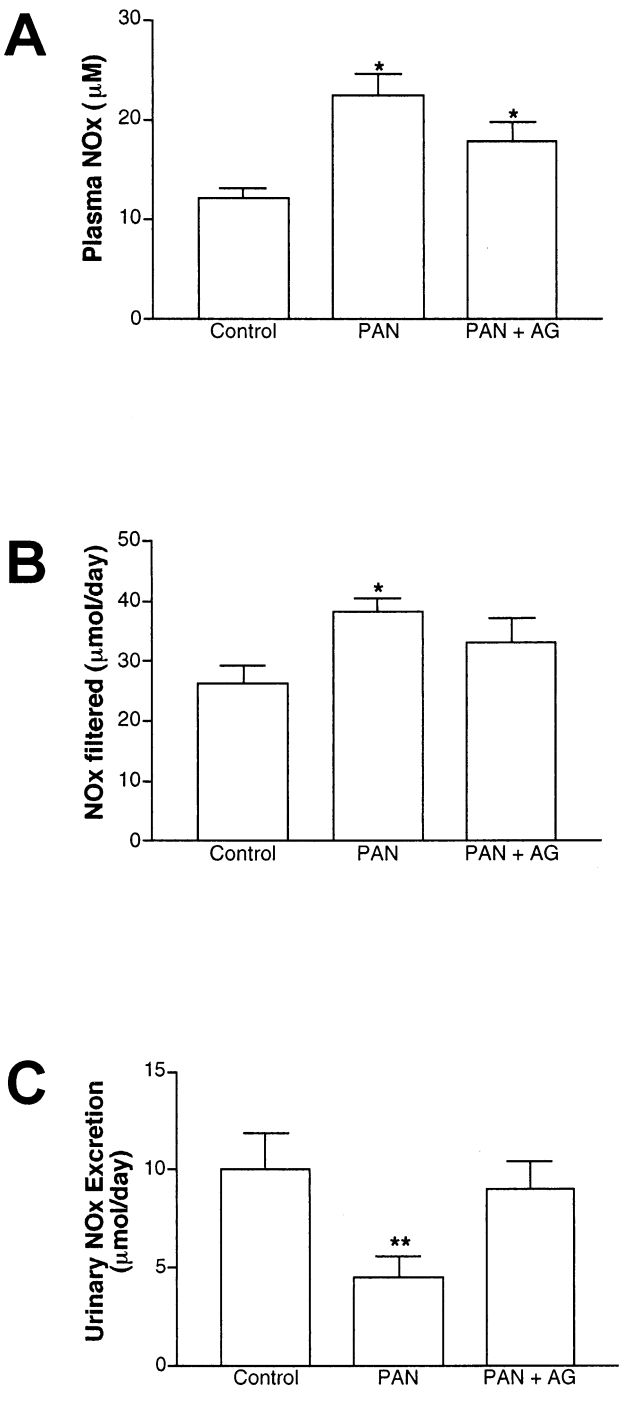

FIG. 2. Plasma NOx concentration, filtered load, and NOx excretion at day 7. NO_3^- + NO_2^- (NOx) are metabolic end products of NO formation. (A) Plasma NOx concentration. Key: (*) $P < 0.05$ vs Control. (B) Filtered load of NOx. Data are based on plasma NOx concentration and creatinine clearance. Key: (*) $P < 0.05$ vs Control. (C) Urinary excretion of NOx. Key: (**) $P < 0.05$ vs Control and PAN + AG. Data are expressed as means ± SEM. N = 9, 14, and 12 for the Control, PAN, and PAN + AG groups, respectively. Statistical significance was determined using a one-way ANOVA followed by the Student–Newman–Keuls test.

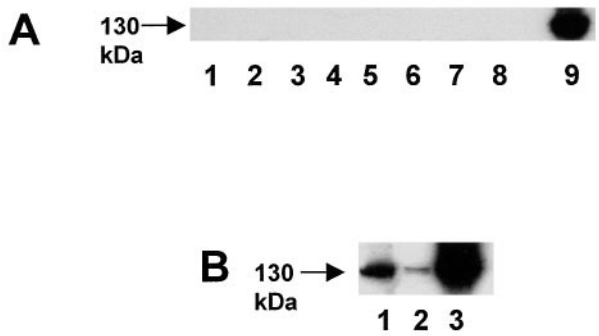

FIG. 3. Western blot analysis for iNOS in glomeruli and kidney cortex samples. As a positive control for iNOS detection, liver, kidney glomeruli, and kidney cortex were isolated from rats treated for 6 hr with LPS from *S. minnesota* (2 mg/kg, i.v.). (A) Kidney glomeruli isolated from PAN-treated animals on day 7. Lanes 1 and 2: Control; lanes 3–5: PAN; lanes 6–8: PAN + AG; lane 9: liver homogenate (25 μg protein) from LPS-treated rat. Results represent one of three independent blots. (B) Positive control for iNOS detection using kidney isolated from LPS-treated rats. Lane 1: kidney cortex (100 μg), lane 2: kidney glomeruli (25 μg), and lane 3: positive iNOS control (2 μg protein from macrophages activated with LPS and interferon-γ).

cortex (data not shown) from any of the groups. As a positive control for iNOS detection in the kidney, kidney glomeruli and kidney cortex were isolated from rats treated for 6 hr with LPS from *Salmonella minnesota* (2 mg/kg, i.v.) (Fig. 3B). An immunoreactive protein was detected at the expected mass of 130 kDa.

Daily urinary protein excretion in presented in Fig. 4. Control animals showed a consistently low level of protein excretion. In the PAN group, urinary protein excretion started to rise on day 4 and reached significant levels on days 5–7 compared with Control. The urinary protein levels in the PAN + AG group were not different from those of the PAN group. In the AG group, urinary excretion of protein was not different from Control (data not shown). These data suggest that iNOS-derived NO does not participate in PAN-induced proteinuria.

DISCUSSION

The present studies show that PAN-induced nephrotic syndrome is associated with an increase in NO_2^- excretion but a decrease in NOx excretion. However, changes in NO_2^- and NO_3^- handling by the kidney do not appear to participate in the development of glomerular injury as measured by proteinuria. The putative iNOS inhibitor AG did reverse the changes in urinary excretion of NO metabolites but did not affect proteinuria. No evidence of iNOS protein expression was found in glomeruli or cortex.

The rationale for examining the role of NO in PAN-induced glomerular injury comes from the recognized interactions between NO and reactive oxygen species. NO reacts with superoxide to produce the potent oxidant peroxynitrite at a rate constant higher than the reaction of superoxide with SOD [27]. Peroxynitrite can react with a wide range of biological targets including lipids [28], proteins, and DNA nitrogenous bases [23]. Thus, under conditions of oxidant stress, both reactive nitrogen and oxygen species may contribute to injury. Oxidant stress in the kidney following PAN administration has been well investigated [14]. Results showing that antioxidants and iron chelators prevent PAN-mediated injury also support the role of oxidants in injury [15–19]. It is important to note that these inhibitors also interfere with the generation of or directly scavenge reactive nitrogen species [22–24].

Our studies found that PAN administration caused significant alterations in NO metabolite excretion in PAN-treated rats that was inhibitable by AG. AG has been shown to be a useful inhibitor of iNOS *in vivo* and is

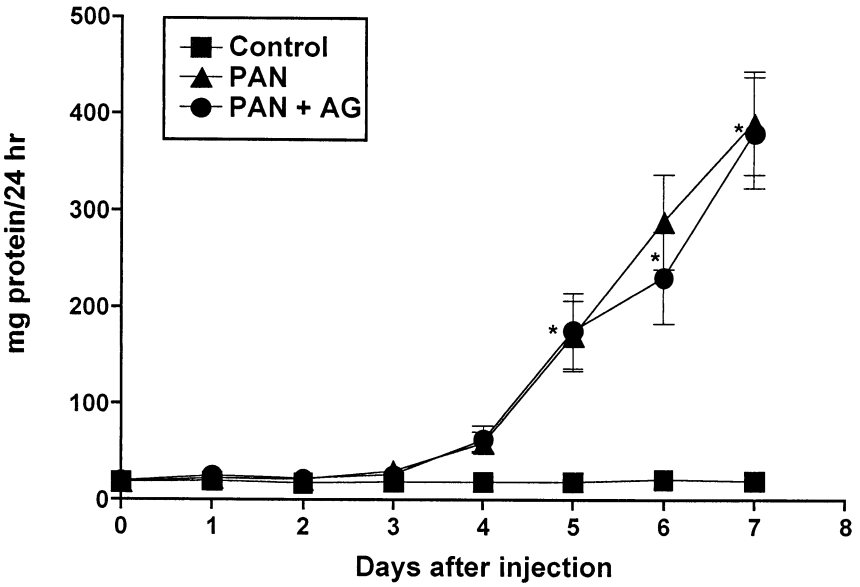

FIG. 4. Urinary protein excretion following PAN administration. Urine was collected daily from all animals, and protein excretion rates were measured using the Coomassie® Plus Protein Reagent. Data are expressed as means ± SEM. N= 9, 14, and 12 for the Control, PAN, and PAN + AG groups, respectively. Statistical significance was determined using a two-way ANOVA followed by Tukey's test. Key: (*) $P < 0.05$ for PAN and PAN + AG vs the Control group.

effective in various models of injury mediated at least in part by iNOS. The dose and dosing interval used in the present study were based on the published pharmacokinetics of AG and its use in other disease models in the rat [29]. Doses in this range retain relative iNOS selectivity *in vivo* [30]. Studies have shown that AG improves vascular function in diabetic animals [31], reduces proteinuria in chronic renal disease [32], and improves heart rate and mean arterial blood pressure in endotoxemia [33]. In chronic renal studies, AG has been used successfully to inhibit iNOS without adverse effects on renal function [34], whereas non-selective NOS inhibition with *N*-nitro-L-arginine methyl ester causes glomerular sclerosis [10]. Although AG reversed the changes in NO_2^- and NO_3^- excretion observed in PAN-treated animals, there was no evidence of iNOS induction in the kidney glomeruli or cortex at day 7. The detection of iNOS expression after LPS administration in rat cortex and glomeruli validated the protocol used to detect iNOS. This suggests that the protocol used could detect iNOS if it was expressed following PAN administration. The possibility remains that iNOS had been induced at an earlier time point and was down to undetectable levels at day 7. It is also possible that renal-derived NO generated from other NOS isoforms or simply changes in NOx handling by the kidney may account for changes seen in the NO_2^- and NO_3^- excretion. The use of other, newer iNOS inhibitors may provide more insight into these issues.

The role of NO has been studied in several models of glomerulonephritis. In anti-Thy-1 glomerulonephritis, iNOS is expressed rapidly by leukocytes infiltrating the glomerulus [11]. Inhibiting NOS blocked the increase in both urinary NO_2^- excretion and protein excretion [12]. In nephrotoxic serum nephritis, iNOS expression is enhanced in glomeruli isolated from nephritic animals [5]; however, the role of NO in this model remains unclear [6]. In anti-myeloperoxidase-associated crescentic glomerulonephritis, inflammatory cells infiltrating the glomerulus express iNOS and generate reactive oxygen and nitrogen species [8]. While NOx excretion correlates with protein excretion, iNOS inhibitors have yet to be evaluated in this model. In anti-glomerular basement membrane glomerulonephritis, iNOS expression is increased in glomeruli, but inhibition of iNOS worsens injury [7]. Clearly, the glomerular microenvironment is a major determinant of the physiology/pathophysiology of NO in the glomerulus. Furthermore, infiltrating inflammatory cells need not be the only source of iNOS. Mesangial cells in the glomerulus will also express iNOS in response to a variety of stimuli [35–37]. iNOS is also up-regulated by reactive oxygen intermediates [38, 39].

In an anti-Thy-1 model of proliferative glomerulonephritis Narita *et al.* [12] found increases in NO_2^- excretion during the first 24 hr following induction of injury. The increase in NO_2^- excretion and subsequent proteinuria were prevented by treatment with the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA). NO_3^- excretion was not examined in this model. In our studies in the PAN model of non-proliferative glomerulonephritis, NO_2^- excretion was also increased, and this increase was inhibitable by AG. In contrast to the anti-Thy-1 model, our studies found that proteinuria was not prevented by iNOS inhibition with AG. By determining both NO_2^- and NO_3^- excretion at day 7 in the PAN model we found differences in NO_2^- and NO_3^- excretion. Although there were increases in NO_2^- excretion, there was a net decrease in total NOx excretion in PAN-treated animals. These results further support the notion that NOx metabolite excretion may not be a reliable marker of NO activity in the kidney [40]. Since the filtered load in PAN-treated animals is elevated compared with Control, this suggests that there are changes in the reabsorption of NO metabolites. The enhanced reabsorption cannot be due to proteinuria alone since AG reversed the decrease in NOx excretion but not proteinuria. This study demonstrated that in contrast to proliferative glomerulonephritis, iNOS-derived NO does not appear to play a role in the proteinuria observed in the PAN model of nephrotic syndrome.

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