Immune suppression blocks sodium-sensitive hypertension following recovery from ischemic acute renal failure

Kimberly R. Pechman,1 David P. Basle,2 Hayley Lund,4 and David L. Mattson1

1Department of Physiology, Medical College of Wisconsin, Milwaukee, Wisconsin; and 2Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, Indiana

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Immune suppression blocks sodium-sensitive hypertension following recovery from ischemic acute renal failure. Am J Physiol Regul Integr Comp Physiol 294: R1234–R1239, 2008. First published February 6, 2008; doi:10.1152/ajpregu.00821.2007.—The present study determined the effect of immune suppression with mycophenolate mofetil (MMF) on sodium-sensitive hypertension following recovery from ischemia reperfusion (I/R)-induced acute renal failure. Male Sprague-Dawley rats fed 0.4% NaCl chow were subjected to 40 min bilateral I/R or control sham surgery. After 35 days of recovery, when plasma creatinine levels had returned to normal, the rats were switched to a 4.0% NaCl chow for 28 days and administered vehicle or MMF (20 mg·kg⁻¹·day⁻¹ ip). High-salt mean arterial pressure was significantly higher in I/R rats (144 ± 16 mmHg) compared with vehicle-treated sham rats (122 ± 2 mmHg). Treatment of I/R rats with MMF during the period of high salt intake prevented the salt-induced increase in arterial pressure (114 ± 3 mmHg). Conscious creatinine clearance was lower in I/R rats (0.27 ± 0.07 ml·min⁻¹·100 g body wt⁻¹) compared with vehicle-treated sham rats (0.58 ± 0.04 ml·min⁻¹·100 g body wt⁻¹). MMF treatment prevented the decrease in creatinine clearance in I/R rats (0.64 ± 0.07 ml·min⁻¹·100 g body wt⁻¹). I/R injury also significantly increased glomerular tissue damage and increased the presence of ED-1 positive (macrophages) and S100A4 positive cells (fibroblasts) in the renal interstitium. The I/R rats treated with MMF exhibited a significant reduction in infiltrating macrophages and fibroblasts and decreased histological damage. The present data indicate that infiltrating immune cells mediate or participate in the development of sodium-sensitive hypertension and renal damage in rats apparently recovered from renal I/R injury.

ACUTE RENAL FAILURE (ARF) is characterized by a rapid decline of renal function that is associated with a clinical mortality rate of 50%-80% (19, 23, 24). Despite the high mortality rate, ARF is considered reversible in surviving patients. Mechanisms of recovery of renal function have been studied extensively in models of ARF such as ischemia reperfusion (I/R) injury. In I/R injury, the primary damage to the kidney is in the proximal tubule, which undergoes a complex repair process (8). Despite the ability of the proximal tubule to regenerate following acute I/R injury, evidence indicates that there is a permanent reduction in renal peritubular capillaries in the recovered kidney (3). Furthermore, we recently demonstrated that rats that have apparently recovered from I/R injury develop hypertension and renal disease when placed on a high-salt diet (3, 21).

Similar to the I/R model, many models of transient or subtle renal injury result in a loss of peritubular capillaries and development of sodium-dependent hypertension (10, 12). In addition, these models of renal injury are characterized by the infiltration of macrophages, T cells, and monocytes in the renal interstitium (1, 13, 15, 18). It has been proposed that the infiltration of renal interstitial inflammatory cells contributes to the development of sodium-dependent hypertension and tubulointerstitial damage in models of renal injury (1, 13, 15, 18).

Recent studies from our laboratory have demonstrated that the sodium-sensitive hypertension and kidney damage in the I/R recovery rats is associated with an increase in the number of fibroblasts and macrophages in the interstitial space of these kidneys (6, 21). The role of these cells in the sodium-sensitive hypertension and renal disease that occurs in rats apparently recovered from I/R injury has not been evaluated. The present studies were therefore performed to assess the influence of infiltrating immune cells in the development of sodium-dependent hypertension and renal injury in rats that have apparently recovered from I/R injury. To perform these experiments, I/R rats were permitted to recover for 5 wk from the I/R injury; the rats were then placed on a high-NaCl diet and treated with vehicle or the immune suppressive drug mycophenolate mofetil (MMF), an inhibitor of inosine 5’-monophosphate dehydrogenase (22, 26), for 4 wk. Indexes of salt-sensitive hypertension and renal disease were then evaluated in the control rats and those treated with the immunosuppressive agent.

METHODS

Animals. Male Sprague-Dawley rats were obtained from Harlan Sprague Dawley (Madison, WI). Animals were fed standard laboratory rat diet (AIN76A; Dyets, Bethlehem, PA) containing 0.4% or 4.0% NaCl, as described below; food and water were available ad libitum. Care of the rats before and during the experimental procedures was conducted in accordance with the policies of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin.

Surgical preparation and experimental protocol. To induce ARF, rats were anesthetized with ketamine (100 mg/kg ip) and pentobarbital sodium (25 mg/kg ip) and placed on a heated surgical table, and a midline incision was made. Blood supply to the kidneys was interrupted for 40 min by applying microvascular clamps on the pedicles of both kidneys. The clamps were then released, and reperfusion was visually confirmed. Additional rats were subjected to sham surgery where the kidneys were exposed but not touched. All animals were allowed to recover from I/R or sham surgery for 5 days (i.e., 5 wk post-I/R or sham surgery) while fed the 0.4% NaCl diet. Plasma creatinine measurements were obtained as an index of renal function at 24 h, 7 days, and 28 days following I/R or sham injury.

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Address for reprint requests and other correspondence: D. L. Mattson, Dept. of Physiology, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226 (e-mail: dmmattson@mcw.edu).
Following 35 days of recovery from I/R or sham surgery, the salt content of the diet was increased to 4.0% NaCl for the final 28 days of the protocol. Postischemic rats or sham-operated controls were administered the immunosuppressive drug MMF (20 mg·kg⁻¹·day⁻¹·ip) or vehicle during the period of high salt intake. For statistical analysis, data are presented as means ± SE. Statistical analysis was performed using one-way ANOVA followed by Tukey’s post-hoc test to determine the significance of differences among groups. Differences were considered significant at P < 0.05.

Histological analysis. Kidneys were obtained for histological analysis from rats at post-I/R or sham surgery day 63 (after ~4 wk of 4.0% NaCl chow). Each rat had been treated with vehicle or MMF for 4 wk. The rats were deeply anesthetized with pentobarbital sodium (50 mg/kg · ip); the kidneys were 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 HM 300), cut in 3-μm sections (Microm HM 355 S), 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 obtained at ×20 and ×40.

Morphometric analyses were performed to quantify glomerular damage and to assess the expansion of the interstitial space. Individual glomeruli (30–40/rat, ×40 magnification) were evaluated using the semiquantitative index method of Raij et al. (17) in which glomeruli were scored from zero (best) to four (worst) on the basis of glomerulosclerosis and mesangial expansion, as we previously described (13, 21). A morphometric analysis was also performed to evaluate the expansion of the interstitial space using an adaptation of our previously described method (21). Four to five representative micrographs (×20 magnification) were obtained from the renal cortex of each animal. A 16 × 11 grid was applied over each image, and intersecting points over the interstitium, tubular epithelium, tubular lumen, and glomeruli were counted. The data are presented as the percent of the total area occupied by each type of structure.

**Fig. 1.** Mean arterial blood pressure and urine albumin-to-creatinine ratio in vehicle-treated sham rats, vehicle-treated ischemia-reperfusion (I/R) rats, mycophenolate mofetil (MMF)-treated sham rats, and MMF-treated I/R rats. These parameters were obtained ~9 wk following renal I/R injury or sham surgery and after 4 wk on a high-salt (4.0% NaCl) diet. The rats were treated with MMF (20 mg·kg⁻¹·day⁻¹·ip) or vehicle during the period of high salt intake. P < 0.05 vs. sham-vehicle group (★) and vs. I/R-vehicle group (#).

Following 35 days of recovery from I/R or sham surgery, the salt content of the diet was increased to 4.0% NaCl for the final 28 days of the protocol. Postischemic rats or sham-operated controls were administered the immunosuppressive drug MMF (20 mg·kg⁻¹·day⁻¹·ip) in 5% dextrose or vehicle (5% dextrose) from day 35 to the end of the study.

At 49 days post-I/R or sham surgery, rats were anesthetized with ketamine hydrochloride (50 mg/kg · im) and acepromazine maleate (5 mg/kg · im). Microrenathane catheters were implanted in the femoral artery, tunneled subcutaneously, and exteriorized at the scapula in a stainless steel spring. Following recovery from anesthesia, all rats were placed in individual stainless steel cages that permit daily measurement of arterial blood pressure and overnight urine collection.

Beginning at day 56 post-I/R or sham surgery (after 3 wk of 4.0% NaCl), daily blood pressure measurements were obtained from 0900 to 1200 as we have previously described (13, 21). After five consecutive days of blood pressure measurements, arterial plasma samples were obtained for measurement of plasma creatinine. An overnight urine sample was also obtained for quantification of urinary sodium, creatinine, and albumin excretion rates.

Analytical procedures. Urine and plasma electrolytes were measured by flame photometry (IL-943; Instrumentation Laboratories, Lexington, MA). Plasma and urine creatinine values were measured with an assay based on the Jaffe reaction by autoanalyzer (ACE; Alfa Wasserman, Fairfield, NJ). Urine albumin was quantified with a fluorescent assay that utilized Albumin Blue 580 dye (Molecular Probes, Eugene, OR) and a fluorescent plate reader (FL600; Bio-Tek, Winooski, VT). Plasma renin activity (PRA) was measured using a modification of the method of Sealey and Laragh (20).

**Fig. 2.** Creatinine clearance and kidney weight in vehicle-treated sham rats, vehicle-treated I/R rats, mycophenolate mofetil (MMF)-treated sham rats, and MMF-treated I/R rats. These parameters were obtained ~9 wk following renal I/R injury or sham surgery and after 4 wk on a high-salt (4.0% NaCl) diet. The rats were treated with MMF (20 mg·kg⁻¹·day⁻¹·ip) or vehicle during the period of high salt intake. P < 0.05 vs. sham-vehicle group (★) and vs. I/R-vehicle group (#).
Assessment of ED-1 and S100A4 positive cells. Immunohistochemistry was performed to localize ED1 and S100A4 in the kidneys of I/R rats treated with MMF or vehicle when fed the 4.0% NaCl diet. Localization of ED-1, a marker for monocytes and macrophages, was performed on 3-μm formalin-fixed paraffin sections. Sections were stained using a robotic DAKO autostainer (S3400; Dako) using a primary antibody obtained from Serotec, as described previously (13) using diaminobenzidine tetrahydrochloride as a substrate. Localization of fibroblasts and myofibroblasts was carried out by staining with antibodies to S100A4 and smooth muscle actin. S100A4 staining was carried out using a primary antibody obtained from Dako as described previously (4) using 3-amino-9-ethylcarbazole as a substrate; colocalization with α-smooth muscle actin was performed with an antibody from Zymed using alkaline phosphatase/nitroblue tetrazolium as a method for detection.

Statistical analysis. Data are presented as means ± SE. A one-way ANOVA with a Holm-Sidak all pairwise multiple comparison post hoc test was used to evaluate the data. The 95% confidence interval was considered significant.

RESULTS

Rats were subjected to sham or I/R surgery and permitted to recover for 35 days while fed a diet containing 0.4% NaCl (n = 9–16 rats/group). Compared with the sham control group, plasma creatinine in the I/R rats was elevated by approximately sixfold after 24 h of recovery from the 40-min period of renal ischemia. Plasma creatinine in I/R rats was approximately two times as high in I/R rats as sham rats 1 wk following surgery, but creatinine levels fully returned to control levels after 4 wk of postsurgical recovery. These data confirmed that the acute I/R injury led to a profound and rapid reduction in renal filtration that was reversible in 4 wk.

Following 35 days of recovery from I/R injury, when the I/R rats had apparently recovered from the initial insult, all rats were fed chow containing high salt (4.0% NaCl) and were treated with MMF or vehicle daily. Indexes of hypertension and renal function were then assessed 4 wk later. Figure 1 illustrates the differences in mean arterial pressure (MAP) in the different groups of rats following 4 wk on the 4.0% NaCl diet. MAP was significantly elevated by 20 mmHg in the vehicle-treated I/R recovery rats compared with sham rats fed the high-salt diet. Treatment with the immunosuppressive drug MMF during the period of high salt intake blocked the increase in MAP in the I/R rats. Moreover, there were no significant differences in MAP detected between the sham-vehicle, sham-MMF, and I/R-MMF groups. No significant differences in heart rate were detected between the four groups of rats; the average heart rate in the vehicle-treated sham rats was 377 ± 4 beats/min (data not shown).

Renal albumin excretion, normalized to creatinine excretion, was used as an index of chronic kidney disease. Figure 1, bottom, indicates that rats recovered from I/R injury had an albumin excretion rate that was approximately sixfold greater than sham rats subjected to the same dietary challenge. Treatment with MMF during the period of high salt intake tended to decrease the albuminuria in the I/R rats, but the difference was not significant. A similar pattern was observed for proteinuria in the I/R and sham rats treated with vehicle or MMF (data not shown).

Conscious creatinine clearance averaged 0.58 ± 0.04 ml/min·100 g body wt−1 in the sham-vehicle rats and was significantly lower in I/R vehicle rats (0.27 ± 0.07 ml/min·100 g body wt−1) following 4 wk on the high-salt diet (Fig. 2). The decrease in creatinine clearance was prevented in I/R rats treated with MMF. Body weight averaged 375 ± 8 g in the sham vehicle group; body weights were not significantly altered by MMF treatment or the I/R surgery (data not shown). Sodium excretion rate was also not different between any of the groups and averaged 9.9 ± 1.2 meq/day in the sham vehicle group. PRA (data not shown) averaged

Fig. 3. Light microscopy images of renal cortex (×40 original magnification) from vehicle-treated sham rats (A), vehicle-treated I/R rats (B), and MMF-treated I/R rats (C). D: glomerular damage index in glomeruli from vehicle-treated sham rats, vehicle-treated I/R rats, MMF-treated sham rats, and MMF-treated I/R rats. The tissue was obtained ~9 wk following renal I/R injury or sham surgery and after 4 wk on a high-salt (4.0% NaCl) diet. The rats were treated with MMF (20 mg·kg−1·day−1 ip) or vehicle during the period of high salt intake. P < 0.05 vs. sham-vehicle group (*) and vs. I/R-vehicle group (#).
0.74 ± 0.22 ng ANG I·ml⁻¹·min⁻¹ in I/R vehicle rats and was not different from this value in the sham vehicle and MMF vehicle groups. Treatment of I/R rats with MMF led to a significant increase in PRA to 3.55 ± 0.83 ng ANG I·ml⁻¹·min⁻¹.

The consumption of a diet containing elevated salt (4.0% NaCl) following apparent recovery from the original I/R injury led to marked changes in renal morphology. Total kidney weight was found to be significantly increased in I/R vehicle rats compared with sham vehicle rats (Fig. 2). Treatment with MMF prevented the renal hypertrophy observed in I/R rats after 4 wk on the high-salt (4.0% NaCl) diet.

Representative photomicrographs of glomeruli from sham-vehicle, I/R-vehicle, and I/R-MMF rats are presented in Fig. 3. Compared with the sham rats, marked glomerular damage occurred in I/R rats fed the high-salt diet. The injury to the glomeruli included the deposition of blue-stained fibrotic tissue and collapsed capillary structure. The glomerular damage was largely attenuated in I/R rats treated with MMF during the high-salt period. The degree of glomerular damage was assessed and is presented in Fig. 3D. Compared with the sham-vehicle group, the glomerular damage index was increased significantly in the I/R vehicle rats, and the renal damage was attenuated in the I/R rats treated with MMF. A morphometric analysis of different tissue compartments in the renal cortex is presented in Fig. 4. The renal hypertrophy of the I/R kidneys was associated with a significant increase in the interstitial compartment and a tendency for increased luminal space; the percentage of total tissue area taken up by epithelial cells was decreased; and there were no detected changes in glomerular area. These effects were prevented by treatment of I/R rats with MMF.

To evaluate the treatment of MMF on the composition of interstitial cells, an immunohistochemical analysis for macrophages, fibroblasts, and myofibroblasts was performed (Fig. 5). Staining of ED-1, a marker for monocytes and macrophages, was not prominent in kidneys of sham-operated control rats treated with vehicle or MMF (not shown) at day 63 postsurgery. Consistent with previous results (21), I/R injury resulted in prominent ED-1 staining in the interstitium and around damaged glomeruli in postischemic vehicle-treated rats; the presence of ED-1 positive cells was decreased in postischemic animals treated with MMF (Fig. 5, A and B). S100A4, a marker for renal fibroblasts, was highly expressed in postischemic vehicle-treated I/R rats (Fig. 5, C and D). The tissue was obtained from rats 9 wk following renal I/R injury and after 4 wk on a high-salt (4.0% NaCl) diet. The rats were treated with MMF (20 mg·kg⁻¹·day⁻¹ ip) or vehicle during the period of high salt intake.
vehicle-treated rats, but treatment with MMF significantly reduced the amount of S100A4 positive cells (red staining, Fig. 5, C and D). Myofibroblasts indicated by α-smooth muscle actin staining were more prominent in postischemic vehicle-treated rats compared with sham-operated control rats and MMF-treated postischemic animals (blue staining, Fig. 5, C and D).

**DISCUSSION**

The present studies demonstrate that administration of the immunosuppressive agent MMF to I/R recovery rats placed on a high-salt diet decreases the number of macrophages and fibroblasts in the renal interstitial space, reduces the degree of renal histological damage, restores creatinine clearance to normal levels, and blunts the increase in arterial blood pressure observed in these animals. These data therefore indicate that the recovery from I/R-mediated ARF is incomplete despite normal tubular morphology 5 wk after the original renal insult. Infiltrating immune cells appear to mediate or participate in the development of sodium-sensitive hypertension in I/R recovery rats.

There is a growing emphasis on the long-term effects of ARF. Recent evidence suggests that renal function is not restored to normal following I/R despite apparently normal renal tubular morphology and creatinine levels. We have reported the development of a renal concentrating defect and a permanent reduction in peritubular capillaries in the recovered kidneys (2, 3). These alterations are largely asymptomatic during conditions of a low or normal sodium intake but may contribute to hypertension with secondary renal disease when sodium intake is increased despite the suppression of plasma renin (21). The sodium-sensitive hypertension is accompanied by renal damage, a reduction of glomerular filtration rate, and the infiltration of inflammatory cells into the renal interstitial space.

ARF induced by I/R has a prominent inflammatory component evident by the infiltration of inflammatory cells and production of proinflammatory cytokines. Many studies have explored the effects of the inflammatory system early in the course of ischemic ARF. Studies by Forbes et al. (6) reported the infiltration of monocytes and macrophages into the renal interstitium as early as 24 h posts ischemia and peaking at day 8 (6). Evidence suggests the inflammatory response may contribute to the pathology of I/R injury. Several investigators have shown that blocking the immune component by genetic manipulation or using immune antagonist ameliorates the injury produced by ischemia (9, 11, 16). The resolution of the inflammation following injury has not been clearly described.

The role of infiltrating immune cells in the sodium-sensitive hypertension and kidney disease that occurs following the apparent recovery from I/R injury has not been examined. Previous experimental evidence indicates that the infiltration of renal interstitial inflammatory cells contributes to the development of sodium-dependent hypertension and tubulointerstitial damage in models of renal injury (1, 13, 15, 18). The present study examined the role of the inflammatory response in the development of sodium-dependent hypertension in the I/R model. The data are consistent with our previous observation showing the development of sodium-sensitive hypertension in postischemic rats. Furthermore, systemic treatment with MMF following recovery from ARF attenuated sodium-dependent hypertension in post-I/R rats. Interestingly, PRA was elevated in the MMF-treated I/R rats compared with I/R sham rats; this likely reflects the altered volume status and different activation of the systemic renin-angiotensin system in MMF-treated I/R rats. The present data suggest a role for infiltrating immune cells in the development of sodium-dependent hypertension and renal disease following recovery from I/R-induced ARF.

The exact mechanism in which the immune system participates in the development of sodium-sensitive hypertension and kidney disease in I/R rats is unknown. It has been proposed that the renal interstitial infiltration of inflammatory cells produces an increase in local ANG II and/or reactive oxygen species within the kidney (5, 7, 18). The present data demonstrate that there is a dramatic reduction in glomerular filtration rate and marked histological damage associated with immune cell infiltration in the kidneys of I/R rats fed a high-salt diet. Moreover, these changes are reversed by treatment with the immunosuppressive drug MMF. The present data are consistent with reports by White and Grollman (25) and by Norman et al. (14) indicating that the secondary or maintenance phase of hypertension in rats with partial renal artery infarction is dependent upon the immune system. It therefore appears likely that the infiltration of immune cells mediates the renal functional changes and histological damage and leads to the retention of sodium and water and the development of hypertension in I/R rats fed a high-salt diet. The cause and effect relationship in this model between the changes in renal function and the development of hypertension, however, remain to be determined.

In summary, these data support the concept that long-term changes occur in the kidney following acute ischemic renal failure that increase the susceptibility to sodium-sensitive hypertension. The predisposition to hypertension and exacerbation of the progression of secondary renal disease with increased sodium intake is abolished by systemic immune suppression. The factor(s) leading to the infiltration of immune cells into the kidney and the pathogenic mediators released by infiltrating immune cells remain to be elucidated.

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