Immune System and Hypertension

Immune Suppression Attenuates Hypertension and Renal Disease in the Dahl Salt-Sensitive Rat

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Abstract—Experiments were performed to determine the importance of activation or infiltration of immune cells in the kidney during the development of hypertension and renal disease in Dahl salt-sensitive rats (SS/Mcw) fed a 4.0% NaCl diet. Compared with vehicle-treated rats, chronic administration of mycophenolate mofetil ([MMF] 30 mg/kg per day, IP), an immunosuppressive agent that has cytostatic effects on T and B cells, decreased cell-specific markers of T and B cells by 50% to 60% in the kidneys of SS/Mcw rats (n=5 per group). Further studies were performed on Dahl SS/Mcw rats, which were instrumented with chronic indwelling catheters and studied after 3 weeks on the 4.0% NaCl diet. Rats were administered MMF or 5% dextrose vehicle daily during the 3-week period of high NaCl intake. Mean arterial blood pressure in the rats administered MMF (122±2 mm Hg; n=11) was significantly decreased compared with vehicle-treated rats (139±4 mm Hg; n=9). Furthermore, the rate of protein (112±13 mg per day) and albumin excretion (15±3 mg per day) in the MMF-treated rats was significantly lower than the protein and albumin excretion rate in vehicle-treated rats (167±25 and 31±7 mg per day, respectively). Creatinine clearance and body weight were not different between the groups, averaging 0.52±0.08 mL/min per gram kidney weight and 322±10 g, respectively, in the MMF-treated group. These experiments indicate that the activation of the immune system or renal infiltration of immune cells plays an important role in the development of hypertension and renal disease in Dahl SS/Mcw rats consuming an elevated NaCl diet. (Hypertension. 2006;48:149-156.)

Key Words: hypertension, sodium-dependent ■ rats ■ blood pressure

The Dahl salt-sensitive (SS) rat is a genetic model of hypertension and renal disease that exhibits many phenotypic characteristics in common with human hypertension.1–3 Similar to traits observed in black hypertensive subjects, this inbred strain of rats has a low-renin, sodium-sensitive form of hypertension that is associated with a progressive decline in renal function.6,7 The renal injury that occurs in Dahl SS rats after exposure to a diet containing elevated salt is similar to that observed in the kidneys of rats with both experimental and genetic forms of hypertension and/or kidney disease.8–12 Of interest, the sodium-sensitive hypertension and renal disease observed in a number of other rodent models is ameliorated by suppression of the immune system.8–11,13,14 The potential role of the immune system in the development of hypertension and kidney disease in the Dahl SS rat has not been examined.

The present experiments were performed to test the specific hypothesis that immune suppression by systemic administration of mycophenolate mofetil (MMF), an inhibitor of inosine-5′-monophosphate dehydrogenase,12,15 would prevent the elevation in arterial blood pressure and development of renal disease associated with increased sodium intake in conscious Dahl SS rats. The NaCl content of the diet was increased from 0.4% to 4.0% at 9 weeks of age; half of the rats were administered vehicle, and half received MMF. After 3 weeks of treatment, hypertension and renal-disease-related phenotypes were quantified.

Methods

Experimental Animals

Experiments were performed on male Dahl SS/Mcw rats obtained from a colony at the Medical College of Wisconsin and male Sprague–Dawley (SD) rats obtained from Harlan–Sprague–Dawley (Madison, WI). The Dahl SS/Mcw breeders and weanlings were fed purified AIN-76A rodent diet (Dyets, Inc) containing 0.4% NaCl. At 9 weeks of age, the salt content of the chow was increased to 4.0% NaCl, and the SS/Mcw rats were studied 3 weeks later. Separate groups of Dahl SS/Mcw rats were studied, which were maintained on the 0.4% NaCl diet from 9 to 12 weeks of age. The SD rats were fed the chow containing 0.4% NaCl throughout the experiment. The Medical College of Wisconsin Institutional Animal Care and Use Committee approved all of the experimental protocols.

Surgical Preparation

Surgical procedures were performed on rats deeply anesthetized with an intraperitoneal injection of ketamine (35 mg/kg), xylazine (10

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mg/kg), and acemprosate (0.5 mg/kg) with supplemental anesthesia administered when needed. Using aseptic technique, polyvinyl catheters were implanted in the femoral artery, tunneled subcutaneously, and exteriorized at the back of the neck in a lightweight tethering spring. Both antibiotic (100 000 U/kg penicillin G, IM) and analgesic (0.1 mg/kg Buprenex, SC) were administered postsurgically, and the rats were allowed to fully awaken from anesthesia on a temperature-controlled pad. After recovery from anesthesia, all of the rats were placed in individual stainless steel cages that permit daily measurement of arterial blood pressure and overnight urine collection.

Protocol 1
A preliminary group of SD rats was prepared as described above to test for nonspecific effects of MMF on arterial blood pressure in normal animals. The rats were fed the 0.4% NaCl diet throughout the protocol. Arterial blood pressure was measured on 3 consecutive days when the rats received vehicle (5% dextrose IP) and on the following 8 days during which each rat received MMF (30 mg/kg IP). An overnight urine collection was obtained in separate groups of rats after 3 weeks of vehicle or MMF administration to quantify albumin and protein excretion rate.

Protocol 2
The Dahl SS/Mcw rats were maintained on the 0.4% NaCl diet from weaning until 9 weeks of age. The salt content of the chow was then increased to 4.0% NaCl, and the rats were studied 3 weeks later. Animals were randomly assigned to receive a daily intraperitoneal injection of vehicle (5% dextrose) or MMF (30 mg/kg per day) in 5% dextrose from the age of 9 weeks until the experiment was concluded. A schematic illustrating the experimental protocol performed in SS/Mcw rats administered vehicle or MMF is presented in Figure 1. After the recovery period from surgery, high-salt blood pressure measurements were obtained from 9:00 AM to 12:00 PM on 3 consecutive days. The rats were studied at 12 weeks of age. Blood pressure measurements were obtained for 3 days when the rats were fed the 4.0% NaCl diet, and urine and blood samples were obtained. The rats were then administered a single dose of furosemide (10 mg/kg, IP) and switched to 0.4% NaCl chow. After a day of equilibration, an additional blood pressure measurement, a urine sample, and a blood sample were obtained.

Histological Analysis of Kidney Tissues
Kidneys (n=4 per group) were obtained for histological analysis from noninstrumented Dahl SS/Mcw rats maintained on the high-salt diet and treated with vehicle or MMF as described above. Separate groups of untreated SS/Mcw rats (n=4/group) fed either the 4% NaCl diet or maintained on the 0.4% NaCl diet were also studied to assess the histological changes caused by increasing the salt intake. The rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, IP); the kidneys were then removed, bisected along the coronal plane, and placed in a 10% formaldehyde solution in phosphate buffer. The tissue was paraffin embedded in an automatic tissue processor (Microm HMP 300), cut in 3 µm sections (Microm HM355S), mounted on silanized/charged slides, and stained with Gomori’s One-Step Trichrome. Slides were photographed using a Nikon E-400 fitted with a Spot Insight camera; digital micrographs were taken at different magnifications. Individual glomeruli (30 to 40 per rat) were evaluated using the semiquantitative index method of Raji et al; glomeruli were scored from 0 (best) to 4 (worst) on the basis of glomerulosclerosis and mesangial expansion as we described previously. The percentage of the outer medullary tissue containing blocked tubules filled with protein was quantified by determining the proportion of red-stained structures in this region using Metamorph Image Analysis software (version 4.6, Universal Imaging Systems Corp) as we described previously. The grading of glomerular and medullary damage was performed in a blinded manner.

Figure 1. Schematic illustrating the final 5 days of the experimental protocol performed on 12-week-old Dahl SS/Mcw rats. The rats were fed 0.4% NaCl chow from weaning until 9 weeks of age. The diet was then switched to 4.0% NaCl chow, and the rats received MMF (30 mg/kg per day, IP) or vehicle for 3 weeks. The rats were studied at 12 weeks of age. Blood pressure measurements were obtained for 3 days when the rats were fed the 4.0% NaCl diet, and urine and blood samples were obtained. The rats were then administered a single dose of furosemide (10 mg/kg, IP) and switched to 0.4% NaCl chow. After a day of equilibration, an additional blood pressure measurement, a urine sample, and a blood sample were obtained.
Immunoblotting Analysis

Immunoblotting protocols were performed to validate the effectiveness of MMF to decrease markers of immune cell infiltration in the kidney. Kidneys were obtained from Dahl SS/Mcw rats maintained on the high-salt diet and treated with vehicle or MMF as described above. The rats were euthanized with an overdose of sodium pentobarbital (100 mg/kg IP), and the kidneys were rapidly removed, separated into cortical and medullary sections, and frozen on dry ice. Isolated tissue was stored at −80°C until protein extraction. In the extraction procedure, pieces of whole tissue were homogenized using a Potter–Elvehjem tissue grinder in a solution containing 250 mmol/L of sucrose, 1 mmol/L of EDTA, and 5 mmol/L of potassium phosphate (pH 7.7). A protease inhibitor mixture was added to give final concentrations of 4-(2-aminoethyl)-benzenesulfonylfluoride (780 μmol/L), aprotinin (0.6 μmol/L), leupeptin (15 μmol/L), bestatin (30 μmol/L), pepstatin A (11 μmol/L), and E64 (10 μmol/L). Large tissue debris and nuclear fragments were removed by a low-speed centrifuge spin (14,000 g, 4°C, 20 minutes). The protein concentration of the tissue homogenate was determined by a Coomassie protein assay with albumin as a standard. Equivalent amounts (10 μg) of total protein from the same tissue of different rats were added to adjacent wells and vacuum blotted onto a nitrocellulose membrane. Homogenates of rat spleen and rat brain were loaded into separate wells as positive and negative controls, respectively. Membranes were washed in Tris-buffered saline (TBS), blocked with 10% nonfat dried milk in TBS overnight for 2 hours, and incubated with an appropriate horseradish peroxidase (HRP)–labeled anti-rabbit or anti-mouse secondary antibody in 4% nonfat dried milk/TBS for 2 hours. Bound antibody was detected by chemiluminescence on x-ray film. As a control for nonspecific binding and to ensure equal loading, separate membranes were incubated for 2 hours with only the HRP-labeled anti-mouse secondary antibody or with an HRP-labeled anti-rat antibody. To control for protein loading, membranes were incubated with Ponceau S for 5 minutes. For purposes of quantification, the developed x-ray films were scanned and subjected to densitometric analysis using commercially available software (Metamorph).

Immunohistochemistry

Immunohistochemistry was performed to localize ED-1 in the kidneys of Dahl SS/Mcw rats fed the 0.4% NaCl diet, the 4.0% NaCl diet, and rats fed the 4.0% NaCl diet and administered MMF (30 mg/kg per day, IP). The excised kidneys were fixed in 10% formaldehyde, paraffin embedded, cut in 3-μm sections, and mounted on silanized/charged slides as described above. Tissue sections were then stained using a robotic DAKO autostainer (S3400; Dako). The sections were deparaffinized with xylene and ethanol and incubated with Proteinase K for antigen retrieval. Endogenous biotin and peroxidase activity were blocked by incubation with avidin and biotin and hydrogen peroxide, respectively. The primary ED-1 monoclonal antibody (MCA341R; Serotec Inc) was used at a dilution of 1:400 and incubated at room temperature for 60 minutes. A biotinylated horse anti-mouse secondary antibody was used for development with avidin-biotinylated HRP complex (Vectastain ABC kits; Vector Laboratory) and 0.02% H2O2 + 0.1% diaminobenzidine tetrahydrochloride. The slides were lightly counterstained with aniline blue dye to assist in tissue visualization and determination whether tissue damage and macrophage infiltration were
The MAP values of the conscious Dahl SS/Mcw rats administered vehicle or MMF while maintained on the 4.0% NaCl diet are illustrated in Figure 2. Unless otherwise noted, group sizes range from 9 to 12 animals for all of the parameters obtained from the conscious SS/Mcw rats. The average MAP was 18 mm Hg lower in MMF-treated rats compared with vehicle-treated rats maintained on the 4.0% NaCl diet. After furosemide administration and placement on the 0.4% NaCl diet, MAP significantly decreased in both vehicle and MMF-treated rats compared with the 4.0% NaCl blood pressure values, and MAP in the MMF-treated rats was significantly lower than in the control group. The absolute decrease in blood pressure after administration of furosemide and placement on the 0.4% NaCl chow (ie, the sodium sensitivity of blood pressure) was not different between the groups. Heart rate was not different in either group on either diet from the average of 399±13 bpm in the vehicle-treated SS/Mcw rats fed the 4.0% NaCl diet. Arterial blood pressure averaged 125±4 mm Hg, and heart rate averaged 390±11 bpm in a separate group of age-matched SS/Mcw rats fed the 0.4% NaCl diet throughout life (n=6; data not shown).

Body weight was not different between the groups, averaging 338±9 g in the vehicle-treated rats. Urine sodium excretion on the 4.0% and 0.4% NaCl intake was also not different between the groups, averaging 11.9±1.5 mEq per day and 1.0±0.5 mEq per day on 4.0% and 0.4% NaCl, respectively, in the vehicle-treated rats. The similar body weights and steady-state sodium excretion rates indicate similar levels of food consumption in the groups. Neither MMF treatment nor dietary salt intake altered plasma sodium or potassium concentrations; plasma sodium and potassium averaged 149.0±1.4 mEq/L and 3.6±0.1 mEq/L, respectively, in the vehicle-treated rats on the 4.0% NaCl diet.

Plasma creatinine was unaltered between the treatments or levels of sodium intake, averaging 0.39±0.04 mg/dL in the MMF-treated rats on 4.0% NaCl. Correspondingly, creatinine clearance was unaltered between the treatments or levels of sodium intake, and averaged 0.52±0.08 mL/min per gram kidney weight in the MMF-treated rats fed 4.0% NaCl (Figure 3). PRA significantly increased from 0.9±0.3 to 3.0±0.4 ng of Angiotensin I per milliliter per hour in the vehicle-treated rats when the NaCl content of the chow was switched from 4.0% to 0.4% (Figure 3). The PRA significantly increased from 1.3±0.1 to 9.0±1.0 ng of Angiotensin I per milliliter per hour when sodium intake was decreased in MMF-treated rats. Whereas PRA was not different between the groups consuming the 4.0% NaCl chow, PRA was significantly elevated in the MMF rats fed 0.4% NaCl food compared with vehicle-treated animals with the same sodium intake.

Albumin and protein excretion, as indices of kidney disease, are illustrated in Figure 4. Chronic treatment with MMF significantly decreased urinary albumin and protein excretion in the SS/Mcw. Total kidney weight was not different between the groups, averaging 4.1±0.1 g in the vehicle-treated rats. In age-matched SS/Mcw rats maintained on the 0.4% NaCl chow throughout life, albumin and protein excretion averaged 14±2 and 94±24 mg per day, respectively (n=8; data not shown).

Representative histological images of kidneys obtained from vehicle- and MMF-treated Dahl SS rats after 3 weeks on the 4.0% NaCl diet are shown in Figure 5. Consistent with previous reports in the Dahl SS/Mcw rat, 16,17 both glomerular damage (blue fibrotic tissue and collapsed capillary structure) and colocalized. The slides were photographed using a Nikon E-400 fitted with a Spot Insight camera.

**Statistical Analysis**

All of the data are presented as the mean±SE. A 2-way ANOVA with a Tukey post hoc test was used to evaluate the effects of both sodium intake and MMF treatment. A t test was used to determine differences in parameters measured between the rats administered MMF and vehicle. The 95% CI was considered significant.

**Results**

**Protocol 1**

Preliminary studies in SD rats (n=6 per group) determined whether MMF (30 mg/kg per day, IP) affected blood pressure or albumin and protein excretion when administered to normal rats. The average mean arterial pressure (MAP) and heart rate (HR) in the SD rats were not altered from the control averages of 114±1 mm Hg and 387±8 bpm, respectively, throughout the 8-day administration of MMF. In separate groups of SD rats, MMF treatment for 3 weeks did not alter protein and albumin excretion from the control levels of 4.1±1.5 mg per day and 55.3±4.2 mg per day, respectively. These data demonstrate that this dose of MMF does not affect arterial blood pressure or albumin and protein excretion in normal rats.

**Protocol 2**

The MAP values of the conscious Dahl SS/Mcw rats administered vehicle or MMF while maintained on the 4.0% NaCl diet were considered significant.
and blocked tubules in the outer medulla (red protein deposition casts) were observed in the kidneys of the vehicle-treated SS/Mcw rats. Visibly less glomerular and tubular injury was observed in the kidneys of the SS/Mcw rats treated with MMF. The glomerular injury index and the percentage of blocked tubules in the outer medulla of the vehicle and MMF-treated rats are presented in Figure 6 (n=4 per group). The glomerular injury index was significantly greater in the vehicle-treated Dahl SS/Mcw rats in comparison with that in the rats treated with MMF. Moreover, 3.3±0.6% of the area of the outer medulla of the vehicle-treated rats stained for protein casts (indicating blocked tubules) compared with 0.6±0.1% of the area of the outer medulla of the rats administered MMF.

To assess the effectiveness of MMF in the kidney, immunoblotting experiments were performed to quantify the expression of CD5, CD25, ED-1, and osteopontin in protein homogenates obtained from the renal cortex of rats treated with vehicle or MMF (Figure 7; n=5 per group). Systemic treatment with MMF resulted in a significant decrease in each marker in the renal cortex of the Dahl SS rats when compared with vehicle-treated Dahl SS rats, indicating that systemic MMF treatment successfully suppressed immune cell infiltration in the kidneys of the SS/Mcw rats. As a loading control, the total amount of protein loaded per well, assessed by Ponceau S staining, was also unaltered between the groups.

In separate experiments, CD5, CD25, and ED-1 immunoreactive protein were increased by 150% to 200% in the renal cortex of Dahl SS/Mcw rats fed the 4.0% NaCl diet compared with age-matched SS/Mcw fed the 0.4% NaCl diet (n=4/group). The kidneys of rats fed the 4.0% NaCl chow had an increased glomerular injury score (2.8±0.1 versus 2.1±0.1 in rats fed 0.4% NaCl chow) and an increased number of protein casts in the outer medulla (16±2% when compared with the kidneys of rats maintained on the 0.4% NaCl diet (5±2%).

To localize the potential site(s) of infiltration of immune cells in the kidneys of SS/Mcw rats fed the 4.0% NaCl diet, an immunohistochemical localization was performed. ED-1–positive staining was observed throughout the kidneys of the rats fed the 4.0% NaCl chow but was found to the greatest extent in the interstitial space surrounding damaged glomeruli and adjacent tubules (Figure 8). In the outer medulla, distinct staining was observed in the interstitium surrounding tubular and vascular structures in the areas adjacent to protein casts. Minimal ED-1–positive staining was observed in the kidneys of MMF-treated rats or in the kidneys of rats maintained on the diet containing 0.4% NaCl.

**Discussion**

The present studies demonstrate that chronic immune suppression achieved by systemic administration of MMF atten-
uates the development of hypertension and kidney damage, which occurs in Dahl SS/Mcw rats after elevation of salt intake by administration of a 4.0% NaCl diet. Blood pressure was significantly decreased in the MMF-treated rats during the intake of both 4.0% and 0.4% NaCl chow. Despite the lack of differences in plasma creatinine, creatinine clearance, or sodium excretion rate, albumin and protein excretion were also significantly decreased in the MMF-treated rats. Kidney tissue damage, as indicated by the glomerular damage index and the percentage of blocked tubules in the outer medulla, was also significantly decreased in the MMF-treated rats. Kidney tissue damage, as indicated by the glomerular damage index and the percentage of blocked tubules in the outer medulla, was also significantly decreased in the MMF-treated rats in comparison with vehicle-treated control rats. Immunoblotting experiments indicated that MMF had a suppressive influence on the infiltration of immune cells into the renal cortex because CD5, CD25, ED-1, and osteopontin, all markers of immune cell infiltration, were significantly decreased in protein homogenates from the renal cortex of MMF-treated rats. Moreover, immunohistochemical staining indicated that the infiltration of ED-1–positive cells in the interstitial spaces surrounding damaged glomeruli and near damaged tubules in the outer medulla was reduced in the kidneys of MMF-treated rats.

A role has been demonstrated for the immune system in the development of hypertension in a number of different experimental and genetic rodent models of hypertension.8–11 The present data indicate that systemic administration of an agent that suppresses the immune system attenuates the hypertension and renal disease in the Dahl SS rat. The present studies do not, however, demonstrate whether a primary change in the immune system is responsible for the hypertension and kidney damage that occurs in the SS/Mcw after placement on a high-NaCl diet or whether the activation of the immune system is secondary to other primary mediators of hypertension and kidney disease. In addition, the mechanisms by which activation of the immune system can participate in the development of hypertension in the Dahl SS rat or other models of hypertension and kidney disease are unclear. It has been suggested that the infiltration of immune cells into the renal interstitial space leads to an increase in Angiotensin II,11 which may mediate sodium retention, increase the synthesis of extracellular matrix and other proteins, and/or lead to increased levels of reactive oxygen and nitrogen species.10,21,22 Our present observations indicate that there is a substantial infiltration of ED-1–positive cells in the interstitial spaces surrounding tubules and blood vessels in the vicinity of damaged glomeruli and blocked tubules in the renal outer medulla. Alternatively, activation of the immune system could lead to immunologically mediated kidney disease in the SS/Mcw.20 Regardless of the role of the immune system in the disease process, inhibition of activation or infiltration of inflammatory cells in the kidney is associated with a marked decrement in hypertension and kidney disease in the SS/Mcw rat.

The immunosuppressive agent used in the present study, MMF, inhibits inosine monophosphate dehydrogenase, an enzyme critical for de novo purine synthesis.15 Whereas most cell types possess salvage pathways that can be used when inosine monophosphate dehydrogenase is inhibited, lymphocytes rely on the de novo pathway.12,15 As such, MMF is an effective immunosuppressive drug. This compound has been administered to a number of other genetic and experimental models of kidney disease and/or hypertension and has effectively reduced the degree of disease in a number of different instances.11,12 Despite these observations, it is possible that the effects of MMF to decrease renal damage in the SS/Mcw rat are mediated by the
fall in blood pressure rather than the direct effect of MMF to inhibit immune cells. Separate experiments in normal SD rats demonstrated that this dose of MMF did not lower blood pressure in normal rats, indicating that the effects observed in the Dahl SS/Mcw rats were likely mediated by immune-suppressive effects and not by direct vascular or other antihypertensive mechanisms.

Perspectives
Activation or infiltration of inflammatory cells in the kidney is associated with hypertension and kidney disease in the Dahl SS/Mcw rat, a rodent model of hypertension with disease phenotypes similar to that observed in humans. The actions of these cell types may result in inflammation and/or the release of vasoactive factors into the kidney, which exacerbate the disease process. Strategies to attenuate the infiltration and action of these immune cells in the kidney of hypertensive patients may prove effective to attenuate the elevation of arterial blood pressure and the damage to the kidney, which occurs in these individuals.

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Disclosures
None.

References


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