Mycophenolate Mofetil Reduces Renal Injury in the Chronic Nitric Oxide Synthase Inhibition Model

Clarice Kazue Fujihara, Denise Maria Avancini Costa Malheiros, Irene de Lourdes Noronha, Gilberto De Nucci, Roberto Zatz

Abstract—We and others have recently shown that mycophenolate mofetil (MMF) reduces renal inflammation and glomerular and interstitial injury in the 5/6 renal ablation model. In the present study, we investigated whether MMF limits renal injury in a model of chronic nitric oxide (NO) inhibition associated with a high-salt diet and characterized by progressive systemic hypertension, albuminuria, glomerular sclerosis and ischemia, interstitial expansion, and progressive macrophage infiltration. Adult male Münch-Wistar rats were distributed among 3 groups: HS, rats receiving a high-salt diet (3.2% Na); HS+N, HS rats orally treated with the NO inhibitor Nω-nitro-L-arginine methyl ester (L-NAME), 25 mg · kg⁻¹ · d⁻¹; and HS+N+MMF, HS+N rats orally treated with MMF, 10 mg · kg⁻¹ · d⁻¹. Renal hemodynamics were studied after 15 days of treatment; histological and immunohistochemical studies were conducted after 30 days of treatment. MMF treatment did not reverse the hemodynamic alterations characteristic of this model.

Renal injury in the HS+N group was associated with macrophage and lymphocyte infiltration. Treatment with MMF reduced glomerular and interstitial injury and associated macrophage and lymphocyte infiltration. These results suggest that renal inflammation is a strong independent factor in the pathogenesis of the nephropathy associated with the HS+N model. (Hypertension. 2001;37:170-175.)

Key Words: mycophenolate mofetil ■ nitric oxide ■ renal inflammation ■ renal hemodynamics

Chronic inhibition of NO synthesis by L-arginine analogues, such as Nω-nitro-L-arginine methyl ester (L-NAME), promotes progressive arterial hypertension associated with proteinuria and severe renal vascular, glomerular, and interstitial injury.1,2 These events are exacerbated by the concomitant administration of a high-sodium diet (HS).3,4 The pathogenesis of renal injury in rats receiving HS and L-NAME (HS+N model) has not been clarified. Hemodynamic factors such as glomerular hypertension are likely to play a role because treatment with drugs that lower glomerular pressure ameliorates renal injury in this model.1,3,5 On the other hand, there is growing evidence that renal inflammation participates in the pathogenesis of renal injury in immune-mediated and nonimmune-mediated progressive nephropathies.6,7 NO inhibition enhances leukocyte adhesion and migration,8,9 the expression of adhesion molecules,9,10 and type I collagen.11,12 Accordingly, NO inhibition stimulates cell proliferation and macrophage infiltration.13 Thus chronic NO inhibition would be expected to favor the development of renal inflammation. We recently reported preliminary evidence that renal injury in HS+N rats is accompanied by a marked macrophage infiltration and renal cell proliferation.14

We and others have shown that the immunosuppressors mycophenolate mofetil (MMF), used to prevent allograft rejection,15,16 reduces renal lymphocyte and macrophage infiltration and attenuates renal injury in rats with 5/6 renal ablation, a nonimmune-mediated model of progressive nephropathy.17,18 In the present study, we investigated whether MMF can similarly reduce inflammation and ameliorate renal injury in HS+N rats.

Methods

Experimental Groups

Fifty-two adult male Münch-Wistar rats, obtained from an established colony at the University of São Paulo, Brazil, and weighing initially 240 to 270 g, were used in this study. The animals were maintained at 23 ± 1°C on a 12/12 hours light/dark cycle, with free access to tap water, and received HS (3.2% Na, Harland Teklad) for 2 weeks. The rats were then divided into 3 groups: Group HS, receiving HS and no drug treatment; Group HS+N, receiving HS and L-NAME (Sigma Co), 0.5 mmol/L dissolved in drinking water, corresponding to a daily intake of ~25 mg/kg; and Group HS+N+MMF, receiving HS, L-NAME, and MMF (Roche Laboratories), 10 mg · kg⁻¹ · d⁻¹. MMF was dissolved in a mixture of dimethylsulfoxide and olive oil, the final concentration of dimethylsulfoxide being 5%. The compound was administered by gavage once daily in a volume of vehicle never exceeding 0.3 mL. The HS and HS+N groups received vehicle only. All experimental procedures were conducted in accordance with our institutional guidelines.

Renal Hemodynamic Studies

Fifteen days after the beginning of treatments, 4 HS rats, 5 HS+N rats, and 5 HS+N+MMF rats were subjected to renal hemodynamic
studies after being anesthetized with Inactin, 100 mg/kg IP, and placed on a temperature-regulated surgical table. Femoral arterial pressure was continuously monitored with a computerized data acquisition system. Saline solution containing \(^{14}C\)-tagged inulin (0.3 \(\mu\text{Ci/mL}\)) and homologous rat plasma was infused through the jugular veins. Urine was collected from the left ureter for measurement of flow rate and inulin concentration. Blood samples were obtained from the left renal vein with a sharpened glass micropipette. Hydraulic pressures in superficial glomeruli (\(P_{\text{OC}}\)), tubules (\(P_{\text{T}}\)), and efferent arterioles (\(P_{\text{E}}\)) were measured with a servo-nulling device. Inulin extraction (equivalent to filtration fraction), glomerular filtration rate, renal plasma flow, and renal vascular resistance were calculated with standard equations. Further details of the methods used in these hemodynamic studies are given elsewhere.19

Long-Term Studies
Twelve HS, 13 HS+N rats, and 13 HS+N+MMF rats were followed-up for 30 days of treatment. At this time, the tail-cuff pressure was measured by an indirect method,17 and rats were placed in metabolic cages for determination of 24-hour urinary albumin excretion rate (\(U_{\text{alb V}}\)) by radial immunodiffusion.17 Rats were thereafter anesthetized with sodium pentobarbital, 50 mg/kg IP, and blood was collected from the abdominal aorta for determination of plasma creatinine concentration (\(P_{\text{crea}}\)). The kidneys were then perfusion-fixed with Dubosq-Brazil solution after a brief washout with saline. After fixation, the renal tissue was weighed and 2 midcoronal sections were postfixed in buffered 4% formaldehyde solution. The material was then embedded in paraffin for assessment of glomerular and renal cortical interstitial injury and for immunohistochemical identification of lymphocytes and macrophages.

Histomorphometry
Sections 2–3 \(\mu\text{m}\) thick were stained with periodic acid-Schiff or Masson-stained sections by a point-counting technique in 25 consecutive microscopic fields, at a final magnification of 100× in \(\mu\text{m}\)-thick Masson-stained sections and 3–4-\(\mu\text{m}\)-thick Masson-stained sections and

Interstitial Injury
The fraction of the renal cortex occupied by interstitial tissue staining positively for extracellular matrix constituents was quantified in Masson-stained sections by a point-counting technique in 25 consecutive microscopic fields, at a final magnification of 100× under a 176-point grid.

Vascular Injury
The frequency of myointimal proliferation and fibrinoid necrosis in renal small arteries and arterioles was evaluated under 400× magnification in 3- to 4-\(\mu\text{m}\)-thick Masson-stained sections and expressed as a percentage of the total number of microvessels examined (55 per section in average). Myointimal fibrosis and microthrombosis were also noted in Group HS+N, but because their frequency was very small, these lesions were not included in the quantitative analysis.

Immunohistochemical Analysis
Macrophages and T-lymphocytes were detected in 4-\(\mu\text{m}\)-thick paraffin-embedded sections. These sections were mounted on glass slides coated with 2% gelatin, deparaffinized in xylene, and rehydrated through graded ethanol and then in deionized water. Sections were then microwave irradiated in citrate buffer to enhance antigen retrieval, and preincubated with 5% normal rabbit serum in Tris-buffered saline or in phosphate-buffered saline to prevent unspecific protein binding.

Optimal working dilutions of the primary antibodies were previously determined in titration experiments. Negative control experiments for all antigens were performed by omitting incubation with the primary antibody.

For specific immunostaining of T-lymphocytes, a monoclonal mouse anti-rat CD-3 antibody (Seralab, Oxford, UK) and an indirect streptavidin-biotin alkaline phosphatase technique were used. Sections were preincubated with normal horse serum to reduce nonspecific staining and then with avidin and biotin solutions to block nonspecific binding of these compounds. The incubation with the primary antibody was performed at room temperature for 90 minutes. Sections were then incubated at room temperature, first with rat-adsorbed biotinylated anti-mouse IgG (Vector Labs, Burlingame, Calif) for 45 minutes, and then with the streptavidin-biotin-alkaline phosphatase complex (Dako Co, Denmark) for 30 minutes.20 Sections were finally incubated with a freshly prepared substrate, consisting of naphthol AS-MX-Phosphate and fast-red dye (Sigma Chemical Co), counterstained with Mayer’s hemalum and covered with Käser’s glycercin-gelatin (Merck).

For detection of macrophages, a monoclonal mouse anti-rat ED-1 antibody (Serotec, Oxford, UK) was used. Incubations were performed overnight at 4°C in a humidified chamber. After the sections were washed, they were incubated with rabbit anti-mouse immunoglobulin (Dako Co, Denmark). To complete the sandwich technique, incubation with a soluble complex of alkaline phosphatase anti-alkaline phosphatase (APAAP, Dako Co, Denmark) was performed. The last 2 steps were repeated to enhance the intensity of the reaction product. Finally, slides were developed with a fast-red dye solution and counterstained as in the lymphocyte detection procedure.

Statistical Analysis
One-way ANOVA with pairwise comparisons according to the Newman-Kuls formulation was used in this study.17 \(P\) levels of 0.05 or less were considered significant.

Results
Renal function and hemodynamic parameters at 15 days of treatment are shown in Table 1. Body growth was similar

| Table 1. Renal Functional and Hemodynamic Parameters at 15 Days of Treatment |
|-----------------------------------|--------|-------|--------|--------|--------|--------|
| Groups                            | BW, g  | LKW, g | MAP, mm Hg | GFR, mL/min | RPF, mL/min | POC, mm Hg |
| HS (n=4)                          | 292±2  | 1.6±0.04 | 112±3 | 1.61±0.06 | 6.0±0.4 | 54±2 |
| HS+N (n=5)                        | 286±7  | 1.6±0.07 | 168±8* | 0.96±0.13* | 2.2±0.4* | 68±4* |
| HS+N+MMF (n=5)                    | 297±4  | 1.7±0.06 | 166±6* | 1.10±0.05* | 2.4±0.2* | 71±3* |

Results are expressed as mean±1 SE. BW, body weight; LKW, left kidney weight; MAP, mean arterial pressure; GFR, glomerular filtration rate; RPF, renal plasma flow; and POC, glomerular hydraulic pressure.

* \(P<0.05\) vs HS.
TABLE 2. Body Weight, Tail-cuff Pressure, Albuminuria, and Plasma Creatinine at 30 Days of Treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>BW, g</th>
<th>TCP, mm Hg</th>
<th>U₆₆₅V, mg/day</th>
<th>Pₖₑₜₜ, µmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS (n=12)</td>
<td>324±3</td>
<td>117±3</td>
<td>1.4±0.2</td>
<td>45.1±3.5</td>
</tr>
<tr>
<td>HS+N (n=13)</td>
<td>304±5*</td>
<td>173±4*</td>
<td>148±33*</td>
<td>80.4±4.4*</td>
</tr>
<tr>
<td>HS+N+MMF (n=13)</td>
<td>303±5*</td>
<td>166±5*</td>
<td>68±15†</td>
<td>64.5±5.3†</td>
</tr>
</tbody>
</table>

Results are expressed as mean±1 SE. BW, body weight; TCP, tail-cuff pressure; U₆₆₅V, daily urinary albumin excretion rate; and Pₖₑₜₜ, plasma creatinine concentration.

*P<0.05 vs HS; †P<0.05 vs HS+N.

TABLE 3. Quantification of Glomerular, Interstitial, and Vascular Injury at 30 Days of Treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>Renal parenchymal</th>
<th>Vascular</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GS</td>
<td>COL</td>
</tr>
<tr>
<td>HS (n=12)</td>
<td>0.4±0.2</td>
<td>2±0.5</td>
</tr>
<tr>
<td>HS+N (n=13)</td>
<td>3.5±0.7*</td>
<td>30±2.0*</td>
</tr>
<tr>
<td>HS+N+MMF (n=13)</td>
<td>0.9±0.2†</td>
<td>17±2.0†</td>
</tr>
</tbody>
</table>

Results are expressed as mean±1 SE. GS, percent glomerulosclerosis; COLL, percent glomerular collapse; GN, percent glomerular necrosis; INT, percent interstitial area; MIP, percent myointimal proliferation; and FN, percent fibrinoid necrosis.

*P<0.05 vs HS; †P<0.05 vs HS+N.

Discussion

As shown in previous studies by this laboratory,3,4 the association of chronic L-NAME treatment and salt overload resulted in systemic hypertension, creatinine retention, and severe renal injury characterized by massive albuminuria, COLL, glomerulosclerosis, glomerular necrosis, interstitial inflammation, and microvascular injury. The marked glomerular hypertension observed in HS+N rats likely contributed to the development of renal injury, as observed in other models.21 Additional damage may have derived from inflammatory phenomena, as suggested by the finding of marked renal lymphocyte and macrophage infiltration in HS+N rats.23 Renal inflammation may have enhanced the production of cytokines and growth factors6,22 and promote podocyte damage with formation of tuft synchiae23 and leakage of glomerular filtrate into the interstitium.24 Additional transmission of glomerular damage to the tissue was 0.5±0.1% in the HS group, increasing to 6.1±0.8% in the HS+N group, whereas MMF treatment reduced this proportion to 3.4±0.8 (P<0.05 versus HS+N and HS). Myointimal proliferation was noted in 9.4±1.8% of microvessels in Group HS+N and in 6.2±2% in Group HS+N+MMF (P<0.05). Fibrinoid necrosis appeared in 3.6±0.9% of microvessels in Group HS+N, as opposed to only 0.6±0.3% in Group HS+N+MMF (P<0.05).

Immunohistochemical analysis at 30 days of treatment is represented in Figure 1. The density of cells staining positively for the lymphocyte-specific CD-3 antigen was significantly increased compared with HS in glomeruli, microvessels, and interstitium. However, the vast majority of these infiltrating cells located at the interstitial area. MMF treatment significantly reduced cell infiltration in all compartments by nearly 50%. Likewise, a pronounced macrophage infiltration was observed in HS+N rats. The predominant location of ED-1-positive cells was again the interstitial area, with only a minority locating at the renal microvessels. No abnormal macrophage infiltration was observed at the glomeruli: the number of macrophages per glomerulus was similar between Groups HS+N and HS. MMF treatment reduced macrophage infiltration by ~40% in both the interstitium and the microvessels. However, no effect was seen at the glomeruli.

among groups. Likewise, no differences in renal mass were observed at this stage. As described previously,3 blood pressure was markedly elevated in Group HS+N. Hypertension was unchanged by MMF treatment. The HS+N treatment reduced glomerular filtration rate by 40%, an effect not reversed by MMF. Changes in renal plasma flow followed a similar pattern. Pₖₑₜₜ was markedly elevated relative to HS in both Group HS+N and Group HS+N+MMF.

Parameters obtained at 30 days after treatment are presented in Table 2. All groups gained weight compared with values measured at 15 days, although growth was blunted in Groups HS+N and HS+N+MMF. Tail-cuff pressure was elevated in HS+N rats, reaching values 100-fold higher than the values of Group HS. Albuminuria was attenuated in Group HS+N+MMF, but remained elevated compared with HS. Likewise, Pₖₑₜₜ was elevated in Group HS+N compared with HS. MMF treatment significantly reduced Pₖₑₜₜ, which nevertheless remained high compared with HS.

Quantitative analysis of renal parenchymal and vascular injury at 30 days of treatment is given in Table 3. GS reached 3.5±0.7% in Group HS+N, compared with 0.4±0.2% in HS. MMF treatment reduced GS to levels indistinguishable from control. COLL represented almost one-third of all glomeruli in Group HS+N. MMF treatment reduced, but did not normalize, the frequency of COLL. Necrotic lesions appeared in 0.8±0.2% of glomeruli in Group HS+N. MMF treatment reduced the frequency of necrotic lesions to negligible values. The fraction of cortical parenchyma occupied by interstitial tissue was 0.5±0.1% in the HS group, increasing to 6.1±0.8% in the HS+N group, whereas MMF treatment reduced this proportion to 3.4±0.8 (P<0.05 versus HS+N and HS). Myointimal proliferation was noted in 9.4±1.8% of microvessels in Group HS+N and in 6.2±2% in Group HS+N+MMF (P<0.05). Fibrinoid necrosis appeared in 3.6±0.9% of microvessels in Group HS+N, as opposed to only 0.6±0.3% in Group HS+N+MMF (P<0.05).

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interstitium may have resulted from the massive proteinuria observed in HS+N rats, which can cause proximal tubular cells to produce inflammatory mediators as a result of their intense protein-absorptive activity. Renal inflammation may also have resulted directly from NO inhibition. This effect is not unexpected, because NO is known to function as an endogenous anti-inflammatory agent by inhibiting cell proliferation, leukocyte adhesion, the expression of adhesion molecules, and platelet adhesion and activation. Accordingly, chronic NO inhibition is associated with enhanced leukocyte-endothelium interaction and increased expression of ICAM-1/VCAM-1. Increasing evidence suggests that chronic inflammation, once regarded as an inseparable element of immune-mediated damage, plays a central role in the pathogenesis of progressive nephropathies of nonimmune origin as well. We and others showed intense renal interstitial lymphocyte infiltration in the 5/6 renal ablation model 1 week after surgery. Accordingly, we have recently shown that treatment of these rats with a nonsteroidal anti-inflammatory agent strongly attenuates renal injury. Even the beneficial effect of suppressors of the renin-angiotensin system in progressive renal diseases, usually attributed to its renal and glomerular hemodynamic effects, may have an anti-inflammatory component. Angiotensin II may enhance inflammation by promoting leukocyte infiltration, increasing the expression of adhesion molecules, and stimulating the cell-mediated immune response through activation of calcineurin phosphatase. Accordingly, recent studies have shown that tacrolimus, a calcineurin inhibitor, limits glomerular injury in the renal ablation model. In the chronic NO inhibition model, ACE inhibition reduced the expression of vascular adhesion molecules, whereas administration of an angiotensin II receptor blocker reduced cell proliferation, macrophage infiltration, and the expression of adhesion molecules.

Treatment of HS+N rats with MMF lowered albuminuria by >50%, reduced the frequency of all modalities of glomerular injury (with the exception of macrophage infiltration), and limited the extent of vascular and interstitial injury. Accordingly, serum creatinine was significantly reduced in MMF-treated rats, indicating relative renal functional preservation.

**Graphical representation** of the extent of infiltrating cells staining positively for CD-3 (lymphocyte-specific, left panels) and ED-1 (macrophage-specific, right panels) antigens and their distribution among glomeruli, microvessels, and interstitium. *P < 0.05 vs HS; #P < 0.05 vs HS+N.
compared with untreated HS+N rats. Renal protection was associated with attenuation of 2 major inflammation markers, namely renal lymphocyte and macrophage infiltration, particularly at interstitial areas. MMF has primarily been used in recent years as an immunosuppressor in the treatment of typically immune-mediated processes such as allograft rejection.\textsuperscript{15,16} In addition, recent experimental\textsuperscript{17,18,32,33} and clinical\textsuperscript{34} evidence suggests that MMF therapy may retard the progression of chronic nephropathies of nonimmunologic nature alone\textsuperscript{17,18} or in combination with suppressors of the renin-angiotensin system.\textsuperscript{32,33} The basic mechanism thought to mediate these beneficial effects is a selective inhibition of lymphocyte proliferation.\textsuperscript{35} Interstitial lymphocyte infiltration is strongly attenuated in MMF-treated HS+N rats, as previously reported in the renal ablation model.\textsuperscript{17} This effect may have limited inflammation by disrupting the interaction between lymphocytes and macrophages, thus reducing macrophage infiltration of the renal interstitium. These results underline the importance of cell-mediated immunity in the development of renal injury in this model. We have shown preliminary evidence of enhanced proliferation of glomerular, tubular, and interstitial cells in the chronic NO inhibition model.\textsuperscript{14} Thus MMF may have conferred additional protection by inhibiting the proliferation of mesangial\textsuperscript{16} and tubular\textsuperscript{17} cells and by reducing the expression of adhesion molecules.\textsuperscript{18} Additional salutary effects may have derived from reduction of proteinuria.

In summary, MMF treatment attenuated renal injury in rats subjected to chronic NO inhibition by a nonhemodynamic mechanism, most likely associated with its anti-inflammatory action. Renal inflammation may be crucial to the development of renal injury in this model.

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References


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