N-Acetyl-Seryl-Aspartyl-Lysyl-Proline Attenuates Renal Injury and Dysfunction in Hypertensive Rats With Reduced Renal Mass

Council for High Blood Pressure Research

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Abstract—N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is a naturally occurring peptide of which the plasma concentration is increased 4- to 5-fold by angiotensin-converting enzyme inhibitors. We reported previously that, in models of both hypertension and postmyocardial infarction, Ac-SDKP reduces cardiac inflammation and fibrosis. However, it is unknown whether Ac-SDKP can prevent or reverse renal injury and dysfunction in hypertension. In the present study, we tested the hypothesis that, in rats with 5/6 nephrectomy (5/6Nx)-induced hypertension, Ac-SDKP reduces renal damage, albuminuria, and dysfunction by decreasing inflammatory cell infiltration and renal fibrosis and by increasing nephrin protein. Ac-SDKP (800μg/kg per day, SC via osmotic minipump) or vehicle was either started 7 days before 5/6Nx (prevention) and continued for 3 weeks or started 3 weeks after 5/6Nx (reversal) and continued for another 3 weeks. Rats with 5/6Nx developed high blood pressure, left ventricular hypertrophy, albuminuria, decreased glomerular filtration rate, and increased macrophage infiltration (inflammation) and renal collagen content (fibrosis). Ac-SDKP did not affect blood pressure or left ventricular hypertrophy in either group; however, it significantly reduced albuminuria, renal inflammation, and fibrosis and improved glomerular filtration rate in both prevention and reversal groups. Moreover, slit diaphragm nephrin protein expression in the glomerular filtration barrier was significantly decreased in hypertensive rats. This effect was partially prevented or reversed by Ac-SDKP. We concluded that Ac-SDKP greatly attenuates albuminuria and renal fibrosis and improves renal function in rats with 5/6Nx. These effects may be related to decreased inflammation (macrophages) and increased nephrin protein. (Hypertension. 2010;55[part 2]:459-467.)

Key Words: Ac-SDKP ■ albuminuria ■ renal dysfunction ■ fibrosis ■ inflammation ■ nephrin

N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is a naturally occurring antiinflammatory and antifibrotic peptide. Ac-SDKP is hydrolyzed almost exclusively by angiotensin (Ang)-converting enzyme (ACE), and its plasma concentration is increased substantially after treatment with ACE inhibitors.1 Ac-SDKP mediates some of the antiinflammatory and antifibrotic effects of ACE inhibitors.2,3 Rats overexpressing cardiac ACE have decreased Ac-SDKP and increased fibrosis in the heart.4 Inhibition of prolyl oligopeptidase, an enzyme responsible for Ac-SDKP release from thymosin-β4, promotes cardiac and renal perivascular fibrosis and nephrosclerosis.5 We and others have shown that in vitro Ac-SDKP inhibits fibroblast and mesangial cell proliferation and collagen synthesis.6–8 Treatment with Ac-SDKP has been shown to reduce inflammation and collagen deposition in the heart, aorta, and kidney in animal models of hypertension, myocardial infarction, and diabetes mellitus.3,9–11

The glomerular filtration barrier is a 3-layered structure consisting of fenestrated endothelial cells lining the renal capillaries, the glomerular basement membrane, and visceral epithelial cells (podocytes) of which the interdigitating foot processes form the slit diaphragm.14 The slit diaphragm is the final barrier that prevents protein leakage into the urinary space. Nephrin is a slit pore protein expressed between foot processes of podocytes in the glomeruli and is critical in maintaining permeability and preventing proteinuria.15,16 Mutation of the nephrin gene leads to congenital nephrotic syndrome of the Finnish type, which specifically affects the kidney and is characterized by massive proteinuria.17 In addition to the glomerular filtration barrier, mesangial cells and their matrix form the central stalk of the glomerulus and are part of a functional unit, which interacts closely with endothelial cells and podocytes. Alterations in 1 cell type can produce changes in the others.18

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We have shown that, in aldosterone-salt-induced hypertension, Ac-SDKP significantly decreased renal cell proliferation and renal fibrosis but only slightly reduced glomerular and tubular injury; however, we did not examine the underlying mechanism(s) or test renal function. In the present study, we tested the hypothesis that Ac-SDKP prevents and reverses renal albuminuria and dysfunction in 5/6 nephrectomy (5/6Nx)-induced hypertension by decreasing inflammatory cell infiltration and renal fibrosis and increasing nephrin protein.

Materials and Methods

Animals

Male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 275 to 300 g were housed in an air-conditioned room with a 12-hour light/dark cycle and received standard laboratory rat chow and tap water. They were allowed 7 days to adjust to their new environment. Before all of the 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.

Surgical Procedure for 5/6Nx

Rats were anesthetized and 5/6Nx was performed by unilateral nephrectomy plus ligation of lower and upper renal arterial branches of the contralateral kidney with a 6-0 silk suture. Ligation was deemed successful when two thirds of the kidney turned dark red. The sham-operated group underwent a similar surgical procedure except that the suture around the renal artery was not tightened. An osmotic minipump filled with Ac-SDKP (800 μg/kg per day) or vehicle (0.01 N acetic acid saline solution) was implanted SC between the shoulder blades.

Experimental Protocols

Rats were randomly divided into 2 protocols: prevention and reversal.

Prevention Protocol

Rats received vehicle or Ac-SDKP beginning 7 days before surgery and continuing for 3 weeks.

Group 1

Group 1 included prevention sham Nx rats given vehicle (sham-vehicle for 3 weeks: n=12).

Group 2

Group 2 included prevention 5/6Nx rats given vehicle (5/6Nx-vehicle for 3 weeks: n=12). This group was also used as a control for the initial point of the reversal protocol.

Group 3

Group 3 included prevention 5/6Nx rats given Ac-SDKP (5/6Nx-Ac-SDKP for 3 weeks: n=12).

Reversal Protocol

Rats received vehicle or Ac-SDKP beginning 3 weeks after surgery and continuing for ≤6 weeks.

Group 4

Group 4 included reversal sham Nx rats given vehicle (sham-vehicle for 6 weeks: n=5).

Group 5

Group 5 included reversal 5/6Nx rats given vehicle (5/6Nx-vehicle for 6 weeks: n=10).

Group 6

Group 6 included reversal 5/6Nx rats given Ac-SDKP (5/6Nx-Ac-SDKP for 6 weeks: n=7).

Group 2 of the prevention protocol (5/6Nx-vehicle for 3 weeks) was used as the treatment baseline for the reversal protocol.

Measurement of Ac-SDKP Plasma Concentration and Urinary Excretion and Albuminuria

Blood was collected from the inferior vena cava at the end of the study using a heparinized syringe containing lisinopril at a final concentration of ~10^{-5} M. Aliquots were centrifuged at 3700 rpm at 4°C for 10 minutes, and the supernatant was collected to measure plasma Ac-SDKP.

After adapting to the metabolic cage for 24 hours, the rats were fasted during the 24-hour urine collection. To prevent Ac-SDKP degradation by urinary ACE, an ACE inhibitor (lisinopril, 10^{-5} M) was sprayed and dried on the funnel and also added to the collecting tubes (200 μL of 10^{-3} M lisinopril per tube). Urinary volume was measured and aliquots were centrifuged twice at 132 000 rpm at 4°C for 10 minutes (Eppendorf centrifuge 5415R). The supernatant was passed through a 25-mm syringe filter (0.2 μm HT Tuffryn membrane, Gelman Laboratories), and stored at −20°C. Urinary and plasma Ac-SDKP were measured by ELISA (SPI Biolaboratories). Urine albumin was measured with an ELISA kit (Cayman Chemical).

Systolic Blood Pressure, Left Ventricle Weight, Body Weight, and Kidney Weight

Systolic blood pressure (SBP) was measured in conscious rats by use of a noninvasive computerized tail-cuff system (model-1231, IITC Inc), as described previously. Animals were trained for 3 days before SBP measurement. At the end points of each study, rats were euthanized and the abdomen opened. The kidney was excised, the capsule was removed and weighed, and the ratio of kidney weight: body weight (BW) was determined. It was then sectioned transversely into 4 sections. One section from the middle of the kidney was fixed in 4% paraformaldehyde for a paraffin-embedded section. A lower midrenal section was embedded in optimal cutting temperature compound and immersed in cold isopentane (VWR), then snap-frozen in liquid nitrogen and stored at −80°C. One section from the apex of the renal cortex was used for a hydroxyproline assay. The remaining section was rapidly frozen in liquid nitrogen and stored at −80°C. The heart was also excised and weighed.

Glomerular Filtration Rate

Glomerular filtration rate (GFR) was measured with fluorescein isothiocyanate–labeled inulin (Sigma). Briefly, rats were anesthetized and placed on a heating pad. Fluorescein isothiocyanate–labeled inulin (10 mg/mL) was injected as a bolus at 3 μL/g of BW, followed by constant infusion of 0.15 μL/min per gram of BW. After a 30-minute stabilization period, urine was collected for 30 minutes, taking a 100-μL sample before and after urine collection. Samples of fluorescein isothiocyanate–labeled inulin standards, plasma, and diluted urine were transferred to a 96-well microplate in triplicate and mixed with 10 mmol/L of HEPES buffer (pH 7.4). Plates were examined with a microplate fluorescence reader (Labsystems Fluoroskan II) at an excitation level of 485 nm and an emission level of 538 nm. GFR was calculated using the following formula: GFR=(urine fluorescence−tube fluorescence)/collection time. GFR was corrected by kidney weight, with units expressed as microliters per minute per 100 mg of kidney weight.

Renal Macrophage Infiltration

Paraffin-embedded sections (6 μm) were deparaffinized and endogenous peroxidase activity blocked with 0.3% hydrogen peroxide. Antigens were revealed by microwave heat-induced epitope retrieval in citrate acid buffer (pH 6.0). First nonspecific binding was blocked with 2.5% normal horse serum; then, a primary monoclonal antibody, mouse antirat CD68 antigen, which is a marker for macrophages (clone: ED-1, 1:200, AbD Serotec), was applied, and samples were incubated overnight at 4°C. The next day, sections were incubated with a secondary biotinylated antibody, horse antimouse IgG. Immunoreactivity was detected with an ABC peroxidase kit.
(Vectastain Elite, Vector Laboratories) and visualized with 3-aminooethylcarbazole (Zymed Laboratories). PBS buffer alone and a nonspecific purified mouse antirat IgG were used as a negative control and an isotype IgG control, respectively. Reddish-brown staining was considered positive. Sections were counterstained with hematoxylin. Twelve regions of the section were examined under the ×40 objective of an inverted microscope (IX81), photographed with a digital camera (DP70, Olympus America), and evaluated by a computerized image analysis system (Microsuite Biological Imaging, Olympus America). All of the images captured and analyzed in this study were obtained using the same system unless otherwise specified. Positive cells in high-power fields were counted for each section and expressed as cells per millimeter squared.

Renal Collagen Content

Collagen content of the renal cortex was determined by hydroxyproline assay, as described previously. 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 micrograms of collagen per gram of renal cortex weight.
milligram of dry weight, assuming that collagen contains an average of 13.5% hydroxyproline.22

Glomerular Matrix and Interstitial and Perivascular Fibrosis

A transmural section was taken from the upper midkidney section. Sequential 4-μm paraffin-embedded sections were stained with periodic acid-Schiff (Sigma)2 to examine the glomerular matrix. Glomeruli (25 to 30) were photographed under the 400× objective, and the glomerular matrix was determined as a percentage of the glomerular area. Picro-sirius red staining was used to quantify the renal interstitial collagen fraction (ICF) and renal perivascular fibrosis.23 For ICF, 12 images were taken with the 200× objective, examining the cortex and outer medulla to avoid interference by large vessels. ICF was expressed as the ratio of collagen area:total area. For perivascular fibrosis, 10 vessels were imaged using the 400× objective, and perivascular fibrosis was expressed as the ratio of the fibrotic area surrounding the vessel:total cross-sectional area.5

Nephrin in the Glomerulus

Frozen sections (4 μm) were immunostained with a nephrin antibody (Fitzgerald Industries) and visualized using a fluorescein isothiocyanate–labeled conjugated secondary antibody. Nonspecific binding was blocked by 10% species-appropriate normal serum. Negative controls were processed in a similar fashion except that sections were incubated with an isotype IgG control instead of the primary antibody. Positive staining in high-power fields was measured in each section of the glomerulus and expressed as a percentage of the glomerular area.

All of the imaging analysis was conducted in a double-blind fashion. The person performing microphotography and computerized imaging analysis did not know which groups he was examining.

Data Analysis

All of the data are expressed as mean±SEM. ANOVA was used to compare mean values of each parameter (Ac-SDKP, BW, left ventricle weight:BW, kidney weight:BW, SBP, albuminuria, GFR, macrophage, renal fibrosis, and nephrin) between different groups. The Hochberg method for multiple comparisons was used to adjust the α level of significance.24

Results

Ac-SDKP Plasma Concentrations and Urinary Excretion

Ac-SDKP plasma concentrations were 3-fold higher in rats infused with Ac-SDKP compared with vehicle, whereas urinary Ac-SDKP excretions were 7- to 11-fold higher in the Ac-SDKP–infused group as compared with the vehicle group (Table).

SBP, BW, and Cardiac and Renal Hypertrophy

Rats with 5/6Nx in the prevention and in the reversal groups had significantly increased SBP and cardiac weight. Ac-SDKP did not affect either SBP or cardiac weight (Figure 1). In the prevention protocol, rats with 5/6Nx had significantly decreased BW compared with sham operation, and this decrease was prevented by Ac-SDKP (Table). In the reversal protocol, BW in the 5/6NX decrease and Ac-SDKP tended to increase; however, these changes did not reach statistical significance. Kidney weight:BW ratio was similar in all of the groups, indicating that 5/6Nx induced hypertrophy of the remaining nephrons (Table).
Albuminuria
Rats with 5/6Nx given vehicle had severe albuminuria compared with sham Nx in both prevention (3 weeks) and reversal protocols (6 weeks). Ac-SDKP prevented or reversed the albuminuria induced by 5/6Nx (Figure 2A).

Glomerular Filtration Rate
Rats with 5/6Nx given vehicle had significantly decreased GFR in both prevention (3 weeks) and reversal protocols (6 weeks). Ac-SDKP significantly improved GFR in both protocols (Figure 2B).

Renal Macrophage Infiltration
Rats with 5/6Nx given vehicle had significant macrophage infiltration in the kidney in both prevention and reversal protocols. Ac-SDKP significantly decreased macrophage infiltration in both prevention and reversal protocols (Figure 3). However, macrophage infiltration was not normalized by Ac-SDKP treatment.

Renal Fibrosis
Rats with 5/6Nx given vehicle had significantly increased renal collagen content (hydroxyproline assay) in both prevention (3 weeks) and reversal protocols (6 weeks). Ac-SDKP significantly prevented and reversed these effects, respectively (Figure 4). Renal ICF and perivascular fibrosis were also investigated by using picro-sirius red staining and computerized imaging analysis. Red color indicated collagen deposition. Renal ICF data were consistent with the collagen content (Figure 5). Ac-SDKP also tended to lower perivascular fibrosis, but this decrease did not reach statistical significance (Table).

Glomerulosclerosis
Effect of Ac-SDKP on glomerulosclerosis was assessed on kidney sections by using periodic acid Schiff staining and computerized image analysis. Dark purple regions indicate renal collagen content (µg/mg dry weight)

Figure 4. Effect of Ac-SDKP on renal collagen content. Renal collagen content increased significantly in 5/6Nx rats given vehicle. Ac-SDKP decreased renal collagen content in both prevention and reversal protocols (n=5 to 8).

Figure 5. Effect of Ac-SDKP on renal interstitial fibrosis. A, Representative images of renal interstitial fibrosis. Red indicates collagen deposition revealed by picro-sirius staining (bar: 200 μm). B, Quantitative data show that renal ICF increased significantly in 5/6Nx rats given vehicle. Ac-SDKP decreased glomerular matrix significantly in both prevention and reversal protocols (n=5 to 6).
extracellular matrix stained by periodic acid-Schiff (Figure 6). Ac-SDKP significantly prevented and reversed glomerular matrix expansion, which is an indicator of glomerulosclerosis.

Nephrin Expression

Rats with 5/6Nx given vehicle had significantly decreased glomerular nephrin expression in both prevention and reversal groups. Ac-SDKP partially restored nephrin expression in both protocols (Figure 7).

Discussion

Using a model of severe hypertension, renal injury, and dysfunction, we found that Ac-SDKP not only prevents but (more importantly) reverses renal injury. The effects of Ac-SDKP were independent of changes in blood pressure. This is in agreement with our previous studies showing that, in various models of hypertension, the anti-inflammatory and antifibrotic effects of Ac-SDKP in the heart, aorta, and kidney were independent of any antihypertensive effect.3,11,12

As reported previously,25–27 the 5/6Nx model is characterized by severe hypertension, nephrosclerosis, renal fibrosis and inflammation (as indicated by macrophage infiltration), proteinuria, and decreased GFR. In addition, we have shown that nephrin is significantly decreased in this model. All of these effects were either prevented or partially reversed by Ac-SDKP. Also, in this model of hypertension and renal disease, an increase in renin-Ang system activity has been described previously.28 Ang II may contribute to renal damage not only by causing hypertension but also by inducing inflammatory cell infiltration and fibroblast and mesangial cell proliferation, resulting in renal fibrosis and nephrosclerosis.28 It has been reported that Ac-SDKP at very high doses inhibits ACE in vitro; thus, this tetrapeptide could have renal protection via inhibition of Ang II production.29 However, it is unlikely that Ac-SDKP acts through inhibition of ACE, because Ac-SDKP in vivo, at similar doses used in the present study, did not affect the conversion of Ang I to Ang II.30 Also, in the present study, in a model of high renin-dependent hypertension, Ac-SDKP did not have an antihypertensive effect, suggesting that it did not act by the blocking of Ang II formation.

The mechanism by which Ac-SDKP decreases renal injury caused by reduction of renal mass could be partially mediated by reducing inflammation, as suggested by the decrease in macrophage infiltration. It is well known that immunologic and inflammatory processes play an important role in end-stage renal disease.31–33 Macrophage infiltration is one of the hallmarks of inflammatory renal injury.33 In the present study, we demonstrated that renal macrophage infiltration was significantly increased in 5/6Nx rats, and this effect was partially prevented or reversed by Ac-SDKP treatment. Ac-SDKP inhibits macrophage infiltration by directly inhibiting macrophage differentiation, activation, and migration in vitro.
and in vivo. Thus, the decrease in renal inflammation caused by Ac-SDKP could involve a direct effect on macrophages either via the release of superoxide or the release of proinflammatory cytokines that may trigger further inflammation and oxidative stress and exacerbate renal lesions. Indeed, we reported previously that, in Ang II–induced hypertension, Ac-SDKP was able to reduce oxidative stress, as evidenced by reduction in 4-hydroxynonenal (a byproduct of lipid oxidation and a marker for oxidative stress) and nitrotyrosine (a marker for superoxide production). Here, we demonstrated that Ac-SDKP decreased macrophage infiltration in both prevention and reversal protocols but did not restore its concentration to baseline. We speculated that a certain level of macrophages could be beneficial for healing injury, because the inflammatory response is one of the body’s defense mechanisms. Completely eliminating macrophage infiltration may lead to infection, tumor growth, and other diseases. The question of how we might balance the inflammatory response needs to be investigated further.

The renal protective effect of Ac-SDKP may also be partly attributed to a reduction in renal fibrosis and nephrosclerosis. We and others have shown that Ac-SDKP inhibited fibroblast and mesangial cell proliferation, as well as collagen synthesis. We also have shown that Ac-SDKP decreases expression of the profibrotic cytokine transforming growth factor-β and that it blocks Smad activation. The present study showed that Ac-SDKP decreased or restored renal collagen content, renal ICF, and glomerulosclerosis, which may be attributed to reduction of fibroblast and mesangial cell proliferation and inhibition of transforming growth factor-β/Smad pathways. We also found that Ac-SDKP tended to decrease perivascular fibrosis but this decrease did not reach statistical significance (Table). The partial effect of Ac-SDKP on perivascular fibrosis could be attributed to either dosage of the peptide or a short period of treatment, especially in the reversal protocol. In addition, we cannot discard the possibility that Ac-SDKP does not inhibit some of the mechanisms leading to perivascular fibrosis, such as mesenchymal transformation of endothelial cells, pericyte migration, and differentiation into myofibroblasts, irreversible transformation of renal adventitial fibroblasts to myofibroblasts, which could require >3 weeks (present experimental protocol) to resolve.

A major finding of the present study was the marked difference in the effect of Ac-SDKP treatment on albuminuria and GFR compared with vehicle in 5/6Nx rats. Ac-SDKP prevented or reversed renal injury and dysfunction. The pathogenesis of proteinuria in hypertension has not been fully delineated. Recent studies implicate the slit pore protein nephrin, which plays an important role in the trafficking of albumin across the glomerular barrier. Nephrin is synthesized by podocytes, glomerular epithelial cells that are reportedly reduced in hypertensive patients, and type II diabetics with nephropathy. Bonnet et al demonstrated...
that reduced nephrin expression in diabetic spontaneously hypertensive rats was accompanied by increased albuminuria. Our results show that 5/6Nx causes a significant decrease in nephrin expression and that Ac-SDKP partially prevents or reverses these decreases. The effects of Ac-SDKP on nephrin could be secondary to a decrease in inflammation or to inhibition of mesangial cell proliferation, which could help maintain balance with the number of podocytes; however, this issue needs to be studied.48

There is evidence that, shortly after the onset of proteinuria, interstitial inflammation develops and fibrosis ensues, indicating that proteinuria itself may elicit proinflammatory and profibrotic effects that directly contribute to renal damage.49 Thus, decreasing proteinuria may also contribute to the anti-inflammatory and antifibrotic effects of Ac-SDKP on subtotal renal ablation.

As discussed above, Ac-SDKP decreased glomerulosclerosis induced by 5/6Nx. Thus, the renal protective effect of Ac-SDKP on GFR could be a consequence of preventing glomerulosclerosis.

In the present study, we found that Ac-SDKP attenuates albuminuria and renal dysfunction in hypertensive rats with subtotal renal ablation. These effects may be related to decreased inflammation, fibrosis, and glomerulosclerosis coupled with an increase in nephrin expression.

Perspectives
The present study demonstrates that Ac-SDKP provided strong renal protective effects in animal models of renal injury and dysfunction despite an absence of any significant effects on blood pressure. Knowing that ACE inhibitors are able to increase circulating, tissue, or excreted ACE inhibitors in angiotensin-converting enzyme is related to enhanced breakdown of N-acetyl-Ser-Lys-Pro and increased phosphorylation of Smad2/3. Circulation. 2004;110:3120–3135.


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