Apelin-13 protects against myocardial infarction-induced myocardial fibrosis

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Abstract. Myocardial infarction is a serious health threat. Apelin is an endogenous ligand of angiotensin II receptor-like 1 (APJ) and the apelin/APJ system is associated with various types of heart disease. However, whether apelin protects against myocardial infarction-induced myocardial fibrosis remains unclear. The present study aimed to investigate the function of apelin-13 during myocardial infarction-induced myocardial fibrosis, and to determine the mechanism underlying the effects of apelin-13. Apelin-13 was demonstrated to improve left ventricular function and results of hematoxylin and eosin staining, Masson's trichrome staining and western blotting showed that apelin-13 attenuated myocardial fibrosis. Further mechanistic investigation was performed by enzyme-linked immunosorbent assay, western blotting and electrophoretic mobility shift assay. The results demonstrated that apelin-13 inhibited the activation of nuclear factor (NF)-κB signaling *in vitro* and *in vivo*. To the best of our knowledge, the present study was the first to demonstrate that apelin-13 may attenuate myocardial infarction-induced myocardial fibrosis, and that this protective function may be mediated by inhibition of NF-κB signaling. The present study suggests a theoretical basis for the effects of apelin-13 and provides insight into the potential clinical application of apelin-13.

Introduction

Myocardial infarction is a serious threat to human health. In addition to the possibility of sudden cardiac death, long-time persistent ischemia reduces survival and induces death of cardiomyocytes, thus leading to heart failure (1). Although the survival of patients with myocardial infarction is increasing as treatment strategies improve, the mortality associated with this disease remains high.

Apelin is synthesized as a 77 amino acid pre-pro-protein, and is sequentially cleaved into four circulating active peptides: Apelin-12, apelin-13, apelin-17 and apelin-36. Apelin is a secreted adipokine involved in the regulation of cardiovascular functions (2). Angiotensin II (Ang-II) receptor-like 1 (APJ) is an endogenous receptor of apelin. APJ is expressed in several cell types, including endothelial cells, vascular smooth muscle cells and cardiomyocytes. Apelin regulates the production of nitric oxide (NO) (3), causes vasodilatation, reduces ventricular preload and afterload, and increases cardiac contractility in failing hearts (4). Furthermore, apelin has previously been demonstrated to exert vasodilatation and positive inotropic effects (5-8), and is regarded as an important therapeutic target in heart failure (9).

Apelin was previously reported to have a protective function during heart failure and ischemia/reperfusion injury (10-12). Growing evidence has demonstrated that apelin is important for left ventricular remodeling (4,9). However, the effects of apelin treatment on myocardial infarction-induced myocardial fibrosis remain unclear. The present study investigated the effects of apelin-13 treatment on myocardial fibrosis.

Materials and methods

Materials. Apelin-13 [(Pyr1)-Apelin-13] was purchased from GL Biochem (Shanghai) Ltd. (Shanghai, China). Apelin-13 was dissolved in sterile normal saline to form a 10 mg/ml stock solution.

Animal experiment protocol. Male Sprague-Dawley rats (age, 8-years-old) weighing 180-220 g were obtained from the Experimental Animal Center of China Medical University (Shenyang, China). Mice were maintained in a temperature-controlled (20-22˚C) environment under a 12 h light-dark cycle. Rats were divided into three groups (n=6/group), as follows: Sham; left anterior descending artery (LAD) ligation; and LAD + apelin-13 groups. Rats in the LAD group underwent a LAD ligation operation (13,14). Briefly, rats were anesthetized with 10% chloral hydrate (3.5 ml/kg; Sinopharm, Shanghai, China) via intraperitoneal injection. In a supine position, endotracheal intubation was performed

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and the rats were ventilated using a rodent ventilator (rate, 80 breaths/min; tidal volume, 6-8 ml/kg). The thoracic cavity was then opened and the heart was exposed. The LAD was ligated using 7-0 silk. Successful LAD ligation was confirmed by myocardial blanching and the thoracic cavity was closed layer-by-layer. Following LAD ligation operation, rats received an intramuscular injection of $2.5x10^4$ U penicillin (MOTIAN, Harbin, China). Rats in the sham group received an equivalent operation, but without ligation. Rats in the LAD + apelin-13 group received an intraperitoneal injection with apelin-13 (200 μg/kg/day) for 4 weeks following the LAD ligation operation. Rats in the sham and LAD groups received an equal amount of normal saline. After 4 weeks, rats in each group were anesthetized with 10% chloral hydrate. The levels of left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), left ventricular maximal rate of pressure rise ($LV+dp/dt_{max}$) and left ventricular maximal rate of pressure decline ($LV-dp/dt_{max}$) were measured. The rats were anesthetized with 10% chloral hydrate (3.5 ml/kg; intraperitoneal injection). The blood was harvested and stored at room temperature for 2-4 h and was centrifuged at 4,000 rpm for 10 min. The supernatant was collected and the serum was obtained. Animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at China Medical University.

Cell culture. The H9C2 rat myoblast cell line was obtained from Type Culture Collection Center of Chinese Academy of Science (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), and maintained in a humidified atmosphere containing 5% CO_2 at 37˚C. Prior to treatment with Ang-II (Cloud-Clone, Wuhan, China), cells were cultured in serum-free medium overnight. The cells were then treated with 100 nM Ang-II for 48 h. Apelin-13 (100 nM) was added to the cell medium 20 min prior to Ang-II treatment. Following treatment with Ang-II and apelin-13, cells were collected for western blotting and electrophoretic mobility shift assay (EMSA).

Histopathology. The hearts of the rats in each group were harvested, fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. Subsequently, the sections were subjected to routine hematoxylin and eosin (HE) and Masson's trichrome staining. Images were captured using an optical microscope (DP73; Olympus, Tokyo, Japan), and the ratio of cardiac tissue fibrosis was analyzed.

Western blot analysis. The hearts of the rats in each group were harvested and homogenized in radioimmunoprecipitation (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) with 1% phenylmethanesulfonyl fluoride (PMSF; Beyotime Institute of Biotechnology). H9C2 cells were also lysed in RIPA lysis buffer with 1% PMSF. Protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein (40 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (either 8, 10 or 13% gels). The separated proteins were then transferred to polyvinylidene fluoride (PVDF; EMD Millipore, Billerica, MA, USA) membranes. The membranes were blocked with 5% skimmed milk and were incubated with the corresponding primary antibodies. The antibodies used were as follows: Rabbit anti-transforming growth factor-β (TGF-β) (1:200; cat. no. sc-146 Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-IκB [nuclear factor (NF)-κB inhibitor] (1:500; cat. no. bs-1287R), rabbit anti-phosphorylated-IκB (p-IκB; 1:500; cat. no. bs5515R), rabbit anti-connective tissue growth factor (CTGF; 1:500; cat. no. bs-0743R), all from BIOSS (Beijing, China), rabbit anti-collagen-I (Col-I; 1:400; cat. no. BA0325), rabbit anti-matrix metalloproteinase-2 (MMP-2; 1:400; cat. no. BA0569), MMP-9 (1:400; cat. no. BA2202) all from Wuhan Boster Biological Technology, Ltd. (Wuhan, China) and mouse anti-β-actin (1:1,000; cat. no. sc-47778 Santa Cruz Biotechnology, Inc.) at 4˚C overnight. Following washing with Tris-buffered saline-0.05% Tween 20, the membranes were incubated with either goat anti-rabbit (cat. no. A0208) or goat anti-mouse (cat. no. A0214) horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000, Beyotime Institute of Biotechnology) at 37˚C for 45 min. Subsequently, the membranes were visualized using an enhanced chemiluminescence (ECL) detection system and analyzed with Gel-Pro-Analyzer 4.5 software (Media Cybernetics, Inc., Rockville, MD, USA). The protein expression levels were normalized to β-actin.

Enzyme-linked immunosorbent assay (ELISA). The concentration of Ang-II in the hearts and serum was measured by ELISA. Hearts of rats in each group were harvested and homogenized. The concentration of total protein in the samples was determined using the BCA protein assay kit, and the concentration of Ang-II in the heart tissues and serum was detected using an ELISA kit for Angiotensin II (USCN Life Science, Inc., Wuhan, China), according to the manufacturer's protocol.

EMSA. NF-κB activity was detected by EMSA. Following treatment, the rat hearts and the H9C2 cells from each group were harvested. The nucleoproteins were extracted using a nucleoprotein and cytoplasmic protein extraction kit (Beyotime Institute of Biotechnology), and EMSA was performed using an NF-κB EMSA kit (Viagene Biotech, Inc., Tampa, FL, USA), according to the manufacturer's protocols. Briefly, equal amounts of nucleoprotein from each group were incubated with a biotin-labeled NF-κB probe at room temperature for 20 min, and were then separated on an EMSA gel. The separated protein was transferred to PVDF membranes and cross-linked under a UV transilluminator (EUV002; Beyotime Institute of Biotechnology). Following incubation with HRP-labeled streptavidin, the signal was detected with an ECL detection system.

Statistical analysis. Each experiment was performed three times. The results are presented as the mean ± standard deviation. Differences between groups were analyzed using one-way analysis of variance and Bonferroni's multiple comparison test. Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Physiological parameters.

Parameter	Sham	LAD	LAD + Apelin-13
LVSP (mmHg)	129.17±16.38	96.50±10.97[a]	117.67±11.29[b]
LVEDP (mmHg)	6.43±2.29	12.38±2.08[a]	7.45±2.60[c]
LV+dp/dt (mmHg/s)	5904.67±868.48	2722.83±1116.19[d]	4468.50±762.94[b]
LV-dp/dt (mmHg/s)	-5230.83±852.97	-2747.33±701.85[a]	-4336.83±1351.82[b]

[a]P<0.01 vs. sham group; [b]P<0.05, [c]P<0.01 vs. LAD group; [d]P<0.001 vs. sham group. Values are presented as the mean ± standard deviation. LAD, left anterior descending ligation; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LV+dp/dt, left ventricular maximal rate of pressure rise. LV-dp/dt, left ventricular maximal rate of pressure decline.

Results

Apelin-13 relieves myocardial infarction-induced left ventricular dysfunction. Left ventricular function was examined following LAD ligation and apelin-13 treatment. As presented in Table I, following LAD ligation, LVSP (P=0.0018), LV+dp/dt_{max} (P<0.0001) and LV-dp/dt_{max} (P=0.0020) were significantly decreased, and LVEDP was significantly increased (P=0.0015) compared with the sham group. However, compared with the LAD group, following treatment with apelin-13, these changes were reversed. These results indicate that apelin-13 may relieve myocardial infarction-induced left ventricular dysfunction.

Apelin-13 attenuates myocardial infarction-induced myocardial fibrosis. HE staining was used to evaluate histopathological changes. As demonstrated in Fig. 1A, hearts in the sham group exhibited normal cardiomyocyte structure, with a clear texture and veins, and plump cytoplasm (less gaps and spaces between myofilaments). However, hearts in the LAD group exhibited disordered structure. The myofilaments were rougher with wave-like changes and the boundary of textures and veins were unclear. Following treatment with apelin-13 (the LAD + apelin-13 group), the above histopathological changes were attenuated compared with the LAD group.

Cardiac fibrosis in each group was evaluated by Masson's trichrome staining. As demonstrated in Fig. 1B, hearts of rats in the LAD group exhibited numerous collagenous fibers (blue) compared with the sham group. However, following treatment with apelin-13, the percentage of fibrosis was significantly decreased compared with the LAD group (P<0.0001; Fig. 1B and C). These results suggest that apelin-13 may attenuate myocardial infarction-induced fibrosis.

An imbalance between extracellular matrix (ECM) and matrix-degrading enzymes is the predominant cause of myocardial fibrosis. TGF-β and CTGF are important factors that promote the synthesis of collagen and myocardial fibrosis. Col-I is an important component of the ECM. MMP-2 and MMP-9 are important for degradation of the ECM. These factors are closely associated with myocardial fibrosis and were detected by western blotting in the present study (Fig. 2A). Results of western blot analysis demonstrated that in the hearts of rats from the LAD group, the protein expression levels of TGF-β, CTGF, Col-I, MMP-2 and MMP-9 were significantly increased compared with the sham group (P=0.0002, P<0.0001, P=0.0007, P=0.0005 and P=0.0004, respectively; Fig. 2B). However, following treatment with apelin-13, these changes were reversed. The results of western blotting and histopathological analysis suggest that apelin-13 may attenuate myocardial infarction-induced myocardial fibrosis.

Apelin-13 reduces Ang-II levels and inhibits the activation of NF-κB in rats with myocardial infarction. Ang-II was previously demonstrated to be associated with myocardial fibrosis. The concentration of Ang-II in the serum and heart tissues was measured by ELISA. In the hearts and serum of rats in the LAD group, the concentration of Ang-II was increased compared with rats in the sham group. However, following treatment with apelin-13, the elevated levels of Ang-II were decreased compared with the LAD group (P=0.0001 and P=0.0003, respectively; Fig. 3A and B). These results suggest that apelin-13 may reduce the increased Ang-II levels induced by myocardial infarction.

The protein expression and phosphorylation levels of IκB, which indicates activation of NF-κB signaling, were analyzed by western blotting. The western blot analysis demonstrated that the protein levels of IκB were decreased and the phosphorylation levels of IκB were increased in the LAD group compared with the control group (P<0.0009); however, following treatment with apelin-13 the levels were significantly reversed compared with the LAD group (P<0.0360 and P<0.0008, respectively; Fig. 3C and D). These results indicate that the NF-κB signaling pathway was activated by LAD ligation and inhibited after apelin-13 treatment. To further measure the activation of NF-κB, an EMSA was carried out. As demonstrated in Fig. 3E, in the LAD group, the level of NF-κB bound to the NF-κB probe, which indicates the activity of NF-κB, was significantly increased compared with the sham group (P=0.0001). However, following treatment with apelin-13, the level of NF-κB bound to the NF-κB probe was decreased compared with the LAD group (P=0.0037), thus suggesting that NF-κB activity was inhibited by apelin-13 (Fig. 3E and F).

Apelin-13 inhibits the activation of NF-κB signaling induced by Ang-II in vitro. To further verify the mechanism underlying the function of apelin-13, *in vitro* experiments were performed. The protein levels of TGF-β, CTGF, Col-I, MMP-2 and MMP-9 were evaluated by western blot analysis. Western blotting results demonstrated that the protein expression levels of TGF-β (P=0.0015), CTGF (P=0.0018), Col-I (P=0.002),

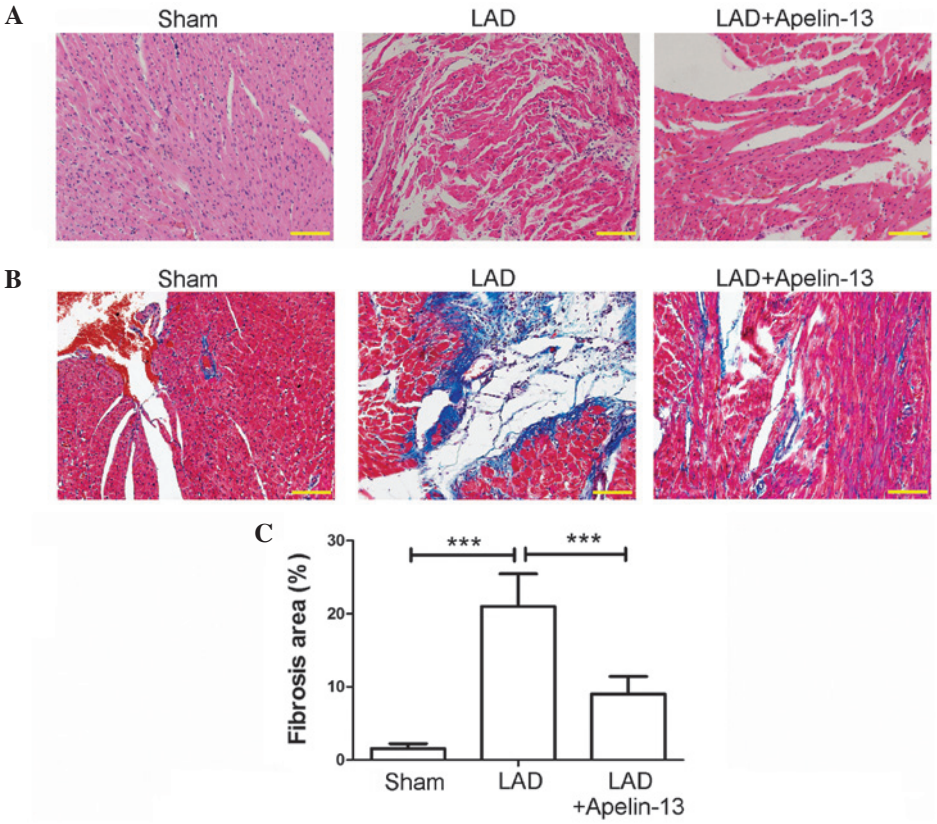

Figure 1. Histopathological changes. (A) Hearts of rats in each group were collected for hematoxylin and eosin staining. (B) Hearts of rats in each group were harvested for Masson's trichrome staining. (C) Percentage of fibrosis was calculated. Results are presented as the mean ± standard deviation. Typical results are presented. Scale bar=100 μm. ***P<0.001, comparison indicated by brackets. LAD, left anterior descending ligation.

MMP-2 (P=0.0003) and MMP-9 (P=0.001) were significantly increased by Ang-II treatment, which was consistent with the results of western blot *in vivo*. However, the elevated protein levels of TGF-β, CTGF, Col-I, MMP-2 and MMP-9 were significantly decreased following apelin-13 treatment compared with the Ang-II treatment group (Fig. 4).

Furthermore, the activation of NF-κB signaling was detected by western blot analysis and EMSA. The western blotting results demonstrated that following Ang-II treatment, the phosphorylation levels of IκB were significantly increased and the protein expression levels were significantly decreased compared with the sham group (P=0.0006). However, compared with the Ang-II group, treatment with apelin-13 attenuated these changes (Fig. 5A and B). EMSA results demonstrated that compared with the sham group, the levels of NF-κB bound to the NF-κB probe were significantly increased following Ang-II treatment (P=0.0031); however, compared with the Ang-II group the levels were significantly reduced following treatment with apelin-13 (P=0.0486; Fig. 5B). These results indicate that the NF-κB signaling pathway was activated by Ang-II, and this effect was inhibited by apelin-13 treatment. The results of the present study suggest that apelin-13 may attenuate myocardial infarction-induced myocardial fibrosis via the regulation of NF-κB signaling.

Discussion

The present study investigated the effects of apelin-13 on myocardial infarction-induced myocardial fibrosis. The study demonstrated that apelin-13 was able to relieve myocardial fibrosis, and reduce Ang-II levels in heart tissues and serum. Further mechanistic analyses demonstrated that the cardioprotective effects of apelin-13 may be mediated via inhibition of NF-κB signaling.

Apelin was previously reported to protect against isoproterenol-induced myocardial injury (15), and exhibited cardioprotective activity in hearts undergoing ischemia and reperfusion (11). Apelin exhibits cardioprotective effects and the present study demonstrated that treatment with apelin-13 was able to attenuate myocardial fibrosis. A previous study also demonstrated that apelin can limit infarct size (16), and the loss of apelin has been reported to exacerbate myocardial infarction-associated adverse remodeling (17). Myocardial infarction also causes myocardial ischemia. Apelin enhances the production of NO, dilates vessels and improves blood supply, thus attenuating myocardial damage (18). Furthermore, apelin-13 can reduce oxidative injury induced by myocardial ischemia (18) and exerts anti-apoptotic effects. Apelin activates phosphatidylinositol 3-kinase (PI3K)/*v*-akt murine thymoma viral oncogene homolog 1 (AKT) signaling, increases the expression of B-cell lymphoma-2 (Bcl-2), and inhibits the expression of Bcl-2-associated X protein, thus reducing cell apoptosis (19,20), which may also contribute to the cardioprotective effects of apelin-13.

Cardiac fibrosis is characterized by an imbalance between ECM proteins and matrix-degrading enzymes, and an excessive accumulation of ECM. In the present study, the protein expression levels of CTGF, Col-I, MMP-2 and MMP-9 were

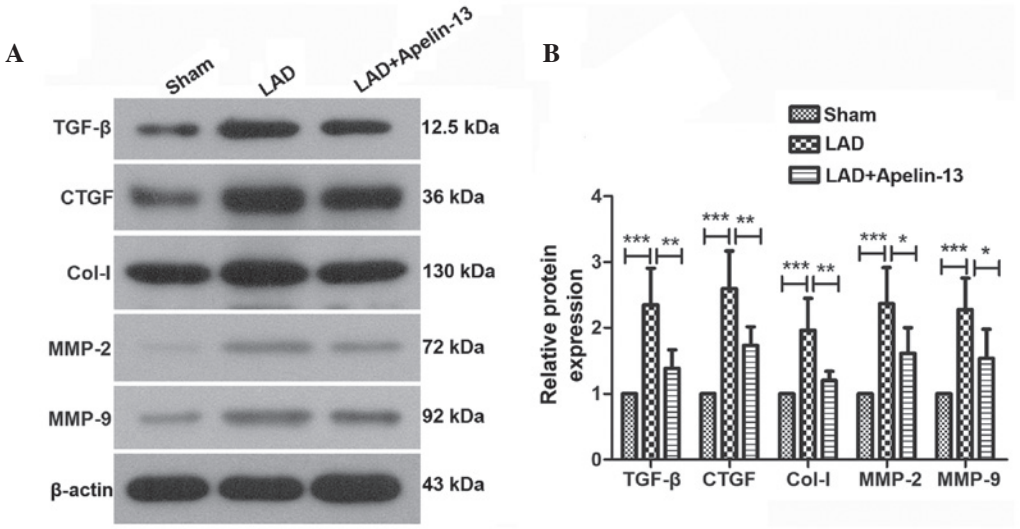

Figure 2. Apelin-13 reduces the protein levels of fibrosis markers in rats with myocardial infarction. (A) Protein expression levels of TGF-β, CTGF, Col-I, MMP-2 and MMP-9 were detected by western blot analysis using β-actin as an internal reference. (B) Relative protein expression levels were calculated by densitometric analysis. Typical results are presented. Results are presented as the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001, comparisons indicated by brackets. LAD, left anterior descending ligation; TGF-β, transforming growth factor-β; CTGF, connective tissue growth factor; Col-1, collagen-1; MMP, matrix metalloproteinase.

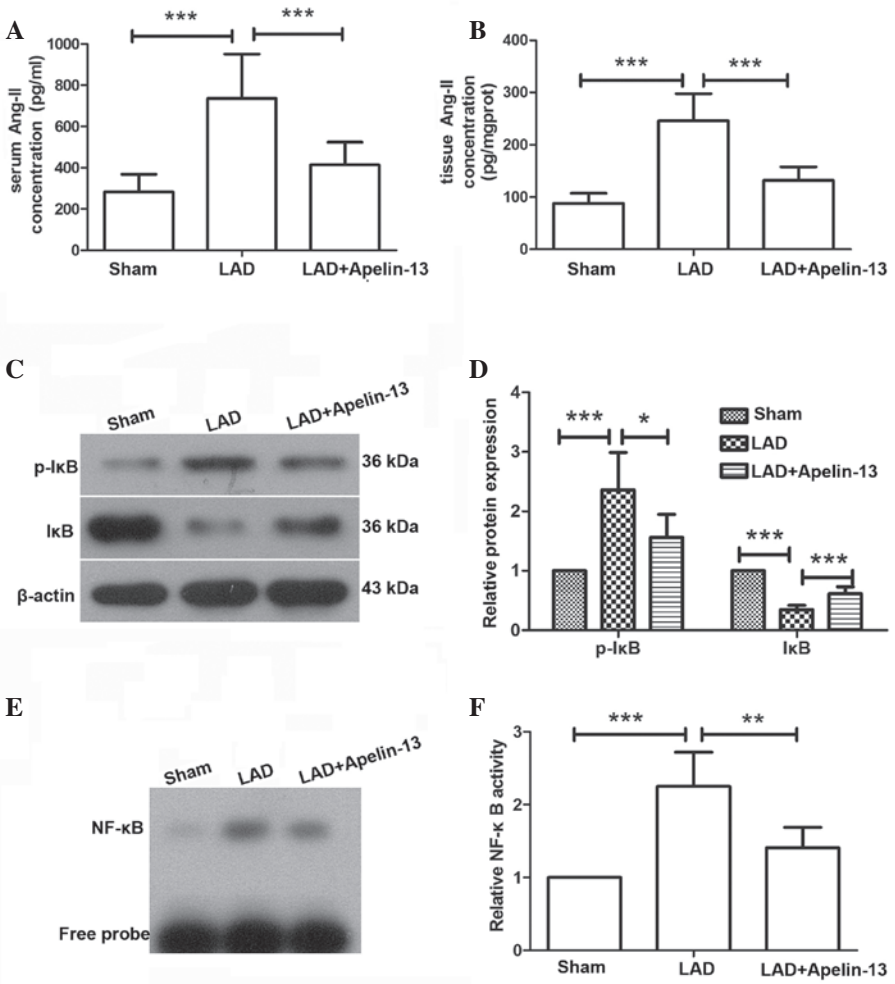

Figure 3. Apelin-13 reduces Ang-II levels and inhibits the activation of NF-κB signaling in rats with myocardial infarction. (A) Concentration of Ang-II in (A) serum and (B) heart tissue was detected by enzyme-linked immunosorbent assay. (C) Protein levels of IκB and p-IκB were detected by western blotting and (D) were quantified using β-actin as an internal reference. (E) NF-κB activity in rat hearts from each group was detected by electrophoretic mobility shift assay and (F) the relative activity of NF-κB was calculated. Typical results are presented. The results are presented as the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001, comparison indicated by brackets. Ang-II, angiotensin II; LAD, left anterior descending ligation; p-IκB; phosphorylated-NF-κB inhibitor; NF-κB, nuclear factor-κB.

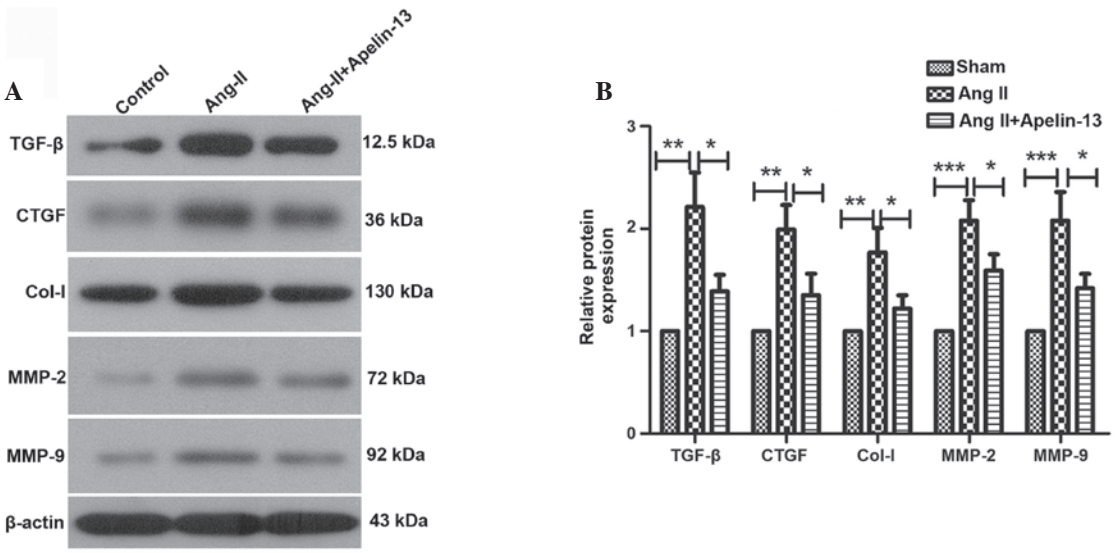

Figure 4. Apelin-13 reduces the protein levels of fibrosis markers induced by Ang-II. (A) Protein expression levels of TGF-β, CTGF, Col-I, MMP-2 and MMP-9 were detected by western blotting using β-actin as an internal reference and (B) the relative protein levels were calculated. Typical results are presented. Results are presented as the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001, comparisons indicated by brackets. Ang-II, angiotensin II; TGF-β, transforming growth factor-β; CTGF, connective tissue growth factor; Col-1, collagen-1; MMP, matrix metalloproteinase.

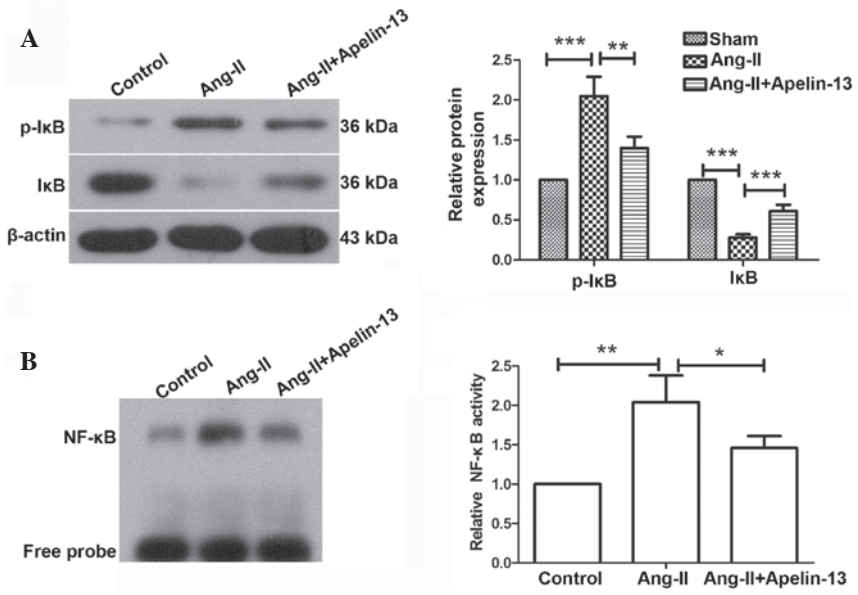

Figure 5. Apelin-13 inhibits the activation of NF-κB signaling induced by Ang-II. (A) Protein levels of IκB and p-IκB were detected by western blotting and the relative protein levels were calculated using β-actin as an internal reference. (B) NF-κB activity was detected by electrophoretic mobility shift assay. Typical results are presented. The results are presented as the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001, comparison indicated by brackets. Ang-II, angiotensin II; p-IκB; phosphorylated-NF-κB inhibitor; NF-κB, nuclear factor-κB.

elevated following LAD ligation or Ang-II treatment, but were reduced by apelin-13 treatment. These results provide further evidence indicating that apelin-13 attenuates myocardial infarction-induced myocardial fibrosis. A similar effect was also demonstrated on the expression pattern of TGF-β, a protein that is closely associated with cell transformation. TGF-β is a crucial mediator of cardiac fibroblast activation and differentiation into myofibroblasts, which produce a large amount of collagen (21). The activation of cardiac fibroblasts and their differentiation into myofibroblasts are key events in the progression of cardiac fibrosis. Pretreatment with apelin has previously been reported to reduce the expression of TGF-β-mediated myofibroblast markers and collagen production (22).

Ang-II is an important factor involved in cardiac fibrosis. Ang-II contributes to cardiovascular injury via the regulation of inflammation and oxidative stress, stimulation of smooth muscle cells growth (23) and promotion of ECM formation (24). In the present study, the levels of Ang-II in heart tissue and serum decreased following apelin-13 treatment, thus indicating that apelin-13 exerts a protective effect. Siddiquee *et al* (25) also reported that apelin protects against Ang-II-induced cardiovascular fibrosis *in vivo*, which is consistent with the results of the present study. Furthermore,

apelin-13 was previously reported to promote the synthesis of NO (3). By contrast with the effects of Ang-II, NO induces vasodilatation and lowers blood pressure.

Apelin was previously reported to perform a protective effect against ischemia-reperfusion injury via the regulation of the PI3K/AKT, extracellular signal-regulated kinase and mitogen-activated protein kinase signaling pathways (26-28). NF-κB is a multifunctional nuclear factor associated with cell growth and inflammatory responses. The present study demonstrated that apelin-13 inhibits the activation of NF-κB signaling. This indicates that apelin-13 may exert its cardioprotective effect through the regulation of NF-κB, however further investigation is required to confirm this hypothesis.

In conclusion, myocardial infarction-induced myocardial ischemia leads to the death of cardiomyocytes and fibrotic lesions. The expression and secretion of apelin was previously reported to be increased in ischemic myocardium (29), and the present study demonstrated that apelin exerted a cardioprotective effect. The current study demonstrated that treatment with apelin-13 may attenuate myocardial infarction-induced myocardial fibrosis, and this cardioprotective effect may be mediated via regulation of NF-κB signaling. The present study provides a theoretical basis for further exploration into apelin, and provides information regarding the clinical application of apelin-13.

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