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Nitric Oxide Inhibition Enhances Platelet Aggregation in Experimental Anti-Thy-1 Nephritis

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In the present paper we studied the role of nitric oxide radicals (NO) on platelet aggregation, fibrinogen deposition, superoxide formation, peroxynitrite formation, hemodynamics, and leukocyte migration in the Thy-1 model of glomerulonephritis. To first study the baseline kinetics of these parameters, groups of anti-Thy-1-treated rats were sacrificed at 1 h, 4 h, 24 h, 3 days, 7 days, and 14 days and compared to controls. Urinary protein excretion was significantly elevated in Thy-1 nephritis at 3 and 7 days. Glomerular macrophages, PMNs, and superoxide anion-positive cells were significantly increased in Thy-1 nephritis. Nitrotyrosine immunoreactivity was absent during the entire study period. Glomerular platelet aggregation was significantly increased in anti-Thy-1 injected rats at 1 h, 4 h, 24 h, and 3 days. Glomerular fibrinogen deposition was significantly elevated at all time points. To elucidate the role of NO in this process, additional groups of anti-Thy-1-injected rats were treated with the NOS inhibitor L-NAME and studied at 24 h. Urinary protein excretion was significantly higher in L-NAME treated Thy-1 rats compared to nontreated Thy-1 rats. Plasma and urine nitrite/nitrate levels were significantly lower in L-NAME-treated Thy-1 rats compared to nontreated Thy-1 rats. Compared to nontreated Thy-1 rats, there were no differences in intraglomerular leukocyte accumulation after treatment with L-NAME. In contrast, we observed a marked increase in platelet aggregation following L-NAME treatment. From these data we conclude that the inflammatory infiltrate in Thy-1 nephritis develops independent of NO radical production, whereas NO radicals prevent the accumulation of platelet aggregates. © 2001 Elsevier Science

Nitric oxide radicals ($NO^{\bullet}$) mediate a variety of physiological and pathophysiological processes. They are produced in various cell types by the enzyme nitric oxide synthase (NOS), which consists of three isoforms: neuronal or brain NOS (type I, nNOS), inducible NOS (type II, iNOS), and endothelial NOS (type III, eNOS). In the kidney, nNOS is abundantly expressed in the macula densa (1) and the $NO^{\bullet}$ produced by these cells may play a crucial role in the regulation of glomerular hemodynamics in response to changes in composition of tubular fluid (2). iNOS is absent under physiologic conditions in most cell types, but can be induced by cytokines and endotoxin (LPS) (3, 4). Under normal conditions, iNOS is expressed in some tubular segments. eNOS is constitutively expressed in endothelial cells and plays a crucial role in vasorelaxation, leukocyte adherence, and inhibition of platelet aggregation. Unlike nNOS and iNOS, which are largely present in the cytosol, eNOS is predominantly localized in the plasma membrane (5).

$NO^{\bullet}$ have been implicated in the induction and progression of several types of experimental kidney diseases (reviewed in 6). Catell *et al.* (7) found increased nitrite production in glomeruli from rats

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with immune complex glomerulonephritis. iNOS mRNA was found to be increased in glomeruli from rats in which so called nephrotoxic nephritis was induced (8). By immunohistochemistry, iNOS has been localized in infiltrating macrophages in *in situ* immune-complex glomerulonephritis (9). In human acute renal transplant rejection we found upregulation of glomerular and interstitial iNOS predominantly in inflammatory cells, whereas glomerular eNOS protein expression was reduced (10). In Thy-1 nephritis, some iNOS expression is found in inflammatory cells in the glomerulus one hour after injection of the anti-Thy-1 antibody (11). In these animals, eNOS mRNA expression is increased biphasically with two peaks at 1 and 4 days (11).

Both *in vitro* and *in vivo,* NOS inhibition is known to result in platelet aggregation accompanied by a reduction in platelet cGMP content (12, 13). However, the contribution of cGMP-independent mechanisms to platelet aggregation has been described as well (14). Kurose *et al.* (15) noticed the formation of platelet-leukocyte aggregates in postcapillary venules from rat mesenteric venules after exposure to NO synthase inhibitors. This process could be inhibited by anti-P-selectin antibodies, suggesting that after stimulation, platelets rapidly mobilize P-selectin to the cell surface. A reduction in $NO^{\bullet}$ synthesis also results in enhanced leukocyte adhesion to the endothelium (16).

The anti-Thy-1 model of mesangio-proliferative glomerulonephritis (16) is associated with early glomerular influx of granulocytes, followed by massive glomerular macrophage infiltration and platelet aggregation (18, 19). There is a complement-dependent lysis of the mesangial cells, followed by a reparative phase that resembles human mesangio-proliferative nephritis. Since iNOS is only expressed at one hour after injection of anti-Thy-1 antibody in a limited number of inflammatory cells (11), eNOS is the main producer of NO radicals in anti-Thy-1 nephritis. We hypothesized that inhibition of NOS in the Thy-1 model of glomerulonephritis results in an increase in glomerular platelet aggregation and granulocyte and macrophage migration to the glomerulus. We therefore first studied the kinetics of platelet aggregation, glomerular leukocyte accumulation, and oxygen free radical production in experimental anti-Thy-1 nephritis. To further elucidate the role of $NO^{\bullet}$ in this process, we studied the effect of inhibition of NOS activity using the arginine analogue N^G-nitro-L-arginine methyl ester (L-NAME).

MATERIAL AND METHODS

Animal Procedures

The anti-Thy-1 antibody-induced glomerulonephritis is a well-established model of acute mesangial proliferative glomerulonephritis in the rat (17). In this study we used PVG/c rats, since mesangiolysis and subsequent mesangial fibrosis in these rats is very mild compared to other rat strains (19). Groups of female PVG/c rats ($n = 5$ per group) 6–8 weeks of age, weighing 150 g, were injected i.v. with 5 mg/kg body weight of monoclonal antibody ER4 (anti-Thy-1) dissolved in 0.5 ml saline. Rats injected with saline alone ($n = 10$), were used as controls. Groups of Thy-1-injected rats ($n = 5$ per time point) were sacrificed at 1 h, 4 h, 24 h, 3 days, 7 days, and 14 days in order to study the kinetics of glomerular granulocyte and macrophage accumulation, glomerular platelet aggregation, glomerular fibrinogen deposition, nitrotyrosine formation, and free radical formation of influxed cells. Subsequently, additional groups of rats were injected with either anti-Thy-1 antibody ($n = 5$) or saline ($n = 5$) and treated with the NOS inhibitor L-NAME (0.75 mg/ml). Rats received 1 ml L-NAME per gastric lavage 1 h prior to anti-Thy-1 injection and subsequently in the drinking water. Control rats received anti-Thy-1 ($n = 5$) or saline ($n = 5$) without L-NAME. All the rats were sacrificed at 24 h.

At sacrifice, serum and urine samples were collected for determination of nitrate/nitrite (serum/urine) and protein concentrations (urine). Systolic blood pressure was measured in the intervention study groups only. For that purpose, rats were anestesized with halothane, the right femoral artery was cannulated, using a polyethylene catheter (PE 50) connected to a pressure recorder (Model 91, Western Laboratories Co.), and systolic blood pressure was recorded during 1 min. Kidneys were flushed with saline and removed for further processing.

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Histologic and Immunohistologic Examinations

Coronal tissue slices were frozen in −80°C isopentane for immunohistology. Five-micrometer cryostat sections were immunohistochemically stained according to standard procedures. Sections were either fixed in 4% paraformaldehyde in PBS for 5 min (for the eNOS immunostaining) or in acetone (for all other immunostaining procedures). The following polyclonal and monoclonal antibodies were used for the study: Mouse monoclonal anti-rat monocyte and macrophage IgG_1 (ED1, Ref. 20, Serotec, Oxford, England); Mouse monoclonal anti-rat granulocytes (HIS 48, Ref. 21, Immunoquality Products, Groningen, The Netherlands); Mouse monoclonal anti platelets (PL-1, Ref. 22); Rabbit polyclonal anti-fibrinogen (Nordic, Tilburg, The Netherlands).

Primary antibodies were dissolved in PBS (pH 7.4) supplemented with 1% bovine serum albumin (BSA). Following incubation with these antibodies, the sections were incubated in appropriate dilutions of peroxidase labeled second step antibodies. All secondary antibodies were dissolved in 1% BSA/PBS containing 5% normal rat serum. Since the injected mouse anti-Thy-1 antibody localizes in the glomerular mesangial cells and will therefore react with secondary antibodies used in the immunosataining, we used isotype specific secondary antibodies for the detection of our monoclonal antibodies. Peroxidase activity was visualized using AEC. Sections were counterstained with Mayers Haematoxilin. Control sections were incubated with omission of the primary antibody. Superoxide anion (O^{2-})-producing cells were visualized using the method of Briggs *et al.* (23).

Thirty glomeruli were counted to determine the average number of glomerular ED1 positive cells, glomerular HIS 48-positive cells, and glomerular O^{2-}-positive cells. Platelet aggregation and fibrinogen deposition was determined by morphometry. Fifty glomeruli per kidney were screened moving from cortex to medulla and vice versa, using a light microscope equipped with a camera device connected to a video screen. The image of a given glomerulus present on the screen was traced with a cursor along the Bowman's capsule over the surface of a graphic tablet connected to the computer. Subsequently, the total surface area with red precipitate was measured and divided by the total surface of the glomerulus.

In order to determine whether L-NAME treatment had any effect on mesangial anti-Thy-1 deposition, sections were fixed in acetone and the injected anti-Thy-1 antibody was visualized by incubation of the sections with a peroxidase conjugated rabbit anti-mouse polyclonal antibody. The peroxidase activity was subsequently developed using AEC. Morphometry was performed in 50 glomeruli per kidney section as described for platelet aggregation.

Analytical Procedures

Nitric oxide metabolites (NOx) in serum and urine were measured according to the Griess method with modifications of Moshage *et al.* (24).

Urinary protein excretion was measured according to standardized methods using the Pyrogalol method.

Statistical Analysis

Reported values in the tables and result sections are group average and standard deviation. The data were evaluated for statistical differences by Mann–Whitney U test combined with Bonferoni correction. The null hypothesis of no difference between two groups was rejected when $P < 0.05$. The statistical package INSTAT was used for computations.

RESULTS

Anti-Thy-1 Antibody Deposition

Using computerized morphometrical analysis we found no differences in anti-Thy-1 deposition between anti-Thy-1 injected rats and L-NAME-treated anti-Thy-1-injected rats (13.6 ± 9.3 vs 9.7 ± 3.9% of total stained surface, respectively, NS), indicating that treatment with L-NAME has no effect on antibody deposition.

Urinary Protein Excretion and Systemic Blood Pressure

Urinary protein was significantly increased 3 days (5.2 ± 4.7 mg/24 h, $P < 0.05$) and 14 days (3.7 ± 0.63 mg/24 h, $P < 0.05$) after anti-Thy-1 injection

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TABLE I

Nitrite/Nitrate Levels in Serum and Urine from Anti-Thy-Treated Rats

	NO_2/NO_3 serum	NO_2/NO_3 urine
Controls	37 ± 9	2224 ± 816
4 h	26 ± 3*	ND
24 h	57 ± 15	1764 ± 446
3 days	41 ± 4	2852 ± 577
7 days	60 ± 8	2349 ± 991
14 days	39 ± 5	1803 ± 417

*$P < 0.05$ compared to control.

compared to controls (2.5 ± 0.6 mg/24 h). No significant differences were observed at 24 h (1.9 ± 0.4 mg/24 h) and 7 days 4.9 ± 1.0 mg/24 h). L-NAME-treated Thy-1 rats had significantly increased levels of proteinuria compared to untreated Thy-1 rats (23.6 ± 8.3 mg/24 h vs 3.7 ± 0.8 mg/24 h, $P < 0.01$). Although there was a numerical increase in proteinuria in control rats as well after L-NAME treatment (9.8 ±10.2 mg/24 h vs 2.7 ± 0.5 mg/24 h in untreated controls), this did not reach statistical significance ($P = 0.056$). During L-NAME treatment, systolic blood pressure rose significantly in anti-Thy-1-injected rats (135 ± 1.6 vs 08 ± 0.9, $P < 0.02$). A similar effect was observed in the saline injected rats after treatment with L-NAME (138 ± 5.4 vs 104 ± 3.5; $P < 0.01$).

Nitrite/Nitrate Analysis

Serum NOx values are presented in Table I. Serum NOx levels were significantly reduced at 4 h after Thy-1 injection compared to controls ($P < 0.05$). No differences were found at 24 h, 3 days, 7 days, and 14 days compared to controls. Serum nitrite/nitrate was not determined at 1 h after anti-Thy-1 injection. No significant differences were observed in nitrite/nitrate levels in urine from anti-Thy-1-injected rats compared to controls. L-NAME-treated Thy-1 rats had significantly lower values for serum nitrite/nitrate compared to nontreated Thy-1 rats ($P < 0.05$, Mann–Whitney) at 24 h. This effect was also seen in L-NAME-treated control rats ($P < 0.05$, Mann–Whitney). No significant differences were observed in urinary NOx levels between L-NAME-treated Thy-1 rats and nontreated Thy-1 rats (Table II).

TABLE II

Nitrite/Nitrate Levels in Serum and Urine from Anti-Thy-1 Rats Treated with L-NAME after 24 h

	NO_2/NO_3 serum	NO_2/NO_3 urine
Thy-1	118 ± 53	594 ± 488
Thy-1/L-NAME	32 ± 4*	124 ± 117
Saline	92 ± 46	873 ± 701
Saline/L-NAME	32 ± 9**	290 ± 475

*$P < 0.05$ compared to Thy-1.
**$P < 0.05$ compared to saline.

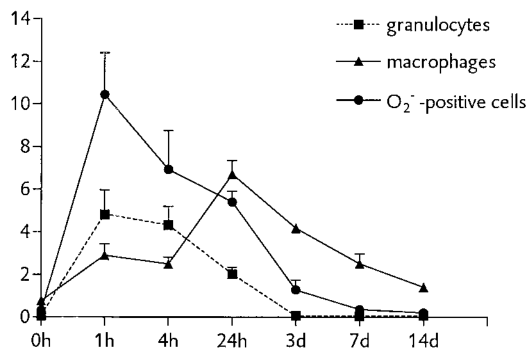

FIG. 1. Time course of glomerular leukocyte accumulation in anti-Thy-1 nephritis. Granulocytes and oxygen free radical producing cells peak at 1 h followed by a major increase in macrophages at 24 h. Most of the inflammatory cells are gone by 14 days.

Immunohistochemistry Nitrotyrosine

Nitrotyrosine-positive cells were largely absent during all time points.

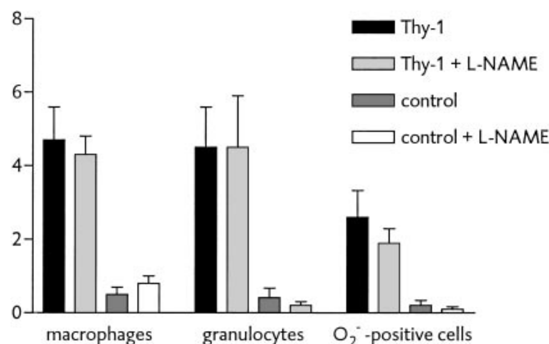

FIG. 2. Glomerular inflammatory cells counts in L-NAME-treated and untreated anti-Thy-1-injected rats and in L-NAME-treated and untreated controls at 24 h. L-NAME treatment did not influence the number of glomerular inflammatory cells.

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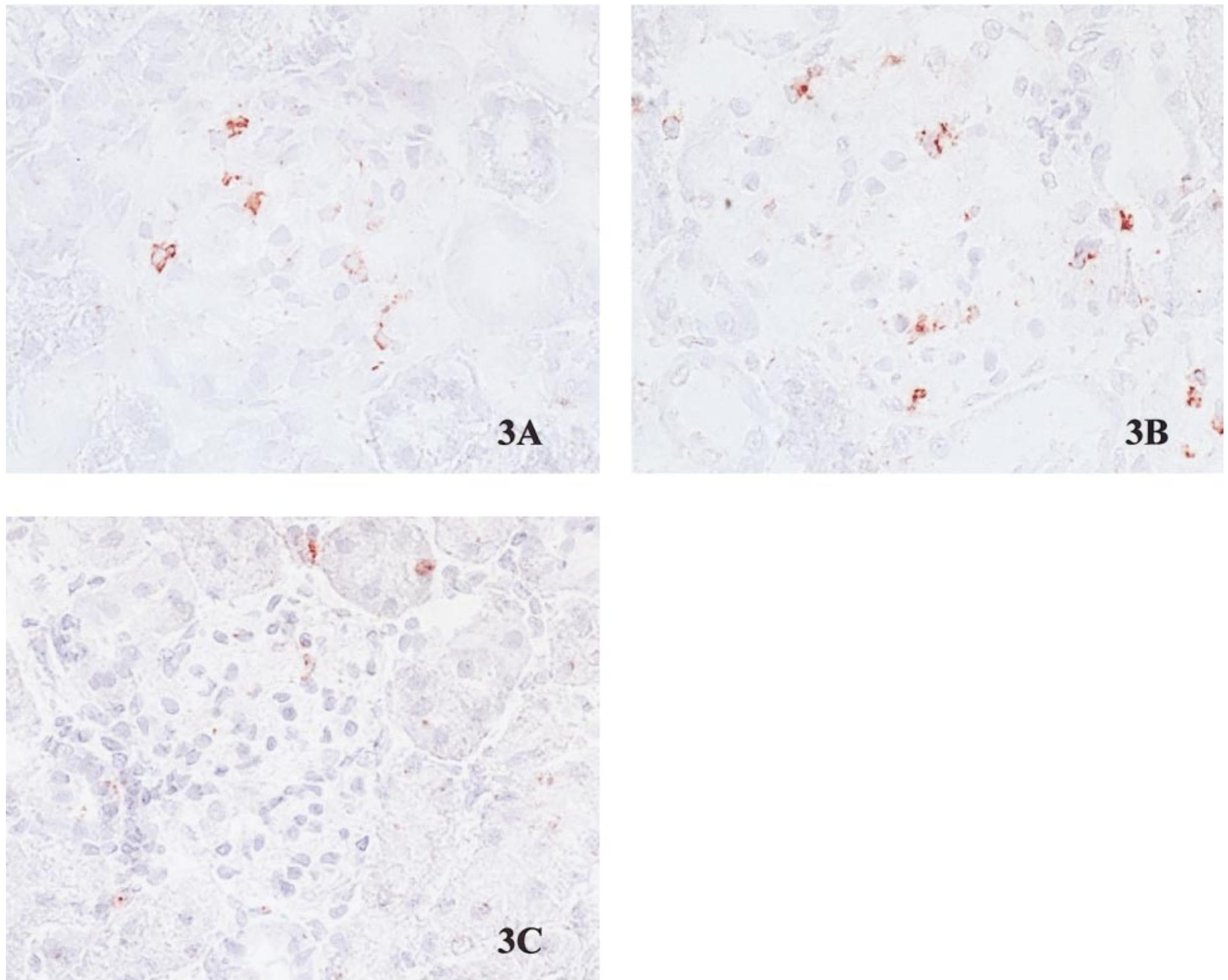

FIG. 3. Representative photograph of glomerular macrophage accumulation in anti-Thy-1-treated rats (A) at 24 h, anti-Thy-1-treated rats treated with L-NAME for 24 h (B) and control rats (C). Equal numbers of macrophages are clearly present (red dots) in the glomerular structures of anti-Thy-1-treated rats (with and without L-NAME treatment). Macrophages are absent in control rats.

Glomerular Leukocyte Accumulation

The time course of glomerular leucocyte accumulation is depicted in Fig. 1. Glomerular granulocytes (HIS 48) were significantly increased at 1 h, 4 h, 24 h, and 3 days ($P < 0.01$). No significant differences were observed at all later time points. Intervention with L-NAME had no effect on the recruitment of granulocytes. Anti-Thy-1-treated rats had 4.5 ± 1.1 granulocyte per glomerular profile and L-NAME-treated anti-Thy-1-injected rats had 4.5 ± 1.4 granulocyte per glomerular profile (Fig. 2, NS). No effects on granulocyte recruitment were seen in control rats following L-NAME treatment compared to nontreated control rats (Fig. 2; 0.2 ± 0.1 vs 0.42 ± 0.2).

Glomerular macrophage influx was significantly increased at all time points after anti-Thy-1 injection compared to saline injected rats (Fig. 1, $P < 0.01$). Intervention with L-NAME had no effect on glomerular macrophage recruitment (Fig. 2). Anti-Thy-1-treated rats had 4.7 ± 0.9 macrophage per glomerular profile and L-NAME-treated anti-Thy-1-injected rats had 4.3 ± 0.5 macrophage per glomerular profile (NS). No significant effects on macrophage recruitment was seen in control rats following L-NAME treatment (Fig. 2) compared to nontreated control rats (0.8 ± 0.2 vs 0.5 ± 0.2). Representative

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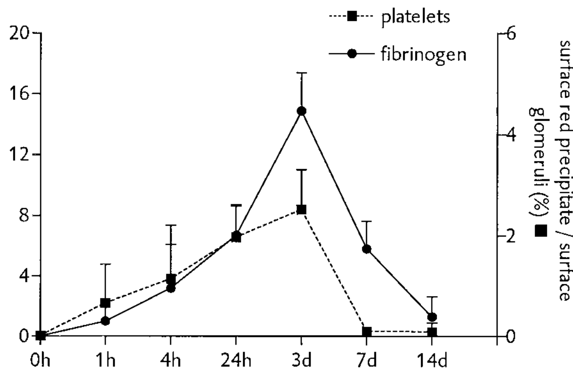

FIG. 4. Time course of glomerular platelet aggregation and fibrinogen deposition in anti-Thy-1 nephritis. Both platelet aggregation and fibrinogen deposition peak at 3 days after injection of anti-Thy-1 antibody.

photographs of controls and anti-Thy-1-treated rats with and without L-NAME treatment are presented in Figs. 3A, 3B, and 3C.

Oxygen free radical producing cells which are basically subsets of granulocytes, which have been activated during glomerular accumulation. O^{2-}-positive cells were significantly increased at 1 h, 4 h, and 24 h (Fig. 1, $P < 0.01$). No significant differences were seen at 3–7 days. Intervention with L-NAME had no effect on the number of O^{2-}-positive cells in the glomerulus (Fig. 2). Anti-Thy-1-treated rats had 2.6 ± 0.7 cells per glomerular profile and L-NAME-treated anti-Thy-1-injected rats had 1.9 ± 0.4 cells per glomerular profile (Fig. 2, NS). No significant effects on the number of O^{2-}-positive cells was seen in control rats following L-NAME treatment compared to nontreated control rats (0.1 ± 0.1 vs 0.2 ± 0.1).

Glomerular Platelets Aggregation and Fibrinogen Deposition

Glomerular platelet aggregation was measured morphometrically. We noted significant increments in platelet aggregation at 1 h ($P < 0.05$), 4 h, 24 h, and 3 days ($P < 0.01$) following anti-Thy-1 injection compared to controls. The time course is depicted in Fig. 4. Intervention with L-NAME in anti-Thy-1-treated rats resulted in a fivefold increase in glomerular platelet aggregation (Fig. 5, $P < 0.02$) compared to control rats (Fig. 5). Representative photographs of platelet staining in controls and anti-Thy-1-treated rats with and without L-NAME treatment (Fig. 6).

Fibrinogen deposition was significantly increased at all time points (Fig. 4, $P < 0.01$). No measurements were performed at 1 h. Treatment with L-NAME resulted in a numerical increase in fibrinogen deposition in both anti-Thy-1 and injected and saline injected rats (Fig. 5). This did not reach statistical significance.

DISCUSSION

The present study provides evidence for the role of nitric oxide radicals in the progression of experimental anti-Thy-1 nephritis. The major finding of the study is that inhibition of NOS results in a marked upregulation of platelet aggregation without changes in leukocyte accumulation. In addition we noted a significant increase in urinary protein excretion following NOS inhibition.

We used the PVG/c rat to study the kinetics of Thy-1 nephritis, since this rat does not develop extensive renal damage at the later time points, although mesangial apoptosis is clearly present in the early time points. This is in contrast to other rat strains in which injection of the anti-Thy-1 antibody leads to massive glomerular damage (18). Therefore, this Thy-1 nephritis model in PVG/c rats enables us to study platelet aggregation and intraglomerular leukocyte influx without the potential influences upon these parameters due to ongoing renal damage.

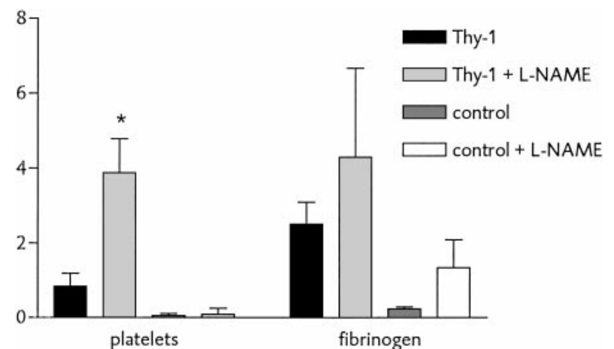

FIG. 5. Glomerular platelet aggregation and fibrinogen deposition in L-NAME-treated and untreated anti-Thy-1-injected rats and in L-NAME-treated and untreated controls at 24 h. L-NAME treatment significanlty increased platelet aggregation in anti-Thy-1-injected rats ($P < 0.05$).

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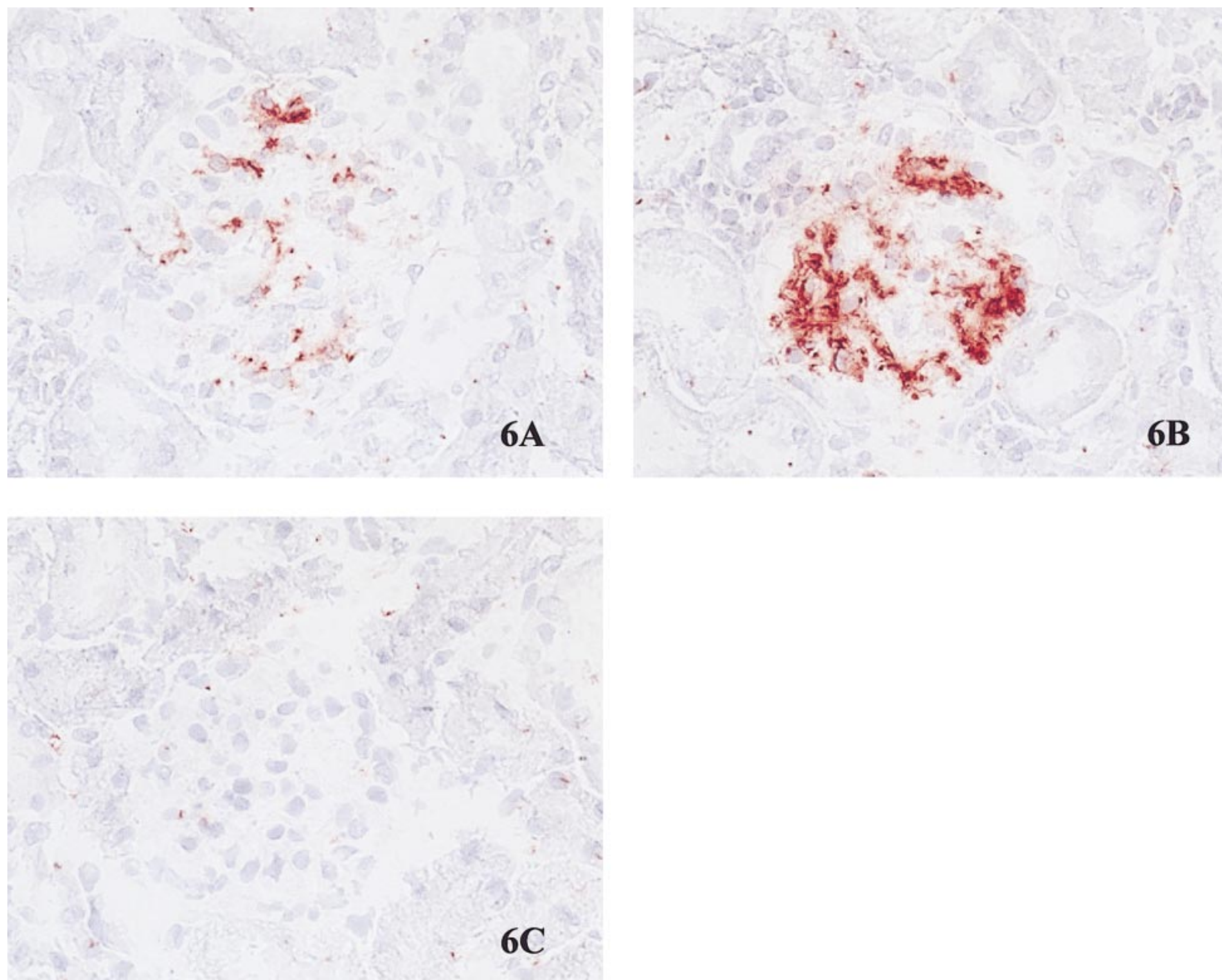

FIG. 6. Representative photograph showing glomerular platelet aggregation in anti-Thy-1-treated rats (A) at 24 h, anti-Thy-1-treated rats treated with L-NAME for 24 h (B) and control rats (C). Platelet agregation is clearly present in untreated rats and massively increasesed after L-NAME treatment. Control glomeruli are negative.

Plasma and urine NOx levels levels did not change in our time course study except for a reduction in plasma nitrate/nitrite at 4 h postinjection of the Thy-1 antibody. In contrast, after L-NAME treatment we noticed a significant reduction in plasma NOx, most probably caused by the inhibition of endothelial NOS. The reduction in plasma NOx did not result in a significant change in urine NOx. Urinary excretion of NOx also depends on tubular handling, since extensive nitrate reabsorbtion in proximal tubules has been described (25). Systolic blood pressure was enhanced in L-NAME-treated rats. This in concordance with the role of eNOS in hemodynamic regulation (26).

Urinary protein excretion was significantly increased at 3 days and 24 days postanti-Thy-1 injection. L-NAME treatment in these rats resulted in sixfold increase in urinary protein excretion. Chronic nitric oxide inhibition promotes progressive arterial hypertension and renal damage (27, 28) associated with markedly increased urinary albumin losses (27, 29). Recent work points to an impairment in both glomerular size and charge selectivity as a cause of glomerular protein loss, reflecting functional rather that structural disruption of the glomerular wall (30). Although we studied L-NAME treatment for only 24 h, hypertension was clearly present, suggesting that similar mechanisms may have played a role in our model as well.

Previous studies have shown that glomerular

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iNOS protein expression is largely absent in this model with only a limited number of iNOS-positive granulocytes in the first hour after injection of the antibody (11). This implicates that endothelial NOS is the major glomerular source of NO radicals in this model. We first studied the kinetics of platelet aggregation and leukocyte accumulation in a time course study, revealing a peak in granulocyte accumulation as early as 1 hour, confirming previous reports (11). $O2^-$-positive cells also peak at 1 h. These cells are mainly granulocytes activated during the inflammatory response. Our data reveal that not all the granulocytes present within the glomerular tuft are activated to such an extent that they produce oxygen-free radicals. Macrophage influx follows the influx of granulocytes, peaking at 24 h after injection of anti Thy-1 antibody. We detected a marked increase of platelets from 1 h to 3 days. Others have described an increase in eNOS mRNA expression at 1 and 3 days (11), suggesting compensatory upregulation of eNOS to prevent platelet aggregation. The accumulation of platelets may play a role in the progression of the disease through release of fibrogenic growth factors such as platelet derived growth factor alpha (31). Platelet aggregation coincided with the deposition of fibrinogen deposits.

To gain better insight in the potential role of NO radicals in the aggregation of platelets and the accumulation of leukocytes, we treated anti-Thy-1-injected rats with the NOS inhibitor L-NAME. Since both leukocyte adherence and platelet aggregation are in part regulated by NO radicals (14–16) we expected increased values for both parameters after treatment with L-NAME. Platelet aggregation was markedly enhanced, suggesting a major role for eNOS derived NO radicals in this process. L-NAME also numerically increased fibrinogen deposition. In contrast, we found no difference in accumulation of both granulocytes and macrophages, suggesting that other factors play a more specific role in the inflammatory response to anti Thy-1 nephritis. Chemokine release from damaged and activated mesangial cells may well play a major role in this process.

We conclude that NO radicals play a crucial role in the development of Thy-1 nephritis by inhibiting the formation of platelet aggregates without affecting the migration of inflammatory cells to the glomerulus.

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