Renal Protective Effect of N-Acetyl-Seryl-Aspartyl-Lysyl-Proline in Dahl Salt-Sensitive Rats

Morel E. Worou, Tang-Dong Liao, Martin D’Ambrosio, Pablo Nakagawa, Branislava Janic, Edward L. Peterson, Nour-Eddine Rhaleb, Oscar A. Carretero

Abstract—N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is a natural tetrapeptide with anti-inflammatory and antifibrotic properties. Its effect on salt-sensitive (SS) hypertension is unknown. We hypothesized that in Dahl SS rats on high-salt (HS) diet, Ac-SDKP prevents loss of nephrin expression and renal immune cell infiltration, leading to a decrease in albuminuria, renal inflammation, fibrosis, and glomerulosclerosis. To test this, Dahl SS rats and consomic SS13BN controls were fed either a low-salt (0.23% NaCl) or HS (4% NaCl) diet and treated for 6 weeks with vehicle or Ac-SDKP at either low or high dose (800 or 1600 μg/kg per day, respectively). HS increased systolic blood pressure in SS rats (HS+vehicle, 186±5 versus low salt+vehicle, 141±3 mmHg; P<0.005) but not in SS13BN rats. Ac-SDKP did not affect blood pressure. Compared with low salt, HS-induced albuminuria, renal inflammation, fibrosis, and glomerulosclerosis in both strains, but the damages were higher in SS than in SS13BN. Interestingly, in SS13BN rats, Ac-SDKP prevented albuminuria induced by HS (HS+vehicle, 44±8 versus HS+low Ac-SDKP, 24±3 or HS+high Ac-SDKP, 8±1 mg/24 h; P<0.05), whereas in SS rats, only high Ac-SDKP dose significantly attenuated albuminuria (HS+vehicle, 94±10 versus HS+high Ac-SDKP, 57±7 mg/24 h; P<0.05). In both strains, Ac-SDKP prevented HS-induced inflammation, interstitial fibrosis, and glomerulosclerosis. In summary, in SS rats on HS diet, at low and high doses, Ac-SDKP prevented renal damage without affecting the blood pressure. Only the high dose of Ac-SDKP attenuated HS-induced albuminuria. Conversely, in SS13BN rats, both doses of Ac-SDKP prevented HS-induced renal damage and albuminuria. (Hypertension. 2015;66:816-822. DOI: 10.1161/HYPERTENSIONAHA.115.05970.) • Online Data Supplement

Key Words: albuminuria • blood pressure • hypertension • inflammation • nephrin

Salt sensitivity (SS) is associated with severe progression of hypertensive target organ damage, including end-stage renal disease.1,2 The underlying mechanism(s) of SS hypertension and associated renal injury are still not clear, but it is thought that inflammation plays an important role.3,4 Previous studies showed that high salt (HS)–induced hypertension and renal injury is associated with increased albuminuria, immune cell infiltration, tubulointerstitial injury, and glomerulosclerosis.4-6

The Dahl SS rat is a model of hypertension and renal disease that exhibits phenotypic traits common for sodium-sensitive hypertension and renal dysfunction observed in the human population. When studying the Dahl SS model, it is valuable to use the consomic SS13BN rat as a control strain. It is generated by substituting chromosome 13 from a normotensive Brown Norway (BN) rat into the SS genome, which makes it 98% genetically identical to the SS rat. Salt-induced hypertension, albuminuria, and renal injury are attenuated in SS13BN rats.7,8

Glomerular filtration barrier integrity and function strongly depend on nephrin, an important transmembrane protein fundamental for the podocyte slit diaphragm function; regulating renal filtration and selectively allowing small molecules like ions to pass through, while excluding the passage of large molecules like proteins.9,10 A decrease in nephrin expression or its function may cause massive proteinuria.11,12

N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is a natural tetrapeptide released from its precursor thymosin β4, by prolyl oligopeptidase.13 Ac-SDKP is found in human plasma and circulating mononuclear cells14 and various organs,15 and is hydrolyzed mainly by angiotensin-converting enzyme (ACE).16 We previously showed that the anti-inflammatory and antifibrotic effects of ACEi are mediated by an increase in endogenous Ac-SDKP.17,18 Animal studies have demonstrated anti-inflammatory and antifibrotic properties of Ac-SDKP,19-21 and that a decrease in endogenous Ac-SDKP levels promotes heart and kidney fibrosis.22 However, the effect of Ac-SDKP on SS hypertension is still unknown. We therefore designed a new study to test the novel hypothesis that the administration of Ac-SDKP to Dahl SS hypertensive or consomic non-hypertensive rats on an HS diet prevents (1) loss of nephrin expression, (2) renal macrophage infiltration, and (3) T-cell
infiltration, and that these changes lead to decreases in albuminuria, renal inflammation, fibrosis, and glomerulosclerosis, without affecting blood pressure.

Methods

Male Dahl SS and consomic SS13BN rats at 4 weeks of age (Charles River Laboratories, Wilmington, MA) were fed either a low-salt (LS=0.23% NaCl) or HS (HS=4% NaCl) diet and treated for 6 weeks with vehicle or Ac-SDKP at either low or high dose (800 or 1600 µg/kg per day, respectively). Measurements of blood pressure and albuminuria and histological studies were performed. This study was approved by the Henry Ford Hospital Institutional Animal Care and Use Committee.

An expanded Methods section is available in the online-only Data Supplement.

Results

Systolic Blood Pressure, Body Weight, and Organ Weight

In Dahl SS rats, 6 weeks of HS diet significantly increased systolic blood pressure compared with LS diet (HS+vehicle, 186±5 versus LS+vehicle, 141±3 mmHg; P<0.005). In contrast, in SS13BN rats, HS did not affect systolic blood pressure (HS+vehicle, 140±3 versus LS+vehicle, 140±2 mmHg). Ac-SDKP did not attenuate the increased blood pressure induced by HS diet in SS rats (Figure 1). HS diet significantly increased kidney weight to body weight ratio and heart weight to body weight ratio in both Dahl SS and SS13BN rats. Ac-SDKP did not prevent renal and cardiac hypertrophy induced by HS diet in either strain (Figure S1 in the online-only Data Supplement).

Urinary Ac-SDKP Excretion

In Dahl SS and SS13BN rats, 24-hour urinary Ac-SDKP excretion was significantly higher (5–11 fold) in Ac-SDKP-treated group than in vehicle-treated group (Figure S2). In the vehicle-treated animals, the urinary Ac-SDKP excretion in SS13BN rats was significantly elevated compared with Dahl SS rats (Figure S3).

Albumin and Protein Excretion

Compared with LS, HS diet increased albuminuria in Dahl SS and in SS13BN rats (Figure 2A). Similar to albuminuria, HS diet increased proteinuria in both strains (Figure 2B). Greater increases were observed in Dahl SS compared with SS13BN rats. In SS13BN rats, low and high doses of Ac-SDKP significantly reduced HS-induced albuminuria and proteinuria (Figure 2A and 2B). In Dahl SS rats, only high Ac-SDKP dose significantly reduced albuminuria and proteinuria (Figure 2A and 2B) induced by HS diet.

Glomerular Nephrin Expression

In Dahl SS and SS13BN rats, HS diet significantly decreased nephrin expression. In Dahl SS rats, only the high dose of Ac-SDKP significantly attenuated HS-induced decrease in nephrin expression, whereas in SS13BN rats on HS diet, both low and high doses of Ac-SDKP significantly prevented decrease in nephrin expression (Figure 2C and 2D).

Renal Macrophage and T-Cell Infiltration

Macrophage and T-helper cell infiltration was examined by immunohistochemistry. In Dahl SS and SS13BN rats, HS diet markedly increased the number of infiltrating macrophages as detected by CD68+ positivity (Figure 3A and 3B), and T-helper cells detected by CD4+ positivity (Figure 3C and 3D). The number of infiltrating renal macrophages and T cells induced by HS were higher in Dahl SS rats than in SS13BN rats. The infiltrated cells are mainly located in the glomerular and interstitial area (Figure S4A and B). Treatments with low and high doses of Ac-SDKP markedly reduced the numbers of infiltrating cells in both strains.

Renal Interstitial Fibrosis and Collagen Content

In both Dahl SS and SS13BN rats, the renal interstitial collagen fraction was higher with HS diet than with LS diet. Treatment with either low or high doses of Ac-SDKP reduced the interstitial collagen fraction compared with animals on HS treated with vehicle (Figure 4A and 4B). In addition, analysis of renal collagen content by the hydroxyproline assay showed that the increases caused by HS diet could be dose-dependently prevented by Ac-SDKP in both Dahl SS and SS13BN rats (Figure 4C).

Glomerulosclerosis

The effect of Ac-SDKP on glomerulosclerosis was assessed by periodic acid–Schiff staining. Dahl SS and SS13BN rats...
on HS exhibited glomerulosclerosis, detected as dark purple regions of extracellular matrix deposition within the glomerular tufts (Figure 5). The glomerulosclerosis noted in response to an HS diet in both strains was significantly attenuated by treatment with either dose of Ac-SDKP.

**Discussion**

In this study, we examined the protective effects of Ac-SDKP on renal damage in SS hypertension. We assessed the effects of either low or high Ac-SDKP dose on renal injury in Dahl SS and consomic SS13BN rats fed an HS diet. Consistent with previous studies, our results showed that in Dahl SS rats, HS diet caused hypertension, accompanied by a decrease in nephrin expression and an increase in albuminuria and proteinuria. This was coupled with renal inflammation (macrophage and T-helper cell infiltration in the glomerular and interstitial area), tubulointerstitial injury (interstitial fibrosis and collagen deposition), and glomerulosclerosis. Interestingly, in consomic SS13BN controls, even without an increase in blood pressure, HS diet also induced a decrease in nephrin expression. This was coupled with renal inflammation, tubulointerstitial injury, and glomerulosclerosis, but to a lesser degree than in Dahl SS rats.

Our results suggest that HS diet per se, even without further increases in blood pressure, can induce renal injury in a non-SS hypertensive model. The underlying mechanisms by which HS diet exerts deleterious effects, even when the blood pressure does not increase, are still not clear. There is new evidence suggesting that NaCl initiates proinflammatory and profibrotic cascades without affecting blood pressure. For example, naive T-cell differentiation to proinflammatory Th17 cells is exacerbated by NaCl.23 Also, dietary salt increases the expression of the fibrogenic growth factor, transforming growth factor-β1, and the production of endothelial nitrite and nitrate (NOx) in rat aortic endothelium through both p38 and p42/44 MAPK pathways.24 DuPont et al25 demonstrated that excess high dietary sodium intake impairs endothelium-dependent dilation in healthy, normotensive, salt-resistant humans independently of changes in blood pressure.

Because the consomic SS13BN controls share the same genetic background with Dahl SS rats (98% genetically identical), it is likely that chromosome 13 plays an important role in the development of salt-induced hypertension. However, the genetic predisposition factors may play a key role in the susceptibility to renal disease independent of increased blood pressure. The higher degree of renal damage observed in Dahl SS rats could be because of a combined effect of hypertension and HS diet in inducing the renal damage, whereas in consomic SS13BN controls, HS was the only factor inducing the renal damage. Because of its anti-inflammatory and antifibrotic properties, the higher endogenous Ac-SDKP levels may be responsible for less renal damage observed in the consomic controls.

Clinical and animal studies have established proteinuria as a marker of glomerular damage and a promoter of tubulointerstitial inflammation and fibrosis leading to renal failure.26–28 The mechanisms by which urinary proteins induce tubulointerstitial damage are not fully understood and are still under investigation. It is generally accepted that during progression of chronic
renal disease, proximal tubular cells are activated after reabsorbing an excessive amount of filtered proteins. This leads to a tubular injury and interstitial inflammation characterized by macrophage and T-cell interstitial infiltration, which seem to play a major role in the upregulation of profibrotic molecules, including transforming growth factor-β.27 Here, we showed that in both Dahl SS and consomic SS13BN rats, HS diet significantly increased albumin and protein excretion. Greater increases were observed in Dahl SS, compared with SS13BN rats. Interestingly, in consomic SS13BN controls, treatment with either low or high dose of Ac-SDKP significantly prevented HS-induced albuminuria and proteinuria, whereas in Dahl SS rats, the same effect was achieved only with the high dose of Ac-SDKP. These data indicate that the effective Ac-SDKP dose for the prevention of HS-induced albuminuria and proteinuria depends on the strain and the stage of renal disease. To further analyze the mechanism of HS-induced albuminuria and proteinuria, we investigated the expression of nephrin. We showed that in both strains on HS diet, the increase in albuminuria and proteinuria was accompanied with a decrease of nephrin expression. In consomic SS13BN controls on HS diet, treatment with either low or high Ac-SDKP dose significantly prevented the decrease in nephrin expression, whereas in Dahl SS rats on HS diet, only a high Ac-SDKP dose had this effect, indicating that the protective effect of Ac-SDKP on proteinuria is achieved by preventing the decrease in nephrin expression.

This study also showed that the high levels of albuminuria and proteinuria are accompanied by renal macrophage and T-helper cell infiltration, fibrosis, and glomerulosclerosis. Infiltrating immune cells play an important role in mediating SS hypertension and renal disease.28,30 Animal studies using immunomodulatory treatments that decreased T-cell infiltration showed amelioration of SS hypertension and renal disease.3,31 However, it is still not clear if the inflammation is a cause or a consequence of the SS hypertension. Our data showed that in Dahl SS and in consomic SS13BN control rats, HS diet markedly increased renal macrophage and T-helper cell infiltration. The number of infiltrating renal macrophages and T cells in rats on HS diet was higher in Dahl SS rats than in consomic SS13BN rats, indicating a correlation between the degree of inflammation and proteinuria. In both strains, low and high doses of Ac-SDKP prevented renal macrophage and T-helper cell infiltration induced by HS diet without a decrease in blood pressure.

Parallel to the macrophage and T-cell infiltration, in Dahl SS and in consomic SS13BN controls, HS-induced renal interstitial fibrosis was accompanied with an increase in renal collagen content and glomerulosclerosis. In both strains, low and high doses of Ac-SDKP significantly prevented HS-induced renal interstitial fibrosis. Ac-SDKP significantly decreased HS-induced renal collagen content, but a greater decrease was observed with the dose of high Ac-SDKP. At low and high doses, Ac-SDKP also significantly abrogated HS-induced glomerulosclerosis in both strains.

We and others have already shown protective effects of Ac-SDKP in the heart, kidney, and brain.32–34 We have previously reported possible mechanism(s) by which Ac-SDKP protects organ damage. These effects are related to a decrease in proinflammatory pathways, such as nuclear factor-κ B activation, cytokine release, and intercellular adhesion molecule-1 expression.35,36 In addition, we also showed that in
vitro, Ac-SDKP exerts anti-inflammatory effects by inhibiting (1) differentiation of bone marrow stem cells to macrophages, (2) activation and migration of macrophages, and (3) release of the proinflammatory cytokine tumor necrosis factor-α by activated macrophages. The anti-inflammatory and antifibrotic effects of Ac-SDKP are observed not only in angiotensin II–induced hypertension but also in angiotensin II–independent models. Thus, more studies are needed to delineate the exact mechanism of the anti-inflammatory and antifibrotic effects of Ac-SDKP in the kidney.

We conclude that, in Dahl SS rats on HS diet, a low dose of Ac-SDKP prevented renal macrophage and T-cell infiltration, renal fibrosis and glomerulosclerosis, but failed to prevent albuminuria and proteinuria, indicating that inflammation and fibrosis may not be the only factors contributing to high albuminuria. In this strain, a higher dose of Ac-SDKP was needed to prevent not only renal inflammation, fibrosis, and glomerulosclerosis, but also albuminuria and proteinuria induced by HS diet. We speculate that in Dahl SS rats, high dose of Ac-SDKP reduced albuminuria and proteinuria because of a complete inhibition of macrophage infiltration and renal fibrosis. These Ac-SDKP effects were independent of changes in blood pressure. However, in consomic SS13BN controls, both low and high Ac-SDKP doses prevented albuminuria and proteinuria, macrophage and T-cell infiltration, renal fibrosis and glomerulosclerosis induced by HS diet.

Figure 4. Effect of N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) on renal interstitial fibrosis and collagen content. A, Representative images of renal interstitial fibrosis. Red color indicates collagen deposition revealed by picrosirius staining. Shown are images captured using ×20 microscope objective. Scale bar, 100 µm. B, Quantitative data analysis. In Dahl salt-sensitive (SS) and consomic SS13BN rats, low or high dose of Ac-SDKP significantly prevented high salt (HS)–induced renal interstitial collagen deposition. Data are calculated as a percentage of the fibrotic area and expressed as mean±SEM; n=6 to 7 in each group. C, Quantitative analysis of total renal collagen content determined by hydroxyproline assay. In SS and consomic SS13BN rats, HS diet significantly increased renal collagen content compared with low-salt (LS) diet. In SS and SS13BN rats, Ac-SDKP significantly decreased HS-induced renal collagen content. Greater decrease was observed with high Ac-SDKP compared with low Ac-SDKP in both strains. Data are expressed as a microgram of collagen per milligram of dry kidney weight and expressed as mean±SEM; n=6 to 7 in each group. Veh indicates vehicle.

Figure 5. Effect of N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) on glomerular matrix deposition. A, Representative images of the glomerular matrix. Dark purple regions indicate extracellular matrix stained within the glomerular tufts by periodic acid–Schiff staining. Shown are images captured using ×40 microscope objective. Scale bar, 25 µm. B, Quantitative data analysis. In Dahl salt-sensitive (SS) and consomic SS13BN, glomerulosclerosis is significantly increased by high-salt (HS) diet compared with low-salt (LS) diet. Ac-SDKP, low or high dose significantly prevented HS-induced glomerulosclerosis in both strains. Data are as expressed as mean±SEM; n=6 to 7 in each group. Veh indicates vehicle.
Perspectives

Our preclinical study demonstrates that HS diet could exert harmful effects not only in SS individuals but also in non-SS individuals and that Ac-SDKP exerts strong renal protective effects without affecting the blood pressure. On the other hand, ACEi is widely used to treat hypertension and related cardiac and renal diseases and dysfunction. However, some patients cannot tolerate ACEi-associated side effects, such as hypotension, hyperkalemia, and angioedema. Moreover, ACEi increases circulating and tissue Ac-SDKP. Ac-SDKP was shown to mediate part of the protective effects of ACEi. Thus, Ac-SDKP or its analog, resistant to enzymatic degradation, could be a novel and useful therapeutic strategy for treating HS-induced renal damages and dysfunction in either SS or non-SS subjects.

Sources of Funding

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Disclosures

None.

References


What Is New?

- This is the first study providing the evidence of the protective effects of *N*-acetyl-serasparyl-lysyl-proline on renal damage not only in salt-sensitive hypertensive but also in consomic nonhypertensive animals.

What Is Relevant?

- Fifty percent of hypertensive and 20% of normotensive patients are salt sensitive. Salt sensitivity is associated with severe progression of hypertensive target organ damage, including end-stage renal disease.

### Novelty and Significance

<table>
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<th>What Is New?</th>
<th>Summary</th>
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<tr>
<td>• This is the first study providing the evidence of the protective effects of <em>N</em>-acetyl-serasparyl-lysyl-proline on renal damage not only in salt-sensitive hypertensive but also in consomic nonhypertensive animals.</td>
<td>Our results showed that in Dahl SS rats on high-salt (HS) diet, at low dose of <em>N</em>-acetyl-serasparyl-lysyl-proline prevented renal damage without lowering the blood pressure, but failed to correct albuminuria. Interestingly, treatment with a high dose of <em>N</em>-acetyl-serasparyl-lysyl-proline was not only able to achieve the anti-inflammatory and antifibrotic effects as seen with low dose but also improved renal function by decreasing HS-induced albuminuria. However, in consomic SS13BN rats, both doses of <em>N</em>-acetyl-serasparyl-lysyl-proline prevented HS-induced renal damage and albuminuria.</td>
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Renal Protective Effect of N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) in Dahl Salt-Sensitive rats

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Running title: Effect of Ac-SDKP on salt-sensitive hypertension

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METHODS

Animals

Male Dahl SS and consomic SS13BN rats at 4 weeks of age (Charles River Laboratories, Wilmington, MA) were housed in an air-conditioned room with a 12-hour light/dark cycle and received standard laboratory rat chow and tap water. Rats were allowed to acclimate to the new environment for 7 days. Then, they underwent blood pressure measure training for another 7 days, before the beginning of the experiment. Before all surgical procedures, rats were given analgesia (2 mg/kg of butorphanol SC) and anesthesia (50 mg/kg of sodium pentobarbital IP). This study was approved by the Henry Ford Hospital Institutional Animal Care and Use Committee.

Experimental Protocols

Dahl SS and SS13BN rats were randomly placed on low salt (LS = 0.23% NaCl) or high salt (HS=4% NaCl) diet (Teklad diets, Harlan, Madison, WI) and subcutaneously infused vehicle (0.01 N acetic acid saline solution) or Ac-SDKP for 6 weeks using osmotic mini-pump (Alzet). Dahl SS and SS13BN rats were divided into 4 groups: 1) LS infused with vehicle (LS+Veh, n=6); 2) HS infused with vehicle (HS+Veh, n=6-7); 3) HS infused with Ac-SDKP (800 μg·kg⁻¹·day⁻¹) (HS+low Ac-SDKP, n=6-7); 4) HS infused with Ac-SDKP (1600 μg·kg⁻¹·day⁻¹) (HS+high Ac-SDKP, n=6-7). Blood pressure was measured weekly by tail-cuff; 24 hours urine was collected for urinary Ac-SDKP, albumin and protein excretion. At the end of the experiment, animals were sacrificed and tissues were collected, weighed and histological studies were performed. The detail of each measurement is as follow:

Systolic Blood Pressure, Body Weight and Organ Weight

Systolic Blood Pressure (SBP) was measured in conscious rats by computerized tail-cuff system (model-1231, IITC Inc), as described¹. At the end of the experiment, rats were euthanized, the abdomen opened and the heart stopped in diastole by injecting 15% potassium chloride solution. The kidneys were weighed after excised and capsules removed. KW to BW ratio was determined. The kidney was then sectioned transversely into 4 sections. The middle section was fixed in 4% paraformaldehyde and paraffin-embedded. A lower mid-renal section was embedded in frozen tissue specimen compound (Tissue-Tek O.C.T. Compound, Sakura Finetek, USA, Inc.), immersed in cold isopentane (VWR), snap-frozen in liquid nitrogen and stored at -80°C. Section from the renal cortex apex was used for a hydroxyproline assay. The remaining section was frozen in liquid nitrogen and stored at -80°C. The heart was also excised, weighed, and HW to BW ratio was determined.

Urinary Ac-SDKP Excretion, Albuminuria and Proteinuria

After 24 hours adaptation to metabolic cages, rats underwent 24 hours urine collection. ACE inhibitor captopril (10⁻⁵ M) was applied to the collecting funnels and tubes to prevent Ac-SDKP degradation by urinary ACE. Total volume of collected urine was measured, aliquots prepared and centrifuged twice at 161,000 rcf at 4°C for 10 minutes. The supernatants were then filtered and stored at -80°C until further analysis. Urine Ac-
SDKP was measured using EIA kit (SPI Biolaboratories, France), as previously described\(^2\). Albuminuria was determined by ELISA kit (Cayman Chem, MI). Proteinuria was measured using Coomassie protein assay kit (Thermo Scientific) following the manufacturer's instructions. Albuminuria and proteinuria were calculated as urine albumin and protein concentration, respectively, multiplied by 24-hour urine volume output.

**Immunohistochemistry**

Frozen sections (6 µm) were fixed with acetone (4°C). Endogenous peroxidase activity blocked with 0.3% H\(_2\)O\(_2\) and nonspecific binding sites were blocked with 1% bovine serum albumin. Primary antibodies specific for CD68 macrophage marker (mouse anti rat clone: ED-1, 1:200, Millipore) and CD4 T helper cell marker (mouse anti rat clone: OX-38, 1:50, BD Pharmingen) were applied overnight at 4°C. The next day, sections were incubated with the biotinylated secondary horse anti-mouse IgGs. Immunoreactivity was detected with the ABC peroxidase kit (Vectastain Elite, Vector Laboratories), counterstained with hematoxylin and visualized with 3-amino-9-ethylcarbazole (AEC) (Zymed Laboratories). Reddish-brown staining was considered as positive. Twelve randomly chosen regions of the section were examined under ×20 objective of a Nikon's Eclipse E600 microscope, photographed with a Nikon's DS-Ri1 digital camera (Nikon Instruments Inc.). Positive cells were counted per mm\(^2\).

**Renal Fibrosis**

Picrosirius red staining was used to quantify the renal interstitial collagen deposition, a marker of fibrosis, as described\(^3\). Sequential 4µm paraffin-embedded sections were stained. Briefly, tissues were postfixed in Bouin’s fluid and stained with 0.1% picrosirius red for 1 hour. Samples were then washed twice in 0.5% acetic acid. Nuclei were counterstained with hematoxylin. For the renal interstitial collagen fraction (ICF), 20 images were taken with ×20 objective of a Nikon’s Eclipse E600 microscope with Nikon’s DS-Ri1 digital camera (Nikon Instruments Inc.). The ICF was analyzed by computerized image analysis (Microsuite Biological imaging software, Olympus America, Center Valley, PA), and expressed as the ratio of the collagen positive area to the entire area of the captured field. All of the images shown in this study were captured and analyzed using the same imaging system, unless otherwise specified.

**Renal Collagen Content by Hydroxyproline Assay**

Total collagen content of the renal cortex was determined by hydroxyproline assay, as described previously\(^4\). Briefly, samples were dried, homogenized, and hydrolyzed with 6 N HCl for 16 hours at 110°C. A standard curve of 0 to 5 µg of hydroxyproline was used. Data were expressed as µg of collagen per mg of dry weight, assuming that collagen contains an average of 13.5% hydroxyproline.

**Glomerular Injury**

The glomerular matrix was evaluated by periodic acid-Schiff (PAS) staining (Sigma), according to the manufacturer’s protocol. Tissue was taken from the upper mid-kidney part, paraffin embedded, cut into 4 µm sections and stained with PAS. Glomeruli (30 to
50) within the randomly chosen fields were photographed by ×40 objective. The dark purple color within the glomeruli was considered as a positive staining for extracellular matrix. The degree of glomerulosclerosis was determined as a percentage of the glomerular tuft area.

**Nephrin Expression in the Glomerulus**

Frozen sections (4 μm) were incubated overnight with a nephrin antibody (R&D Systems) at 4°C and visualized using a fluorescein isothiocyanate-labeled conjugated secondary antibody for 1 hour at room temperature. Nonspecific binding was blocked by 1% bovine serum albumin. Positive staining in high-power fields was measured in each section of the glomerulus and expressed as a percentage of the glomerular area.

**Data Analysis**

All data are expressed as mean ± SEM. ANOVA and nonparametric Wilcoxon tests was used to compare mean values of the various parameters between different groups. Specifically, comparisons were done as follows: LS+Veh vs. HS+Veh; HS+Veh vs. HS+low Ac-SDKP; HS+Veh vs. HS+high Ac-SDKP, and HS+low Ac-SDKP vs. HS+high Ac-SDKP. Hochberg’s method for multiple comparisons was used to adjust the alpha level of significance.

**REFERENCES**

Table S1. Baseline systolic blood pressure (SBP), body weight and albuminuria in Dahl SS and consomic SS13BN rats.

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<td>Body weight (g)</td>
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<td>Albuminuria (mg/24h)</td>
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Data are expressed as mean ± SEM. N=6-7 in each group.
Figure S1. Effect of Ac-SDKP on renal and cardiac hypertrophy. In both Dahl salt-sensitive (SS) rats and consomic SS13BN rats, HS diet significantly increased left kidney weight-to-body weight ratio (LKW/BW) (A) and heart weight-to-body weight ratio (HW/BW) (B) compared to LS diet. Ac-SDKP had no effect on LKW/BW and HW/BW ratios. Data are expressed as mean ± SEM. N=6-7 in each group.
Figure S2. 24-hour urinary Ac-SDKP excretion. 24-hour urinary Ac-SDKP excretion was measured by enzymatic immunoassay. In both Dahl salt-sensitive (SS) and consomic SS13BN rats, 24-hour urinary Ac-SDKP excretion was significantly higher in Ac-SDKP treated groups compared to vehicle groups. Data are expressed as mean ± SEM. N=6-7 in each group.
Figure S3. 24-hour urinary Ac-SDKP excretion comparison between Dahl SS and consomic SS13BN rats. 24-hour urinary Ac-SDKP excretion was measured by enzymatic immunoassay. In vehicle treated animals, and low Ac-SDKP treated animals, 24-hour urinary Ac-SDKP excretion was significantly elevated in SS13BN compared to SS rats. Data are expressed as mean ± SEM. N=6-7 in each group.
Figure S4. Enlarged representative images of renal macrophage (A) and T helper cell infiltration (B) showing the infiltrated glomerular (black arrow) and interstitial (yellow arrow) area. Red staining in the cytoplasm indicates a positive immunohistochemistry staining for macrophages (anti-CD68 antibody) and T helper cells (anti-CD4 antibody). Shown are images captured using x20 microscope objective.
Scale bar = 100 µm. HS diet markedly increased macrophages and T helper cells in the kidney. Low or high dose of Ac-SDKP significantly reduced HS-induced renal macrophage and T helper cell infiltration.