Apelin-13 alleviated cardiac fibrosis via inhibiting the PI3K/Akt pathway to attenuate oxidative stress in rats with myocardial infarction-induced heart failure

Shan Zhong\textsuperscript{a}, Hongli Guo\textsuperscript{a}, Hui Wang\textsuperscript{a}, Dan Xing\textsuperscript{a}, Tingting Lu\textsuperscript{a}, Jing Yang\textsuperscript{a}, Chen Wang\textsuperscript{b, c}\textsuperscript{*}

\textsuperscript{a} Department of Anesthesiology, Children’s Hospital of Nanjing Medical University, Nanjing, China

\textsuperscript{b} Department of Anesthesiology, The Affiliated Suzhou Science & Technology Town Hospital of Nanjing Medical University, Suzhou, China

\textsuperscript{c} Department of Anesthesiology, Suzhou Hospital (West District) Affiliated to Nanjing Medical University, Suzhou, China

Running Title: Apelin-13 alleviated heart failure

*Corresponding author:

Chen Wang, M.D.

Department of Anesthesiology, The Affiliated Suzhou Science & Technology Town Hospital of Nanjing Medical University, 1 Lijiang Road, Gaoxin District Suzhou 215153, China

Tel: +86-0512-33322120, Fax: +86-0512-33322120

Email: chen_wangnj@163.com
Abstract

This study aimed to determine whether apelin-13 could attenuate cardiac fibrosis via inhibiting the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway to inhibit reactive oxygen species in heart failure (HF) rats. HF models were established by inducing ischemia myocardial infarction (MI) through ligation of the left anterior descending artery in Sprague-Dawley rats. MI-induced changes in hemodynamics and cardiac function were reversed by apelin-13 administration. The increases in the levels of collagen I, collagen III, α-smooth muscle actin (SMA), and transforming growth factor-β (TGF-β) in the heart of MI rats and cardiac fibroblasts (CFs) treated with angiotensin (Ang) II were inhibited by apelin-13. The levels of PI3K and p-Akt increased in Ang II–treated CFs, and these increases were blocked by apelin-13. The PI3K overexpression reversed the effects of apelin-13 on Ang II–induced the increases of collagen I, collagen III, α-SMA, and TGF-β, NADPH oxidase activity and superoxide anions in CFs. Apelin-13 reduced the increases in the levels of NADPH oxidase activity and superoxide anions in the heart of MI rats and CFs with Ang II treatment. The results demonstrated that apelin-13 improved cardiac dysfunction, impaired cardiac hemodynamics, and attenuated fibrosis of CFs induced by Ang II via inhibiting the PI3K/Akt signaling pathway to inhibit oxidative stress.

Key words Apelin-13, fibrosis, heart failure, phosphatidylinositol 3-kinase, protein kinase B, oxidative stress
Introduction

Chronic heart failure (CHF) was commonly caused by myocardial infarction (MI) [1]. Heart failure (HF) is preceded by ventricular remodelings such as changes of left ventricular (LV) mass and myocardial size after alterations in pressure-overload conditions [2]. Rats with coronary artery ligation-induced HF showed significantly impaired cardiac hemodynamics and cardiac dysfunction [3].

Cardiac fibrosis is a major driver of disease progression in CHF [4], and excessive fibrosis causes large infarct scars, resulting in cardiac dilatation and cardiac dysfunction [5,6]. Cardiomyocytes produce extracellular matrix proteins and therefore contribute to fibrosis. However, resident cardiac fibroblasts (CFs) are currently considered as the main source of fibrosis in the myocardium in response to ischemic injury [7]. CFs played a critical role in postinfarction remodeling, which can ultimately lead to pathological fibrosis and HF [8]. Cardiac fibrosis is a hallmark of HF for which no effective pharmacological therapy is available.

Apelin is a hormone peptide widely found in cardiovascular [9], adipose [10], cerebral [11], and pulmonary tissues [12]. Diverse active apelin peptides exist under the form of 36, 17 or 13 amino acids which originated from a preproteins consisted of 77 amino acid residues. Apelin-13 has the highest activity of these three active peptides, followed by apelin-17 then apelin-36 [13]. Apelin, together with its receptor APJ, is involved in cardiovascular diseases, diabetes, obesity, and cancer [10,14]. The expression levels of apelin were increased in many pathological states or disease processes such as cardiovascular and metabolic disorders [15]. Increased nitric oxide production through the apelin/APJ/protein kinase B (Akt)/endothelial
nitric oxide synthase (eNOS) pathway may, at least in part, contribute to the alleviating effect of losartan in unilateral ureteral obstruction–induced renal fibrosis in mice [16]. Apelin can be an important mediator of fibrogenesis in human liver diseases [17]. However, whether apelin is involved in regulating cardiac fibrosis in MI-induced HF is not well known.

The expression level of phosphatidylinositol 3-kinase (PI3K) and the phosphorylation level of Akt increased in infarcted myocardial tissues of mice [18]. The inhibition of PI3K/Akt signaling activity on treatment with LY294002 markedly reversed the protective effect of erythropoietin on the abdominal aortic constriction–induced myocardial fibrosis [19]. Apelin-13 promotes H9C2 rat cardiomyocyte hypertrophy via the PI3K/Akt signaling pathway and the autophagy induced by PI3K [20]. Angiotensin (Ang) II–stimulated collagen production is mediated through reactive oxygen species (ROS) generation in adult rat cardiac fibroblasts (CFs). Ang II activates ROS-sensitive kinases that are critical in mediating fibrotic remodeling of the heart [21]. This study was performed to determine whether apelin-13 improved cardiac function, hemodynamics, and fibrosis in rats with HF and whether apelin-13 attenuated cardiac fibrosis via inhibiting the PI3K/Akt signaling pathway to attenuate oxidative stress.

Materials and Methods

Animals

The experiments were carried out using male Sprague–Dawley (SD) rats (weighing 160–200 g; Vital River Biological Co., Ltd, Beijing, China). The experiments were carried out at Animal Core Facility of Nanjing Medical University. All procedures were approved by the Experimental Animal Care and Use Committee of Nanjing Medical University and conducted...
in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996). The rats were kept in a temperature-controlled room with a 12-h light–dark cycle with free access to standard chow and tap water.

**MI rat model**

The MI rat model in the present study was induced by coronary artery ligation using sterile techniques as reported in a previous study [22]. Briefly, the rats were anesthetized with isoflurane (2.5%). A ventilator connection with the gas anesthesia machine was used during establishing the MI rat model. Rats were randomly subjected to the ligation of the left anterior descending coronary artery and sham operation (Sham). The heart was exposed through a left intercostal thoracotomy, and the left coronary artery was looped with a single nylon suture. Finally, the heart was quickly repositioned into the chest. The rats in the sham group were treated in the same way as the rats with coronary ligation, except that their coronary arteries were not ligated.

**Animal grouping**

Rats were subjected to MI or Sham. At the same time, apelin-13 (10 nmol/kg/day, i.p., Phoenix Pharmaceuticals, CA, USA) [23] or saline was administered for 28 days. After 28 treatment with apelin-13, transthoracic echocardiography and hemodynamic monitoring were performed then sacrificed with an overdose of pentobarbital (100 mg·kg⁻¹, I.V.). The left ventricle (LV) was sectioned for masson staining and the remaining tissue was used for quantitative reverse-transcription PCR (qRT-PCR) and western blotting.

**Echocardiography**

After 4-weeks of MI and apelin-13 treatment, transthoracic echocardiography was
performed under isoflurane anesthesia using an ultrasound (Vevo 2100, VisualSonics, Toronto, Canada) with a 21-MHz probe. The left ventricular end-systolic diameter (LVESD), end-diastolic diameter (LVEDD), LV volumes in systole (LVVs) and diastole (LVVd), and LV mass were measured. The LV ejection fraction (EF) and fractional shortening (FS) were calculated. Measurements over three consecutive cardiac cycles were averaged.

**Hemodynamic monitoring**

The rats were anesthetized with isoflurane (2.5%), and a 1.4-F-conductance micromanometer-tip catheter (Millar Instruments, TX, USA) was inserted via the right carotid artery across the aortic valve and into the LV chamber. The LV end-diastolic pressure (LVEDP), LV systolic pressure (LVSP) and maximum of the first derivative of LV pressure (LV +dP/dt_{max}) were obtained on a PowerLab data acquisition system (AD Instruments, Sydney, Australia).

**Masson trichrome staining**

The rats were killed with an overdose of pentobarbital (100mg/kg, I.V.). The heart was removed after PBS Perfusion. Sections of the LV (5 µm) were examined by Masson’s trichrome staining (Biochannel Biotechnology Co., Ltd., Nanjing, China) according to the manufacturer’s protocol to determine the extent of fibrosis. Tissue sections from rat hearts were observed under a light microscope (Zeiss, Oberkochen, Germany). Images were analyzed using the Image-Pro Plus software (Media Cybernetics, Inc., MD, USA).

**Culture of CFs isolated from adult rats**

Adult CFs were obtained from male SD rats using two independent isolation procedures. The ventricular tissue was dissected, washed, minced, and subjected to seven times repeated
digestions at 37°C for 20 min in a solution containing a mixture of 1 mg/mL of collagenase A and 0.5 mg/mL hyaluronidase after an initial digestion step in a proteinase bacterial solution (4 U/mL) for 15 min. After each cycle of digestion, the tissue was mechanically dissociated using a wide-mouth pipet, the supernatant containing dissociated cells was collected, and the cells were resuspended in Iscove’s modified Dulbecco’s medium (IMDM). The cells from all digestions were pooled and resuspended in IMDM supplemented with 20% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), nonessential amino acids (1%), and 2-mercaptoethanol (0.1mM). The cells were plated and incubated for 2 h to allow for the preferential attachment of fibroblasts. CFs were used for experiments between passages 3 and 5. CFs were incubated with Ang II (10^{-6} M, Sigma, MO, USA) for 24 h to induce the fibrotic phenotype. CFs were assigned to four groups, including PBS group, Ang II group, Apelin-13 group, Ang II + Apelin-13 (10 μM) group.

**Western blot analysis**

The LV or cultured CFs were sonicated in RIPA lysis buffer and homogenized. The debris was removed, and the supernatant was obtained by centrifugation at 12,000g for 10 min at 4°C. Approximately 30–50 μg protein was separated by electrophoresis, transferred to a PVDF membrane, and probed with primary antibodies against collagen I, collagen III, transforming growth factor-β (TGF-β), and α-smooth muscle actin (SMA) (Abcam, MA, USA); PI3K, Akt, and p-Akt (Cell Signaling Technology, MA, USA); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Abcam) was used as an internal control. Images were analyzed using the Image-Pro Plus software.

**Real-time polymerase chain reaction**
RNA was isolated from cultured CFs using TRIzol (Thermo Fisher Scientific, Shanghai, China). Total RNA (0.5 μg) was reverse transcribed to cDNA. Real-time polymerase chain reactions were performed on an ABI Prism 7900 system. TaqMan probes to detect apelin, APJ, collagen I, collagen III, TGF-β, and α-SMA were purchased from Roche. All samples were amplified in triplicates for 45 cycles in a 384-well plate. The relative level of mRNA expression was expressed as $2^{-\Delta\Delta Ct}$. The primers are shown in Table 1.

**Measurement of NADPH oxidase activity**

The NAD(P)H oxidase activity in the heart was measured by enhanced lucigenin chemiluminescence. Briefly, the LV or CFs were sonicated in RIPA lysis buffer and homogenized. The debris was removed, and the supernatant was obtained by centrifugation at 12,000g for 10 min at 4°C. NAD(P)H (100 μM) was added to the supernatant as a substrate to react with NAD(P)H oxidase and generate superoxide anions. The light emission produced by the reaction of lucigenin (5 μM) with superoxide anions was measured with a microplate reader (BioTek, VT, USA) once every minute for 10 min. The values represented the NAD(P)H oxidase activity and were expressed as the mean light units (MLU) per minute per milligram of protein. The total protein in the supernatant was measured by a BCA protein assay kit (BioChannel Biotechnology Co., Ltd, Nanjing, China).

**Measurement of superoxide anions**

Superoxide anions level in the heart was determined by lucigenin-derived chemiluminescence. Briefly, the LV or CFs were sonicated in RIPA lysis buffer and homogenized. The debris was removed, and the supernatant was obtained by centrifugation at 12,000g for 10 min at 4°C. the reaction with superoxide anions was started by adding
dark-adapted lucigenin (5μM) to each supernatant to cause photon emission, which was measured with a microplate reader (BioTek, VT, USA) once every minute for 10 min. The values representing the superoxide anions level were expressed as the MLU per minute per milligram of protein. The total protein in the supernatant was measured by a BCA protein assay kit (BioChannel Biotechnology Co., Ltd, Nanjing, China).

**PI3K overexpression**

For overexpression experiments, adenovirus carrying PI3K (Ad-PI3K) coding sequence (GeneChem, Shanghai, China) was diluted in PBS and added into the media. The adenovirus carrying green fluorescent protein (GFP, Ad-GFP) was used as a control. Generally, CFs were infected with adenovirus at 50 multiplicity of infection (MOI) for 24h.

**Statistical analyses**

Data were presented as mean ± standard error of the mean. Using GraphPad Prism 5.0 (GraphPad software Inc., CA, USA), statistical significance among multiple groups was evaluated by one-way analysis of variance with the Bonferroni post-hoc test. A two-tailed P-value <0.05 was considered statistically significant.

**Results**

**Expression levels of apelin and APJ in the heart of MI rats**

The expression of apelin in the heart of MI rats was higher than sham rats. In addition, the level of APJ was increased in the heart of MI rats (Fig. 1).

**Effects of apelin-13 on cardiac function in rats with MI**

In rats with MI, LV \(+dP/dt_{max}\), LVSP, EF, and FS were reduced, and apelin-13 treatment enhanced the decreases of LV \(+dP/dt_{max}\), LVSP, EF, and FS in rats with MI. LVEDP, LVESD,
LVEDD, LVVs, and LVVd increased in rats with MI, which were reversed by apelin-13 treatment (Fig. 2).

**Effects of apelin-13 on cardiac fibrosis in rats with MI**

The Masson staining results showed that cardiac fibrosis increased in rats with MI, which was prevented by apelin-13 (Fig. 3A). The mRNA levels of collagen I, collagen III, and TGF-β increased in the heart of rats with MI, which was inhibited by apelin-13 treatment (Fig. 3B). The protein levels of collagen I, collagen III, and TGF-β increased in the heart of MI rats, and apelin-13 treatment attenuated the increases in the protein levels of collagen I, collagen III, and TGF-β in the heart of MI rats (Fig. 3C).

**Effects of apelin-13 on fibrosis in CFs**

The mRNA expression levels of collagen I, collagen III, TGF-β, and α-SMA were higher in the Ang II group compared with the phosphate-buffered saline (PBS) group in CFs. Treatment with apelin-13 inhibited the increases in the mRNA levels of collagen I, collagen III, TGF-β, and α-SMA induced by Ang II administration in CFs (Fig. 4A). The protein levels of collagen I, collagen III, TGF-β, and α-SMA were higher in the Ang II group, which was inhibited by apelin-13 treatment in CFs (Fig. 4B).

**Levels of PI3K/Akt**

The levels of PI3K and p-Akt increased in Ang II–treated CFs, and these increases were blocked by apelin-13CFs. However, no significant difference in the Akt level was found in the four groups (Fig. 5).

**Effects of PI3K overexpression**

PI3K expression level in Ad-PI3K-treated CFs was 3.14 times of that in control CFs. PI3K
overexpression reversed the effects of apelin-13 on Ang II-induced increases in the mRNA levels of collagen I, collagen III, TGF-β, and α-SMA in CFs (Fig. 6B and C). Furthermore, PI3K overexpression reversed the effects of apelin-13 on Ang II-induced increases in the protein levels of collagen I, collagen III, TGF-β, and α-SMA in CFs (Fig. 6D).

Effects of apelin-13 on ROS in CFs

NADPH oxidase activity and superoxide anions levels were higher in the heart of MI rats. Apelin-13 reduced the increases in the levels of NADPH oxidase activity and superoxide anions in the heart of MI rats (Fig. 7A). The levels of NADPH oxidase activity and superoxide anions were higher in CFs treatment with Ang II, and these increases were blocked by apelin-13 administration (Fig. 7B). The PI3K overexpression reversed the effects of apelin-13 on Ang II–induced increases in the levels of NADPH oxidase activity and superoxide anions in CFs (Fig. 7C).

Discussion

CHF is preceded by ventricular remodeling, and cardiac fibrosis is a major driver of disease progression in CHF [4]. Excessive fibrosis causes large infarct scars, resulting in cardiac dilatation and cardiac dysfunction [5,6]. Apelin is a detrimental mechanism that promotes liver fibrosis mainly via upregulating the expression of collagen I and platelet-derived growth factor receptor β. On the contrary, apelin is beneficial for renal fibrosis and pulmonary fibrosis [24]. The present study showed that apelin-13 improved cardiac dysfunction, impaired cardiac hemodynamics, and attenuated fibrosis of CFs in HF via inhibiting the PI3K/Akt signaling pathway.
In rats with left coronary artery ligation, the LV function reduced as indicated by the decreases in EF, infarct thickness, ± LV dP/dt, LV developed pressure, and end-diastolic pressure [25]. Apelin in the hypothalamic paraventricular nucleus can improve the cardiac function of rats with thoracic surgical trauma [26]. (3R)-5,6,7-Trihydroxy-3-isopropyl-3-methylisochroman-1-one protected cardiomyocytes against isoproterenol-induced MI, potentially via the apelin/apelin receptor signaling pathway [27]. The apelin/APJ system is vital in the regulation of myocardial contractility and blood pressure [27]. In the present study, the results showed that LV +dP/dt\textsubscript{max}, LVSP, EF, and FS reduced in rats with MI, and apelin-13 treatment enhanced the decreases in LV +dP/dt\textsubscript{max}, LVSP, EF, and FS. LVEDP, LVESD, LVEDD, LVVs, and LVVd increased in rats with MI, which were reversed by apelin-13 treatment. These results indicated that apelin-13 improved cardiac dysfunction and impaired cardiac hemodynamics in rats with HF, which is supported by previous study that apelin-13 treatment improved left ventricular function of MI rats [28].

Cardiac remodeling is an important mechanism for the occurrence and development of CHF [29]. CFs played a key role in postinfarction remodeling, which can ultimately lead to pathological fibrosis and HF [8]. Apelin ameliorated the expression of Ang II–induced TGF-β in primary cardiomyocytes, accompanied by reduced hypertrophy [30]. The present study found that the expression levels of collagen I, TGF-β, and α-SMA increased in the hearts of rats with MI, which were inhibited by apelin-13 treatment. The mRNA levels of collagen I, collagen III, TGF-β, and α-SMA were higher in the Ang II group compared with the PBS group in CFs. Treatment with apelin-13 inhibited the increases in the mRNA levels of collagen I, collagen III, TGF-β, and α-SMA induced by Ang II administration in CFs. These
results demonstrated that the increase in cardiac fibrosis in rats with HF was attenuated by apelin-13 treatment.

MI was associated with decreased activities of PI3K and signal transducer and activator of transcription 3 (STAT3) in aging rats compared with young rats [31]. The Akt signaling pathway was enhanced in CFs after Ang II treatment [32]. Apelin-13 blocked cisplatin-induced H9C2 cell apoptosis via the regulation of mitogen-activated protein kinases (MAPKs) and PI3K/Akt signaling pathway [33]. The present study showed that the levels of PI3K and p-Akt increased in Ang II–treated CF, and these increases were blocked by apelin-13. PI3K overexpression reversed the effects of apelin-13 on Ang II–induced increases in the mRNA levels of collagen I, collagen III, TGF-β, and α-SMA in CFs. The results indicated that apelin-13 alleviated fibrosis induced by Ang II via inhibiting the PI3K/Akt signaling pathway. However, previous study revealed that apelin-13 promoted the phosphorylation of PI3K and Akt to induce cardiomyocyte hypertrophy of H9C2 [20]. These findings indicated that apelin-13 showed protective effects in CFs on attenuating fibrosis via inhibiting PI3K/Akt pathway; while apelin-13 had adverse effects in cardiomyocytes on promoting hypertrophy via enhancing PI3K/Akt signaling.

Oxidative stress, defined as an excess production of reactive oxygen species (ROS), has been shown to play important roles in the pathophysiology of HF and cardiac remodeling [34]. Apelin/APJ played important roles in oxidative stress-related inflammatory diseases [35]. Whether apelin can attenuate HF via inhibiting ROS is not well known. In the present study, NADPH oxidase activity and superoxide anions levels were higher in the heart of MI rats and in CFs treatment with Ang II, and these increases were blocked by apelin-13 administration.
The PI3K overexpression reversed the effects of apelin-13 on Ang II–induced increases of NADPH oxidase activity and superoxide anions in CFs. The results demonstrated that apelin-13 attenuated oxidative stress via inhibiting the PI3K/Akt signaling pathway.

In conclusion, apelin-13 improved cardiac dysfunction, attenuated impaired cardiac hemodynamics, and alleviated fibrosis in rats with HF, and apelin-13 attenuated fibrosis of CFs induced by Ang II via inhibiting the PI3K/Akt signaling pathway to attenuate oxidative stress.

Conflict of interest
The authors declare that they have no competing interests.

Acknowledgments
This work was supported by the 23 group of the Science and Technology Program of Nanjing, China (No.201723005).

Author contribution
S.Z. and HL.G: conceptualization, method; H.W., D.X., TT.L.: analysis, investigation; S.Z. and C.W.: manuscript written; J.Y. and C.W.: manuscript revision.

Abbreviations
Akt, protein kinase B; Ang, angiotensin; CFs, cardiac fibroblasts; CHF, Chronic heart failure; EF, ejection fraction; eNOS, endothelial nitric oxide synthase; FS, fractional shortening; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HF, heart failure; IMDM, Iscove’s modified Dulbecco’s medium; LV, left ventricular; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVVd, left ventricular volumes in diastole; LVVs left ventricular volumes in systole; MI, myocardial infarction; MOI,
multiplicity of infection; PI3K, phosphatidylinositol 3-kinase; SD, Sprague-Dawley; SMA, smooth muscle actin; TGF, transforming growth factor

**Figure Legends**

Figure 1. Apelin and APJ expression in the heart of myocardial infarction (MI) rats. A, The expression of apelin was increased in the heart of MI rats. B, The expression of APJ was increased in the heart of MI rats. The results are expressed as mean ± standard error (N = 8). *P < 0.05 versus the Sham group.

Figure 2. Effects of apelin-13 on cardiac function in rats with myocardial infarction (MI). Apelin-13 reversed MI-induced the decreases in the maximum of the first derivative of left ventricular pressure (LV +dP/dtmax), LV systolic pressure (LVSP) ejection fraction (EF) and fractional shortening (FS), and the increases in the LV end-diastolic pressure (LVEDP), LV end-systolic diameter (LVESD), LV end-diastolic diameter (LVEDD), LV volumes in systole (LVVs), LV volumes in diastole (LVVd). The results are expressed as mean ± standard error (N = 8). *P < 0.05 versus the Sham + Saline group; #P < 0.05 versus the MI + Saline group.

Figure 3. Effects of apelin-13 on cardiac fibrosis in rats with myocardial infarction (MI). A, Apelin-13 attenuated cardiac fibrosis (blue) in MI rats. B, Apelin-13 reduced the mRNA levels of collagen I, collagen III, and transforming growth factor-β (TGF-β) in the heart of MI rats. C, Apelin-13 reduced the protein levels of collagen I, collagen III, and TGF-β in the heart of MI rats. The results are expressed as mean ± standard error (N = 8). *P < 0.05 versus the Sham + Saline group; #P < 0.05 versus the MI + Saline group.

Figure 4. Effects of apelin-13 on fibrosis induced by angiotensin (Ang) II in cardiac fibroblasts (CFs). A, Apelin-13 reduced the mRNA levels of collagen I, collagen III,
transforming growth factor-β (TGF-β), and α-smooth muscle actin (SMA) in the CFs induced by Ang II. B, Apelin-13 reduced the protein levels of collagen I, collagen III, TGF-β, and SMA in the CFs induced by Ang II. The results are expressed as mean ± standard error. *P < 0.05 versus the PBS group; #P < 0.05 versus the Ang II group.

Figure 5. Levels of phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling molecules. The levels of PI3K and p-Akt were increased in angiotensin (Ang) II–treated cardiac fibroblasts (CFs), and these increases were blocked by apelin-13. The results are expressed as mean ± standard error. *P < 0.05 versus the PBS group; #P < 0.05 versus the Ang II group.

Figure 6. Effects of phosphatidylinositol 3-kinase (PI3K) overexpression. A, The level of PI3K was increased in cardiac fibroblasts (CFs) treated with adenovirus carrying PI3K (Ad-PI3K). B and C, The increases in the mRNA levels of collagen I, collagen III, transforming growth factor-β (TGF-β), and α-smooth muscle actin (SMA) induced by angiotensin (Ang) II were reversed after PI3K overexpression in CFs. D, The increases in the protein levels of collagen I, collagen III, TGF-β, and SMA induced by Ang II were reversed after PI3K overexpression in CFs. The results are expressed as mean ± standard error. *P < 0.05 versus the PBS group; #P < 0.05 versus the Ang II group; &P < 0.05 versus the Ang II + apelin-13 group.

Figure 7. Effects of apelin-13 on reactive oxygen species levels. A, The increases in the levels of NADPH oxidase activity and superoxide anions induced by angiotensin (Ang) II were reduced after apelin-13 treatment in the heart of myocardial infarction (MI) rats. B, The increases in the levels of NADPH oxidase activity and superoxide anions induced by Ang II
were reduced after apelin-13 treatment in the cardiac fibroblasts (CFs). C, The PI3K overexpression reversed the effects of apelin-13 on Ang II–induced increases in the levels of NADPH oxidase activity and superoxide anions in CFs. The results are expressed as mean ± standard error. *P < 0.05 versus the Sham + Saline group (A) or PBS group (B and C); #P < 0.05 versus the MI + Saline group (A) or Ang II group (B and C); &P < 0.05 versus the Ang II + apelin-13 group.

References

1. Go AS, Mozaffarian D, Roger VL et al. Heart disease and stroke statistics--2014 update: a report from the American Heart Association. Circulation 2014; 129: e28-e292 DOI: 10.1161/01.cir.0000441139.02102.80

2. Raso A, Dirkx E, Philippen LE et al. Therapeutic Delivery of miR-148a Suppresses Ventricular Dilation in Heart Failure. Molecular therapy : the journal of the American Society of Gene Therapy 2019; 27: 584-599 DOI: 10.1016/j.ymthe.2018.11.011

3. Gan XT, Ettinger G, Huang CX et al. Probiotic administration attenuates myocardial hypertrophy and heart failure after myocardial infarction in the rat. Circulation Heart failure 2014; 7: 491-499 DOI: 10.1161/CIRCHEARTFAILURE.113.000978

4. Tarone G, Balligand JL, Bauersachs J et al. Targeting myocardial remodelling to develop novel therapies for heart failure: a position paper from the Working Group on Myocardial Function of the European Society of Cardiology. European journal of heart failure 2014; 16: 494-508 DOI: 10.1002/ejhf.62

5. Kong P, Christia P, Frangogiannis NG. The pathogenesis of cardiac fibrosis. Cellular and molecular life sciences : CMLS 2014; 71: 549-574 DOI:
6. Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. Nature reviews Molecular cell biology 2006; 7: 589-600 DOI: 10.1038/nrm1983

7. Tallquist MD, Molkentin JD. Redefining the identity of cardiac fibroblasts. Nature reviews Cardiology 2017; 14: 484-491 DOI: 10.1038/nrcardio.2017.57

8. Philip JL, Xu X, Han M et al. Regulation of cardiac fibroblast-mediated maladaptive ventricular remodeling by beta-arrestins. PloS one 2019; 14: e0219011 DOI: 10.1371/journal.pone.0219011

9. Yang P, Kuc RE, Brame AL et al. [Pyr(1)]Apelin-13(1-12) Is a Biologically Active ACE2 Metabolite of the Endogenous Cardiovascular Peptide [Pyr(1)]Apelin-13. Front Neurosci 2017; 11: 92 DOI: 10.3389/fnins.2017.00092

10. Wysocka MB, Pietraszek-Gremplewicz K, Nowak D. The Role of Apelin in Cardiovascular Diseases, Obesity and Cancer. Front Physiol 2018; 9: 557 DOI: 10.3389/fphys.2018.00557

11. Harford-Wright E, Gavard J. Apelin, the Devil Inside Brain Tumors. J Exp Neurosci 2018; 12: 1179069518759680 DOI: 10.1177/1179069518759680

10.1177_1179069518759680 [pii]

12. Fan XF, Xue F, Zhang YQ et al. The Apelin-APJ axis is an endogenous counterinjury mechanism in experimental acute lung injury. Chest 2015; 147: 969-978 DOI: S0012-3692(15)38945-5 [pii]

10.1378/chest.14-1426
13. Tatemoto K, Hosoya M, Habata Y et al. Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. Biochemical and biophysical research communications 1998; 251: 471-476 DOI: 10.1006/bbrc.1998.9489

14. Leung OM, Li J, Li X et al. Regulatory T Cells Promote Apelin-Mediated Sprouting Angiogenesis in Type 2 Diabetes. Cell Rep 2018; 24: 1610-1626 DOI: S2211-1247(18)31109-4 [pii] 10.1016/j.celrep.2018.07.019

15. O'Carroll AM, Lolait SJ, Harris LE et al. The apelin receptor APJ: journey from an orphan to a multifaceted regulator of homeostasis. J Endocrinol 2013; 219: R13-35 DOI: 10.1530/JOE-13-0227 JOE-13-0227 [pii]

16. Nishida M, Okumura Y, Oka T et al. The role of apelin on the alleviative effect of Angiotensin receptor blocker in unilateral ureteral obstruction-induced renal fibrosis. Nephron extra 2012; 2: 39-47 DOI: 10.1159/000337091

17. Melgar-Lesmes P, Casals G, Pauta M et al. Apelin mediates the induction of profibrogenic genes in human hepatic stellate cells. Endocrinology 2010; 151: 5306-5314 DOI: 10.1210/en.2010-0754

18. Yang W, Wu Z, Yang K et al. BMI1 promotes cardiac fibrosis in ischemia-induced heart failure via the PTEN-PI3K/Akt-mTOR signaling pathway. American journal of physiology Heart and circulatory physiology 2019; 316: H61-H69 DOI: 10.1152/ajpheart.00487.2018

19. Liu F, Wen Y, Kang J et al. Regulation of TLR4 expression mediates the attenuating
effect of erythropoietin on inflammation and myocardial fibrosis in rat heart.

International journal of molecular medicine 2018; 42: 1436-1444 DOI: 10.3892/ijmm.2018.3707

20. Xie F, Liu W, Feng F et al. Apelin-13 promotes cardiomyocyte hypertrophy via PI3K-Akt-ERK1/2-p70S6K and PI3K-induced autophagy. Acta biochimica et biophysica Sinica 2015; 47: 969-980 DOI: 10.1093/abbs/gmv111

21. Ohtsu H, Frank GD, Utsunomiya H et al. Redox-dependent protein kinase regulation by angiotensin II: mechanistic insights and its pathophysiology. Antioxidants & redox signaling 2005; 7: 1315-1326 DOI: 10.1089/ars.2005.7.1315

22. Gan XB, Duan YC, Xiong XQ et al. Inhibition of cardiac sympathetic afferent reflex and sympathetic activity by baroreceptor and vagal afferent inputs in chronic heart failure. PLoS One 2011; 6: e25784 DOI: 10.1371/journal.pone.0025784

PONE-D-11-12568 [pii]

23. Azizi Y, Imani A, Fanaei H et al. Post-infarct treatment with [Pyr1]apelin-13 exerts anti-remodelling and anti-apoptotic effects in rats' hearts. Kardiologia polska 2017; 75: 605-613 DOI: 10.5603/KP.a2017.0022

24. Huang S, Chen L, Lu L et al. The apelin-APJ axis: A novel potential therapeutic target for organ fibrosis. Clinica chimica acta; international journal of clinical chemistry 2016; 456: 81-88 DOI: 10.1016/j.cca.2016.02.025

25. Zhou Y, Richards AM, Wang P. MicroRNA-221 Is Cardioprotective and Anti-fibrotic in a Rat Model of Myocardial Infarction. Molecular therapy Nucleic acids 2019; 17: 185-197 DOI: 10.1016/j.omtn.2019.05.018
26. Zhang HH, Wang YJ, Zheng C et al. Apelin in the hypothalamic paraventricular nucleus improves cardiac function in surgical trauma rats. Sheng li xue bao: [Acta physiologica Sinica] 2018; 70: 99-105

27. Yeganeh-Hajahmadi M, Najafipour H, Farzaneh F et al. Effect of apelin on cardiac contractility in acute reno-vascular hypertension: The role of apelin receptor and kappa opioid receptor heterodimerization. Iran J Basic Med Sci 2018; 21: 1305-1315 DOI: 10.22038/IJBMS.2018.31361.7555

28. Zhang X, Hu W, Feng F et al. Apelin-13 protects against myocardial infarction-induced myocardial fibrosis. Molecular medicine reports 2016; 13: 5262-5268 DOI: 10.3892/mmr.2016.5163

29. Liu M, Ai J, Feng J et al. Effect of paeoniflorin on cardiac remodeling in chronic heart failure rats through the transforming growth factor beta1/Smad signaling pathway. Cardiovascular diagnosis and therapy 2019; 9: 272-280 DOI: 10.21037/cdt.2019.06.01

30. Sato T, Kadowaki A, Suzuki T et al. Loss of Apelin Augments Angiotensin II-Induced Cardiac Dysfunction and Pathological Remodeling. International journal of molecular sciences 2019; 20: DOI: 10.3390/ijms20020239

31. Lin CC, Chen SY, Lien HY et al. Targeting the PI3K/STAT3 axis modulates age-related differences in macrophage phenotype in rats with myocardial infarction. Journal of cellular and molecular medicine 2019: DOI: 10.1111/jcmm.14526

32. Wang L, Liu C, Chen X et al. Alamandine attenuates longterm hypertension-induced cardiac fibrosis independent of blood pressure. Molecular medicine reports 2019; 19:
33. Zhang P, Yi LH, Meng GY et al. Apelin-13 attenuates cisplatin-induced cardiotoxicity through inhibition of ROS-mediated DNA damage and regulation of MAPKs and AKT pathways. Free radical research 2017; 51: 449-459 DOI: 10.1080/10715762.2017.1313414

34. Tsutsui H, Kinugawa S, Matsushima S. Oxidative stress and heart failure. American journal of physiology Heart and circulatory physiology 2011; 301: H2181-2190 DOI: 10.1152/ajpheart.00554.2011

35. Zhou Q, Cao J, Chen L. Apelin/APJ system: A novel therapeutic target for oxidative stress-related inflammatory diseases (Review). International journal of molecular medicine 2016; 37: 1159-1169 DOI: 10.3892/ijmm.2016.2544
**A**

Sham

Saline

Apelin-13

**B**

Relative collagen I expression

Sham-Saline  MI-Saline  Sham+Apelin-13  MI+Apelin-13

Relative collagen III expression

Sham-Saline  MI-Saline  Sham+Apelin-13  MI+Apelin-13

Relative TGF-β expression

Sham-Saline  MI-Saline  Sham+Apelin-13  MI+Apelin-13

**C**

Collagen I

Sham-Saline  MI-Saline  Sham+Apelin-13  MI+Apelin-13

Collagen III

Sham-Saline  MI-Saline  Sham+Apelin-13  MI+Apelin-13

TGF-β

Sham-Saline  MI-Saline  Sham+Apelin-13  MI+Apelin-13

GAPDH

**D**

α-SMA

Sham-Saline  MI-Saline  Sham+Apelin-13  MI+Apelin-13

TGF-β

Sham-Saline  MI-Saline  Sham+Apelin-13  MI+Apelin-13
The images depict Western blot analysis and bar graphs comparing the expression of PI3K, p-Akt, Akt, and GAPDH under different treatments: PBS, Ang II, Apelin-13, Ang II + Apelin-13.

**Western Blot Analysis:**
- **PI3K:** The intensity of PI3K bands is compared across treatments with PBS showing the lowest expression, followed by Ang II, Apelin-13, and Ang II + Apelin-13 with the highest expression.
- **p-Akt:** The intensity of p-Akt bands follows a similar trend, with PBS having the lowest, Ang II intermediate, Apelin-13 and Ang II + Apelin-13 having the highest.
- **Akt:** The intensity of Akt bands decreases as follows: PBS > Ang II > Apelin-13 > Ang II + Apelin-13.
- **GAPDH:** The intensity of GAPDH bands is consistent across all treatments, indicating normalization for protein loading.

**Bar Graphs:**
- **PI3K/GAPDH:** The ratio of PI3K to GAPDH shows a significant increase with Ang II + Apelin-13 compared to PBS.
- **p-Akt/Akt:** The ratio of p-Akt to Akt decreases in a similar trend as the Western blot analysis, with Ang II + Apelin-13 showing a significant decrease.
- **Akt/GAPDH:** The ratio of Akt to GAPDH remains relatively constant across treatments.

These results suggest that the combination of Ang II and Apelin-13 has a significant impact on the expression of PI3K and p-Akt compared to other treatments.
| Gene     | Species | Forward primer      | Reverse primer              |
|----------|---------|---------------------|-----------------------------|
| Collagen I | Rat     | TCAAGATGGTGCGCGTTAC | CTGCAGTTGTTCTCAATCTG         |
| Collagen III | Rat    | CGAGATTAAGCAAGAGGAA | GAGGCTTTCTTACATACCAC        |
| α-SMA    | Rat     | GTCCAGACATCAGGGAGTAA| TCGGATACTTCAGCGTCAGGA        |
| TGF-β    | Rat     | CAGGGAGTAAGGGACACGA | ACAGCATCAGGAACCCAGAT         |
| GAPDH    | Rat     | GGCACAGTGCAAGGCTGGAATG | ATGGTGCTGAAGACGCCAGTA       |

α-SMA, α-smooth muscle actin; TGF-β, transforming growth factor-β; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.