Agmatine Inhibits Cell Proliferation and Improves Renal Function in Anti–Thy-1 Glomerulonephritis

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Abstract. Changes in the expression of alternate arginine metabolic pathways have been implicated in the pathogenesis of experimental glomerulonephritis. Agmatine, decarboxylated arginine, has been shown in vitro to suppress both inducible nitric oxide synthase and the rate-limiting enzyme of polyamine biosynthesis, ornithine decarboxylase (ODC). This study was undertaken to determine whether agmatine administration could reduce tissue injury by decreasing nitric oxide, and reduce cell proliferation, by diminishing ODC activity, in experimental mesangial proliferative glomerulonephritis (Thy-1 nephritis). Agmatine treatment (50 mg/kg per d intra-peritoneally) in Thy-1 nephritis rats prevented a reduction in GFR at day 1. Agmatine treatment decreased nitric oxide production in Thy-1 nephritis rats by 23% and 41% at days 1 and 4, respectively. Agmatine treatment also reduced ODC activity and glomerular \(^{3}H\)-thymidine incorporation on days 1, 4, and 7. Histologic evaluation revealed a decline in mesangial cell proliferation and extracellular matrix accumulation associated with agmatine treatment administered before or 24 h after Thy-1 antibody, and this was confirmed by a reduction in the number of cells expressing proliferating cell nuclear antigen on days 4 and 7. These studies provide the first in vivo evidence that agmatine administration can reduce cellular proliferation in Thy-1 nephritis and attenuate the initial reduction in renal function associated with this model.

Two well-described pathways of L-arginine metabolism in inflammation include the conversion of arginine to nitric oxide (NO) by nitric oxide synthase (NOS), and the breakdown of L-arginine to urea and L-ornithine by arginase. These pathways are temporally regulated in inflammatory models of wound healing and glomerulonephritis (GN) (1,2). In the early injury phase of inflammation, there is induction of inducible NOS (iNOS) expression and generation of NO (3–5). High-output NO generation from iNOS during periods of cellular stress is known to exert cytostatic and cytotoxic effects. This is followed by the production of ornithine, which initiates the repair phase (1). Ornithine is converted to polyamines via the rate-limiting polyamine biosynthetic enzyme, ornithine decarboxylase (ODC). Polyamines are required components of cell cycle entry and progression and thus are essential for cellular proliferation (6). Ornithine is also metabolized by ornithine aminotransferase (OAT) to proline, an important constituent of extracellular matrix (3). Therefore, the production of ornithine and its subsequent metabolism to polyamines and proline are important elements of the repair phase.

A third arginine metabolic pathway, the conversion of arginine to arginine by arginine decarboxylase (ADC), has recently been described in mammals (7). ADC activity is prevalent in the kidney, liver, and brain. Similarly, agmatine is present in the plasma and has been observed in multiple tissues and cell types (8–10). Agmatine exerts functional effects within the kidney, elevating single nephron filtration rate (SNGFR) via vasodilatation by constitutive NOS (cNOS) and ryanodine channel-dependent mechanisms (10,11). Agmatine also suppresses NO generation by iNOS in vitro (12,13; Satriano et al., submitted). Studies have recently shown that agmatine inhibits ODC activity and polyamine transport by an ODC-antizyme–dependent mechanism (14). These data suggest that agmatine could exert important effects on both NO production and cell proliferation in inflammatory conditions.

It is widely recognized that NO production is increased after induction of accelerated nephrotoxic-serum GN, active Heymann GN, \textit{in situ} immune complex GN, and anti–Thy-1 antibody–induced GN (Thy-1 nephritis) (4,5,15). Thy-1 nephritis is a model of acute mesangial proliferative GN in rats that is characterized by early mesangial cell (MC) injury, followed by MC proliferation (16,17). Augmentation of arginase activity and ODC and OAT expression in the repair phase result in cellular proliferation and extracellular matrix production in this...
showing slight lucency (0 to 25% disruption of MC); 2 = MA showing moderate lucency (25 to 50% disruption of MC) with preservation of the underlying glomerular tuft architecture; 3 = MA showing severe lucency (50 to 75%) with degeneration and disruption of MC; 4 = MA showing complete dissolution (75 to 100%) with disappearance of MC, usually in association with microaneurysm formation. Matrix score: 0 = decrease in mesangial matrix (MM); 1 = no increase in MM; 2 = slight increase in MM; 3 = moderate increase in MM; 4 = almost confluent appearance of MM. Each score reflects changes in the extent rather than in the intensity of MM staining. Proliferation score: 0 = no nests of proliferating cells (PC); 1 = normal number of PC; 2 = slight increase in PC; 3 = moderate increase in PC; 4 = substantial increase in PC. To compare the glomerular cell number quantitatively, we individually counted nuclei and polymorphonuclear leukocytes (PMN) in 30 glomeruli from each rat.

Materials and Methods

Disease Model and Experimental Protocol

Animal experiments were performed using male Sprague-Dawley rats that weighed 200 to 225 g. Rats were allowed free access to normal rat chow and tap water. The mouse monoclonal antibody against rat Thy-1.1 (OX7) was a generous gift from Dr. T. Yamamoto (Niigata University School of Medicine, Japan). This antibody binds to the Thy-1 antigen expressed on the MC membrane and leads to mesangiolysis followed by marked MC proliferation (16,17). The animals were divided into three groups. The Thy-1 nephritis group (Thy-1, n = 18) received a single 0.1 ml/kg body wt intravenous injection of anti-Thy-1 antibody on day 0. The agmatine-treated Thy-1 nephritis group (Thy-1+Ag, n = 18) received a 50 mg/kg body wt intraperitoneal injection of agmatine (Sigma Chemical Co., St. Louis, MO) 2 h before administration of anti-Thy-1-antibody and then daily. The agmatine group (Ag, n = 18) served as a positive control and received the same daily intraperitoneal injections of agmatine as the Thy-1+Ag group. Untreated rats were used as a normal control group (Control, n = 6). In a separate group of animals, agmatine was administered 24 h after disease induction to confirm that the alterations in proliferation were not due to agmatine’s ability to limit initial tissue injury (N = 5). At each time point, rats were anesthetized with sodium brevital (65 mg/kg intraperitoneally) and killed immediately after the kidneys were removed at days 0 (Control), 1 (24 h), 4, and 7 after anti-Thy-1 antibody injection. The kidneys were immediately fixed for microscopic studies. Kidney cortical slices were obtained for measurement of ODC activity, and glomeruli isolated from the cortex were used for the cell proliferation, nitrre, and guanosine 3′, 5′-cyclic monophosphate (cGMP) assays.

Awake Animal Studies of GFR and Urinary Protein Excretion

To measure 3H-inulin clearance, cannulated rats were prepared as previously described (21,22). In brief, animals were anesthetized and catheters were placed in the left femoral artery, vein, and bladder. After a 3-d recovery from the cannulation procedure, rats were divided into three experimental groups: Thy-1–treated rats, Thy-1+Ag–treated rats, and agmatine-only–treated rats (n = 6 per group). Three measurements of GFR in each animal were performed at days 0, 1, 4, and 7. For determination of urinary protein excretion, a 24-h urine collection was filtered, precipitated by 10% trichloroacetic acid (TCA), collected by centrifugation, and measured by the method of Lowry et al. (23).

Renal Morphology

Kidney tissue was fixed in 10% buffered formalin and embedded in paraffin. The paraffin-embedded tissues were sectioned and stained with periodic acid Schiff. Three observers, blinded to the treatments, semiquantitatively graded glomerular injury, extracellular matrix accumulation, and cellular proliferation of each quadrant in 30 glomeruli per kidney on a scale from 0 to 4 using the following scales. Injury score: 0 = absence of mesangiolysis; 1 = mesangial area (MA) showing slight lucency (0 to 25% disruption of MC); 2 = MA showing moderate lucency (25 to 50% disruption of MC) with preservation of the underlying glomerular tuft architecture; 3 = MA showing severe lucency (50 to 75%) with degeneration and disruption of MC; 4 = MA showing complete dissolution (75 to 100%) with disappearance of MC, usually in association with microaneurysm formation. Matrix score: 0 = decrease in mesangial matrix (MM); 1 = no increase in MM; 2 = slight increase in MM; 3 = moderate increase in MM; 4 = almost confluent appearance of MM. Each score reflects changes in the extent rather than in the intensity of MM staining. Proliferation score: 0 = no nests of proliferating cells (PC); 1 = normal number of PC; 2 = slight increase in PC; 3 = moderate increase in PC; 4 = substantial increase in PC. To compare the glomerular cell number quantitatively, we individually counted nuclei and polymorphonuclear leukocytes (PMN) in 30 glomeruli from each rat.

ODC Activity Assay

The activity of ODC was determined by measuring the conversion of L-(14C)-ornithine to 14CO2. After the kidneys were removed, sections of the cortex were immediately suspended in ODC reaction buffer (10 mM Tris [pH 7.4], 2.5 mM dithiothreitol, 0.3 mM pyridoxal-5-phosphate, 0.1 mM ethylenediaminetetraacetate) and homogenized for 10 s. The homogenate was centrifuged at 30,000 x g for 40 min, and the supernatants were assayed for ODC activity as described (14). Briefly, large bore tubes capped with rubber stoppers fitted with metabolic wells (Kontes, Vineland, NJ) containing trapping agent
MgCl₂, 10 mM glucose, and 15 mM NaHCO₃. Then the suspension was passed through a 75-μm sieve. The aliquots were incubated for an additional 4 min, then 100 μM 1-methyl-xanthine (Sigma Chemical Co.) to inhibit degradation of KCl, 1 mM CaCl₂, 0.75 mM NaH₂PO₄, 0.75 mM Na₂HPO₄, 0.5 mM MgCl₂, 10 mM glucose, and 15 mM NaHCO₃. Then the suspension was passed through a 75-μm sieve. The glomeruli trapped on the sieve were washed and pelleted by centrifugation at 3000 RPM for 1 min. After graded sieving, glomeruli were suspended in Dulbecco’s modified Eagle’s medium (DMEM) without phenol red for the cell proliferation and nitrite assays. The remaining pellet was resuspended in Krebs buffer, aliquoted, and used for the cGMP assay.

Isolation and Incubation of Glomeruli

After the kidneys were removed, they were decapsulated and bisected and the cortex was carefully dissected free. Cortices were gently pressed through a 180- then a 106-μm stainless steel sieve. The resulting material was suspended in Krebs buffer at 4°C and gassed with 95% O₂/5% CO₂. The buffer consisted of 125 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.75 mM NaH₂PO₄, 0.75 mM Na₂HPO₄, 0.5 mM MgCl₂, 10 mM glucose, and 15 mM NaHCO₃. Then the suspension was passed through a 75-μm sieve. The glomeruli trapped on the sieve were washed and pelleted by centrifugation at 3000 RPM for 1 min. After graded sieving, glomeruli were suspended in Dulbecco’s modified Eagle’s medium (DMEM) without phenol red for the cell proliferation and nitrite assays. The remaining pellet was resuspended in Krebs buffer, aliquoted, and used for the cGMP assay.

Glomerular Cell Proliferation Assay

The proliferation assay was performed using the method described by Ketteler et al. (3). After graded sieving, cell strainers were placed in a six-well plate and glomeruli resuspended in DMEM (3 ml/well) were pipetted onto the cell strainers. [³H]-thymidine (1 μCi/well) was added to the cell strainers and incubated for 24 h. The cell strainers were then removed from the six-well plate, rinsed with cold PBS, placed into the six-well plate again, and washed three times for 10 min with 2 ml of 5% TCA per well. Then 2 ml of 0.5 M NaOH/0.1% sodium dodecyl sulfate was added to each well, and the plates were agitated at 100 RPM for 30 min. Samples were neutralized with 0.6 mol/L HCl, and radioactivity in 500-μl aliquots was determined.

Nitrite Assay

Nitrite was measured using the Griess reaction (24). After the isolated glomeruli were incubated for 24 h in DMEM in a 24-well plate, the supernatants were harvested and centrifuged at 14,000 × g for 5 min to remove the glomeruli. Total nitrite was then measured at 550 nm in an autoplatter reader. Isolated glomeruli in the Thy-1 group were also incubated for 24 h in DMEM with 100 μM L-NIL or 100 μM agmatine to assess in vitro their inhibitory effects on NO production. Assays were done in triplicate.

cGMP Assay

We measured cGMP, a second messenger of NO, to estimate indirectly glomerular NO activity. The aliquots (50 μl/tube) of glomeruli resuspended in Krebs buffer were incubated at 37°C for 10 min and then were added to 50 μl of Krebs buffer with 1 mM 3-isobutyl-1-methyl-xanthine (Sigma Chemical Co.) to inhibit degradation of cGMP. The aliquots were incubated for an additional 4 min, then 100 μl of 10% TCA was added to terminate the reaction. The aliquots were centrifuged at 3000 RPM at 4°C for 20 min. The pellet was extracted in 2 N NaOH and used for protein determination. The aliquots in the Thy-1 group were also preincubated for 10 min with 10 mM sodium nitroprusside (SNP; Sigma Chemical Co.) to assess whether a direct NO donor could elicit an increase in cGMP levels. The supernatants obtained from the aliquots were ether extracted and lyophilized, and the remaining pellets were used for RIA determination of cGMP with an RIA kit (Dupont-NEN).

Statistical Analyses

All data are expressed as the mean ± SEM. Histologic data were averaged across three raters, across 30 glomeruli within each quadrant, and across quadrants to yield mean injury, proliferation, and matrix scores for each animal. The data were then evaluated with a two-way ANOVA and post hoc Tukey test. GFR was measured repeatedly in each animal, and the data were analyzed by repeated measures ANOVA. The remainder of the data were analyzed by two-way ANOVA followed by Tukey test for multiple group comparisons.

Results

GFR and Urinary Protein Excretion

In Figure 1, the GFR in each group is compared with its control. On day 1, the GFR in the Thy-1 group declined to 83% of control levels. However, in the Thy-1 rats treated with agmatine, there was a significant increment in the GFR (P < 0.05). This elevation in GFR persisted in the Thy-1 + Ag group on day 4, but the values did not reach statistical significance. In the agmatine-only–treated group, there was no appreciable change in GFR. Proteinuria in the Thy-1 and Thy-1 + Ag groups was similar on day 1 (167 ± 32 versus 158 ± 28, respectively); however, by days 5 through 7, there was a significant decline in protein excretion (day 7: 113 ± 5 versus 86 ± 8, P < 0.05, Thy-1 versus Thy-1 + Ag).

Histologic Studies

The glomeruli on day 1, as assessed by light microscopy, showed signs of injury with diminished cell numbers in both

![Figure 1. GFR in Thy-1–treated and –untreated rats and effects of agmatine administration. Each point is the mean ± SEM of six rats, and the control value is the mean GFR of each group on day 0. On day 1, there was a significant increase in GFR in the Thy-1 rats treated with agmatine as compared with the Thy-1 group. The agmatine-only–treated group had no significant change in GFR during the study period. * P < 0.05 compared with Thy-1 group.](image)
Figure 2. Light microscopic assessment of renal morphology in Thy-1 rats. (A) A normal glomerulus. Glomeruli from rats in the Thy-1 group developed severe mesangiolysis (B, day 1), characterized by reduced nuclear number (arrowhead). On day 4 (C), there was focal and segmental hypercellularity in the mesangium and increased mesangial matrix (arrowhead). By day 7, there was a marked increase in mesangial cell (MC) number and extracellular matrix (D, nests of proliferating cells [arrowhead]). Mesangiolysis in the Thy-1 + Ag group (E, day 1, microaneurysm formation [arrowhead]) was similar as compared with the Thy-1 group. However, there was a significant decrease in MC proliferation and extracellular matrix accumulation in the Thy-1 + Ag group on days 4 and 7 (F, G). Periodic acid-Schiff (PAS), ×500.
the Thy-1 and Thy-1+Ag groups as compared with the controls (Figure 2, B and E, versus Figure 2A, respectively), and the histologic appearance of the two Thy-1–treated groups was similar. Our evaluation showed no significant difference in the injury score in the two groups at day 1, but the sample size may have limited the power to detect a small difference (Figure 3). However, other studies support that there was an equivalent degree of injury in both treatment groups. First, immunofluorescence studies revealed no qualitative difference in distribution or intensity of the staining of anti–Thy-1 antibody or in the number of ED-1–positive cells between the Thy-1 and Thy-1+Ag groups (Table 1). Furthermore, there was no difference in the total number of nuclei and PMN on day 1 (Table 1), and there was no alteration in the degree of proteinuria (167 ± 32 versus 158 ± 28, Thy-1 versus Thy-1+Ag), suggesting that agmatine did not influence the initial degree of injury. By day 4, the glomeruli in the Thy-1 group were characterized by focal and segmental hypercellularity in the mesangium with occasional disintegration of the mesangial architecture (Figure 2C). This was followed at day 7 by a marked increase in extracellular matrix and MC proliferation (Figure 2D). In contrast, there was a decrease in the proliferation and matrix scores at day 4, which became statistically significant at day 7 in the Thy-1+Ag group (Figures 2, F and G, and 3). Reductions both in the number of cells that stained for PCNA (Figure 4) and in 3H-thymidine incorporation (Figure 5A) in the Thy-1+Ag group as compared with the Thy-1 group provide additional evidence that agmatine treatment diminished cellular proliferation at days 4 and 7.

Administration of Agmatine after Induction of Thy-1 Nephritis

Agmatine was also administered 24 h after induction of Thy-1 nephritis to provide evidence that the alterations in proliferation and matrix accumulation induced by agmatine were not secondary to changes in the initial degree of injury. The matrix and proliferation scores again were reduced in the agmatine-treated group, and ODC activity was reduced by almost 50% by agmatine treatment (Table 2). These findings suggest that agmatine’s ability to reduce cellular proliferation and matrix accumulation in this model is not due to a reduced degree of injury.

Glomerular Cell Proliferation and ODC Activity

Proliferation in isolated glomeruli was measured by 3H-thymidine incorporation. Glomerular cell proliferation in the Thy-1 group was significantly increased on day 4 (3403 ± 183 versus 2424 ± 54, Thy-1 versus Control, P < 0.01), and the Thy-1+Ag group exhibited significantly reduced cellular proliferation on days 1, 4, and 7 as compared with the Thy-1 group (2130 ± 52, 2630 ± 175, 2197 ± 57, P < 0.05 versus Thy-1, Figure 5A). The group that was treated only with agmatine served as a time control on days 1 through 7. We also measured ODC activity, the rate-limiting polyamine biosynthetic enzyme, as another index of the effect of agmatine on MC proliferation. ODC activity in the Thy-1 group achieved significant peak levels on day 1 (25559 ± 2243 versus 1168 ± 252 cpm/mg protein, day 1 versus Control, P < 0.01) and was maintained above control levels on days 4 and 7 (22000 ± 2211, 16719 ± 1923, P < 0.01). ODC activity in the Thy-1+Ag group was also markedly elevated above the control during the experimental period (11430 ± 527, 11536 ± 726, 2151 ± 213, P < 0.01 versus Control) but was significantly lower than that of the Thy-1 group (P < 0.05 versus Thy-1, Figure 5B) on all days evaluated. Addition of L-NIL to the supernatants from the Thy-1 group did not cause a significant difference in ODC activity during the experimental period (data not shown). This suggests that reductions in NO were not responsible for alterations in ODC activity.

Table 1. The number of glomerular cells, infiltrating cells, and quantitative assessment of the binding of anti–Thy-1 antibody

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>No. of Nuclei Per Glomerulus</th>
<th>No. of PMN Per Glomerulus</th>
<th>No. of ED-1 Cells Per Glomerulus</th>
<th>Goat Anti-Mouse IgG Staining</th>
</tr>
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<tbody>
<tr>
<td>Thy-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 1</td>
<td>3</td>
<td>56.5** ± 2.3</td>
<td>1.75 ± 0.50</td>
<td>12.5 ± 1.4</td>
<td>4+</td>
</tr>
<tr>
<td>day 4</td>
<td>3</td>
<td>61.2 ± 2.3</td>
<td>0.55 ± 0.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 7</td>
<td>3</td>
<td>77.1 ± 8.8</td>
<td>0.73 ± 0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thy-1+Ag</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>day 1</td>
<td>3</td>
<td>56.4** ± 1.6</td>
<td>1.48 ± 0.28</td>
<td>10.8 ± 1.2</td>
<td>4+</td>
</tr>
<tr>
<td>day 4</td>
<td>3</td>
<td>55.3 ± 2.4</td>
<td>0.3 ± 0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 7</td>
<td>3</td>
<td>65.7 ± 3.9</td>
<td>0.5 ± 0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>3</td>
<td>64.0 ± 1.3</td>
<td>0.43 ± 0.02</td>
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</tbody>
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PMN, polymorphonuclear leukocytes. The number of nuclei and PMNs were counted individually in 30 glomeruli in PAS stained slides. There was no significant difference in histologic variables on day 1 between the Thy-1 and Thy-1+Ag groups. However, both groups on day 1 had a reduced nuclear number as compared with the control. The values are expressed as mean ± SEM of three rats. **P < 0.05 compared with control group.
Nitrite Production in Isolated Glomeruli

Nitrite production in glomeruli isolated from rats in the Thy-1 group achieved significant peak levels on day 1 (172.9 ± 22.2 versus 6.5 ± 6.5 nmol/mg protein, Thy-1 versus Control, P < 0.01), remained high on day 4 (73.1 ± 9.7, P < 0.01), and declined by day 7 (17 ± 1.4, not significant). Nitrite production in the Thy-1 Ag group was also elevated above control, but these levels were only 77% and 59% of those measured in the Thy-1 group on days 1 and 4, respectively (Figure 6). Addition of L-NIL to glomeruli isolated from the Thy-1–treated rats greatly reduced NO products to 17% on day 1 and 11% on day 4 (data not shown), suggesting that the increase in NO production was due to iNOS activity. When compared with the L-NIL effects, in vitro addition of agmatine demonstrated only weak inhibitory effects on NO production (79% at day 1 and 76% at day 4 of Thy-1 levels).

Intracellular Glomerular cGMP Concentration

cGMP, the second messenger of NO, was measured to assess indirectly the alterations in NO production. Paradoxically, despite the high levels of NO measured in the Thy-1–treated groups at days 1 and 4, there was no physiologically significant increase in cGMP as compared with the controls (agmatine-only–treated group, Figure 7). To examine the response of guanylate cyclase to an NO donor, SNP was added to glomeruli isolated from the Thy-1 group on days 1 and 4, respectively (Figure 6). Addition of L-NIL to glomeruli isolated from the Thy-1–treated rats greatly reduced NO products to 17% on day 1 and 11% on day 4 (data not shown), suggesting that the increase in NO production was due to iNOS activity. When compared with the L-NIL effects, in vitro addition of agmatine demonstrated only weak inhibitory effects on NO production (79% at day 1 and 76% at day 4 of Thy-1 levels).

Discussion

Agmatine, decarboxylated arginine, has been shown to exert multiple effects on arginine metabolic pathways (10,14,25–27; Satriano et al., submitted). The majority of this work defining the actions of agmatine has been performed in vitro. Herein we describe that agmatine can exert beneficial renal functional and glomerular cellular effects in vivo, in the Thy-1 nephritis model.

Thy-1 nephritis has an early injury phase characterized functionally and histologically by a reduction in GFR and by mesangiolysis. This is followed by the repair phase in which there is extensive cellular proliferation and matrix accumulation. In this study, administration of agmatine significantly attenuated the decline in GFR without appreciably altering the initial degree of injury. During the repair phase, which is associated with markedly elevated ODC activity, agmatine substantially reduced ODC activity in the Thy-1–treated rats. Similarly, there was a decrease in cellular proliferation and matrix accumulation in the rats treated with agmatine, and these alterations did not seem to be attributable to reduced binding of anti–Thy-1 antibody to MC or to reduced glomerular infiltration by monocytes/macrophages.

The first phase of Thy-1 nephritis lasts up to 1 d after administration of the Thy-1 antibody. Glomerular injury is

day 1 of the Thy-1 GN model, it is likely that despite elevated NO, the decline in cGMP levels is more likely caused by a reduction in GTP availability or altered redox environment due to mesangial injury. By day 7 in both the Thy-1 and Thy-1 + Ag groups, there was a significant increase in cGMP levels as compared with the control group (P < 0.01), suggesting restoration of guanylate cyclase activity or GTP levels during the MC proliferative phase.

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The first phase of Thy-1 nephritis lasts up to 1 d after administration of the Thy-1 antibody. Glomerular injury is

Figure 3. Semiquantitative analysis of glomerular tissue injury, cellular proliferation, and extracellular matrix scores. Each quadrant in 30 glomeruli per rat was graded on a scale from 0 to 4 on PAS-stained slides. There was no difference in the injury score between the Thy-1 and Thy-1 + Ag groups on day 1. However, the Thy-1 + Ag group exhibited significant declines in both proliferation and matrix scores on day 7. The histogram represents the average score per quadrant of 30 glomeruli assessed in each rat. Each point is mean ± SEM of three rats. *, P < 0.05 compared with the Thy-1 group.

Figure 4. Quantification of the number of cells stained with proliferating cell nuclear antigen (PCNA) in 30 glomeruli per animal. There was a significantly fewer number of cells stained with PCNA in the Thy-1 + Ag group at days 4 and 7 as compared with the Thy-1 group. *, P < 0.05 compared with Thy-1 group. Each point is mean ± SEM of four rats.
partially due to the effects of high-output NO generation by iNOS, and the expression of this inducible enzyme has been localized to infiltrating macrophages and polymorphonuclear leukocytes rather than intrinsic glomerular cells (3,5,19). This suggests that the arginine/NO pathway is involved in the pathogenesis of tissue injury. In fact, inhibition of NO synthesis with NG -monomethyl-D-arginine (L-NMMA), a nonspecific NOS inhibitor, has been reported to reduce MC lysis significantly (28).

Agmatine has been shown in vitro to inhibit iNOS, resulting in decreased NO production (12,13; Satriano et al., submitted). However, in this study, there was only a 23% and 41% decline in NO production in the agmatine-treated Thy-1 rats as compared with untreated Thy-1 rats on days 1 and 4. The lack of a significant alteration in the degree of injury may have been due to this limited inhibition of NO production. However, it is possible that the levels of NO measured in this study may have underestimated in vivo levels, because they were performed ex vivo on isolated glomeruli, incubated for 24 h in DMEM without exogenous agmatine.

In the present study, agmatine treatment significantly reversed the decline in GFR in the Thy-1–treated rats (Figure 1) and the reduction in NO production by agmatine treatment on day 1 was probably not sufficient to explain the preservation of GFR observed (Figure 6). Similarly, the agmatine-mediated improvement in GFR cannot be attributed to a difference in initial injury (Figures 2 and 3) or to a reduction in the recruitment of macrophage/monocytes into glomeruli (Table 1) on day 1. It is interesting that the control rats treated with 50 mg/kg of agmatine intraperitoneally did not exhibit an increase in GFR, which seems at odds with our earlier findings that agmatine increases SNGFR (10). The most likely reason for this finding is that the doses of agmatine achieved intrarenally in this study probably were much lower than the 1 μM urinary space concentrations achieved in the previous microperfusion experiments.

Table 2. Evaluation on day 7 of effect of administration of agmatine 24 hours after induction of Thy-1 nephritis

<table>
<thead>
<tr>
<th></th>
<th>Thy-1 Group</th>
<th>Thy-1 + Agmatine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear number</td>
<td>82.5 ± 7.2</td>
<td>73.6 ± 4.5</td>
</tr>
<tr>
<td>Proliferation score</td>
<td>2.4 ± 0.08</td>
<td>1.9 ± 0.13*</td>
</tr>
<tr>
<td>Matrix score</td>
<td>2.3 ± 0.11</td>
<td>1.9 ± 0.14*</td>
</tr>
<tr>
<td>ODC levels</td>
<td>20164 ± 2565</td>
<td>11187 ± 2340*</td>
</tr>
</tbody>
</table>

* ODC, ornithine decarboxylase. Values expressed are means ± SEM of five rats. * P < 0.05 compared with Thy-1 group.
The present study demonstrated that agmatine administration can improve GFR and, thus, renal function in this in vivo model of inflammatory renal disease. Agmatine can also mark-

Figure 7. Glomerular intracellular guanosine 3′, 5′-cyclic monophosphate (cGMP) concentrations. In isolated glomeruli on day 1, there was a small increase in cGMP generation in the Thy-1+Ag group as compared with the Thy-1 group. Because the NO levels were highest at this time, it is unlikely that this increase was physiologically significant. By day 7, cGMP levels were elevated in both the Thy-1 and Thy-1+Ag groups as compared with the control and agmatine groups. Each point is mean ± SEM of six rats. #, P < 0.01 compared with control.

We previously demonstrated that microperfusion of agmatine into the renal interstitium or into the urinary spaces of surface nephrons produces an increase in nephron filtration rate, which is largely mediated by an increase in plasma flow caused by vasodilatation (10). These effects of agmatine were prevented by coadministration of a nonselective NOS blocker, indicating that the vasodilatation partially depends on constitutive NOS (11). Other groups have shown that agmatine may function as an endothelial NOS (eNOS) agonist (29). In the Thy-1 nephritis model, we recently demonstrated that pretreatment with L-NIL, a specific eNOS inhibitor, did not prevent a reduction in GFR at day 1, whereas the nonselective inhibitor L-NAME further decreased GFR, suggesting that activation of cNOS may preserve GFR (Ishizuka et al., unpublished observations). Likewise, Goto et al. (19) reported an increase in eNOS activity in the early phase of this model. Recently, we demonstrated that production of high levels of NO in the lipopolysaccharide (LPS) model can downregulate the eNOS enzymes (isoform switching) (30). Pretreatment with agmatine can prevent the reduction in BP and GFR in the LPS model (Satriano et al., submitted). These data suggest that agmatine preserves eNOS effects in pathologies associated with high NO production, such as GN.

In addition to its effects on NOS isoforms, agmatine has been shown to alter polyamine synthetic pathways, important in cellular proliferation. Increased ODC expression has been described in animal models of diabetes and LPS-induced inflammation (31–33). Agmatine administration results in the inhibition of ODC activity and the concurrent inhibition of polyamine transport by promoting translation of the ODC antizyme (14). The resulting depletion of intracellular polyamine levels by agmatine administration suppresses proliferation (14).

The data from the present studies demonstrate that ODC activity is markedly increased as early as day 1 after anti-Thy-1 antibody administration, and these high levels of activity persisted during the later cell proliferation phases (Figure 5B). Agmatine treatment in Thy-1 rats significantly attenuated the increase in ODC activity and resulted in reduced glomerular cell proliferation (Figures 3 and 5B). The histologic observation of a decrease in cellular proliferation was supported by a decrease in both the number of PCNA-positive staining cells and in glomerular 3H-thymidine incorporation at days 4 and 7 (Figures 4 and 5A). On the basis of these data, it is likely that agmatine reduces MC proliferation via suppression of ODC.

Agmatine therapy clearly reduced cell proliferation by day 7 in Thy-1 nephritis. We have argued that this reduction in cellular proliferation is a direct effect of agmatine therapy. However, the amelioration of cell proliferation also could have derived from lesser cell injury on day 1 as a consequence of agmatine administration. Therefore, we made considerable efforts to assess this issue and did not discern any difference in initial glomerular injury between untreated and agmatine-treated Thy-1 nephritis rats, using histologic scoring, evaluation of the number of nuclei, infiltrating PMN and macrophages, and extent of binding of anti-Thy-1 antibody. ODC levels were reduced by agmatine therapy on day 1, but we interpret these findings as early indicators of cell proliferation rather than as indices of cellular injury. Nevertheless, we acknowledge that small differences in initial injury may be difficult to detect despite our objective efforts. Because of this possibility, we designed other studies that obviate the possibility of an agmatine effect on initial MC injury. In a second group of rats, we began agmatine therapy 24 h after administration of the Thy-1 antibody, thereby guaranteeing that the initial injury was equivalent in both untreated and agmatine-treated rats. When these results are evaluated (Table 2), we observed reductions in parameters of cell proliferation including histologic evaluation and ODC activity in the rats that received agmatine beginning 24 h after the initial immune insult. We conclude from these separate studies that agmatine exerts a significant antiproliferative effect that is independent of any influence on initial glomerular injury.

Herein we provide the first in vivo results of the effects of agmatine on cellular injury and repair. Agmatine influences MC proliferation and the accumulation of extracellular matrix in the Thy-1 nephritis rats, when given before and after disease induction. Although this model was not performed to assess effects of agmatine on different NOS isoforms, further studies should be undertaken to examine how the agmatine pathway affects the balance of NOS isoform activities in GN and regulates NO and polyamine synthesis to mediate the antiproliferative effects.
edly reduce proliferative changes by inhibiting ODC activity, an effect previously demonstrated \textit{in vitro}.

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**References**