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***N*-acetyl-seryl-aspartyl-lysyl-proline treatment protects heart against excessive myocardial injury and heart failure in mice**

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Abstract

Myocardial infarction (MI) in mice results in cardiac rupture at 4–7 days after MI, whereas cardiac fibrosis and dysfunction occur later. *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) has anti-inflammatory, anti-fibrotic, and pro-angiogenic properties. We hypothesized that Ac-SDKP reduces cardiac rupture and adverse cardiac remodeling, and improves function by promoting angiogenesis and inhibiting detrimental reactive fibrosis and inflammation after MI. C57BL/6J mice were subjected to MI and treated with Ac-SDKP (1.6 mg/kg per day) for 1 or 5 weeks. We analyzed (1) intercellular adhesion molecule-1 (ICAM-1) expression; (2) inflammatory cell infiltration and angiogenesis; (3) gelatinolytic activity; (4) incidence of cardiac rupture; (5) p53,

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Conflict of interest

The authors declare that there is no conflict of interest associated with this work.

the endoplasmic reticulum stress marker CCAAT/enhancer binding protein homology protein (CHOP), and cardiomyocyte apoptosis; (6) sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2) expression; (7) interstitial collagen fraction and capillary density; and (8) cardiac remodeling and function. Acutely, Ac-SDKP reduced cardiac rupture, decreased ICAM-1 expression and the number of infiltrating macrophages, decreased gelatinolytic activity, p53 expression, and myocyte apoptosis, but increased capillary density in the infarction border. Chronically, Ac-SDKP improved cardiac structures and function, reduced CHOP expression and interstitial collagen fraction, and preserved myocardium SERCA2 expression. Thus, Ac-SDKP decreased cardiac rupture, ameliorated adverse cardiac remodeling, and improved cardiac function after MI, likely through preserved SERCA2 expression and inhibition of endoplasmic reticulum stress.

RÉSUMÉ:

L'infarctus du myocarde (IM) chez la souris entraîne des ruptures cardiaques de 4 à 7 jours après l'IM, mais la fibrose et le dysfonctionnement surviennent plus tard. La N-acétyl-séryl-aspartyl-lysyl-proline (Ac-SDKP) dispose de propriétés anti-inflammatoires, anti-fibrotiques et angiogéniques. Nous avons formulé l'hypothèse selon laquelle l'Ac-SDKP permet de réduire la fréquence des ruptures, d'atténuer le remodelage néfaste et d'améliorer le fonctionnement du cœur en favorisant l'angiogenèse et en inhibant la fibrose et l'inflammation nuisibles en réaction à l'IM. Nous avons créé un IM chez des souris C57BL/6J auxquelles nous avons ensuite administré de l'Ac-SDKP (1,6 mg/kg par jour) pendant 1 ou 5 semaines. Nous avons étudié les phénomènes suivants : (1) expression de la molécule 1 d'adhérence intercellulaire (ICAM-1); (2) infiltration de cellules inflammatoires et angiogenèse; (3) activité gélatinolytique; (4) fréquence des ruptures cardiaques; (5) p53, protéine CHOP (pour « CCAAT/enhancer binding protein homology protein ») comme marqueur du stress imposé au réticulum endoplasmique et apoptose des cardiomyocytes; (6) expression de la pompe Ca^{2+} ATPase dans le réticulum sarcoplasmique (SERCA2); (7) fraction de collagène et densité des capillaires interstitiels; et (8) fonctionnement et remodelage du cœur. À court terme, l'Ac-SDKP permettait de réduire la fréquence des ruptures cardiaques, de diminuer l'expression de l'ICAM-1 et le nombre de macrophages infiltrés, de diminuer l'activité gélatinolytique et l'expression de p53, ainsi que d'atténuer l'apoptose des myocytes, mais le produit permettait d'augmenter la densité des capillaires en bordure de l'infarctus. À long terme, l'Ac-SDKP permettait d'améliorer le fonctionnement du cœur et ses structures, de diminuer l'expression de la protéine CHOP et la fraction de collagène interstitiel, ainsi que de préserver l'expression de la SERCA2 dans le myocarde. Par conséquent, l'Ac-SDKP permettait de réduire la fréquence des ruptures cardiaques, d'atténuer le remodelage cardiaque néfaste, ainsi que d'améliorer le fonctionnement du cœur après un IM, probablement par l'intermédiaire de la préservation de l'expression de la SERCA2 et de l'inhibition du stress imposé au réticulum endoplasmique. [Traduit par la Rédaction]

Keywords

Ac-SDKP; mice; myocardial infarction; SERCA2; endoplasmic reticulum stress; cardiac function

Mots-clés

Ac-SDKP; souris; infarctus du myocarde; SERCA2; stress imposé au réticulum endoplasmique; fonction cardiaque

Introduction

Myocardial infarction (MI) is a leading cause of human death. MI is associated with high rates of acute death caused by ventricular wall rupture, arrhythmias, or cardiogenic shock, and with long-term complications such as heart failure (HF) (Gao et al. 2012; Pouleur et al. 2010). Incidences of myocardial rupture were initially reported to range from 0.3% to 1.1% (Brathwaite et al. 1990; Lopez-Sendon et al. 2010; Shamshad et al. 2010). However, some studies report that the percentage of autopsy-proven rupture in patients who died of acute MI is actually higher, ranging from 12.4% (Gao et al. 2012; Pouleur et al. 2010) to almost 25% (Shamshad et al. 2010). Others report even higher cardiac rupture incidences, reaching 36%–65% on autopsied chest trauma patients (Co et al. 2011). This high percentage of autopsy-confirmed rupture deaths suggests a lack of effective strategies for preventing post-MI rupture. Furthermore, both clinical and experimental studies provide strong evidence that maladaptive late-stage cardiac remodeling mainly exhibits as cardiomyocyte hypertrophy and reactive interstitial fibrosis, causing left ventricular (LV) dysfunction and HF (Jessup and Brozena 2003; Sutton and Sharpe 2000). Excessive inflammatory response, insufficient myocardial capillary density, and cardiomyocyte apoptosis after MI were also identified as critical events in both wall rupture (Kameda et al. 2006; Matsui et al. 2011; Tao et al. 2004) and LV adverse remodeling and dysfunction (Damas et al. 2000; Prech et al. 2006; Sam et al. 2000). Cardiomyocyte apoptosis and cardiac dysfunction were reportedly mediated by reduced sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA2), resulting in an impaired cardiomyocyte Ca^{2+} homeostasis, and increased endoplasmic reticulum (ER) stress in HF. ER stress directly activates endothelial cells to express adhesion molecules for inflammatory cell infiltration (Abdullaev et al. 2008; Kassan et al. 2012; Kolattukudy and Niu 2012), activates cardiac fibroblasts to produce collagen (Humeres et al. 2014), and activates cardiomyocytes to induce apoptosis and abnormal contractility/relaxation and calcium cycling (Dong et al. 2013; Mao et al. 2007; Neuber et al. 2014; Okada et al. 2004; Zhang and Ren 2011). Hypertension and MI are potent inducers of ER stress, resulting in cardiac injury and dysfunction (Kassan et al. 2012; Mitra et al. 2013; Rivard et al. 2007; Toko et al. 2010).

N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is an endogenous tetrapeptide found in the circulation and various organs, including the heart (Pradelles et al. 1990,1991). Ac-SDKP is endogenously produced from the 43-amino acid thymosin β 4 (T β 4) by 2 successive enzymes, meprin α and prolyl oligopeptidase (Cavasin et al. 2007; Cavasin et al. 2004; Kumar et al. 2016; Myohanen et al. 2011).

Ac-SDKP is hydrolyzed mainly by angiotensin-converting enzyme (ACE), and its circulating levels were found to increase more than 5-fold in patients treated with ACE inhibitors (Azizi et al. 1996). Using animal models of hypertension and MI, studies from our

group have shown that Ac-SDKP exerts anti-inflammatory and anti-fibrotic effects in the heart (Peng et al. 2007; Rhaleb et al. 2001b; Yang et al. 2004). However, it remains to be elucidated whether treatment with Ac-SDKP can improve cardiac function. Moreover, Ac-SDKP also promotes angiogenesis following either MI (Wang et al. 2004) or limb ischemia (Waeckel et al. 2006). Therefore, we hypothesized that Ac-SDKP prevents cardiac rupture post-MI, and chronically ameliorates maladaptive LV remodeling, thereby improving cardiac function, in part, through its anti-inflammatory and pro-angiogenic actions.

The adult mouse infarct model replicates the cardiac response of human patients to an ischemic injury (Camacho et al. 2016). Indeed, this experimental model exhibits both clinical and pathological features of post-MI changes found in human heart, such as cardiac dysfunction and cardiac rupture (Gao et al. 2012). Using C57BL/6J mice, we examined whether Ac-SDKP (1) reduces the incidence of cardiac rupture during acute MI, (2) reduces cardiac fibrosis and improves cardiac function during the chronic phase of MI, and (3) inhibits ER stress and rescues SERCA2.

Materials and methods

Animals

All animal procedures were reviewed and approved by the Henry Ford Hospital Institutional Animal Care and Use Committee, and the investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Twelve-week-old male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were studied.

Experimental design

Ac-SDKP (1.6 mg/kg per day) infused intraperitoneally (i.p.) by ALZET osmotic pump in sham-operated mice has no effect on cardiac geometry and function (H. Peng, J. Xu, X.-P. Yang, K.M. Kassem, I.A. Rhaleb, E. Peterson, and N.-E. Rhaleb; unpublished observation). In the present study, we selected the dose of 1.6 mg/kg per day based on our previous studies and reported equivalent surface area dosing conversion factors (Freireich et al. 1966; Liu et al. 2009; Nakagawa et al. 2010; Peng et al. 2005; Rasoul et al. 2004; Rhaleb et al. 2001b, 2011). Animals were divided into 3 groups: (1) sham, (2) MI + vehicle, and (3) MI + Ac-SDKP.

To examine the effect of Ac-SDKP on MI-induced cardiac rupture (protocol 1, Fig. 1) and on cardiac remodeling and function in the late phase of MI (protocol 2, Fig. 1), mice were treated with Ac-SDKP at 1.6 mg/kg per day (Genscript, Piscataway, New Jersey, USA) i.p. by ALZET osmotic minipump (Durect, Cupertino, California, USA) for 2 or 5 weeks, respectively. In protocol 2, we implanted the minipump 7 days before MI to ensure that Ac-SDKP reached steady high circulating levels by the time of MI, as the half-life of Ac-SDKP in the circulation is 4.5 min (Ezan et al. 1994). Note that cardiac rupture occurs as early as 2 days after MI (Cavasin 2006; Cavasin et al. 2000). In protocol 2, osmotic minipumps filled with Ac-SDKP solution were implanted immediately after MI surgery.

At week 1 after MI surgery (protocol 1), mice were assessed for the following: (1) cardiac remodeling and function by echocardiography; (2) changes in the number of infiltrating neutrophils and macrophages, gelatinolytic activity, and capillary density in the infarcted border regions; (3) apoptotic myocytes; and (4) ICAM-1 and p53 expression in the myocardium. Five weeks after MI surgery (protocol 2), cardiac remodeling/function and interstitial collagen and capillary density in the LVs were evaluated. Due to the small size of the mouse left ventricles, some mice were used only for 1 or 2 analytical parameter(s), as indicated in the Results and in the figure legends.

Implantation of osmotic minipump containing Ac-SDKP

ALZET osmotic minipumps were implanted (i.p.) to deliver Ac-SDKP. Briefly, mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the abdominal area was shaved and disinfected with 70% alcohol and betadine. A small incision (<1 cm) was made through the skin and abdominal muscle, and the osmotic minipump was inserted into the intraperitoneal cavity. The muscle was closed with 3–0 resorbable suture, and the skin incision site was closed with wound clips (MikRon Precision, Inc., Gardena, California, USA). Betadine mixed with collodion was applied topically to cover the wound.

Induction of acute MI

MI was surgically induced by ligating the left anterior descending coronary artery, as described previously (Yang et al. 1999). Briefly, mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), intubated, and ventilated with room air using a positive-pressure respirator. A left thoracotomy was performed via the fourth intercostal space. The left anterior descending coronary artery was ligated with a 8–0 silk suture near its origin between the pulmonary outflow tract and the edge of the left atrium. Acute myocardial ischemia was deemed successful when the anterior wall of the left ventricle became cyanotic. The lungs were then inflated by increasing positive end-expiratory pressure, and the thorax was closed. Sham-operated mice were prepared in the same manner but did not undergo coronary artery ligation. All animals resumed spontaneous respiration and recovered from anesthesia. Mice were then housed in a temperature- and humidity-controlled environment with ad libitum access to food and water.

Identification of cardiac rupture

Necropsy was performed on each animal that died after the MI. The presence of a large blood clot in the chest cavity and a perforation in the infarct border zone area were considered to indicate rupture.

Cardiac remodeling and function by echocardiography

Cardiac geometry and function were determined in non-anesthetized mice after MI. Diastolic left ventricular dimensions (LVDd) and areas (LVAd), diastolic posterior wall thickness (PWTd), LV ejection fraction (EF), and shortening fraction (SF) were measured using a Doppler echocardiograph with a 15 MHz linear transducer (Acuson c256; Siemens, Mountain View, California, USA), as described previously (Yang et al. 1999).

Plasma Ac-SDKP levels

At the end of each experimental protocol, mice were euthanized by an overdose of sodium pentobarbital (100 mg/kg, i.p.). Blood was withdrawn via the vena cava with a 1 mL syringe coated with heparin and containing captopril (final concentration 10^{-5} M), and centrifuged at 1600g for 20 min at 4 °C. Plasma was collected and stored at -20 °C. Plasma Ac-SDKP concentrations at 1 or 5 weeks after MI were quantified using an Ac-SDKP enzyme immunoassay kit (SPI-BIO, Montigny-le-Bretonneux, France) and expressed in nanomoles per litre, as previously described (Azizi et al. 1996; Peng et al. 2007; Peng et al. 2005; Rhaleb et al. 2001b).

Histopathological analysis

At the end of the experiments (1 or 5 weeks), mice were weighed and euthanized with sodium pentobarbital (100 mg/kg, i.p.). The heart was stopped at diastole by intraventricular injection of 15% KCl, and rapidly excised and weighed. The left ventricle, including the septum, was sectioned transversely into 3 slices from apex to base, and the slices were rapidly frozen in isopentane precooled in liquid nitrogen, and then stored at -80 °C until use. Cryosections of left ventricle (6 µm) were used for histological studies. For the histology/morphology studies listed below, the images of the sections were captured using a microscope (IX81; Olympus American, Melville, New York, USA) equipped with a digital camera (DP70; Olympus American) and quantified with Microsuite Biological imaging software (Olympus).

Assessment of infarct size—A 6 µm LV cryosection was cut from each slice and stained with Masson's trichrome. The images of the whole LV sections were captured at low magnification and processed with Microsuite Biological imaging software. The endocardial and epicardial circumferences, as well as the length of the scar, were measured for each slice. The ratio of scar length to ventricular circumference of the endocardium and epicardium for each of the 3 slices was determined, and the 3 ratios were averaged and expressed as a percentage to define infarct size (Liu et al. 1997a). Mice with infarct size < 20% were excluded from the analysis.

Immunohistochemistry—LV cryosections were fixed in cold acetone for 10 min and rinsed in phosphate-buffered saline (PBS) for 5 min. Sections were incubated in 3% hydrogen peroxide in distilled water to quench endogenous peroxidase activity and rinsed in PBS. Sections were then pre-incubated with blocking serum (2% normal serum) for 30 min, then incubated at 4 °C overnight with the following primary antibodies: (1) rat anti-mouse CD68 (a macrophage marker, 1:200; AbD Serotec, Raleigh, North Carolina, USA); (2) rat anti-mouse Ly-6B.2 alloantigen (a neutrophil marker, 1:200; AbD Serotec, Raleigh, North Carolina, USA); and (3) rabbit polyclonal antibody against CD31 (a marker for endothelial cells, 1:200; Abcam, Cambridge, Massachusetts, USA). Each section was washed 3 times in PBS and assayed with a Vectastain ABC kit using 3-amino-9-ethylcarbazole as substrate (Vector Laboratories, Burlingame, California, USA). The sections were counterstained with hematoxylin to show the nuclei in blue. The images of the sections were captured at high magnification (×400). Positive staining was recognized by the reddish-brown color. The number of positive cells or capillaries was counted with Microsuite Biological imaging

software (Olympus) and expressed as the number of cells or vessels per square millimetre of myocardium, respectively.

In situ zymography—Gelatinolytic activity in the left ventricle was determined in cryosections using in situ zymography (Gkantidis et al. 2012). Briefly, DQ-gelatin (Molecular Probes, Eugene, Oregon, USA) was dissolved in the concentration of 1 mg/mL in distilled water and then diluted 1:10 in 1% (w/v) low-gelling-temperature agarose (Sigma, St. Louis, Missouri, USA) in PBS. Subsequently, 25 µL of this mixture was placed on air-dried LV sections and incubated for 5 h at room temperature after the placement of a 22 mm × 22 mm coverslip. As a control, autofluorescence in sections was measured by incubating sections in an agarose-containing medium lacking DQ-gelatin. Specific in situ zymography was tested by preincubating the sections with 20 mM ethylenediaminetetraacetic acid in PBS at room temperature for 1 h, and then incubating the sections with incubation medium. With appropriate background and autofluorescence correction, the images of the sections showing the borders of the infarction were captured and processed with ImageJ (NIH, <http://rsbweb.nih.gov/ij/>) to assess fluorescence intensity. The intensity is expressed as the mean tissue pixel intensity in infarct borders/tissue area.

In situ detection of cardiomyocyte apoptosis by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-X nick end labeling (TUNEL)—LV cryosections were analyzed by immunohistochemistry and TUNEL to identify α-actinin (Sarcomeric, a marker of cardiomyocytes) and apoptotic cells, respectively. Briefly, 4% formaldehyde-fixed sections were permeabilized with proteinase K (1:50) for 15 min at room temperature. Sections were incubated overnight at 4 °C with monoclonal anti-α-actinin (Sarcomeric) antibody (EA-53, 1:800, Sigma, St. Louis, Missouri, USA), and then with Cy3-labeled goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA). TUNEL assay was performed using a TdT-Fluor in situ apoptosis detection kit according to the manufacturer's instructions (Trevigen Inc., Gaithersburg, Maryland, USA). Sections were counterstained with 4, 6-diamidino-2-phenylindole to show the nuclei (blue). The images of the sections showing the myocardium were captured and processed with Microsuite Biological imaging software to assess the area of the myocardium. TUNEL-positive cardiomyocytes with bright green nuclei surrounded by red striations (α-actinin) were counted and expressed as the number of TUNEL-positive myocytes per square millimetre.

Histochemistry—LV sections were cut from each frozen slice and stained separately with (1) fluorescein-labeled peanut agglutinin (Vector Laboratories, Burlingame, California, USA) and (2) rhodamine-labeled *Griffonia simplicifolia* lectin I (Vector Laboratories, Burlingame, California, USA) for determination of interstitial collagen fraction and capillary density in the heart, as described previously (Liu et al. 1997b). Briefly, 3 slices per frozen section were pretreated with 3.3 U/mL neuroaminidase type V (Sigma, St. Louis, Missouri, USA) for 50 min at room temperature. Tissue samples were then washed 3 times in PBS, and then incubated with mixing solution of fluorescein-labeled peanut agglutinin to delineate the interstitial space (collagen and capillaries, 0.12 mg/mL; Vector Laboratories, Inc., Burlingame, California, USA) and rhodamine-labeled *Griffonia simplicifolia* lectin I,

showing only the capillaries (0.02 mg/mL; Vector Laboratories, Burlingame, California, USA). Total surface area (microscopic field), interstitial space (collagen plus capillaries), and the area occupied by capillaries alone were measured with Microsuite Biological imaging software. Interstitial collagen fraction was calculated by percent total surface area occupied by the interstitial space minus the percent total surface area occupied by the capillaries. Capillary density was taken as the number of capillaries in a given microscopic field using Microsuite Biological imaging software and expressed as number of capillaries per square millimetre. The average interstitial collagen fraction was calculated using data obtained from all 3 slices. Twelve fields were chosen randomly from each specific region, including non-infarct and infarct border areas.

Western blot analysis

About 20 mg of snap-frozen LV tissue from the base of the heart was thawed in 250 μ L lysis buffer (Cell Signaling Technology, Danvers, Massachusetts, USA) containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, Indiana, USA) and 1 mM phenylmethanesulfonyl fluoride that was added immediately before lysing the tissues. Small pieces were disrupted and homogenized with a Polytron, keeping samples at 4 °C throughout the procedures. Homogenized LV samples were centrifuged at 14 000g for 10 min at 4 °C, taking the supernatant as total LV tissue lysate. Protein in the supernatant was measured with Coomassie reagent (Thermo Scientific, Rockford, Illinois, USA). Aliquots of protein (60 μ g each) were kept at -80 °C. LV lysates were subjected to 10% SDS-PAGE under reducing condition and electro-transferred to nitrocellulose membrane. Intercellular adhesion molecule-1 (ICAM-1), transcriptional factor p53, GADD153 (CHOP), and SERCA2 ATPase were detected by immunoblotting the membranes overnight at 4 °C with the following primary antibodies: mouse antibody against ICAM-1 (0.1 μ g/mL, R & D Systems, Minneapolis, Minnesota, USA), p53 (1C12, 1:1000, Cell Signaling Technology, Danvers, Minnesota, USA), GADD 153 (B-3, 1:500, Santa Cruz Biotechnology, Santa Cruz, California, USA), or rabbit antibody against SERCA2 ATPase (1:2500, Abcam, Cambridge, Massachusetts, USA). Bound antibodies were visualized using a secondary antibody conjugated to horseradish peroxidase (Cell Signaling Technology, Danvers, Minnesota, USA) and enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, New Jersey, USA). After detecting ICAM-1, p53, GADD 153, or SERCA2 ATPase, the same membranes were blotted with a rabbit monoclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (1:3000, Cell Signaling Technology, Danvers, Massachusetts, USA). Band intensity was quantified by densitometry. All detected parameters were normalized to glyceraldehyde 3-phosphate dehydrogenase and the results were expressed as a fold increase compared with sham.

Statistical analysis

Binary data are expressed as proportions, and groups are compared using a χ^2 test for two-by-two tables. Continuous data are expressed as means $\pm$ standard errors and groups were compared using the two-sample Wilcoxon test. A nonparametric method was chosen if the variances differed substantially between groups. In all settings where multiple testing was used, a Hochberg's method was utilized to determine significance. An adjusted p value less than 0.05 was considered significant.

Results

Effects of Ac-SDKP on the heart at 7 days after MI (protocol 1)

Incidence of cardiac rupture, and cardiac remodeling and function after MI—

All 73 mice with MI (MI + vehicle, $n = 43$, and MI + Ac-SDKP, $n = 30$) survived for 24 h after surgery. Seven days after MI, 60.5% of vehicle-treated mice died after suffering from rupture of the LV free wall. The incidence of cardiac rupture was significantly reduced to 23.3% with treatment of Ac-SDKP (Fig. 2). The time window of rupture was 3–6 days after MI. Infarct size was similar between the vehicle and the Ac-SDKP treated groups (Table 1; Supplementary Fig. S1¹). The Ac-SDKP plasma levels were nearly 5-fold higher in the Ac-SDKP-treated mice compared with the vehicle-treated mice (Table 1).

The echocardiography data from mice surviving to 1 week after MI are shown in Table 1. MI caused LV chamber dilatation, as shown by increased LVDd and LVAd. Ac-SDKP tended to reduce LV dilatation by 1 week after MI, but the difference did not reach statistical significance. Cardiac function, indicated by EF and SF, was markedly reduced after MI, but Ac-SDKP treatment had no effect on these parameters. Mice with MI had increased LV mass, which was not affected by Ac-SDKP treatment (Table 1).

Inflammatory cell infiltration in the left ventricle—Seven days after MI, immunohistochemical analysis of the LV frozen sections showed markedly increased infiltration of both macrophages and neutrophils, mostly in the infarct border (Fig. 3). In vehicle-treated mice, the number of macrophages was almost double compared with the number of neutrophils. Ac-SDKP significantly reduced the number of infiltrating macrophages (Figs. 3A and 3B) but had no effect on neutrophil infiltration compared with the vehicle-treated group (Figs. 3A and 3C).

ICAM-1, expressed by vascular endothelial cells, initiates recruitment of inflammatory immune cells including macrophages (Etienne-Manneville et al. 1999). Thus, we decided to test whether Ac-SDKP-mediated inhibition of macrophage infiltration was associated with reduced expression of ICAM-1 in the left ventricle. We found that ICAM-1 expression significantly increased in vehicle-treated mice with MI, and this effect was diminished by treatment with Ac-SDKP (Figs. 3A and 3D).

Angiogenesis in the infarct border of the left ventricle—The number of CD31-positive capillary vessels was markedly decreased in the infarct border of vehicle-treated mice compared with sham group. Ac-SDKP treatment significantly increased the number of CD31-positive capillary vessels in the left ventricle after MI (Fig. 4).

Gelatinolytic activity in the heart—In situ zymography analysis revealed that gelatinolytic activity was not detectable in the hearts of sham-operated mice. However, there was a substantial increase in gelatinolytic activity (detected as increased field fluorescence

¹Supplementary data are available with the article through the journal Web site at <http://nrcresearchpress.com/doi/suppl/10.1139/cjpp-2019-0047>.

brightness) at the infarct border at 1 week after MI. Ac-SDKP significantly inhibited this activity, compared with vehicle-treated mice (Fig. 5).

Cardiomyocyte apoptosis—TUNEL-positive cells were barely found in the myocardium of sham-operated mice. MI caused a significant increase of the number of TUNEL-positive cardiomyocytes, and these positive cells were mainly located near the infarct border. Ac-SDKP treatment for 1 week markedly prevented cardiomyocyte apoptosis in mice after MI (Figs. 6A and 6B).

The tumor suppressor protein p53 mediates cell apoptosis through its activity as a transcription factor (Vousden and Lu 2002). Expression of p53 in the myocardium was barely detectable by Western blot in the hearts of sham-operated mice. MI strongly induced p53 protein expression in the left ventricle at 1 week after MI. Ac-SDKP significantly downregulated p53 expression after MI (Fig. 6C).

Effects of Ac-SDKP on the heart at 5 weeks after MI (protocol 2)

Cardiac remodeling and function—The echocardiography data from surviving mice at week 5 after MI are shown in Table 2. MI caused LV chamber dilatation and marked reduction of EF/SF, both of which were significantly improved by 5-week treatment of Ac-SDKP. Mice with MI had increased LV mass, which was not affected by Ac-SDKP (Table 2). Infarct size was not significantly different between the MI + vehicle versus MI + Ac-SDKP mouse groups (Table 2; Supplementary Fig. S1¹).

There was no difference in plasma Ac-SDKP levels between sham and MI + vehicle groups; however, Ac-SDKP treatment increased plasma levels more than 5-fold (Table 2).

Capillary density and collagen deposition—Analysis of the LV section located remotely from the infarct zone shows that the capillary density in the myocardium was significantly diminished in the MI + vehicle group compared with the sham-MI group. This detrimental effect on capillaries was slightly, but significantly, counteracted by treatment with Ac-SDKP (Figs. 7A (top panel) and 7B). MI also caused cardiac fibrosis, as indicated by increased myocardial interstitial collagen fraction. Ac-SDKP significantly inhibited collagen deposition in the heart (Figs. 7A (lower panel) and 7C).

CHOP (GADD 153) and SERCA2 ATPase expression—Although CHOP was detected in the myocardium of mice from the sham group, it was highly elevated in the left ventricle of mice 5 weeks after MI. Treatment with Ac-SDKP significantly inhibited CHOP expression, almost to the levels observed in sham-operated mice (Fig. 8A). Cardiac SERCA2 ATPase in vehicle-treated mice was less than half the level observed in sham-operated mice. Thus, Ac-SDKP significantly preserved SERCA2 ATPase in the left ventricles of mice with MI (Fig. 8B).

Discussion

The main findings of the present study are as follows. (1) Mice treated with Ac-SDKP exhibited reduction in the incidence of cardiac rupture secondary to acute MI and mortality

rate. (2) MI was associated with an excessive inflammatory response (increased neutrophil and macrophage infiltration and augmented ICAM-1 expression), increased gelatinolytic activity, reduced capillary number, and increased cardiomyocyte apoptosis. All these adverse responses (except neutrophil infiltration) were prevented by Ac-SDKP. (3) Five-week treatment with Ac-SDKP reduced LV dilatation and, more importantly, improved cardiac function, along with reduced interstitial fibrosis, increased capillary density in the myocardium, decreased CHOP expression (ER stress marker), and preserved SERCA2 protein expression. Importantly, the infarct size was similar among all groups. Most of the above listed observations were previously presented at the 2013 Council of High Blood Pressure Meeting (Peng et al. 2013). These effects are roughly similar to those previously reported when T β 4 was used (Peng et al. 2014), except that the dose of T β 4 (molecular mass, 5000 Daltons) used in that study was likely too low (1.6 mg/kg per day) to generate significant increases in circulating Ac-SDKP (molecular mass, 487 Daltons) (Supplementary Fig. S2¹). We have previously shown that only optimal increases in circulating Ac-SDKP obtained during administration of high doses of Ac-SDKP or an ACE inhibitor could block inflammation and fibrosis (Peng et al. 2005, 2007; Rasoul et al. 2004). Thus, the biological effects of T β 4 could be directly mediated through its putative receptor Ku80 (Bednarek et al. 2008), whereas those of Ac-SDKP may be mediated via a yet unidentified receptor. However, we previously demonstrated that their cardiac fibroblasts and myocardial tissue have functional Ac-SDKP receptor binding sites (Zhuo et al. 2007), by which Ac-SDKP exerts its anti-fibrotic and anti-inflammatory effects. More studies are needed to identify, characterize, and clone the Ac-SDKP receptors.

Nevertheless, in the acute MI mouse model, our findings are consistent with published studies showing that cardiac rupture mainly occurs at 3–5 days after MI in mice. In addition, within the first 2 days after MI, neutrophils constitute the most infiltrating cells. After 2 days, the number of infiltrating neutrophils declines, and this is followed by a significant macrophage accumulation at days 3 and 4 that persists up to 4 weeks (Yang et al. 2002, 2008). MI evokes intense inflammatory responses by the infarcted myocardium. The peak time of rupture occurrence and macrophage infiltration in the infarcted myocardium overlap, indicating macrophages as an important inflammatory cell population relevant to cardiac rupture. Indeed, clinical studies also show that hearts from patients with cardiac rupture reveal a higher density of inflammatory cells near the infarcted myocardium, compared with non-ruptured hearts (Kameda et al. 2006). Although inflammatory responses are essential for post-MI wound healing and scar formation, excessive and unregulated inflammatory responses can lead to adverse consequences (Frangogiannis 2008), such as thinning of the LV free wall, cardiac rupture, adverse LV remodeling, and HF. Importantly, Ac-SDKP significantly inhibited ICAM-1 expression in the infarcted myocardium, along with reducing macrophage infiltration, and lowering the incidence of cardiac rupture. ICAM-1 is expressed on the cell surface of leukocytes where it binds to integrins type CD11a/CD18 (on lymphocytes) or CD11b/CD18 (on macrophages), and it is essential for inflammatory cell adhesion (Rothlein et al. 1986; Yang et al. 2005). ICAM-1 signaling is key for the recruitment of inflammatory cells such as macrophages (Etienne-Manneville et al. 1999). The serum levels of ICAM-1 in patients within the first week of MI are also significantly higher than that in normal control subjects (Elahi et al. 2001). The increases in ICAM-1

expression in our study coincided with macrophage infiltration, indicating its key role in the recruitment of macrophages in the heart. Interestingly, we reported earlier that the inhibitory effects of Ac-SDKP on the tumor necrosis factor- α activation of canonical, nuclear factor kappa B pathway and subsequent decrease in ICAM-1 expression is achieved via inhibition of nuclear factor kappa- β kinase (Zhu et al. 2016). Tumor necrosis factor- α is one of the major pro-inflammatory cytokines released in the myocardium after MI (Marketou et al. 2010; Meldrum et al. 2005). Thus, these findings indicate that Ac-SDKP may decrease macrophage infiltration after MI, in part by the inhibition of ICAM-1 expression. This event could be likely associated with the reduction of cardiac rupture by Ac-SDKP, even though we did not monitor inflammatory cell infiltration daily during the first week after MI.

Inflammatory cell infiltration is also associated with enhanced expression and activities of gelatinolytic proteinases (Fang et al. 2008), such as matrix metalloproteinase (MMP)-2 and MMP-9. Significantly, the deletion of MMP-9 has been shown to lower cardiac rupture in mouse models of MI (Ducharme et al. 2000), and this effect coincided with a moderate reduction in leukocyte infiltration as measured at day 4 after MI (Yang et al. 2004). Similarly, MMP-2 expression in the mouse heart has been reported to be elevated at day 7 following MI; and inhibition of MMP-2 activity delayed post-MI remodeling through a reduction in macrophage infiltration and improved survival rate after acute MI (Matsumura et al. 2005). Therefore, these results suggest that excessive leukocyte infiltration and gelatinolytic proteinase expression could be closely related to cardiac rupture. Ac-SDKP exerts an antiinflammatory effect in the rat model of MI (Yang et al. 2004) and in experimental autoimmune myocarditis (Nakagawa et al. 2012) as evidenced by reduction in ICAM-1 expression and cardiac infiltrating macrophages, and by reduction in the gelatinolytic activity. In the present study, we found that acute treatment with Ac-SDKP decreased gelatinolytic activity in the infarct border region and corresponded with a low incidence of cardiac rupture. Our findings are in agreement with previous studies using MMP-2 or MMP-9 knockout mice, in which the deletion of either of these gelatinolytic proteinases prevented cardiac rupture without acutely improving cardiac function (Ducharme et al. 2000; Hayashidani et al. 2003; Matsumura et al. 2005). Thus, our findings are consistent with those previously reported using a mouse model of MI that showed that Ac-SDKP protected the heart, in part, by reducing macrophage infiltration (Ma et al. 2014; Nakagawa et al. 2018). However, mechanistically, Ma et al.'s and Nakagawa et al.'s studies differ as Ma et al. reported that in vivo or in vitro application of Ac-SDKP or its analog Ac-SD_DK_DP decreased M2 activation of macrophages with no effect on M1 subtype, whereas Nakagawa et al. reported that Ac-SDKP prevented cardiac rupture by decreasing pro-inflammatory M1 macrophage infiltration and MMP-9. Therefore, further studies are needed to clarify this part of the antiinflammatory mechanism induced by Ac-SDKP. Nevertheless, both clinical and experimental results show that excessive cardiac inflammation and gelatinolytic activity constitute the central mechanisms in the pathogenesis of cardiac rupture.

Angiogenesis/neovascularization in the heart is important for the viability of the myocardium after MI. Pro-angiogenic signaling participates in cardiac repair and positively correlates with the improvement of post-MI cardiac function (McSweeney et al. 2010). Ac-SDKP promotes angiogenesis both in vitro and in vivo (Liu et al. 2003; Wang et al. 2004).

Here, Ac-SDKP exerted a pro-angiogenic effect at an early stage of MI, as evidenced by the increased number of both CD31-positive cells and capillary density in the infarction border. As newly formed capillaries are a critical component of tissue granulation and repair, the increased angiogenic response may contribute to not only the reduced incidence of cardiac rupture in the acute phase of MI, but also to the improved cardiac remodeling and function in the late phase. The exact mechanism via which Ac-SDKP triggers angiogenesis/neovascularization remains unclear. Nevertheless, T β 4 or Ac-SDKP mediate angiogenesis via upregulation of vascular endothelial growth factor (Cha et al. 2003; Kim et al. 2015). Also, AKT, a serine/threonine protein kinase has been proposed to be essential in vascular endothelial growth factor mediated angiogenesis (Chaanine and Hajjar 2011). Thus, it remains essential to identify the receptor(s) for Ac-SDKP to better understand the cellular and downstream signaling(s) to ensure adequate elucidation of the mechanisms behind angiogenesis.

Both animal (Oskarsson et al. 2000; Sam et al. 2000) and human (Olivetti et al. 1996; Saraste et al. 1997) studies have detected an increased number of TUNEL-positive cardiomyocytes, a hallmark of apoptosis, in hearts either acutely or chronically after MI. These TUNEL-positive cells were accompanied with LV dilation and contractile dysfunction, suggesting that apoptotic cell death may have an important role in early and late LV remodeling and dysfunction after MI (Sam et al. 2000; Wencker et al. 2003). The tumor suppressor protein p53 mediates cell apoptosis through its activity as a transcription factor (Vousden and Lu 2002). Indeed, p53^{-/-} mice with MI had improved survival that was shown to be entirely due to a reduced rupture incidence, which was associated with markedly reduced numbers of apoptotic myocytes in the infarct region (Matsusaka et al. 2006). Notably, these mice had also been reported to exhibit better LV function after MI compared with wild type mice. These studies demonstrated that p53-dependent myocyte apoptosis is involved not only in early cardiac rupture (Matsusaka et al. 2006), but also in the late remodeling and dysfunction after MI (Sam et al. 2000). Consistent with other studies, we observed more apoptotic myocytes and higher levels of p53 expression in the myocardium of mice 1 week after MI; these effects were significantly inhibited by Ac-SDKP. Reduced incidence of cardiac rupture and (or) restored cardiac function by Ac-SDKP observed in the present study might be associated with its negative regulation of p53, and thus with the reduced myocyte apoptosis by Ac-SDKP. Ac-SDKP blocked the expression of CHOP, the hallmark ER stress/apoptosis protein, suggesting it may be essential for the observed inhibition of ER stress observed in this study. This finding is in agreement with previous reports that prolonged activation of ER stress results in increased apoptotic myocytes during cardiac injury and dysfunction in HF (Minamino et al. 2010; Okada et al. 2004). Moreover, Ac-SDKP has also been shown to suppress SiO₂-induced apoptosis in type II alveolar epithelial cells by attenuating the Caspase-12 and the ER stress pathway activation to prevent silicotic fibrosis (Zhang et al. 2018). Thus, Ac-SDKP could be an important regulator of cellular ER stress, but further studies are needed to determine whether this effect could be directly mediated via Ac-SDKP receptors.

Insufficient myocardial capillary density and cardiac fibrosis after MI have been identified as one of the critical events in the development of adverse LV remodeling and dysfunction after MI (Biner et al. 2002; Prech et al. 2006) that eventually leads to HF (Jessup and

Brozena 2003). Importantly, Ac-SDKP treatment for 5 weeks significantly ameliorated LV dilatation and improved cardiac function as evidenced by reduced LVDd/LVAd and increased SF/EF, which were associated with significantly increased capillary density, reduced cardiac fibrosis, and rescued SERCA2 expression in the left ventricle of mice subjected to MI. The mechanisms by which Ac-SDKP regulates fibrotic healing after MI could be mainly due to (1) its anti-inflammatory effects demonstrated by reduced ICAM-1 expression and the numbers of infiltrating macrophages, which limit exacerbated fibrotic remodeling; and (2) its direct inhibition of myofibroblast transformation (Peng et al. 2010) and collagen synthesis (Rhaleb et al. 2001a). Improved EF/SF of mice with MI by Ac-SDKP could also be mediated by rescued expression of SERCA2, preserving the normal contraction/relaxation cycle of surviving cardiomyocytes. Similar observations have been reported when mice with MI were rescued by initial treatment with an ACE inhibitor (Zalvidea et al. 2012). Thus, it is possible that part of the beneficial effect of ACE inhibitor could be mediated by Ac-SDKP, because ACE inhibition results in an approximate 5-fold increase in the circulating concentrations of Ac-SDKP, as we have previously demonstrated in hypertensive experimental models (Peng et al. 2005, 2007). Taken together, Ac-SDKP exerts profound anti-inflammatory, anti-fibrotic, anti-apoptotic, and pro-angiogenic properties, as well as preservation of SERCA2 expression that may contribute greatly to its cardioprotective effects after MI.

This type of mechanistic interaction between SERCA2 and ER stress signaling has been well recognized elsewhere (Bousette et al. 2014; Humeres et al. 2014; Zhang and Ren 2011). Indeed, disturbing ER structure has led to increased expression of ER stress proteins, together with abnormal cardiomyocyte viability, dysregulated Ca^{++} cycling, reduced SERCA2 expression, and increased apoptosis (Bousette et al. 2014). Moreover, thapsigargin, a SERCA2 inhibitor, has been shown to induce ER stress in cardiac fibroblasts, resulting in cell deaths, apoptosis, and intracellular procollagen accumulation (Humeres et al. 2014). Also, thapsigargin induced CHOP protein expression and collagen production in in vitro cultured rat cardiac fibroblasts; these effects were diminished in cells treated with Ac-SDKP (Supplementary Fig. S3¹). Our present data and published findings, thus, suggest a close reciprocal interaction between SERCA2 and ER stress in cardiac cells; and Ac-SDKP tilted such interaction in favor of SERCA2. More studies are needed to closely delineate the cellular and molecular mechanisms by which Ac-SDKP inhibits ER stress and favors SERCA2.

In conclusion, the present study shows for the first time that (1) Ac-SDKP reduces the incidence of cardiac rupture after MI, and markedly improves cardiac function at 5 weeks after MI in mice; and (2) the cardiac protective effects of Ac-SDKP after MI are associated with a significant reduction in ER stress of excessive inflammatory response, cardiomyocyte apoptosis and fibrosis, and an enhancement of angiogenesis and SERCA 2 expression in the heart. Thus, Ac-SDKP or its analogue could potentially be a useful therapeutic agent in protecting patients with acute MI from cardiac injury. The present research provides the essential pre-clinical data for translation to a clinical trial.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Experimental protocols. (A) N-acetyl-seiyl-aspartyl-lysyl-proline (Ac-SDKP) was administrated 7 days before myocardial infarction (MI) surgery to ensure its stable concentration in circulation. The incidence of cardiac rupture was assessed during the first week after MI (protocol 1). (B) Ac-SDKP administration started right after MI surgery. Cardiac remodeling and function were evaluated 5 weeks after MI (protocol 2). For both protocols, echocardiography was performed prior to euthanasia, which was followed by tissue harvest.

Fig. 2.

The percentage of cardiac rupture deaths in C57BL/6J mice up to 1 week of myocardial infarction (MI). N-acetyl-seiyl-aspartyl-lysyl-proline (Ac-SDKP) significantly reduced incidence of cardiac rupture induced by MI. *** $p < 0.005$, χ^2 test.

Fig. 3.

(A) Representative images of immunohistochemically stained macrophages (upper panel) and neutrophils (lower panel) in the left ventricle at 1 week of myocardial infarction (MI). (B) Quantitative analysis of CD68-positive cells (marker for macrophage), showing that *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) significantly reduced numbers of infiltrating macrophages. (C) There were no differences in the numbers of Ly-6b.2-positive cells (a marker for neutrophils) between the MI + vehicle and MI + Ac-SDKP groups. (D) Increased intercellular adhesion molecule-1 (ICAM-1) expression after MI was significantly inhibited by Ac-SDKP. $n = 6/\text{group}$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, two-sample Wilcoxon test with a Hochberg correction for multiple testing. [Color online.]

Fig. 4. (A) Representative images of capillary vessels detected by positive staining for CD31 (brown color). (B) Quantitative analysis of number of capillary vessels at 1 week of myocardial infarction (MI), showing that the number of capillary vessels was significantly increased in the MI + *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) group compared with MI + vehicle group. $n = 6-8/\text{group}$, $*p < 0.05$, $**p < 0.01$, $***p < 0.005$, two-sample Wilcoxon test with a Hochberg correction for multiple testing. [Color online.]

Fig. 5.

(A) Representative images of in situ zymography for gelatinolytic activity in the infarct border (green fluorescent color); no clear activity was detected in the sham-operated heart. (B) Semiquantitative analysis shows that myocardial infarction (MI) increased gelatinolytic activity, which was significantly blunted by *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP). $n = 4-5/\text{group}$, $*p < 0.05$, $**p < 0.01$, $***p < 0.005$, two-sample Wilcoxon test with a Hochberg correction for multiple testing. [Color online.]

Fig. 6.

(A) Representative images of terminal deoxynucleotidyl transferase mediated dUTP-X nick end labeling (TUNEL)-positive cardiomyocytes in myocardium. (B) The number of TUNEL-positive cells increased significantly at 1 week after myocardial infarction (MI), which was reduced by N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP). (C) p53 protein expression in the myocardium was markedly induced by MI, which was significantly inhibited by Ac-SDKP. $n = 6/\text{group}$, $*p < 0.05$, $^{\dagger}p < 0.005$, two-sample Wilcoxon test with a Hochberg correction for multiple testing. LV, left ventricle. [Color online.]

Fig. 7.

(A) Representative images of capillaries (red stained, upper panel) and interstitial collagen (green stained, lower panel) in the left ventricle 5 weeks after myocardial infarction (MI). Quantitative analysis of capillary density and interstitial collagen fraction (ICF), showing that MI caused a decrease in capillary density (B) and an increase in ICF (C), and these adverse effects were significantly prevented by *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP). $n = 6-8/\text{group}$, $*p < 0.05$, $***p < 0.005$, two-sample Wilcoxon test with a Hochberg correction for multiple testing. [Color online.]

Fig. 8.

(A) CCAAT/enhancer binding protein homology protein (CHOP) expression in the left ventricle was markedly increased 5 weeks after myocardial infarction (MI), and was significantly blunted by *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) treatment. (B) SERCA2 ATPase was dramatically decreased in the left ventricle s of mice with MI, which was significantly prevented by Ac-SDKP. $n = 5/\text{group}$, $*p < 0.05$, $**p < 0.01$, two-sample Wilcoxon test with a Hochberg correction for multiple testing.

Table 1.

Plasma *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) concentration, infarct size, echocardiographic measurements, and left ventricular mass (LVM) and body mass (BM) from protocol 1.

Parameter	Sham	7 days after MI	
		Vehicle	Ac-SDKP
Ac-SDKP, nM	0.92±0.16	1.01±0.11	5.12±1.25 [‡]
Infarct size, %	NA	37.4±4.0	39.5±2.3
LVDd, mm	2.60±0.08	5.10±0.04 [*]	4.43±0.34 [‡]
LVAd, mm ²	4.94±0.58	23.06±4.08 [*]	18.75±4.18 [‡]
PWTd, mm	0.82±0.02	0.82±0.03	0.80±0.02
HR, bpm	676±16	677±16	685±16
EF, %	75.16±3.25	27.54±3.87 [*]	30.84±3.48 [‡]
SF, %	51.88±2.12	18.61±2.89 [*]	25.37±3.61 [‡]
LVM, mg/10 g BM	32.36±1.35	49.26±1.74 [*]	43.45±2.00 [‡]
BM, g	27.11±0.86	25.43±0.85	23.53±1.16

Note: Values are means ± standard error. MI, myocardial infarction; NA, not applicable; LVDd, left ventricular diastolic dimensions; LVAd, left ventricular diastolic area; PWTd, diastolic posterior wall thickness; HR, heart rate; bpm, beats per minute; EF, left ventricular ejection fraction; SF, shooting fraction.

^{*}
 $p < 0.005$ versus sham;

[‡]
 $p < 0.001$ or

[‡]
 $p < 0.005$ versus vehicle. $n = 8-10$ /group.

Table 2.

Plasma *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) concentration, infarct size, echocardiographic measurements, and left ventricular mass (LVM) and body mass (BM) from protocol 2.

Parameter	Sham	5 weeks after MI	
		Vehicle	Ac-SDKP
Ac-SDKP, nM	1.34±0.13	1.10±0.11	6.35±1.45 [‡]
Infarct size, %	NA	38.9±3.1	37.0±2.2
LVDd, mm	2.48±0.03	4.07±0.18 [*]	3.47±0.16 ^{‡, *}
LVAd, mm ²	4.15±0.16	18.26±2.30 [*]	11.14±2.10 ^{‡, #}
PTWd, mm	0.83±0.02	0.85±0.02	0.87±0.02
HR, bpm	680±11	642±20	661±11
EF, %	77.61±1.15	30.51±2.95 [*]	47.91±3.15 ^{‡, *}
SF, %	54.93±0.77	26.47±2.59 [*]	39.05±2.74 ^{‡, #}
LVM, mg/10 g BM	31.42±0.76	47.50±2.35 [*]	42.98±2.54 [#]
BM, g	28.14±0.75	27.84±0.68	26.62±0.53

Note: Values are means ± standard error. MI, myocardial infarction; NA, not applicable; LVDd, left ventricular diastolic dimensions; LVAd, left ventricular diastolic area; PTWd, diastolic posterior wall thickness; HR, heart rate; bpm, beats per minute; EF, left ventricular ejection fraction; SF, shooting fraction.

^{*}
 $p < 0.0005$ or

[#]
 $p < 0.005$ versus sham;

[‡]
 $p < 0.05$ or

[‡]
 $p < 0.005$ versus vehicle. $n = 10-16$ /group.