Decreased Endogenous Levels of Ac-SDKP Promote Organ Fibrosis

Maria A. Cavasin, Tang-Dong Liao, Xiao-Ping Yang, James J. Yang, Oscar A. Carretero

Abstract—There is convincing evidence that chronic treatment with N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), a peptide normally found in tissues and biological fluids, reduces collagen deposition in the heart and kidneys of hypertensive rats and rats with myocardial infarction. However, it is not known whether endogenous Ac-SDKP at basal concentrations has any physiological function related to collagen deposition. Prolyl oligopeptidase is responsible for release of Ac-SDKP from its precursor thymosin-β4. When we treated rats with a specific oral prolyl oligopeptidase inhibitor, Ac-SDKP decreased significantly in the plasma, heart, and kidney. In the present study, we tested the hypothesis that endogenous Ac-SDKP at basal levels plays a physiological role, antagonizing and/or preventing excessive collagen deposition. We studied whether chronic blockade of Ac-SDKP promotes collagen accumulation and/or accelerates this process in the presence of a profibrotic stimulus such as angiotensin II. We found that decreased basal levels of Ac-SDKP increased cardiac and renal perivascular fibrosis and promoted glomerulosclerosis. Moreover, in the presence of angiotensin II decreasing basal levels of Ac-SDKP accelerated interstitial cardiac fibrosis attributable to an increase in cells that produce collagen. We concluded that Ac-SDKP participates in the regulation of collagen content under normal conditions. We believe this is the first study showing that this peptide plays a physiological role at basal concentrations, preventing organ collagen accumulation. (Hypertension. 2007;50:130-136.)

Key Words: prolyl endopeptidase □ thymosin β4 □ angiotensin-converting enzyme □ heart □ kidney □ collagen

In hypertension and cardiovascular diseases, excessive collagen accumulation in the heart and kidney leads to pathological remodeling with important functional consequences. The net content of extracellular matrix proteins under normal conditions results from the delicate balance between synthesis and degradation. There is convincing evidence that N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), a tetrapeptide normally present in organs and biological fluids of humans and experimental animals,1,2 can reduce collagen deposition in the heart and kidneys when it is chronically infused into hypertensive rats and rats with myocardial infarction.3–6 More recently, it has been shown to mediate the antifibrotic effects of angiotensin-converting enzyme inhibitors.7 However, it is not known whether endogenous Ac-SDKP at concentrations normally found in plasma and organs has any physiological function related to collagen deposition, especially in the heart and kidneys.

We recently found that prolyl oligopeptidase (POP), a cytosolic enzyme involved in the metabolism of many peptide hormones and neuropeptides,8 is responsible for release of Ac-SDKP from its precursor thymosin-β4. When we treated rats with a specific oral POP inhibitor (POPi), Ac-SDKP decreased significantly in the plasma, heart, and kidneys.9 In the present study, we tested the hypothesis that endogenous Ac-SDKP at basal levels has a physiological role, antagonizing and/or preventing excessive collagen deposition. We tested whether chronic blockade of Ac-SDKP release using a POpi promotes collagen accumulation and/or accelerates this phenomenon in the presence of a profibrotic stimulus such as angiotensin II (Ang II).

Methods

Animals

The study was approved by the Institutional Animal Care and Use Committee (IACUC) of Henry Ford Health System, and all procedures were conducted in accord with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. We used 50 10-week-old male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass). All surgical procedures were conducted with the animals under pentobarbital anesthesia (50 mg/kg, IP); and buprenorphine (0.05 mg/kg, SQ) was administered after osmotic mini-pump implantation.

Tissue Levels of Ac-SDKP, Arg8-Vasopressin, Substance-P, and Ang II

We performed a pilot study to test the effects of POpi on tissue levels of some peptides, which were reported to be POP substrates. Rats...
were treated with either vehicle (n = 9) or POPi (n = 9; S17092, 40 mg/kg per d, a gift of P. Vanhoutte, Institut de Recherches Internationales Servier, France). POPi was mixed with peanut oil and administered daily by gavage. After 1 week rats were euthanized and the heart, kidneys, and brain were rapidly frozen in liquid nitrogen (LN2) and kept at −70°C until processing. We used commercially available enzyme immunoassay kits to measure Ac-SDKP (Cayman Chemicals) as well as Arg8-vasopressin and substance-P (Assay Designs) and Ang II (Peninsula Laboratories). Samples were processed according to the manufacturer’s recommendations. Tissue was weighed, homogenized with cold methanol, and centrifuged; the supernatant was evaporated to dryness and dried samples kept at 20°C until assay, when they were reconstituted with ELA buffer.

**Experimental Protocol**

Rats were divided into the following groups: (1) Ang II vehicle + POPi vehicle (n = 8), (2) Ang II vehicle + POPi (n = 9), (3) Ang II (100 μg/kg/d) + POPi vehicle (n = 8), and (4) Ang II + POPi (n = 9). Ang II or its vehicle (0.01N acetic acid) was infused by osmotic mini-pump implanted SQ. We used a low dose of Ang II because we did not want to reach the maximum fibrotic effect with this treatment. Systolic blood pressure was measured by tail cuff every other week. After 8 weeks, rats were euthanized, blood was collected for Ac-SDKP measurements (to ensure treatment administration), and the heart and kidneys were removed and weighed. They were cut transversely into 3 pieces: one was used for total collagen determination, the second was kept in formalin for 24 hours and then embedded in paraffin, and the third was frozen and kept in isopentane at −70°C.

**Total Collagen Determinations**

Total cardiac and renal collagen were measured using the established hydroxyproline (HP) method as described by Woessner. Briefly, tissue samples were dried, weighed, and pulverized. Each sample was hydrolyzed in 0.5 mL 6N HCl for 16 hours at 120°C. All samples were dried using a vacuum centrifugation device with 10 kDa cutoff. HD content was determined by a color-based reaction. Results are expressed as the ratio of collagen to vessel cross-sectional area. Cardiac interstitial collagen fraction was calculated in a percentage of total area. To measure glomerulosclerosis, 6-μm paraffin-embedded kidney slices were stained with periodic acid-Schiff (PAS) and mesangial matrix expansion expressed as a percentage of positive PAS staining compared with total glomerular area. To assess interstitial cell proliferation and prolyl 4-hydroxylase-expressing cells, we incubated frozen heart sections with monoclonal anti-rat Ki-67 antigen antibody (1:50, clone MIB-5, Dako) and mouse anti-rat prolyl 4-hydroxylase (β-subunit, Chemicon), respectively, at 4°C overnight, using a Vectastain ABC kit (Vector Laboratory) to visualize immunoreactivity. Sections were developed with diamobenzidine substrate (Vector) and counterstained with hematoxylin. Cells were counted and expressed as cells/mm².

**Data Analysis**

Three pairwise comparisons were conducted: (1) POPi versus vehicle, (2) Ang II versus vehicle, and (3) Ang II + POPi versus Ang II. The first comparison was done to test whether Ac-SDKP has any physiological role under normal conditions; the second and third comparisons were done to ascertain whether Ac-SDKP antagonizes a pro-fibrotic stimulus. Two sample t test was used for each pairwise comparison. To control the type I error rate while conducting multiple comparisons, Hochberg multiple comparison procedure was used to identify significant difference. The family-wise significant level was set at 0.05 and the tests with adjusted probability value less than 0.05 were considered statistical significant. All analysis results are presented as mean±SE.

### Results

**Effect of POPi on Endogenous Levels of Ac-SDKP and Other POP Substrates: Arg8-Vasopressin, Substance-P, and Ang II**

Table 1 shows amounts of these peptides in the heart, kidney, and brain (as control for the neuropeptides). Endogenous levels of Ac-SDKP decreased significantly after 1 week of chronic treatment with POPi. Ang II content was not affected by chronic POPi administration. Levels of Arg8-vasopressin and substance-P in the heart and kidneys are normally very low compared with the brain, and chronic treatment with POPi had no significant effect.

**Effect of POPi on Cardiac and Renal Morphology and Systolic Blood Pressure in the Presence or Absence of Ang II**

There were no differences in heart and kidney weight corrected by body weight between any groups (Table 2). POPi alone did not affect SBP (Figure 1A); only Ang II significantly increased SBP, and the combination of Ang II and POPi had no further effect compared with Ang II alone. Both groups treated with POPi treatment had significantly less Ac-SDKP in the heart and kidneys compared with their controls, indicating that POPi treatment was successful. Ang II infusion had no effect on organ levels of Ac-SDKP; however, it decreased plasma Ac-SDKP compared with vehicle.

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Heart</th>
<th>Kidney</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>POPi</td>
<td>Vehicle</td>
</tr>
<tr>
<td>Ac-SDKP, pg/mg tissue</td>
<td>34.3±3.3</td>
<td>5.7±1*</td>
<td>113.0±24</td>
</tr>
<tr>
<td>Ang II, pg/mg tissue</td>
<td>0.185±0.05</td>
<td>0.144±0.02</td>
<td>0.382±0.10</td>
</tr>
<tr>
<td>Substance-P, pg/mg tissue</td>
<td>0.329±0.12</td>
<td>0.249±0.03</td>
<td>0.099±0.01</td>
</tr>
<tr>
<td>Arg8-vasopressin, pg/mg tissue</td>
<td>0.017±0.00</td>
<td>0.020±0.01</td>
<td>0.087±0.02</td>
</tr>
</tbody>
</table>

*P<0.01 and †P<0.05 vs vehicle.
Effect of POPi on Cardiac and Renal Collagen in the Presence or Absence of Ang II

Figure 1B shows total hydroxyproline content in the heart and kidneys after 8 weeks of treatment. POPi alone significantly increased total collagen content. A low dose of Ang II increased total collagen deposition slightly, and the combined treatment increased collagen content similarly to POPi alone. An additional group of rats was treated with a higher dose of Ang II (750 μg/kg per d); these rats had renal and cardiac hydroxyproline content of 4.89 ± 0.36 and 4.44 ± 0.53 mg/mg dry tissue, respectively, not significantly different from the POPi and Ang II POPi groups. This indicates that the maximum fibrotic effect may be achieved by POPi. Figure 2 shows representative images of PVF from heart and kidney sections stained with picrosirius red as well as the cumulative data. POPi alone significantly increased PVF. A low dose of Ang II increased PVF slightly but the difference was not significant, and the combined treatment increased perivascular collagen similarly to POPi alone, which was not significantly different from Ang II alone. Figure 3 shows interstitial cardiac fibrosis, and Figure 4 shows interstitial cell proliferation and number of prolyl 4-hydroxylase expressing cells. POPi or Ang II alone did not have any significant effect; however, the combined treatment significantly increased interstitial collagen deposition, cell proliferation, and prolyl 4-hydroxylase expressing cells compared with Ang II alone.

Effect of POPi on Mesangial Matrix Deposition in the Presence or Absence of Ang II

Paraffin-embedded kidney sections were stained using PAS, which is used to examine mesangial matrix deposition. Figure 5 shows representative images and cumulative data, expressed as per cent of glomerular area positively stained by PAS. POPi or Ang II alone significantly increased PAS staining compared with vehicle; however, the combined treatment had no additional effect.

Discussion

We have accumulated extensive evidence of the antifibrotic properties of Ac-SDKP when administered chronically in rat models of hypertension and myocardial infarction4,14,15; however, we questioned whether this peptide would have any physiological role at normal concentrations. In this study we found that chronic treatment with an oral POPi, which blocked the release of Ac-SDKP from its precursor thymosin-9, significantly decreased cardiac and renal endogenous levels of Ac-SDKP in normal rats and promoted collagen deposition, perivascular fibrosis, and glomerulosclerosis, suggesting that this peptide helps regulate normal collagen content.

Prolyl oligopeptidase (POP, EC 3.4.21.26), also called prolyl endopeptidase, is widely distributed in mammalian tissues and has been isolated from several organs (brain, kidney, liver, muscle) from various species (human, rat, mouse, and chicken). It is important to note that POP has been shown to have a role in the degradation of other peptides, such as angiotensin II, which may contribute to its antifibrotic properties. In this study, we investigated the effects of POPi on cardiac and renal collagen deposition in the presence or absence of Ang II, a powerful vasoconstrictor and fibrogenic agent.

Table 2: Morphology and Ac-SDKP Levels After 60 Days of Chronic Treatment With POPi in the Presence or Absence of Ang II

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vehicle</th>
<th>POPi</th>
<th>Ang II</th>
<th>Ang II + POPi</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>482 ± 14</td>
<td>438 ± 8</td>
<td>493 ± 9</td>
<td>460 ± 17</td>
</tr>
<tr>
<td>Heart/BW, mg/g</td>
<td>2.60 ± 0.09</td>
<td>2.63 ± 0.06</td>
<td>2.61 ± 0.09</td>
<td>2.63 ± 0.11</td>
</tr>
<tr>
<td>R. Kidney/BW, mg/g</td>
<td>2.98 ± 0.11</td>
<td>2.97 ± 0.11</td>
<td>2.84 ± 0.09</td>
<td>2.99 ± 0.07</td>
</tr>
<tr>
<td>Plasma Ac-SDKP, nmol/L</td>
<td>1.67 ± 0.21</td>
<td>0.68 ± 0.05*</td>
<td>1.11 ± 0.07†</td>
<td>0.78 ± 0.08‡</td>
</tr>
<tr>
<td>Heart Ac-SDKP, pg/mg tissue</td>
<td>36.24 ± 4.5</td>
<td>5.33 ± 0.61*</td>
<td>31.55 ± 4.96</td>
<td>9.51 ± 1.71†</td>
</tr>
<tr>
<td>Kidney Ac-SDKP, pg/mg tissue</td>
<td>159.5 ± 9.4</td>
<td>29.5 ± 6.3*</td>
<td>145.9 ± 16.3</td>
<td>35.3 ± 6.7‡</td>
</tr>
</tbody>
</table>

*P < 0.01 and †P < 0.05 vs vehicle; ‡P < 0.01 vs Ang II.

Figure 1. Systolic blood pressure of rats chronically treated with POPi in the presence or absence of Ang II. Data are expressed as mean ± SE. *P < 0.01 vs vehicle (A). Total collagen content in the heart and kidneys of rats chronically treated with POPi in the presence or absence of Ang II. Hydroxyproline content directly indicates collagen amount. Data are expressed as mean ± SD. *P < 0.05, NS vs vehicle (B).
rabbit, lamb, cow).16 POP is involved in the degradation of various neuropeptides,17 such as Arg8-vasopressin and substance-P, whose levels may rise during POPi treatment. Because Arg8-vasopressin and substance-P reportedly stimulate fibroblast proliferation in vitro,18–20 which could increase collagen deposition, we measured cardiac and renal levels of Arg8-vasopressin and substance-P. In a pilot study, we found that endogenous levels of these profibrotic neuropeptides were not affected by POPi in the heart and kidneys whereas they were slightly increased in the brain, in agreement with other studies11,21; however, this increase did not reach significance, possibly because of the fact that we homogenized the entire brain and the neuropeptides are reportedly significantly increased only in some regions of the brain (frontal cortex and hypothalamus) after treatment with S 17092.21,22 On the contrary, chronic POPi administration significantly decreased Ac-SDKP, suggesting that the increase in collagen deposition observed in rats treated with POPi is not likely caused by a possible increase in profibrotic neuropeptides but rather to decreased endogenous levels of Ac-SDKP. POP is also one of the enzymes (along with neutral endopeptidase 24.11, thimet oligopeptidase, prolyl carboxypeptidase23 and ACE224) involved in the degradation of Ang I and Ang II forming Ang 1-7. Although contribution to the conversion of Ang 1-7 by different enzymes was dependent on tissue and biochemical environment, current data reported by several investigators suggest that POP does not contribute significantly to the formation of Ang 1-7 in vivo.25–27

In this study, we used a low dose of Ang II, because we wanted to avoid maximal fibrotic effects. We found that collagen content and perivascular fibrosis were slightly increased in the rats given a low dose of Ang II, and the combination of Ang II and POPi had no further effect compared with POPi alone, suggesting that the maximum fibrotic effect may be reached with the POPi, as it was confirmed with a higher dose of Ang II.

The fact that decreasing normal endogenous levels of Ac-SDKP promoted excessive collagen deposition may indicate that this tetrapeptide plays a role in normal collagen turnover, either decreasing the rate of collagen synthesis or promoting its degradation. This hypothesis has been also suggested by Pokharel et al, who recently showed that...
enhanced breakdown of endogenous Ac-SDKP attributable to cardiac ACE overexpression (because Ac-SDKP is a natural ACE substrate) is responsible for the increase in cardiac collagen. One interesting finding was that interstitial collagen fraction was not increased with POPi alone, in contrast to PVF; however, the combined treatment of POPi plus Ang II significantly increased interstitial collagen fraction compared with Ang II alone, and this was accompanied by an increase in interstitial cell proliferation and prolyl 4-hydroxylase-expressing cells. These observations may suggest that Ac-SDKP participates in maintenance of the collagen balance, so that a decrease in endogenous levels would affect remodeling of the myocardium similarly to hypertension, which occurs initially in the perivascular portion and progressively extends to cause widespread interstitial fibrosis; this process may be accelerated in the presence of a profibrotic stimulus, such as Ang II. Although the increase in interstitial collagen in the combined Ang II+POPi group is significant by itself, it does not represent a high percentage of total organ collagen content, which is derived mainly from the perivascular portion, perhaps explaining why no additive effect was seen when total collagen was assessed.

We previously showed that chronic administration of Ac-SDKP prevents interstitial collagen deposition in hypertension and myocardial infarction possibly by decreasing interstitial cell proliferation, monocyte/macrophage infiltration, and transforming growth factor (TGF)-β expression7 as well as fibroblast proliferation. In the present study we found that interstitial cell number was increased in the rats given the combined treatment, and this corresponded to increased interstitial collagen in the heart. Although we could not identify the type of proliferating interstitial cells, it is possible that some would be fibroblasts, because the number of prolyl 4-hydroxylase-positive cells (a marker for fibroblasts) likewise increased.

**Figure 3.** Cardiac interstitial collagen fraction. Interstitial collagen is expressed as percent of myocardial area. Interstitial cells are expressed per mm². Data are expressed as mean±SE. *P<0.05 vs Ang II alone. Representative images of cardiac interstitial fibrosis are shown on the right. Magnification 400×.

**Figure 4.** Cardiac interstitial cell proliferation and prolyl 4-hydroxylase-expressing cells. Number of Ki-67 and r-PH positive cells are expressed per mm². Data are expressed as mean±SE. *P<0.05 and **P<0.01 vs Ang II alone.
We also found that decreased endogenous levels of Ac-SDKP promoted glomerulosclerosis, suggesting that Ac-SDKP is involved in normal mesangial matrix turnover. This is supported by a recent report showing that chronic treatment with Ac-SDKP prevented kidney damage and reduced mesangial matrix expansion in diabetic mice.6

It is worth noting that the effects of POPi treatment on collagen deposition were not attributable to changes in blood pressure, because POPi did not increase systolic blood pressure as reported previously.9 The low dose of Ang II used in this study reduced basal plasma levels of Ac-SDKP. Although we do not currently have a good explanation for this observation, we do not believe it negates our observations or confounds our interpretation of the results, because tissue contents were not affected by Ang II treatment, which is the relevant parameter to consider when organ fibrosis is analyzed.

Limitations of Our Study
In vitro studies using the purified enzyme revealed that POP has many substrates, including substance-P, Arg8-vasopressin, and Ang I and II (POP action on Ang I and II results in formation of Ang 1-7).32 A wide range of K_m values has been reported, depending on the species and tissues (see Welches et al33 for review). Although angiotensins reportedly have the lowest K_m for POP, to conclude that they are the best substrates, possessing the highest affinity in vivo, would be an oversimplification. We designed a pilot study to evaluate the effects of S 17092 on levels of natural POP substrates; however, we were more concerned with the profibrotic neuropeptides substance-P and Arg8-vasopressin, because they were reportedly increased significantly by S17092 in some regions of the brain such as the hypothalamus and frontal cortex,11 and if the same is true in other organs, that may favors an increase in fibrosis.21

We are not aware of any available data showing the effects of chronic POPi treatment on angiotensin peptides. We only measured Ang II after 7 days (pilot study) and there was no change in organ levels with POPi. Because we observed a similar reduction in organ Ac-SDKP content after 7 and 60 days of POPi treatment (Tables 1 and 2), we have no reason to believe that Ang II would be affected after 60 days.

Conclusion
We concluded that significant reduction of endogenous Ac-SDKP in the heart and kidneys promotes collagen deposition and perivascular fibrosis as well as glomerulosclerosis, suggesting that this peptide helps regulate collagen content under normal conditions. We believe this is the first study showing that Ac-SDKP has a physiological role at basal concentrations, preventing organ collagen accumulation.

Perspectives and Significance
In this study we found that Ac-SDKP plays an important physiological role in maintenance of the collagen balance, because decreasing endogenous levels of Ac-SDKP increased net collagen content. Extracellular matrix proteins exhibit continuous turnover, and we still do not know whether Ac-SDKP participates in slowing collagen synthesis, accelerating degradation, or both. However, we believe our findings contribute to knowledge of the mechanisms that regulate collagen accumulation and may help in selecting potential targets for treatment of fibrosis.

Source of Funding
This work was supported by a National Institutes of Health grant K01 HL076581 (to M.A.C.).

Disclosures
None.

References


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Hypertension. 2007;50:130-136; originally published online April 30, 2007;
doi: 10.1161/HYPERTENSIONAHA.106.084103
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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