Prevention of myocardial fibrosis by N-acetyl-seryl-aspartyl-lysyl-proline in diabetic rats

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ABSTRACT

Ac-SDKP (N-acetyl-seryl-aspartyl-lysyl-proline) is a physiological tetrapeptide hydrolysed by ACE (angiotensin-converting enzyme). In experimental models of hypertension, Ac-SDKP has antifibrotic effects in the heart; however, the role of Ac-SDKP in diabetic cardiomyopathy is currently unknown. The aim of the present study was to evaluate the effect of Ac-SDKP on cardiac systolic and diastolic function, and interstitial and perivascular fibrosis in the heart of diabetic rats. Diabetes was induced in 55 Sprague–Dawley rats by streptozotocin injection. Control rats (n = 18) underwent only buffer injection. Out of the 55 diabetic rats, 19 were chronically treated with insulin and 13 with the ACEI (ACE inhibitor) ramipril (3 mg·kg⁻¹·day⁻¹). At 2 months after the onset of diabetes, Ac-SDKP (1 mg·kg⁻¹·day⁻¹) was administered by osmotic minipumps for 8 weeks to eight control rats, 13 diabetic rats, seven diabetic rats treated with ramipril and nine insulin-treated diabetic rats. Diabetic rats had a significant increase in blood glucose levels. Left ventricular interstitial and perivascular fibrosis, and TGF-β1 (transforming growth factor-β1) protein levels were increased in diabetic rats, but not in insulin-treated diabetic rats and ramipril-treated diabetic rats, compared with control rats. Ac-SDKP administration significantly reduced left ventricular interstitial and perivascular fibrosis in diabetic rats and in diabetic rats treated with ramipril. This was accompanied by a significant reduction in active TGF-β1 and phospho-Smad2/3 protein levels in myocardial tissue of diabetic rats. Echocardiography showed that diabetes was associated with increased end-systolic diameters, and depressed global systolic function and diastolic dysfunction, as assessed by transmitral Doppler velocity profile. These changes were completely reversed by insulin or ramipril treatment. Ac-SDKP treatment partially restored diastolic function in diabetic rats. In conclusion, Ac-SDKP administration in diabetic rats reduces left ventricular interstitial and perivascular fibrosis, active TGF-β1 and phospho-Smad2/3 levels, and improves diastolic function. Taken together, these findings suggest that, by inhibiting the TGF-β1/Smad pathway, Ac-SDKP protects against the development of diabetic cardiomyopathy.

Key words: angiotensin-converting enzyme inhibitor (ACEI), diabetic cardiomyopathy, echocardiography, fibrosis, N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), Smad, transforming growth factor-β1 (TGF-β1).

Abbreviations: A, late diastolic peak velocity; ACE, angiotensin-converting enzyme; ACEI, ACE inhibitor; Ac-SDKP, N-acetyl-seryl-aspartyl-lysyl-proline; AngII, angiotensin II; a.u., arbitrary units; BP, blood pressure; E, early diastolic peak velocity; HR, heart rate; LV, left ventricular; RAS, renin–angiotensin system; SBP, systolic BP; TGF, transforming growth factor.

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INTRODUCTION

Diabetes is a major cause of mortality and morbidity [1]. It represents not only a well known risk factor for ischaemic heart disease, but it may also directly affect cardiac structure. In fact, ‘diabetic cardiomyopathy’ indicates a clinical condition characterized by ventricular dysfunction leading to heart failure in the absence of atherosclerotic coronary heart disease and hypertension [2–4]. Molecular abnormalities underlying this condition are complex and caused by the concomitant presence of metabolic alterations such as hyperglycaemia, hyperlipidaemia and hyperinsulinaemia [5]. Finally, the central role of the activation of the RAS (renin–angiotensin system) in the development of diabetic cardiomyopathy has been shown [6].

In line with this concept, ACEIs [ACE (angiotensin-converting enzyme) inhibitors] are presently among the drugs of choice for the treatment of hypertension, heart failure and renal disease, both in diabetic and non-diabetic patients [7,8]. The effects of ACEIs cannot be explained only by their antihypertensive actions [9].

Ac-SDKP (N-acetyl-seryl-aspartyl-lysyl-proline) is an endogenous tetrapeptide hydrolysed by ACE. ACEIs increase plasma Ac-SDKP concentrations in healthy subjects [10] through the inhibition of its degradation. Ac-SDKP has antifibrotic effects in the left ventricle of AngII (angiotensin II)-treated rats [11] and in aldosterone-salt hypertensive rats [12]. In these experimental models, the antifibrotic effect of ACEIs is partially mediated by Ac-SDKP [13,14].

The role of Ac-SDKP in diabetic cardiomyopathy is presently still unknown. Of interest, this complication is characterized by an alteration of collagen and extracellular matrix synthesis [15], a possible target of Ac-SDKP [13,14].

The aim of the present study was to investigate the effect of Ac-SDKP administration on cardiac function, and interstitial and perivascular fibrosis in the heart of diabetic rats, treated without or with insulin. The effect of Ac-SDKP in diabetic rats treated with ACEIs was also evaluated.

MATERIALS AND METHODS

Animal and experimental design

Experiments were conducted in accordance with Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Experiments were performed in conscious 12-week-old male Sprague–Dawley rats (body weight, 250–275 g; Charles River). Animals were individually housed in cages in a temperature-controlled room with a 12 h/12 h light–dark cycle for the whole experimental period and were allowed to become accustomed to the experimental procedures. Rats had free access to a standard rat chow and tap water.

Diabetes was induced in Sprague–Dawley rats (n = 55) through a single intraperitoneal injection of streptozotocin (75 mg/kg of body weight; Sigma) dissolved in 0.1 mol/l citrate buffer. Sham-treated rats (n = 18) underwent only buffer injection. At 2 days after streptozotocin administration, blood glucose was measured using a glucometer (Bayer) and only rats with blood glucose levels > 13.88 mmol/l entered the experimental protocols. A group of 36 diabetic rats were kept hyperglycaemic and received only 2–4 units of insulin twice a week to prevent ketoacidosis. An ACEI, ramipril (3 mg · kg⁻¹ of body weight · day⁻¹, in drinking water), was administered to 13 diabetic rats. A group of 19 diabetic rats, 2 days after the onset of diabetes, received chronic administration of insulin (2–4 units/24 h; Limplant sustained-release insulin implants; Linshin Canada) to control glycaemia. After 2 months, the rats were anaesthetized with sodium pentobarbital (40 mg/kg of body weight, intraperitoneally) and osmotic minipumps (Alzet 2004) were implanted subcutaneously. Osmotic minipumps delivered Ac-SDKP (Primm) at the dose of 1 mg · kg⁻¹ of body weight · day⁻¹ for 2 months to eight control rats, 13 diabetic rats, seven diabetic rats treated with ramipril and nine insulin-treated diabetic rats. In the corresponding sham-treated rats (ten control, ten diabetic, six diabetic rats treated with ramipril and ten insulin-treated diabetic rats), osmotic minipumps delivered saline solution.

SBP [systolic BP (blood pressure); in mmHg] was measured twice a month, and was assessed using the tail cuff method (average of six recordings; BP recorder; Ugo Basile Instruments) by a single investigator who was unaware of the specific treatment. Body weight (in g) was measured twice a week, and blood glucose levels (in mmol/l) were measured once a week during the whole experimental period. After 8 weeks, echocardiography was performed as described previously [16,17]. Briefly, the rats were anaesthetized with sodium pentobarbital, placed in the prone position and scanned via a 12-MHz transducer connected to an Esaote Sonosite Titan echocardiograph. Two-dimensional guided M-mode images of the left ventricle were obtained in the parasternal short-axis view at the level of the papillary muscles and were digitally stored for subsequent analysis. Doppler-derived mitral inflow velocities were obtained in the apical four-chamber view at the tip of the valve leaflets. LV (left ventricular) end-diastolic and endsystolic wall thicknesses and chamber dimensions were measured by a single operator, according to the American Society of Echocardiography recommendations. All echocardiographically derived measures were obtained by averaging the readings of three consecutive beats.

At the end of the experiments, the rats were killed by an overdose of sodium pentobarbital. Blood samples were
collected to measure Ac-SDKP by immunoassay (Inalco). Hearts were immediately excised and weighted. The apex of the heart was cut, frozen in liquid nitrogen and stored at −80 °C until protein extraction. The heart, sectioned into three transverse slices from the apex to the base, was fixed with 10 % formalin, embedded in paraffin and used for light microscopic examination and morphometric analysis of interstitial and perivascular collagen.

**Histological and morphometric analysis**

Changes in cardiac structure were assessed in at least five consecutive sections (4 μm thickness, stained with haematoxylin/eosin) of the three transverse slices from each group studied, using a light microscope under normal light.

For all rats, a section (4 μm) of the left ventricle equatorial slice, representative of the whole left ventricle, was chosen for morphometric analysis. The sections were deparaffinized, rehydrated and stained with collagen-specific Sirius Red. The slides were examined with a Leica microscope using normal light. A computerized digital camera (Olympus Camedia 5050) was used to capture 5 megapixel (24-bit colour depth) images that were then analysed for collagen volume fraction in interstitial and perivascular cardiac areas with computerized imaging software (MetaMorph® 6.2; Universal Imaging Corp.). Image analysis was performed by a pathologist blinded to the experimental source of the samples.

The total collagen volume fraction was measured on pictures of the whole left ventricle equatorial slice (×100 magnification) and was automatically calculated as the ratio between the red-stained interstitial area and the total area of the whole heart section.

For analysis of the perivascular collagen volume fraction, all vessels from the equatorial slice were traced and measured semi-automatically (×200 magnification). The collagen immediately surrounding each intramyocardial vessels was considered to represent perivascular collagen deposition and was expressed as the ratio between the perivascular collagen area and luminal media area.

**Collagen analysis by polarized light microscopy**

For each group, the Sirius-Red-stained tissue sections of the left ventricle equatorial slice were also analysed using light microscopy under polarized light to evaluate the different type of collagen content occupying the interstitial and perivascular areas. In fact, analysis using polarized light microscopy allowed the visualization of collagen fibres with different colours [18]. Collagen type I appears yellow/orange, whereas collagen type III appears green.

For each rat, five randomly selected microscopic fields of both interstitial and perivascular cardiac areas were analysed with a light microscope using a ×10 objective (Leitz). The images were captured by a computerized digital camera (Olympus Camedia 5050) using SPOT (Diagnostic Instruments), and were analysed for different types of collagen content by two pathologists blinded to the experimental source of the samples.

**Measurement of TGF-β1 (transforming growth factor-β1) protein**

Heart tissue (apical portion) was homogenized in 20 mmol/l Tris/HCl (pH 7.4), 150 mmol/l NaCl and 1 % (v/v) Triton X-100 containing protease inhibitors. Samples were centrifuged at 15 700 g for 30 min, and the supernatants were used for the assay. TGF-β1 levels were quantified using a commercially available ELISA (Promega), according to the manufacturer’s instructions. This assay measures biologically active TGF-β1 in tissue extracts. For each experiment, a TGF-β1 standard curve was constructed using various concentrations of recombinant human TGF-β1, and a curve-fitting software program (Curve Expert 1.3; http://curveexpert.webhop.net/) was used to quantify TGF-β1 protein concentrations in hearts. Results were corrected for heart protein content, measured using the Bradford assay (Bio-Rad Laboratories), and are expressed in pg/mg of protein.

**Western blot analysis**

Heart protein samples were diluted 1:2 with a solution containing 60 % (v/v) glycerol, 30 % (v/v) 2-mercaptoethanol and 10 % (w/v) Bromophenol Blue. A portion of the sample (75 μg of total protein) was loaded on to a 9 % (w/v) polyacrylamide gel and was separated by SDS/PAGE. The proteins were then blotted onto nitrocellulose membranes (Schleicher and Shull) at 120 mA overnight. The blots were probed with anti-Smad2/3 (Transduction Laboratories), anti-(phospho-Smad2/3) (Transduction Laboratories), anti-(phospho-Smad2/3) (Santa Cruz Biotechnology) or anti-(β-catenin) (Transduction Laboratories) as the primary antibodies. Peroxidase-conjugated anti-(mouse IgG) (for Smad2/3 and β-catenin) (Jackson Laboratories) or biotin-conjugated anti-(goat IgG) (Jackson Laboratories) antibodies and peroxidase-conjugated streptavidin (for phospho-Smad2/3) (Jackson Laboratories) were used as the secondary reagents. The anti-Smad2/3 antibody detects both the phosphorylated and non-phosphorylated Smad2/3 protein, whereas the anti-(phospho-Smad2/3) antibody detects only the phosphorylated protein. The bands were visualized with an enhanced chemiluminescence Western blotting detection system (PerkinElmer Life Science), and the signal was quantified by densitometry using NIH IMAGE 1.59 software. Protein loading was also confirmed by staining membranes with Ponceau solution (results not shown). Results were collected from two different blots, and are expressed as arbitrary units (a.u.) of the Smad2-3/β-catenin and phospho-Smad2-3/β-catenin ratio.
At the end of the experimental period, blood glucose levels were significantly increased in diabetic rats, whereas insulin treatment was effective in maintaining blood glucose levels similar to control levels (Table 1). Ac-SDKP did not modify blood glucose levels (Table 1) in control, diabetic, ramipril-treated diabetic and insulin-treated diabetes groups of rats (control, control + Ac-SDKP, diabetes, Ac-SDKP, ramipril-treated diabetes, ramipril, insulin-treated diabetes, insulin-treated diabetes + Ac-SDKP, ramipril, insulin-treated diabetes + Ac-SDKP) for body weight, SBP, echocardiographic parameters, blood glucose, insulin and phospho-Smad2/3, cardiac TGF-β1, fibrosis and perivascular collagen content.

Table 1  Effects of experimental diabetes and chronic treatment with ramipril, Ac-SDKP and insulin at the end of the experimental period on blood glucose levels, SBP, plasma Ac-SDKP levels, body weight and heart weight/body weight

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 6)</th>
<th>Control + Ac-SDKP (n = 6)</th>
<th>Diabetes (n = 8)</th>
<th>Diabetes + Ac-SDKP (n = 6)</th>
<th>Diabetes + ramipril + Ac-SDKP (n = 6)</th>
<th>Diabetes + ramipril + Ac-SDKP, insulin-treated diabetes (n = 7)</th>
<th>Insulin-treated diabetes + Ac-SDKP (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mmol/l)</td>
<td>4.89 ± 0.26</td>
<td>5.76 ± 0.49</td>
<td>21.52 ± 1.24*</td>
<td>21.56 ± 1.82*</td>
<td>22.62 ± 1.91*</td>
<td>21.16 ± 1.87*</td>
<td>6.82 ± 1.42</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>117.5 ± 3.9</td>
<td>121.1 ± 8.7</td>
<td>128.2 ± 5.1</td>
<td>134.1 ± 5.6</td>
<td>132.5 ± 4.8</td>
<td>134.0 ± 5.1</td>
<td>127.3 ± 7.6</td>
</tr>
<tr>
<td>Ac-SDKP (nmol/l)</td>
<td>2.38 ± 0.07</td>
<td>5.64 ± 1.24*</td>
<td>1.71 ± 0.14</td>
<td>3.94 ± 0.30§</td>
<td>2.98 ± 0.39</td>
<td>6.26 ± 1.38§</td>
<td>2.15 ± 0.24</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>575 ± 20.1</td>
<td>593 ± 13.9</td>
<td>421 ± 9.3</td>
<td>428 ± 12.5</td>
<td>416 ± 8.7*</td>
<td>430 ± 11.2*</td>
<td>566 ± 21.8</td>
</tr>
<tr>
<td>Heart weight/body weight (mg/g)</td>
<td>2.45 ± 0.14</td>
<td>2.26 ± 0.15</td>
<td>2.67 ± 0.09</td>
<td>2.87 ± 0.12</td>
<td>2.99 ± 0.16</td>
<td>2.87 ± 0.14</td>
<td>2.44 ± 0.12</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. *P < 0.01 compared with the control, control + Ac-SDKP, insulin-treated diabetes, insulin-treated diabetes + Ac-SDKP groups; §P < 0.05 compared with the corresponding sham-treated group.
Effects of Ac-SDKP on myocardial fibrosis

As shown in Figure 2, cardiac interstitial collagen content was significantly increased in diabetic rats, whereas in ramipril-treated diabetic rats and insulin-treated diabetic rats it was similar to the control group. Chronic Ac-SDKP administration in diabetic rats and ramipril-treated diabetic rats significantly reduced cardiac collagen content, despite the high blood glucose levels. Collagen analysis using polarized light microscopy identified the presence of type I collagen fibres (yellow/orange staining) and type III collagen fibres (green staining).

Similar results were obtained for cardiac perivascular fibrosis, as shown in Figure 3. A significant increase in cardiac perivascular fibrosis, characterized mainly by type I collagen fibres, was observed in diabetic rats. In diabetic rats and ramipril-treated diabetic rats, Ac-SDKP administration significantly reduced perivascular fibrosis.

Effects of Ac-SDKP on the TGF-β/Smad pathway

Diabetic rats had a significant increase in active TGF-β1 expression in myocardial tissue compared with control rats (Figure 4A). In diabetic rats treated with Ac-SDKP, an increase in TGF-β1 levels was not observed, resulting in similar results compared with control rats. No differences in cardiac TGF-β1 levels in ramipril-treated diabetic rats, insulin-treated diabetic rats and the corresponding groups treated with Ac-SDKP were observed.

We examined whether Ac-SDKP antagonizes TGF-β signalling, leading to fibrosis through the modulation of the Smad pathway. Western blot analysis showed an increase, although not statistically significant, in Smad2/3 expression in diabetic rats and ramipril-treated diabetic rats. Ac-SDKP reduced Smad2/3 expression only in ramipril-treated diabetic rats (control, 235.3 ± 44.6 a.u.; control + Ac-SDKP, 263.2 ± 45.5 a.u.; diabetes, 347.8 ± 37.5 a.u.; diabetes + Ac-SDKP, 350.0 ± 124.7 a.u.; ramipril-treated diabetes, 478.6 ± 171.1 a.u.; ramipril-treated diabetes + Ac-SDKP, 199.6 ± 55.8 a.u. (P < 0.05 compared with ramipril-treated diabetes); insulin-treated diabetes, 257.1 ± 56.6 a.u.; and insulin-treated diabetes + Ac-SDKP, 416.0 ± 21.0 a.u.). A significant increase in phosho-Smad2/3 was observed only in diabetic rats compared with control rats (Figure 4). In diabetic rats treated with Ac-SDKP, an increase in phosho-Smad2/3 levels was not observed, resulting in similar findings compared with control rats. No differences in cardiac
Table 2  Effects of experimental diabetes and chronic treatment with ramipril, Ac-SDKP and insulin on echocardiographic parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 6)</th>
<th>Diabes − Ac-SDKP (n = 8)</th>
<th>Diabes + Ac-SDKP (n = 6)</th>
<th>Diabetes + ramipril − Ac-SDKP (n = 6)</th>
<th>Diabetes + ramipril + Ac-SDKP (n = 6)</th>
<th>Insulin-treated diabetes + Ac-SDKP (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDD (mm)</td>
<td>62.58 ± 2.08</td>
<td>60.83 ± 3.17</td>
<td>59.50 ± 1.35</td>
<td>59.20 ± 1.71</td>
<td>61.17 ± 0.71</td>
<td>59.05 ± 0.83</td>
</tr>
<tr>
<td>ESD (mm)</td>
<td>26.18 ± 0.87</td>
<td>28.83 ± 3.17</td>
<td>31.22 ± 1.71</td>
<td>30.13 ± 1.41†</td>
<td>25.72 ± 1.09†</td>
<td>24.43 ± 0.91†</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>20.50 ± 0.22</td>
<td>19.50 ± 0.50</td>
<td>19.56 ± 0.25</td>
<td>20.33 ± 0.28</td>
<td>20.44 ± 0.27</td>
<td>20.57 ± 0.20</td>
</tr>
<tr>
<td>PWD (mm)</td>
<td>20.92 ± 0.39</td>
<td>20.50 ± 0.50</td>
<td>20.17 ± 0.33</td>
<td>19.87 ± 0.25</td>
<td>20.94 ± 0.36</td>
<td>21.00 ± 0.15</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>386 ± 13</td>
<td>328 ± 5</td>
<td>374 ± 25</td>
<td>358 ± 18</td>
<td>320 ± 33</td>
<td>327 ± 30</td>
</tr>
<tr>
<td>E (cm/s)</td>
<td>107 ± 7</td>
<td>73 ± 1</td>
<td>85 ± 10</td>
<td>89 ± 13</td>
<td>82 ± 7</td>
<td>80 ± 2</td>
</tr>
<tr>
<td>A (cm/s)</td>
<td>54 ± 8</td>
<td>72 ± 14</td>
<td>25 ± 4*</td>
<td>67 ± 4†</td>
<td>40 ± 7</td>
<td>39 ± 4†</td>
</tr>
</tbody>
</table>

Although a much more precise method of assessing diastolic function is represented by the use of in vivo pressure-volume relationships, the echo-Doppler technique is widely used in the clinical as well as in the experimental setting. At comparable HR and loading conditions, an altered transmitral flow velocity profile may suggest the presence of diastolic dysfunction. In the experiments in the present study, HR was comparable in the different groups, and we did not observe any significant change in LV end-diastolic diameter. Therefore the altered Doppler filling pattern suggests diastolic dysfunction, which was present in diabetic animals, abolished in insulin-treated diabetic rats and partially blunted in Ac-SDKP-treated diabetic animals.

Our present results demonstrate that chronic Ac-SDKP administration in diabetic rats reduces myocardial fibrosis, evaluated by qualitative morphology, both at the interstitial and perivascular levels. No significant differences in myocardial fibrosis were observed in insulin-treated diabetic rats, in which myocardial fibrosis was not increased, and control rats. On the contrary, in ramipril-treated diabetic rats, even if myocardial fibrosis was not increased with respect to controls, Ac-SDKP administration resulted in a further reduction in fibrosis, strongly suggesting an additive antifibrotic effect of Ac-SDKP in diabetic cardiomyopathy. In the hearts of diabetic rats, a significant increase was measured in the main pro-fibrotic cytokine TGF-β1, in its active form, which, on the contrary, did not increase in diabetic rats treated with Ac-SDKP and in diabetic rats treated with ramipril. The increase in TGF-β expression in myocardial tissue of diabetic rats is not surprising. In fact, clinical and experimental evidence has demonstrated an activation of cardiac RAS in diabetes [24,25], and AngII is a potent stimulus for TGF-β expression. It is known that the TGF-β1/Smad pathway induces the production of extracellular matrix leading to tissue fibrosis [26]. TGF-β1 was suggested
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Figure 2 Effect of Ac-SDKP administration on LV interstitial fibrosis

(A) Representative photomicrographs of the LV interstitial collagen deposition in control (a), diabetic (c), ramipril-treated diabetic (e) and insulin-treated diabetic (g) rats, and in the corresponding Ac-SDKP-treated groups (b, d, f and h). For each group, Sirius Red staining (left-hand panels) and Sirius-Red-stained images under polarized light (right-hand panels) are shown. Original magnification, ×100. Picrosirius staining shows an increase in cardiac interstitial collagen content in diabetic rats (c) compared with control (a), ramipril-treated diabetic (e) and insulin-treated diabetic (g) rats. Ac-SDKP administration reduced interstitial collagen content in diabetic rats (d). The polarized light image shows an increase in both types of collagen fibres (type I collagen in yellow/orange, and type III collagen in green) only in diabetic rats (c). (B) Quantification of interstitial fibrosis in the different groups of rats. Values are means ± S.E.M. *P < 0.05 and §P < 0.01. Control, n = 10; control + Ac-SDKP, n = 7; diabetes, n = 10; diabetes + Ac-SDKP, n = 11; ramipril-treated diabetes, n = 6; ramipril-treated diabetes + Ac-SDKP, n = 6; insulin-treated diabetes, n = 6; and insulin-treated diabetes + Ac-SDKP, n = 6.

as a major player in cardiac fibrosis by activating the intracellular pathways linking TGF-β1 signals to plasma membrane serine/threonine kinase receptors with the final activation of cytoplasmic effectors, such as Smad proteins. Phosphorylation of Smad2 and its subsequent translocation to the nucleus are the critical steps in cell signalling through this pathway [26,27]. The effects of TGF-β1-induced Smad2 activation on cell
growth depend on the types of cell involved. In the heart, TGF-β1 stimulates the growth of cardiac fibroblasts, thus increasing fibrosis [28].

In the heart of diabetic rats, we have observed a significant increase in phospho-Smad2/3 levels, indicating an activation of the Smad pathway. This could explain why cardiac fibrosis is not increased in diabetic rats treated with Ac-SDKP, and is in line with other experimental findings demonstrating that Ac-SDKP antagonizes TGF-β signalling through the modulation of Smad pathway [14,27,28].

The pathogenesis of diabetic cardiomyopathy is complex, and many factors participate in the development and progression of this condition, such as hyperglycaemia, hyperlipidaemia and the activation of the RAS [3,5,6]. In streptozotocin-induced diabetic rats, it has been shown that blocking the RAS by ACEIs is able to reduce cardiac dysfunction [29] and perivascular fibrosis [30]. In addition to the inhibition of the conversion of AngI (angiotensin I) into AngII and kinin hydrolysis, blocking the hydrolysis of Ac-SDKP has been described as a potential mechanism involved in the cardioprotective effects of ACEIs [13,14].

From a pharmacological point of view, Ac-SDKP, by acting directly on diabetes-specific cardiac damage such as tissue fibrosis, could perspective represent a therapeutic tool complementary to classic cardioprotective treatment based on antihypertensive drugs.

In our experimental conditions, we have observed an increase in LV collagen content and perivascular fibrosis in diabetic rats that was not present in insulin-treated diabetic animals. This is not surprising because, during the whole experimental period, insulin treatment was effective in maintaining blood glucose levels near control values, and it is well known that hyperglycaemia represents an important stimulus for myocardial fibrosis and for the activation of the RAS [3,5]. Conversely, in clinical practice, an adequate blood glucose control in diabetic patients is still a very difficult target to reach [31], and the uncontrolled increases in glycaemia often result in worsening of the fibrotic process linked to diabetic cardiomyopathy. In addition, insulin and, in particular, hyperinsulinism can promote cardiac hypertrophy and fibrosis [32,33]. However, in our experimental conditions, the aim of insulin administration was to normalize glucose metabolism and it did not induce hyperinsulinism. This is confirmed by the monitoring of glycaemia; in insulin-treated rats, no hypoglycaemic events (and therefore no hyperinsulinaemia) were observed during the entire duration of the experimental procedure.

Taken together, the findings of our present study strongly support the search for new therapeutic approaches focused specifically on the prevention/remission of diabetic cardiomyopathy. Ac-SDKP, in particular, appears to be, along with hypoglycaemic treatment and ACE inhibition, a powerful tool to counteract the development of fibrosis in diabetic cardiomyopathy.

In summary, the results of our present study demonstrate that Ac-SDKP reduces cardiac fibrosis and diastolic dysfunction, as assessed by transmitral flow velocity profile, in diabetic rats, indicating a potential protective role of Ac-SDKP in diabetic cardiomyopathy.

**Perspectives**

Cardiac fibrosis is an important determinant of the increase in diastolic LV stiffness in diabetic patients with reduced LV ejection fraction [34]. Present therapy is based on strict glycaemic control and, when appropriate, on antihypertensive treatment and lipid-lowering drugs. These pharmacological approaches ameliorate the prognosis of diabetic cardiomyopathy by reducing...
risk factors and improving the ‘environment’, but do not directly affect the underlying fibrosis. A direct antifibrotic agent is still far from being identified and tested in the clinical arena. In the present study, we have shown how early treatment with Ac-SDKP prevents cardiac fibrosis in an experimental model of Type 1 diabetes without affecting other clinical parameters. Our results support the testing of Ac-SDKP in clinical trials with the final aim of clarifying whether the addition of a direct antifibrotic intervention may further strengthen the current therapy for diabetic cardiomyopathy.

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