N-acetyl-seryl-aspartyl-lysyl-proline attenuates renal inflammation and tubulointerstitial fibrosis in rats

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Abstract. It has been reported that N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) attenuates renal and cardiac inflammation as well as fibrosis in hypertensive rats. In this study, we investigated these effects using a unilateral ureteral obstruction (UUO) model. Eighteen male Wistar rats were randomly divided into three groups: control, UUO/vehicle and UUO/Ac-SDKP groups. Animal models of renal inflammation and tubulo-interstitial fibrosis were established with unilateral ureteral ligation in rats. Ac-SDKP and vehicle were infused subcutaneously by using osmotic mini pumps for two weeks. On the 14th day post-injection, kidney histological changes of each group were observed by hematoxylin-eosin and Masson’s stain. Renal macrophage infiltration, together with protein expression and localization of monocyte chemoattractant protein-1 (MCP-1), nuclear factor-kappa B (NF-κB), α-SMA and transforming growth factor-ß1 (TGF-ß1) in renal tissue was assessed by immunohistochemical staining. Gene expression of MCP-1 and TGF-ß1 was analyzed with reverse transcription-polymerase chain reaction. Ac-SDKP-treated animals demonstrated less severe renal inflammation and tubulointerstitial fibrosis. Interstitial fibrosis was significantly attenuated with Ac-SDKP. ED-1 was expressed in the interstitium of the UUO/vehicle group kidneys and decreased with Ac-SDKP treatment. MCP-1, NF-κB, α-SMA and TGF-ß1 were increased in the renal interstitium and tubular epithelial cells of the UUO/vehicle group. Ac-SDKP significantly reduced their expressions. Gene expressions of MCP-1 and TGF-ß1 were upregulated in the UUO/vehicle group kidneys and were significantly inhibited by Ac-SDKP. In conclusion, in the rat UUO model Ac-SDKP administration protected against renal inflammation and tubulointerstitial fibrosis. The inhibitory effect of Ac-SDKP was mediated by the reduction in the expression of MCP-1, NF-κB, α-SMA and TGF-ß1.

Introduction

N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) was originally isolated from calf bone marrow and was identified as a negative regulator of hematopoietic stem cell proliferation (1). Long-term administration of Ac-SDKP, a tetrapeptide normally present in tissues and biological fluids, can reverse renal and cardiac inflammation and fibrosis in hypertensive rats (2-4). Ac-SDKP effects include inhibition of DNA and collagen synthesis in renal and cardiac fibroblasts as well as reduction of inflammatory cell infiltration (2-5). The beneficial properties of Ac-SDKP have been described in experimental models of renal disease accompanied by hypertension. However, the mechanism underlying the inhibition by Ac-SDKP of renal inflammation and tubulointerstitial fibrosis in a model of pure tubulointerstitial renal injury remains incompletely understood.

Renal tubulointerstitial fibrosis, the severity of which could be regarded as the major indicator of end-stage renal disease, is characterized by mononuclear infiltration, epithelial-mesenchymal transdifferentiation (EMT) and the accumulation of extracellular matrix (6,7). Unilateral ureteral obstruction (UUO) is a representative experimental model of progressive inflammation and tubulointerstitial fibrosis. Renal inflammation is believed to play a critical role in the initiation and progression of kidney tubulointerstitial diseases. Monocyte chemoattractant protein-1 (MCP-1), one of the most important indicators of the inflammatory process within the renal tubulointerstitial tissue, is a potent chemokine in the regulation of interstitial inflammation. Increasing evidence suggests that MCP-1 expression is correlated with the activation of nuclear factor-kappa B (NF-κB) (8,9), which could facilitate the transcription of a large number of genes, including cytokines and a variety of inflammatory and proliferative proteins involved in the pathogenesis of renal disease. In addition, identifed EMT is able to account for a large number of myofibroblasts in the UUO model (10-12). α-smooth muscle actin (α-SMA) is considered the most well known and widely used marker of myofibroblasts. Transforming growth factor-ß1 (TGF-ß1) (12-14), which promotes fibrogenic processes,
induces the matrix-producing myofibroblast activation and the EMT. In this study, we investigated the efficacy of Ac-SDKP in a model of UUO. In addition, we examined the expression levels of MCP-1, NF-κB, α-SMA and TGF-ß1. For the first time, we demonstrate that Ac-SDKP is effective in the treatment of renal inflammation and tubulointerstitial fibrosis using the rat UUO model.

Materials and methods

Experimental protocol. All animal experiments were approved by the animal care and ethics committee of Harbin Medical University. Eighteen male Wistar rats weighing 230-250 g, purchased from the institutional animal care unit of the First Affiliated Hospital of Harbin Medical University, were used in this study. Ac-SDKP was provided by Sigma. These rats were randomly divided into three groups (6 animals per group): a control group, a UUO/vehicle group and a UUO/ Ac-SDKP group. All animals received a mid-abdominal incision after anesthesia with a 50 mg/kg sodium pentobarbital injection intraperitoneally. Then, the UUO/vehicle group and the UUO/vehicle group underwent ligation of the left ureter intraperitoneally. Two days after surgery, the UUO/vehicle group and the UUO/ Ac-SDKP group underwent ligation of the left ureter next to the ureteropelvic junction twice with 4-0 nylon silk. The abdominal incision was closed with sutures. Osmotic mini pumps (Alzet, Cupertino, CA) were surgically implanted in the neck for subcutaneous delivery of 400 μg/kg/day of Ac-SDKP (2-4) or vehicle (saline plus 0.01 N acetic acid). Ac-SDKP and vehicle were infused subcutaneously for two weeks. The control group underwent a mock surgery. Animals were maintained with free access to regular food and water in a temperature-controlled room (22±1˚C) under a 12/12 h light/dark cycle. On the 14th day after surgery, the animals were sacrificed, and the left kidney was removed quickly from each animal. Next, the kidney samples were prepared for analysis by either of two methods. One method was to paraffin embed the kidney in 10% neutral buffered formalin for histopathological and immunohistochemical examination. The other method was to snap-freeze the kidney in liquid nitrogen and store it at -80˚C for further mRNA and protein analysis.

Morphology. Paraffin-embedded renal tissue for light microscopy was dewaxed using standard sequential techniques, and 3-μm sections were stained with hematoxylin-eosin and Masson’s trichrome. Briefly, the percentage of the renal cortical area occupied by interstitial tissue was estimated in the Masson-stained sections using an image analyzer (Leica, Leica Micros Imaging Solutions, Cambridge, UK). Using 10 high-power fields (x400) on each slide, the widening of the interstitial space and interstitial fibrosis were assessed by the percentage of the total area on each section at a x200 magnification (excluding areas with blood vessels) using Lumina Vision 2.20 (Mitani Corporation, Osaka, Japan). In each section of the kidney, the intensity of immunostaining for MCP-1, NF-κB and TGF-ß1 was scored separately by two observers as follows (15): (0) no specific staining (no cytoplasmic or nuclear staining); (1) weakly positive (mild cytoplasmic staining and no nuclear staining); (2) moderately positive (moderate cytoplasmic staining and/or nuclear staining); and (3) strongly positive (severe cytoplasmic and nuclear staining). For each section, at least 30 consecutive renal cortical fields were examined at x200 magnification.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from rat kidney cortices with Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The cDNA was synthesized from 2 μg of total RNA with the RT system for RT-PCR. The primer pairs were chosen from the published cDNA sequences of the rat: MCP-1 (primers: sense 5'-TCTCAAGCCAGATGCAGTTAA-3' and antisense 5'-TACAGCTTTCTTGAGACC-3', 126 bp product), TGF-ß1 (primers: sense 5'-GACTCTCCACCTGCAAGACC-3' and antisense 5'-TGTGCGGTCCACCATTAG-3', 267 bp product) and β-actin (primers: sense 5'-CGTCTCCCTCCATGG-3' and antisense 5'-GGGGTGATGAAAGTCTCAG-3', 303 bp product). PCR was performed by mixing 2 μl of RT reaction product (cDNA), 10 μl of buffer (100 mM Tris.HCl, 500 mM KCl, 0.1% Triton X-100, 250 mM MgCl2), 2 μl of 10 mM dNTP mix, 5 μl of each primer (10 pmol/μl) and 2.5 U of Taq DNA polymerase (Promega) with water up to 100 μl. CDNs were amplified according to the following conditions: 94˚C for 3 min, 94˚C for 30 sec, 66.5˚C for 30 sec, 72˚C for 30 sec, and 72˚C for 10 min. The number of cycles was predetermined for each pair of primers to avoid the PCR plateau phase. Each sample was tested in triplicate. The amplified DNA fragments were screened and analyzed using quantitation analysis computer.
software (Scion Image; Scion Corporation, MD, USA). β-actin expression was used as an internal control to assure equal cDNA among samples. Negative controls were routinely included by omitting the primers in the PCR reaction. All PCRs resulted in the amplification of a single product of the predicted size.

Statistical analysis. All data are presented as the mean ± SD. Comparisons between groups were done by using analysis of variance. Statistical significance was defined as P<0.01.

Results

Morphological findings. The non-obstructed contralateral kidney from rats and from the control group did not show any morphological alterations. The obstructive kidneys developed severe tubulointerstitial injury consisting of tubular dilatation and atrophy, interstitial inflammation and interstitial fibrosis. Glomeruli and vessels were well preserved. Analysis of the interstitium by Masson’s trichrome staining showed that Ac-SDKP administration significantly attenuated the interstitial fibrosis (10.46±1.36 vs. 20.0±2.01%, P<0.01, Ac-SDKP vs. vehicle) (Figs. 1 and 2).

Table I. The immunohistochemical staining score in control, UUO/Ac-SDKP and UUO/vehicle groups.

<table>
<thead>
<tr>
<th>Score</th>
<th>Control group</th>
<th>UUO/Ac-SDKP group</th>
<th>UUO/vehicle group</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>0.17±0.41</td>
<td>1.50±0.55 a,b</td>
<td>2.67±0.32 a</td>
</tr>
<tr>
<td>NF-κB</td>
<td>0.33±0.52</td>
<td>1.17±0.41 a,b</td>
<td>2.50±0.55 a</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>0.33±0.52</td>
<td>1.67±0.41 a,b</td>
<td>2.83±0.41 a</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. *Significantly different from the control group (P<0.01). †Significantly different from the UUO/vehicle group (P<0.01).

Figure 1. Interstitial renal fibrosis. Sections of control group kidneys, UUO/vehicle group obstructed kidneys and UUO/Ac-SDKP group obstructed kidneys stained with hematoxylin-eosin (A, B, and C) and Masson’s trichrome (D, E, and F) at 14 days after the procedure. Each image was randomly acquired from the cortex area and is representative for 6 rats. Original magnification x400.

Figure 2. Quantitative analysis of interstitial fibrosis (%IF) in the control group, UUO/vehicle group and UUO/Ac-SDKP group. Values are expressed as mean ± SD. *P<0.01 vs. control group kidneys, †P<0.01 vs. UUO/vehicle group kidneys.
Effects of Ac-SDKP on ED-1, MCP-1, NF-κB, α-SMA and TGF-β1 immunohistochemistry expression. We analyzed the expressions of key markers during the course of renal damage induced by UUO. One of the key features was macrophage infiltration, as assessed by ED-1. No signal for macrophage infiltration was detected in kidneys of the control group. The number of ED-1-positive monocytes/macrophages infiltrating in the periglomerular and peritubular interstitium was significantly increased in the UUO/vehicle group kidneys. Surprisingly, the number of ED-1-positive monocytes/macrophages in the UUO kidneys of Ac-SDKP-treated rats was reduced significantly from an average of 24.17±4.46 cells per field (UUO/Ac-SDKP group) to 50.00±5.67 cells per field in the untreated kidney (P<0.01; Figs. 3 and 4). The expression of MCP-1 was rare in the tubuloepithelial cells of the control group kidney. MCP-1 was strongly expressed in the tubuloepithelial cells and the interstitial area in the UUO/vehicle group. Treatment with Ac-SDKP significantly resulted in a reduction of the tubuloepithelial cell expression of MCP-1 and the matrix score (1.50±0.55 vs. 2.67±0.52, P<0.01, Ac-SDKP vs. vehicle). The control group kidneys had weak staining for NF-κB that was mainly located in the glomerular endothelial and tubular epithelial cells. NF-κB was markedly expressed in the tubular cells and interstitium of the UUO/vehicle group. Ac-SDKP treatment significantly suppressed the expression of NF-κB and the matrix score (1.17±0.41 vs. 2.50±0.55, P<0.01, Ac-SDKP vs. vehicle). The expression of α-SMA, a major profibrogenic mediator of EMT, denoted the intensity of the fibrogenic response after UUO. α-SMA was expressed in the smooth muscle cells of the renal arterioles but was rare in the interstitium of the control group kidneys. Semiquantification of the immunostaining of α-SMA was strikingly increased in the interstitium of the UUO/vehicle group kidneys. Ac-SDKP administration significantly reduced the expression level of α-SMA, and there was a significant reversal in the size of the α-SMA-positive stained area (5.71±1.41 vs. 12.01±2.31%, P<0.01, Ac-SDKP vs. vehicle). As shown in Fig. 5, the obstructed kidneys significantly expressed high levels of TGF-β1, whereas the administration of Ac-SDKP reduced the expression of this profibrogenic cytokine (1.67±0.41 vs. 2.83±0.41, P<0.01, Ac-SDKP vs. vehicle). Although ureteral obstruction resulted in an increase in the tubulointerstitial expression of ED-1, MCP-1, NF-κB, α-SMA and TGF-β1, Ac-SDKP treatment suppressed all their expressions (Figs. 5 and 6, Table I).

Effects of Ac-SDKP on MCP-1 and TGF-β1 RT-PCR expression. To better understand the mechanisms of the Ac-SDKP attenuation of renal inflammation and tubulointerstitial fibrosis, we examined the mRNA levels of the typical macrophage chemokine, MCP-1. Analysis of MCP-1 mRNA expression in the kidneys showed a marked increase in the UUO/vehicle group, which was significantly down-regulated with Ac-SDKP treatment (0.86±0.04 vs. 0.50±0.05, P<0.01, Fig. 7). Our results suggest that Ac-SDKP significantly modulated renal inflammation by blocking expression of MCP-1. The expression of TGF-β1 mRNA in the UUO/vehicle group increased after UUO. In contrast, Ac-SDKP treatment significantly inhibited TGF-β1 mRNA expression (0.79±0.05 vs. 0.42±0.04, P<0.01, Fig. 8), which could indicate that Ac-SDKP attenuated tubulointerstitial fibrosis by inhibition of TGF-β1 expression.

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tension and myocardial infarction, prevented interstitial collagen deposition (4,16) by decreasing the interstitial cell proliferation, monocyte/macrophage infiltration and TGF-ß1 expression. Lin et al (17) reported that Ac-SDKP has an aortic antifibrotic effect due to a reduced expression of the profibrotic cytokine TGF-ß1 in the ANG II-induced hypertension rat model and that long-term Ac-SDKP treatment (18) could ameliorate renal insufficiency and glomerulosclerosis in db/db mice via inhibition of the TGF-ß/Smad pathway. In previous in vitro studies, Ac-SDKP has been demonstrated (2) to block the proliferation of renal fibroblasts.
and to suppress DNA and collagen synthesis in cardiac fibroblasts. However, it is not known whether Ac-SDKP also has anti-inflammatory and antifibrotic effects on pure tubulointerstitial renal injury. To clarify this issue, we used a UUO model, characterized by renal inflammation and striking fibrosis limited to the tubulointerstitial area, to test our hypothesis that Ac-SDKP has anti-inflammatory and antifibrotic effects.

Renal interstitial inflammation, which plays a critical role in the genesis and progression of chronic kidney disease, is characterized by monocyte/macrophage infiltration. Monocyte/macrophage infiltration plays an important role in the renal inflammatory response during UUO (19,20). Rhaeleb et al (3) previously reported that Ac-SDKP inhibits monocyte/macrophage infiltration within the left ventricular interstitial space of rats with 2K-1C hypertension. As shown in Fig. 3, obstructed kidneys significantly expressed high levels of ED-1, and the administration of Ac-SDKP reduced this expression. To investigate the mechanisms of Ac-SDKP blockade of interstitial inflammation, we examined the levels of the typical macrophage chemokine, MCP-1. MCP-1, a protein primarily produced by tubular cells in response to renal injury, is involved in interstitial inflammation. In our study, MCP-1 level showed a significant increase in the UUO/vehicle group kidneys; Ac-SDKP not only significantly decreased MCP-1 immunohistochemical staining and mRNA levels in the obstructed kidneys but also led to less severe renal interstitial inflammation, which was demonstrated by a decrease in the ED-1-positive cells in the interstitium. Thus, the down-regulated MCP-1 immunohistochemical staining and mRNA levels could explain the effect of Ac-SDKP on the reduction of macrophage infiltration.

The expression of a large number of genes important to the inflammatory responses is primarily mediated by NF-κB activation, which leads to the synthesis of inflammatory mediators, including MCP-1. NF-κB activation (21,22) has been demonstrated in association with various renal diseases in which inflammation is a major feature of the pathological findings. It has been reported that angiotensin-converting enzyme inhibitors prevented NF-κB activation in a rabbit model of arteriosclerosis and in a rat model of immune complex nephritis (23,24). Morrissey et al (25) showed that ACE inhibitors suppressed NF-κB activation, MCP-1 expression and macrophage infiltration in a UUO rat model. Ac-SDKP, a ubiquitous tetrapeptide hydrolyzed almost exclusively by the angiotensin-converting enzyme, has been shown to have similar anti-inflammatory effects as angiotensin-converting enzyme inhibitors (26,27). In the present study, Ac-SDKP markedly decreased the activation of NF-κB in the UUO/vehicle group kidneys as shown by immunohistochemical staining. Therefore, Ac-SDKP treatment prevented renal inflammation in UUO rats, in agreement with studies showing that inhibition of NF-κB activity suppresses renal inflammation induced by obstruction. Because NF-κB activation is upstream of the synthesis of inflammatory cytokines, the reduction of MCP-1 expression via the NF-κB pathways may be responsible for the protective effect of Ac-SDKP in the UUO model. The mechanism by which Ac-SDKP affects the NF-κB pathways remains to be determined.

Macrophage infiltration induced by tubulointerstitial cell-derived MCP-1 might be a trigger of progressive interstitial fibrosis, which is characterized by a significant increase in the expression of some cytokines. It is well known that TGF-ß1, which is produced by epithelial cells and/or infiltrated cells, is a cytokine that plays an important role in the development and maintenance of fibrosis in ureteral obstruction kidneys (28-31). TGF-ß1 has been shown to stimulate the transformation of interstitial fibroblasts into activated fibroblasts. TGF-ß1 is a potent inducer of EMT, which is considered to be a contributor to fibrogenesis by disrupting the polarized tubular epithelial layers and increasing fibrotic scar formation. TGF-ß1 also increases the expression of α-SMA (32,33), which is recognized as a signal of myofibroblast appearance and an indicator of renal fibrosis disease severity (34,35).

Because we have shown an inhibitory effect on inflammatory factors after Ac-SDKP administration in the UUO model, we propose that Ac-SDKP could attenuate renal interstitial fibrosis induced by ureteral obstruction through the down-regulation of certain cytokines involved in fibroblast activation. As demonstrated in Fig. 5, the immunohistochemical staining levels of α-SMA significantly increased in the UUO/vehicle group kidneys, which could be partly explained by the migration of de-differentiated tubular cells through the basal membrane into the interstitium. In the present study, Ac-SDKP also markedly decreased the activation of TGF-ß1 in the UUO/vehicle group kidneys, as shown both by immunohistochemical staining and by measurement of TGF-ß1 mRNA levels. Using the UUO model, we proposed that Ac-SDKP could attenuate renal injury through the inhibition of the TGF-ß1 gene involved in the development and progression of interstitial fibrosis. In addition, TGF-ß1 directly affects macrophage infiltration within the interstitium (36). Thus, the down-regulated TGF-ß1 may be another mechanism to explain the reduction of macrophage infiltration in this UUO model. Hence, further intensive investigations are necessary to explain the exact mechanism of Ac-SDKP on renal inflammation and fibrosis in pure tubulointerstitial renal injury.

In conclusion, by using the UUO model we have demonstrated for the first time that Ac-SDKP significantly attenuates interstitial inflammation and fibrosis. The anti-inflammatory effects can be explained by the down-regulation of macrophage infiltration into the tubular interstitium and simultaneous inhibition of the expression of MCP-1 and NF-κB. Similarly, the decrease in renal tissue fibrosis results from the suppression of TGF-ß1 and α-SMA expression. We are currently engaged in identifying the cellular and molecular mechanisms involved in the Ac-SDKP-induced inhibition of interstitial fibrosis and inflammation using the UUO model, testing different doses and observing the outcomes in more detail. With evidence from further studies, Ac-SDKP may become a potential candidate for the treatment of chronic renal disease.

References