Long-Term Effect of N-Acetyl-Seryl-Aspartyl-Lysyl-Proline on Left Ventricular Collagen Deposition in Rats With 2-Kidney, 1-Clip Hypertension

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Background—N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is a natural inhibitor of pluripotent hematopoietic stem cell proliferation. Ac-SDKP plasma concentration is increased 5-fold after angiotensin-converting enzyme inhibition. Here we studied the effect of Ac-SDKP on monocyte/macrophage infiltration, fibroblast proliferation, and collagen deposition in the rat heart in renovascular hypertension.

Methods and Results.—We investigated whether long-term Ac-SDKP administration would prevent left ventricular (LV) hypertrophy and interstitial collagen deposition in rats with 2-kidney, 1-clip (2K-1C) hypertension. Ac-SDKP (400 μg · kg⁻¹ · d⁻¹) did not affect development of hypertension. Mean blood pressure was similar in rats with 2K-1C hypertension whether they were given vehicle or Ac-SDKP and was higher than in controls. Both LV weight and cardiomyocyte size were significantly increased in rats with 2K-1C hypertension compared with controls and were unaffected by Ac-SDKP. Proliferating cell nuclear antigen- and monocyte/macrophage-positive cells were increased in the LV of 2K-1C hypertensive rats; this increase was significantly blunted by Ac-SDKP (P<0.001). LV interstitial collagen fraction was also increased in 2K-1C hypertensive rats given vehicle (10.1±0.8%) compared with sham (5.3±0.1%, P<0.001), and this increase was prevented by Ac-SDKP (5.4±0.4, P<0.001).

Conclusions.—Ac-SDKP reduced monocyte/macrophage infiltration, cell proliferation, and collagen deposition in the LV of hypertensive rats without affecting blood pressure or cardiac hypertrophy, suggesting that it may be partly responsible for the cardioprotective effect of angiotensin-converting enzyme inhibitors. (Circulation. 2001;103:3136-3141.)

Key Words: angiotensin ■ hypertension, renal ■ blood pressure ■ inhibitors ■ collagen ■ myocardium
creased interstitial collagen deposition within the myocardium in hypertensive patients, spontaneously hypertensive rats, and Lewis rats with heart failure induced by myocardial infarction. Decreased Ang II generation associated with plasma Ac-SDKP levels are greatly increased after short- and long-term ACE inhibition and Ac-SDKP inhibits adult rat cardiac fibroblast proliferation and collagen synthesis in vitro. Therefore, the present study was designed to test the hypothesis that long-term Ac-SDKP administration prevents cardiac monocyte/macrophage infiltration, fibroblast proliferation, and collagen deposition in the left ventricle (LV) of rats with 2-kidney, 1-clip (2K-1C) hypertension.

Methods

2K-1C Hypertension

Male Sprague-Dawley rats (150 to 175 g; Charles River, Wilmington, DE) were anesthetized with pentobarbital sodium (50 mg/kg IP). The left renal artery was exposed by retroperitoneal flank incision and dissected free of the renal vein and connective tissue. A silver clip with a lumen of 0.22 mm was placed around the artery for partial occlusion; in sham operations, the artery was not clipped. After surgery, an osmotic minipump (Alzet 2 ML4, Alza Corp) was implanted subcutaneously in the neck to deliver either Ac-SDKP (Bachem) or saline plus 0.01N acetic acid, basing the route of administration and dosage on previous studies. Rats were subdivided into 4 groups: (1) sham, (2) 2K-1C plus vehicle (saline plus 0.01N acetic acid), (3) 2K-1C plus low-dose Ac-SDKP (400 μg · kg⁻¹ · d⁻¹), and (4) 2K-1C plus high-dose Ac-SDKP (800 μg · kg⁻¹ · d⁻¹). This study was approved by the Henry Ford Hospital Care of Experimental Animals Committee.

Blood Pressure and Heart Weight

Systolic blood pressure was measured by the tail-cuff method twice a week for 8 weeks. At the end of the experiment, rats were anesthetized with sodium hexobarbital (50 mg/kg IP), and a catheter (PE-10 fused to PE-50, Clay Adams) filled with saline plus heparin (100 IU) was inserted into the abdominal aorta through the femoral artery; the distal end was tunneled under the skin and exteriorized on the neck. Twenty-four hours later, each conscious rat was placed in a restrainer, and mean blood pressure was recorded on a 4-channel recorder (Brush 220, Gould) once a minute for 60 minutes using a pressure transducer and processor (model P10EZ, Ohmeda). Animals were again anesthetized with 50 mg/kg pentobarbital sodium; the heart was stopped at diastole by intraventricular injection of 15% KCl, excised, cleaned of blood, and then blotted to dryness. The LV plus septum, right ventricle, and left and right atria were weighed, and the LV was sectioned transversely into 3 slices. The slices were rapidly frozen in isopentane precooled in liquid nitrogen until the hydroxyproline assay was performed. The LV plus septum, right ventricle, and left and right atria weights were normalized to 100 g of body weight.

Histological and Histochemical Analysis of Myocyte Cross-Sectional Area and Interstitial Collagen Fraction in the LV

Sections 10 μm thick from each frozen slice were stained separately with (1) fluorescein-labeled peanut agglutinin (Vector Laboratories) after pretreatment with 3.3 U/mL neuroaminidase type V (Sigma) to delineate myocyte cross-sectional area (an indicator of myocyte volume) and the interstitial space (consisting of collagen and capillaries) and (2) rhodamine-labeled Griffonia simplicifolia lectin I to show only the capillaries, because G simplicifolia lectin I selectively binds to capillaries. Three radially oriented microscopic fields were selected at random from each section and photographed. We used a fluorescence microscope (Nikon) equipped with a Spot digital camera (×100, Diagnostic Instruments).

Cross-Sectional Area of LV Myocytes

Stained sections were photographed on 35-mm film. Each field contained ≥100 myocytes. Images were projected with a photomag- nifier, and myocyte cross-sectional area (radial fields) and length (longitudinal fields) were determined by computer-based planimetry (SigmaScan). An average cross-sectional area was calculated using data from all 3 slices.

LV Interstitial Collagen Fraction

Total surface area (microscopic field), interstitial space (collagen plus capillaries), and area occupied by capillaries alone were measured by computer-assisted videodensitometry (JAVA, SPSS Inc). The interstitial collagen fraction was calculated as percent surface area occupied by the interstitial space minus the percent surface area occupied by capillaries. The interstitial collagen fraction was averaged using data from all 3 slices.

LV Cell Proliferation (PCNA) and Inflammatory Cell Infiltration (ED1)

LV samples were isolated 2 weeks after the 2K-1C procedure, fixed in 2.5% paraformaldehyde, and mounted in a paraffin block. Four-micron-thick sections were deparaffinized, rehydrated, boiled in 0.2% citric acid (pH 6.0) for 10 minutes for antigen retrieval, washed 3 times in phosphate-buffered saline for 5 minutes each, preincubated with blocking serum (1% normal serum) for 30 minutes, and finally incubated with a mouse monoclonal antibody against either proliferating cell nuclear antigen (PCNA, 1:250 dilution) or rat monocytes and macrophages (ED1, 1:1000 dilution; Chemicon) at room temperature for 30 minutes. Each section was washed 3 times in phosphate-buffered saline, and PCNA or ED1 was assayed (Vectastain ABC kit, Vector Laboratories). Sections were developed with diaminobenzidine substrate (Vector) and counterstained with hematoxylin. We used a Nikon microscope equipped with a color-sensitive digital video camera (Optronics). The microscopic image was imported to a computer fitted with a Bioquant NOVA image analysis system (R&M Biometrics). Stained cells were counted at ×40, and because every selected image occupied the entire window, we measured window size with the same objective. The window area was fixed at 22,194 μm² (0.022194 mm²). Cell density was calculated as the number of cells per window area (ie, number of PCNA- or ED1-positive cells per square millimeter). For each sample, 24 randomly selected fields were examined.

Hydroxyproline Assay

Myocardial collagen was measured by hydroxyproline assay. LV samples from 2K-1C rats (180 to 200 mg) were freeze-dried, weighed, and pulverized. Each sample was homogenized in 0.1 mol/L NaCl and 5 mmol/L NaHCO₃, washed with the same solution 5 times, and hydrolyzed in 1 mL of 6N HCl for 16 hours at 110°C. Samples were filtered, vacuum-dried, and then dissolved in distilled water. Hydroxyproline was measured using a colorimetric assay and a standard curve of 0 to 5 μg hydroxyproline. Data were expressed as micrograms of collagen per milligram dry weight, assuming that collagen contains an average of 13.5% hydroxyproline.

Ac-SDKP Plasma Levels

After mean blood pressure was measured, arterial blood from 4 rats per group was collected in a heparinized tube containing captopril (final concentration, 10 μmol/L) and centrifuged at 2000g for 15 minutes at 4°C. Recovered plasma was stored at −70°C until the Ac-SDKP assay was performed. Plasma Ac-SDKP was quantified with a competitive enzyme immunoassay and expressed as nanomoles per liter.
Statistical Analysis

The mean response in the 2K-1C/vehicle group was compared separately with each Ac-SDKP group (400 or 800 \( \text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1} \)) and controls. Significance was evaluated with Student’s \( t \) test and Holm’s procedure, which adjusts individual rejection criteria to ensure a family-wise \( P \) value of 0.05. Procedural assumptions were verified, and data are expressed as mean±SEM.

Results

Effect of Long-Term Ac-SDKP Infusion on Blood Pressure and Heart Rate

Ac-SDKP at 400 or 800 \( \text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1} \) for 8 weeks had no effect on mean blood pressure or the development of hypertension in 2K-1C rats (Figure 1). At the end of the experiment, mean blood pressure was significantly higher in rats with 2K-1C/vehicle (161±5 mm Hg, \( n=10 \)) than in sham (118±2 mm Hg, \( n=14; P<0.001 \)). However, heart rate was unchanged (Figure 2).

Effect of Long-Term Ac-SDKP Infusion on LV Hypertrophy and Cardiomyocyte Cross-Sectional Area

LV hypertrophy was significantly greater in 2K-1C rats than in sham (\( P<0.05 \)). Ac-SDKP did not reduce LV hypertrophy in hypertensive rats (Figure 3). Myocyte cross-sectional area was also significantly increased in 2K-1C/vehicle rats versus sham (\( P<0.05 \)) and was similar in Ac-SDKP–treated hypertensive rats.

Effect of Long-Term Ac-SDKP Infusion on Cell Proliferation (PCNA) and Monocyte/Macrophage (ED1) Infiltration in the LV Interstitium

PCNA-positive cells were found only in the LV interstitial space in controls but were distributed diffusely throughout both interstitial and perivascular spaces in the 2K-1C/vehicle rats (Figure 4). PCNA-positive cells were significantly increased in the 2K-1C/vehicle group (\( P<0.0001 \)). Ac-SDKP significantly reduced the number of PCNA-positive cells in the LV interstitial and perivascular spaces, resembling control levels (\( P<0.01 \)). There were also significantly more ED1-positive cells in the LV in the 2K-1C/vehicle group than in

![Figure 1](image1.png)

![Figure 2](image2.png)

![Figure 3](image3.png)

![Figure 4](image4.png)
Ac-SDKP kept monocytes/macrophages (ED1) at normal levels in 2K-1C rats.

Effect of Long-Term Ac-SDKP Infusion on LV Interstitial Collagen Deposition

LV collagen estimated by hydroxyproline content was significantly increased in untreated 2K-1C rats versus controls (Figure 5). Hypertensive rats treated with Ac-SDKP (400 μg · kg⁻¹ · d⁻¹) had less LV collagen than did untreated rats (7.56 ± 0.44 vs 11.85 ± 1.05 μg/mg dry LV, P<0.005). Higher doses of Ac-SDKP did not appear to reduce LV collagen more than lower doses (8.82 ± 1.3 μg/mg dry LV). The interstitial collagen fraction was significantly increased in untreated 2K-1C rats versus sham (10.1 ± 0.8% vs 5.3 ± 0.1%, P<0.0001) but normalized in 2K-1C rats given 400 μg · kg⁻¹ · d⁻¹ Ac-SDKP (5.4 ± 0.4%) and was somewhat less reduced at higher doses (6.78 ± 0.81%, Figure 5), confirming the hydroxyproline data. Myocyte cross-sectional area and interstitial collagen deposition are shown in Figure 6.

Ac-SDKP Plasma Concentration

Ac-SDKP plasma concentration was the same in 2K-1C/vehicle and sham groups (Figure 7) but nearly doubled in 2K-1C rats treated with Ac-SDKP versus vehicle, although this difference was significant only at the 800 μg · kg⁻¹ · d⁻¹ dose (P<0.02).

Discussion

We found that Ac-SDKP inhibits collagen deposition, cell proliferation (cardiac fibroblasts), and infiltration by inflammatory cells (monocytes and macrophages) within the LV interstitial space of rats with 2K-1C hypertension without affecting blood pressure, cardiac hypertrophy, or myocyte cross-sectional area (an indicator of myocyte volume). At the end of the experiment, the plasma Ac-SDKP level was
doubled in rats given Ac-SDKP compared with untreated hypertensive 2K-1C rats, values similar to those found in patients on long-term enalapril (an ACEI) for 25 days (3.97±0.75 nmol/L), normal rats given captopril for 28 days (3.51±0.87 nmol/L), or irradiated mice given lisinopril (another ACEI) for 72 hours. Higher doses (800 μg kg⁻¹ d⁻¹) were slightly less effective than lower doses, exhibiting almost the same pharmacological profile as our in vitro studies as well as others.\(^3\)\(^1\),\(^2\)

The antifibrotic effect of Ac-SDKP in vivo could result from inhibition of fibroblast proliferation and collagen synthesis, as shown in our in vitro study.\(^1\) The present results may indirectly indicate that the beneficial effect of ACEIs in hypertension could be partially mediated by Ac-SDKP. In fact, long-term ACE inhibition has been associated with (1) a significant reduction in cardiac collagen deposition in spontaneously hypertensive rats,\(^1\) in rats with renovascular\(^3\) or aldosterone-salt–induced hypertension,\(^4\) or in hypertensive patients\(^5\) and (2) a significant increase in plasma Ac-SDKP in patients, normal rats, or irradiated mice.\(^2\),\(^9\)\(^2\),\(^8\)\(^3\)\(^\_\)\(^0\) Long-term ACE inhibition in hypertension is associated with decreased collagen deposition, blood pressure, total peripheral resistance, and cardiac hypertrophy and improved cardiovascular function. However, these ACEI effects do not necessarily indicate whether reduced LV collagen deposition is due to hemodynamic changes, inhibition of conversion of Ang I to Ang II, increased tissue and/or circulating kinins, and/or increased Ac-SDKP plasma levels per se. Nicolleti et al\(^1\)\(^4\) likewise reported that myocardial macrophages (ED1–positive cells) were significantly increased in rats with 2K-1C hypertension and co-localized with collagen-synthesizing fibroblasts. Inflammatory cells could promote fibrosis by releasing growth factors or cytokines, which act on fibroblasts. Therefore, Ac-SDKP may prevent LV collagen deposition in hypertensive rats through inhibition of fibroblast proliferation and collagen synthesis and/or increased metalloproteinase activity, either directly or indirectly by inhibiting myocardial inflammatory cells. Both antihypertensive and non-antihypertensive doses of ACEIs abolished myocardial collagen deposition in hypertension, and the fact that this antifibrotic effect was unaffected by icatibant, a potent B₂ kinin receptor antagonist,\(^6\) suggests that kinins do not mediate the antifibrotic effect of ACEIs in hypertensive rats and that Ac-SDKP may be a good candidate for this therapeutic effect. This hypothesis is based on our results as well as those of Junot et al,\(^7\) who found that at 0.1 to 0.3 mg/kg, captopril inhibited Ac-SDKP hydrolysis without affecting the renin-angiotensin system or blood pressure. Such an association between the therapeutic effect of ACEIs and Ac-SDKP is not unique to the cardiovascular system, perhaps occurring also after radiotherapy and/or chemotherapy. Indeed, ACEI prevented entry of murine hematopoietic stem cells into the cell cycle after irradiation in vivo, and this inhibitory effect correlated positively with a 6-fold increase in plasma Ac-SDKP.\(^3\)\(^0\)\(^2\) Ac-SDKP also blunts the cytotoxic effect of 3'-azido-3'-deoxythymidine (AZT), commonly used to treat AIDS patients.\(^3\)\(^9\) Moreover, long-term ACEI treatment inhibits renal fibroblast proliferation and fibrosis and improves function in animal models of renal disease; thus, one has to wonder whether this renal protective effect of ACEIs could be mediated by Ac-SDKP. An abnormal increase in cardiac fibroblasts and extracellular matrix proteins during cardiac remodeling may be a major cause of increased myocardial stiffness and cardiac dysfunction.\(^1\)\(^2\),\(^7\),\(^1\)\(^8\) Collagen deposition in the myocardium of hypertensive rats begins within the adventitia of intramyocardial coronary arteries (perivascular fibrosis). As hypertension progresses, perivascular collagen radiates outward into neighboring intramuscular spaces, leading to established perivascular and interstitial fibrosis. ACEIs impair fibroblast proliferation, reverse excessive interstitial and perivascular fibrosis, and restore function.\(^1\) Because ACE activity has been found in various cells of the heart, including fibroblasts, and because Ac-SDKP is also found in the heart,\(^6\) a tissue ACE/ACE-SDKP system, acting as a paracrine hormone, might be involved in local regulation of myocardial Ac-SDKP levels, and thus regulation of fibroblast activity. The attractive feature of the antifibrotic effect of Ac-SDKP is that it occurs in the absence of decreased blood pressure or LV hypertrophy. The pharmacological effect of Ac-SDKP may be considered a novel mechanism whereby part of the cardioprotective effect of ACEIs in hypertension is achieved.

Acknowledgments

This study was supported by American Heart Association Grant-in-Aid 29GS978, American Heart Association Scientist Development Award 0130128N, and grant HL 298217 from the National Institutes of Health, Bethesda, Md.

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Circulation. 2001;103:3136-3141
doi: 10.1161/01.CIR.103.25.3136

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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