

## High Dietary Protein Exacerbates Hypertension and Renal Damage in Dahl SS Rats by Increasing Infiltrating Immune Cells in the Kidney

Carmen De Miguel, Hayley Lund, David L. Mattson

**Abstract**—The present study evaluated the influence and mechanism of action of dietary protein intake in Dahl SS hypertension and renal disease. Rats were fed isocaloric diets with low (6%), normal (18%), or high (30%) amounts of protein and 0.4% NaCl from 5 to 12 weeks of age; the NaCl content of the diets was then increased to 4.0% NaCl from 12 to 15 weeks of age. Rats fed the high-protein diet developed the highest mean arterial blood pressure and urine albumin-to-creatinine ratio when fed the 4.0% NaCl diet ( $153 \pm 7$  mm Hg and  $8.0 \pm 2.4$ , respectively) compared to rats fed normal protein ( $132 \pm 3$  mm Hg,  $1.2 \pm 0.3$ ) or low-protein ( $132 \pm 6$  mm Hg,  $0.3 \pm 0.1$ ) diets. Significantly greater numbers of infiltrating T lymphocytes were observed in kidneys of SS rats fed the high-protein diet ( $18.9 \pm 3 \times 10^5$  cells) than in rats fed the low-protein diet ( $9.1 \pm 3 \times 10^5$  cells). Furthermore, treatment of SS rats fed the high-protein diet with the immunosuppressant agent mycophenolate mofetil (20 mg/kg per day, ip) significantly reduced the number of infiltrating T cells in the kidneys (from  $18.9 \pm 2.7$  to  $10.6 \pm 2.0 \times 10^5$  cells) while decreasing blood pressure (from  $133 \pm 3$  to  $113 \pm 4$  mm Hg) and the albumin/creatinine ratio (from  $10.9 \pm 2.3$  to  $5.4 \pm 1.2$ ). These results demonstrate that restriction of protein intake protects the Dahl SS rats from hypertension and kidney disease and indicates that infiltrating immune cells play a pathological role in Dahl SS rats fed a high-protein diet. Moreover, the results show that hypertension in Dahl SS rats is sensitive to both NaCl and protein intake. (*Hypertension*. 2011;57:269-274.)

**Key Words:** hypertension ■ kidney disease ■ albuminuria ■ T lymphocytes ■ immunosuppressive agents

Dietary nutrients have a significant influence on arterial blood pressure in humans and experimental animals. Consumption of cholesterol, saturated fats, or carbohydrates is associated with elevated arterial blood pressure, while high-protein diets are linked to decreased blood pressure in the general human population.<sup>1-5</sup> Evidence in patients with renal insufficiency, however, indicates that elevated protein intake may accelerate the decline in renal function.<sup>6-9</sup> The effects of changes in dietary fat, protein, and carbohydrate on blood pressure have also been examined in experimental animal models. The development of hypertension is accelerated in genetic models of hypertension depending on the protein,<sup>10</sup> carbohydrate,<sup>11-14</sup> and fat<sup>14,15</sup> composition of the diet. These clinical and experimental results indicate that various dietary components can influence arterial blood pressure and kidney damage independently of sodium intake.

Recent studies from our laboratory have demonstrated that the source of protein in the chow fed to Dahl SS rats modified the degree of sodium-sensitive hypertension and the associated damage to the kidney observed with elevated NaCl intake.<sup>16,17</sup> Additional experiments demonstrated that the renal damage in Dahl SS rats is associated with infiltration of macrophages and T lymphocytes and that suppression of

immune cell infiltration attenuates sodium-sensitive hypertension and kidney damage.<sup>18,19</sup> Since characteristics of salt-dependent hypertension and kidney damage in the SS rat strain are similar to those observed in human populations,<sup>20</sup> an understanding of environmental effects that modify disease phenotypes can provide insight into human disease. The present study was specifically designed to test the hypothesis that the amount of protein in the diet can modify sodium-sensitive hypertension and kidney damage in Dahl SS rats. Additional experiments were designed to examine the infiltration of immune cells into the kidneys of rats fed elevated NaCl and to determine the importance of these infiltrating cells in the development of these protein- and sodium-sensitive disease phenotypes.

To address these questions, rats were fed custom diets containing different amounts of protein. The custom diets consisted of simple modifications of the AIN-76A diet formulation with low (6%), normal (18%), or high (30%) amounts of protein. The rats were fed the different diets containing 0.4% NaCl from  $\approx 4$  to 12 weeks of age; the amount of salt in the diet was then increased to 4.0% NaCl for the final 3 weeks of the study. The influence of the dietary protein content on the development of sodium-sensitive

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hypertension and kidney damage and the potential role of infiltrating immune cells in this process was assessed in the final week of the high-NaCl intake period.

## Methods

### Experimental Animals

Experiments were performed on inbred Dahl SS rats obtained from Charles River Laboratories (SS/JrHsdMewiCrl) and outbred Sprague Dawley (SD) rats obtained from Harlan Sprague Dawley. The SS rats were received at  $\approx$ 4 to 5 weeks of age and randomly placed and maintained on 1 of the 3 (low, normal, or high) protein diets described below. The salt content of each diet was 0.4% NaCl from 4 to 12 weeks of age. At 12 weeks of age, the salt content of the chow was increased to 4.0% NaCl, and the rats were maintained on this diet for an additional 3 weeks. A subset of SS rats fed the high-protein/high-salt diet was treated with the immunosuppressant agent mycophenolate mofetil (20 mg/kg per day, ip) or vehicle daily throughout the period of elevated salt intake. The SD rats, which served as a normotensive control strain in this experiment, were fed either a normal protein or a high-protein diet from 4 to 15 weeks of age. Both the normal protein and high-protein diet contained 0.4% NaCl from 4 to 12 weeks for all SD rats; the NaCl content of the diet was then either maintained at 0.4% NaCl in a group fed the normal protein chow or increased to 4.0% NaCl in groups fed the normal protein or high-protein chow from 12 to 15 weeks of age. The Medical College of Wisconsin Institutional Animal Care and Use Committee approved all experimental protocols.

### Diets

The diets were obtained from Dyets Inc. The standard AIN-76A chow containing 18% casein served as normal protein chow. Custom formulations were also obtained containing 6% and 30% casein and served as low- and high-protein diets, respectively. The different percentages of protein in each diet were substituted for or replaced with carbohydrate (sucrose) and were isocaloric.

### Surgical Procedure

Surgical procedures were performed on the first day of the 2-week experimental protocol and occurred 2 weeks following the transition from low (0.4%) to high (4.0%) NaCl chow. The rats were deeply anesthetized with an intraperitoneal injection of ketamine (35 mg/kg), xylazine (10 mg/kg), and acepromazine (0.5 mg/kg) with supplemental anesthesia administered when needed. Using an 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 and analgesic agents were administered postsurgically, and the rats were allowed to fully awaken from anesthesia on a temperature-controlled pad. 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.

### Experimental Procedures

The rats were permitted to recover for 1 week following surgery. During this time, they were maintained on the individual diets containing 4.0% NaCl. After the recovery period, high-salt blood pressure measurements were obtained from 9:00 AM to 12:00 PM on 3 consecutive days as described previously.<sup>16,17</sup> Following the second day of blood pressure measurement, an overnight urine collection (from 4:00 PM to 8:00 AM) was obtained for measurement of urinary sodium, creatinine, and albumin excretion rates. The data are normalized to a 24-hour period and may therefore provide an overestimate of excretory parameters in these nocturnal animals. On the final day, arterial plasma samples were obtained for measurement of plasma creatinine concentration while the rats were maintained on the high-NaCl diet.

Urine electrolytes were measured by flame photometry (IL-943; Instrumentation Laboratories). Plasma and urine creatinine values were measured with an assay based on the Jaffé reaction by

autoanalyzer (ACE; Alfa Wasserman). Urine albumin was quantified with a fluorescent assay that utilized Albumin Blue 580 dye (Molecular Probes) and a fluorescent plate reader (FL600; Bio-Tek).

### Histological and Immunohistochemical Analysis

Kidneys were obtained for histological and immunohistochemical analysis using methods we have described previously.<sup>17–19</sup> The rats were anesthetized with sodium pentobarbital (50 mg/kg, ip); the kidneys were then removed and placed in a 10% formaldehyde solution. The tissue was paraffin embedded (Microm HMP 300), cut in 3- $\mu$ m sections (Microm HM355S), mounted, and stained with Gomori's One-Step Trichrome. Individual glomeruli (average of 37 per rat) were evaluated with the method of Raji et al.<sup>21</sup> For immunohistochemistry, slides were deparaffinized and incubated with proteinase K for antigen retrieval. The primary monoclonal antibody used to detect T cells was anti-CD43 (Abcam). A biotinylated horse anti-mouse secondary antibody was used for development with avidin-biotinylated horseradish peroxidase complex (Vectastain ABC kits; Vector Laboratories). The slides were lightly counterstained with aniline blue dye and photographed.

### T-Cell Isolation

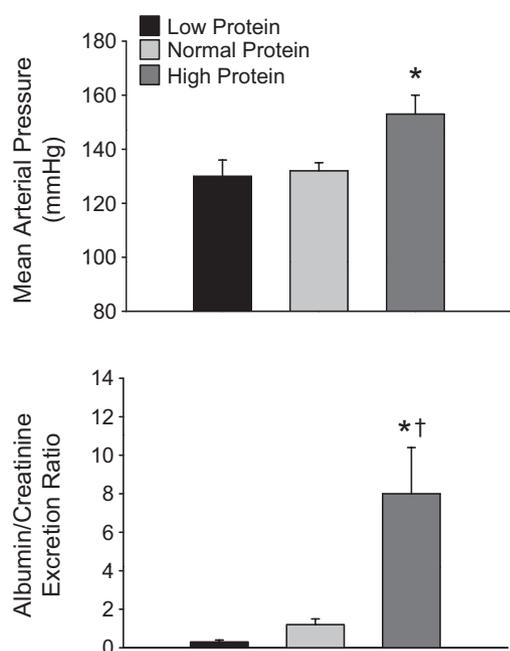
Isolation of infiltrating cells was performed using methods we described previously.<sup>19</sup> Rats were anesthetized with sodium pentobarbital (50 mg/kg, ip), the abdominal aorta was isolated and cannulated, and the kidneys were perfused with a solution containing 154 mmol/L NaCl and 100 U/mL heparin. The kidneys were removed and cut into 1- to 2-mm-thick sections; the sections were incubated at 37°C for 60 minutes in dissection solution containing the following: 135 mmol/L NaCl, 3 mmol/L KCl, 2 mmol/L  $\text{KH}_2\text{PO}_4$ , 5.5 mmol/L glucose, 20 mmol/L HEPES (pH 7.2), and 0.85 mg/mL collagenase (573 U/mg; Sigma). During incubation, the samples were gently shaken and bubbled with 95%  $\text{O}_2$ /5%  $\text{CO}_2$ . The digested solution was then filtered through 100- $\mu$ m and 70- $\mu$ m filters and centrifuged at 300g for 10 minutes. The pellet was resuspended in PBS containing DNase 1 (20 U/mL). The isolated cells were layered over 5 mL of Histopaque (Sigma) and centrifuged at 400g for 30 minutes at room temperature, and the mononuclear cell layer was collected. The separated mononuclear cells were resuspended, washed, and incubated with a rat Pan T cell antibody coupled to magnetic microbeads (MACS Rat Pan T Cell Microbeads; Miltenyi Biotec) for 15 minutes at 4°C to 8°C. The cells were then washed and resuspended in labeling buffer, and the cell suspension was applied to a magnetic column (MACS Separation Columns; Miltenyi Biotec) to isolate T lymphocytes.

### Statistical Analysis

Data are presented as the mean  $\pm$  SE. Experiments were performed on 5 to 10 rats per group. A 1-way ANOVA was utilized to determine the differences in parameters between the rats maintained on the different diets. A Tukey post hoc test was used when appropriate. An unpaired *t* test was used to evaluate differences between two groups. The 95% confidence interval was considered significant.

## Results

High-salt mean arterial blood pressure was significantly elevated in SS rats fed the high-protein diet ( $153 \pm 7$  mm Hg) compared to those fed the low-protein or normal protein diet (Figure 1, top). No differences were detected between the rats fed the low-protein and normal protein diet. The heart rate in rats fed the high-NaCl diet was not altered in the low- or high-protein diet groups from the value measured in the normal protein rats ( $389 \pm 14$  bpm). As an index of kidney disease, it was observed that urinary albumin excretion rate in the high-salt rats was directly related to the protein content of the diet. The ratio of albumin to creatinine in urine was  $\approx$ 10-fold greater in the rats fed the high-protein diet than that

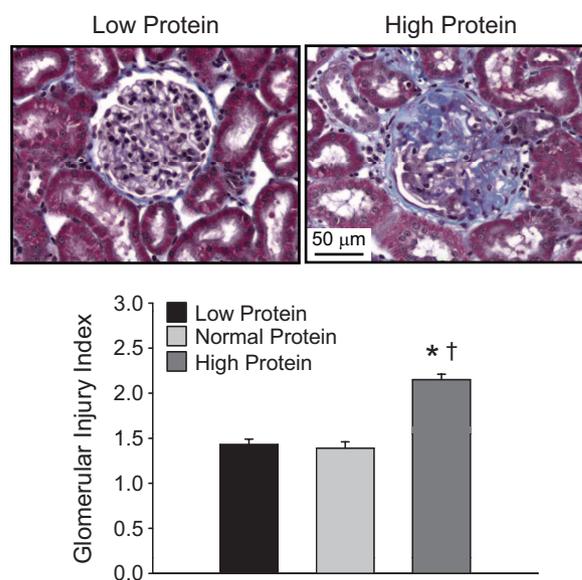


**Figure 1.** Mean arterial blood pressure (top) and urine albumin-to-creatinine excretion ratio (bottom) in SS/Mcw rats maintained on high-protein, normal protein, or low-protein diets for 14 weeks. The measurements were made following 3 weeks on a high-NaCl (4.0%) diet. \* $P < 0.05$  vs the low-protein diet group; † $P < 0.05$  vs the normal protein diet group.

observed in rats fed the low-protein diet (Figure 1, bottom). Albumin and protein excretion rates averaged  $114 \pm 37$  and  $220 \pm 45$  mg/d, respectively, in the high-protein rats. The steady-state sodium excretion rate was not different between the groups and averaged  $10 \pm 2$  mEq/d in the normal protein rats fed the high-NaCl chow.

Representative examples of histological differences observed between the kidneys of rats fed the low- and high-protein diet are illustrated in Figure 2. Consistent with previous reports,<sup>18,19</sup> the presence of large amounts of blue fibrotic tissue with collapsed glomerular capillaries are apparent in the kidneys of SS/Mcw fed the high-protein diet. Visibly less glomerular damage is evident in the kidneys of rats fed the low-protein diet, despite similar levels of sodium intake. The glomerular injury index (Figure 2, bottom) was significantly greater in the Dahl SS/Mcw rats fed the high-protein diet ( $2.2 \pm 0.1$ ) than in the rats fed the normal protein ( $1.4 \pm 0.2$ ) or low-protein ( $1.5 \pm 0.3$ ) diet. Despite the influence of dietary protein content on glomerular histological damage and on albumin and protein excretion, no differences were detected in creatinine clearance. Though the conscious creatinine clearance rate on high salt tended to decrease with the increased protein intake, it was not different in the groups fed the high-protein ( $0.48 \pm 0.12$  mL/min per gkwt) or low-protein ( $0.66 \pm 0.10$  mL/min per gkwt) diet compared to the average of  $0.62 \pm 0.06$  mL/min per gkwt measured in the Dahl SS rats fed the normal protein diet.

Body weight was not different between rats fed the high-protein ( $379 \pm 5$  g) and the normal protein ( $374 \pm 9$  g) diet; rats fed the low-protein diet were significantly smaller ( $313 \pm 13$  g) than rats fed the normal protein or high-protein

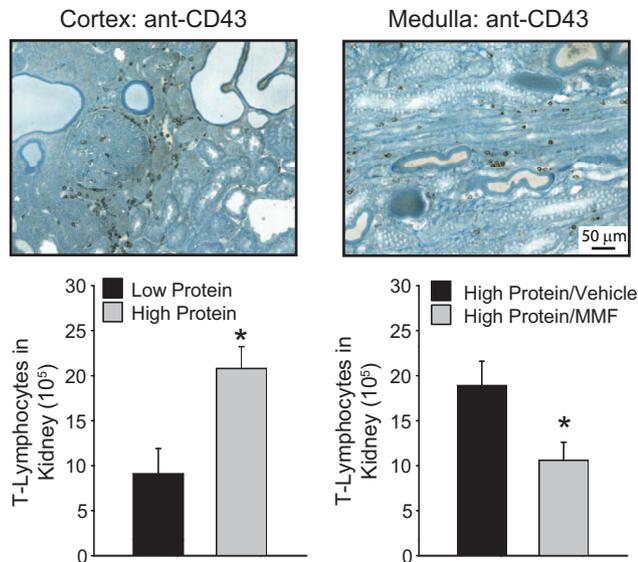


**Figure 2.** Top, Light microscopy images of representative glomeruli ( $\times 40$  original magnification, bottom) from kidneys obtained from male Dahl SS/Mcw rats fed the low-protein or high-protein diets. Bottom, Changes in the glomerular injury score in rats fed the low-protein, normal protein, or high-protein diet. The NaCl content of each diet was increased from 0.4% to 4.0% for the final 3 weeks of the experiment. \* $P < 0.05$  vs the low-protein diet group; † $P < 0.05$  vs the normal protein diet group.

diets. Kidney weight was directly related to the protein intake; total kidney mass of rats fed the high-protein diet ( $4.8 \pm 0.2$  g) was significantly greater than that observed with rats fed the normal protein ( $3.4 \pm 0.2$  g) or low-protein diet ( $2.7 \pm 0.2$  g). No differences were detected in plasma protein or albumin concentration between groups. Plasma protein and albumin concentration averaged  $5.6 \pm 0.1$  and  $2.9 \pm 0.1$  mg/dL in rats fed the high-protein diet,  $5.5 \pm 0.1$  and  $2.9 \pm 0.1$  mg/dL in rats fed the normal protein diet, and  $5.7 \pm 0.1$  and  $3.0 \pm 0.1$  mg/dL in rats fed the low-protein diet.

The high-protein/4% NaCl diet had no influence on blood pressure or renal albumin excretion in normotensive SD rats compared to values obtained from rats fed normal protein/4% NaCl or normal protein/0.4% NaCl chow. The mean arterial pressure ( $111 \pm 3$  mm Hg,  $n = 5$ ) and urine albumin-to-creatinine excretion ratio ( $1.2 \pm 0.3$ ) in SD rats fed the high-protein diet containing 4.0% NaCl were not different from the values obtained in the SD rats fed the normal protein diet containing 0.4% NaCl ( $108 \pm 2$  mm Hg and  $0.8 \pm 0.2$ , respectively) or the rats fed the normal protein/4.0% NaCl diet ( $109 \pm 2$  mm Hg and  $0.5 \pm 0.2$ , respectively).

Results of additional studies to examine the infiltration of T lymphocytes into the kidneys of SS rats fed the low- or high-protein diet are summarized in Figure 3. Figure 3 (top) provides an illustration of the localization of infiltrating CD43-positive cells in the tissue surrounding damaged glomeruli in the renal cortex (left) and damaged tubules and vasa recta bundles (right) in the renal outer medulla. Significantly greater numbers of infiltrating T lymphocytes were observed in the kidneys of SS rats fed the high-protein diet following 3 weeks of elevated NaCl intake ( $18.9 \pm 3 \times 10^5$  cells per two kidneys) than in rats fed the low-protein/high-NaCl diet



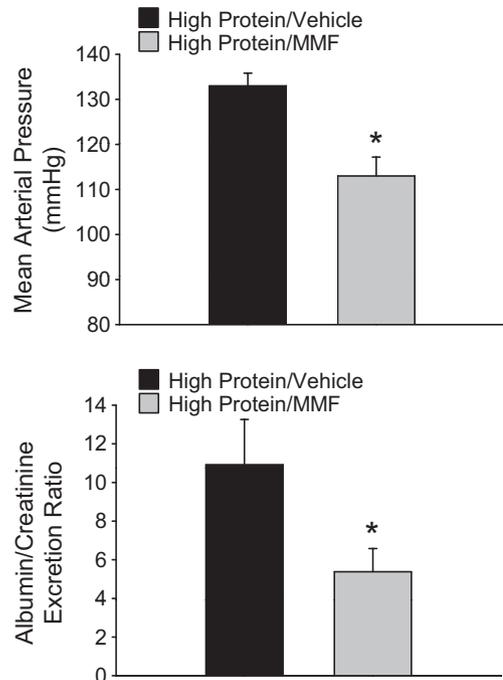
**Figure 3.** Top, Immunohistochemical localization of T cells in the renal cortex (left) and medulla (right) of Dahl SS rats fed 4.0% NaCl chow. T cells were localized in the renal tissue with antibodies directed against the cell surface marker CD43 (original magnification  $\times 20$ ). Bottom left, Infiltrating T lymphocytes in the kidneys of Dahl SS/Mcw rats maintained on high- or low-protein diets for 14 weeks with 4.0% NaCl for the final 3 weeks. Bottom right, Influence of vehicle or mycophenolate mofetil treatment (20 mg/kg per day, ip) during the high-salt period on infiltrating T lymphocytes in the kidneys of Dahl SS/Mcw rats maintained on a high-protein diet for 14 weeks with 4.0% NaCl for the final 3 weeks. \* $P < 0.05$  vs other group.

( $9.1 \pm 3 \times 10^5$  cells per two kidneys) (Figure 3, bottom left). To elucidate a role for the infiltrating T cells in the hypertension and renal disease, separate groups of rats fed the high-protein diet were treated with the immunosuppressive agent mycophenolate mofetil (20 mg/kg per day) or vehicle during the period of elevated NaCl intake. Treatment of SS rats fed the high-protein diet with mycophenolate mofetil (MMF) significantly reduced the number of infiltrating T cells in the kidneys by  $\approx 44\%$  (Figure 3, bottom right). Moreover, the MMF treatment significantly attenuated the development of hypertension and albuminuria in Dahl SS rats fed high salt and maintained on the high-protein diet. The high-salt mean arterial blood pressure value was reduced by 20 mm Hg, whereas the albumin/creatinine excretion ratio was reduced by 50% in rats treated with MMF (Figure 4).

### Discussion

The present experiments demonstrate that hypertension and renal disease phenotypes in Dahl SS/Mcw rats are sensitive to elevated dietary protein intake. Rats fed the high-protein diet developed higher blood pressure, had greater albumin and protein excretion rates, and demonstrated greater renal hypertrophy and histological damage compared to rats fed diets containing low or normal protein. The results of this study therefore indicate that the hypertension and kidney disease phenotypes are sensitive to both NaCl and protein intake in the Dahl SS/Mcw rat.

A mechanism for the increased development of disease in rats fed an elevated protein diet is unclear. The present



**Figure 4.** Influence of MMF (20 mg/kg per day, ip) during the high-salt period on mean arterial blood pressure (top) and urine albumin-to-creatinine excretion ratio (bottom) in SS/Mcw rats maintained on the high-protein diet for 14 weeks with 4.0% NaCl administered for the final 3 weeks. \* $P < 0.05$  vs other group.

studies, however, provide some insight into the development of disease in this genetic model. In addition to the kidney damage observed following 3 weeks of the high NaCl intake, rats fed the high-protein diet demonstrated an increased renal infiltration of T lymphocytes compared to rats maintained on the low-protein diet. Renal infiltration of immune cells has been demonstrated in nonimmune models of hypertension and kidney disease.<sup>22–27</sup> Lymphocytes and macrophages infiltrate the kidney in experimental and genetic models of hypertension disease,<sup>22–28</sup> and immunosuppression through genetic or pharmacological means has been demonstrated to attenuate hypertension and renal disease.<sup>29–32</sup> The mechanisms utilized by infiltrating immune cells to increase blood pressure and renal disease are unclear. It has been proposed that infiltrating immune cells can participate in the disease phenotype by releasing free radicals, cytokines, or other vasoactive factors. It is possible that the infiltrating immune cells are exerting their deleterious effects in the kidney; alternatively, infiltration of T cells into systemic blood vessels, the brain, or other organs may mediate the deleterious effects of immune cells in hypertension. Moreover, the factors mediating infiltration of immune cells in salt-sensitive hypertension remain to be elucidated.

The present data may reveal mechanisms whereby a high-protein diet can exacerbate renal disease and hypertension. Though a high-protein diet has not been convincingly demonstrated to induce renal disease in normal individuals, epidemiological and experimental data indicate that an elevated protein diet is deleterious in subjects with preexisting kidney disease.<sup>6–9</sup> This observation is consistent with the hypothesis posed by Brenner et al<sup>33</sup> in which it was postulated

that an elevated protein intake can lead to renal vasodilation and elevated glomerular capillary hydrostatic pressure with resultant glomerular damage. Protein-induced renal dilation in the Dahl SS rat, which is susceptible to glomerular damage and hypertension, could accelerate the disease process.

The blood pressure effects of varying protein intake have also not been consistently noted.<sup>34</sup> A high-protein diet has been demonstrated to increase blood pressure in a genetic model of hypertension.<sup>10</sup> Clinical data indicate that high-protein diets may also increase arterial blood pressure in patients<sup>35,36</sup> although other human data indicate an inverse association between blood pressure and protein intake.<sup>37,38</sup> Of note, a previous study in rats demonstrated that protein overloading of normal Lewis rats by intraperitoneal injection of large amounts of bovine serum albumin led to infiltration of immune cells, renal damage, and salt-sensitive hypertension.<sup>39</sup> Of interest, the description of the histological changes in the renal medulla of these rats was similar to that observed in Dahl SS rats fed high salt, though the glomerular damage in the protein-overload rats appeared to be less severe than observed in the Dahl SS rats. The present data support the concept that elevated dietary protein intake enhances hypertension in a genetic animal disease model.

The Dahl SS rats fed the normal protein/high-salt diet in the present experiment have attenuated hypertensive and renal disease phenotypes when compared to previous data we have published with Dahl SS/Mcw rats fed the same AIN-76A diet.<sup>16–19</sup> Although the Dahl SS rats used in the present study are genetically identical to those studied previously, the animals in the present study were obtained from a commercial vendor. Based on our previous work in this area that indicated that the diet fed to the mothers during pregnancy and weaning can have a marked impact on the final disease phenotype,<sup>16,17</sup> we speculate that the difference in the degree of the final disease phenotype may be attributable to the diet fed to the breeding stock during pregnancy and nursing at Charles River Laboratories (Purina 5L79, a grain-based diet) compared to the AIN-76A diet fed to the breeding colony at Medical College of Wisconsin.

## Perspectives

The present experiments illustrate the harmful effects of a high-protein diet in an animal model of salt-sensitive hypertension. The results indicate that the deleterious effects of high NaCl intake are exacerbated in Dahl SS/Mcw rats when superimposed on the background of a high-protein intake. One potential mechanism identified in this study may be related to factors promoting the infiltration of immune cells, particularly T lymphocytes. Further study will be necessary to elucidate the mechanisms leading to the infiltration of T cells in the kidneys of these hypertensive animal models as well as the potential mechanisms that these cells may use to alter disease phenotypes.

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## Disclosures

None.

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