Apelin-13 promotes cell proliferation in the H9c2 cardiomyoblast cell line by triggering extracellular signal-regulated kinase 1/2 and protein kinase B phosphorylation

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Abstract. Apelin-13 (APL-13), a peptide hormone that serves as a ligand for G-protein coupled receptors, has been demonstrated to be highly expressed in left ventricular hypertrophy rat models. It has been implicated in cardio-protection under pathological states. The present study aimed to assess the physiological proliferation effect of APL-13 in cultured H9c2 cardiomyoblast cells, and to elucidate the underlying mechanisms. Cell proliferation was determined by MTT assay. The extracellular signal-regulated kinase (ERK) 1/2 and protein kinase B (Akt) signaling pathway was identified, and protein expression levels were detected using western blot analysis. The results demonstrated that APL-13 markedly increased cell proliferation. Western blotting results suggested that APL-13 significantly enhanced the expression of phosphoinositide ERK1/2 and Akt activation in a dose-dependent manner. U0126 (10 μM; ERK1/2 inhibitor) and/or 10 μM LY294002 (Akt inhibitor) were used to help to determine the APL-signaling mechanism. As a result, LY294002 and U0126 partially blocked the APL-13 induced H9c2 proliferation. In conclusion, these data suggested that APL-13 has a proliferative effect on myocardium cells via the Akt and ERK1/2 signaling pathways, and provide potential novel pharmaceutical targets for cardiovascular disease.

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Introduction

Due to the limited ability of cardiac myocytes to proliferate, low levels of apoptosis can result in profound structural and functional consequences in the myocardium, leading to cardiac dysfunction and heart failure (HF). Therefore, novel therapeutic targets are required to investigate the reversal of HF pathogenesis.

Apelin (APL), isolated from the digestive juice of cattle by Tatemoto *et al* (1) in 1998, is a protein hormone derived from the adipose tissue family. APL-13 is a member of the APL endogenous peptide family, with powerful inotropic and cardio-protective properties (2). APL exerts its effects by binding and activating APL receptor (APJ, gene symbol APLNR), a member of the G-protein coupled receptor (GPCR) super-family. GPCRs are central to many endocrine pathways in the body, and represent a major therapeutic target class. Both APL and APJ receptors are widely distributed in most tissues, including the lung, heart, brain, skeletal muscle, kidney and liver. APL can promote proliferation in various cell types, as retinal Müller and retinal endothelial cells (3-7). Furthermore, APL-13 exerts a cardio-protective effect of the myocardium under pathological states, including myocardial infarction (MI) (8,9). However, to the best of our knowledge, there is currently no information about the effect of APL in H9c2 cells physiological conditions.

The signaling cascades of the mitogen activated protein kinase (MAPK) family, and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt), are believed to be involved in cell-cycle regulation, apoptosis and inflammation (10,11). A growing body of evidence suggests that the Akt and extracellular signal-regulated (ERK) signaling pathway serve important roles in the development of cardiac hypertrophy and progression to HF. Therefore, it was hypothesized that APL-13 may exert proliferative effects on H9c2 cells under physiological conditions, which may be mediated by the Akt and ERK1/2 signaling pathways.

The present study investigated the potential proliferative role of APL under physiological conditions in H9c2. The effect of exogenous recombinant APL on cell proliferation and phosphorylation of ERKs and Akt, and the underlying mechanisms, were examined.

Materials and methods

Reagents. APL-13 trifluoroacetate salt and MTT were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) was purchased from Hyclone; GE Healthcare Life Sciences (Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). LY294002 (Akt inhibitor) and U0126 (ERK1/2 inhibitor) were purchased from Selleck Chemicals (Houston, TX, USA). An anti-β-actin primary antibody (ab8226; 1:5,000) was purchased from Abcam (Cambridge, MA, USA,). Antibodies for phosphorylated (p)-Akt (Ser473; 4060S; 1:1,000), Akt (2920S; 1:1,000), p-ERK1/2 (4370S; 1:1,000) and ERK1/2 (9102S; 1:1,000) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. H9c2 cells were purchased from the Chinese Academy of Sciences (Shanghai, China) and were cultured in DMEM supplemented with FBS, 2 mM glutamine and 1% penicillin/streptomycin (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA). Cells were allowed to reach 80% confluence in complete DMEM and were incubated for an additional 24 h in serum-free medium prior to experimental treatments. Following this, H9c2 cells were treated with 0, 5, 25, 50, 100, 200, 400, 600, 800 or 1,000 nM APL-13 for 24 h (5).

In experiments involving kinase inhibitors, LY294002 and U0126 were dissolved in dimethyl sulfoxide (DMSO) and diluted with DMEM. The final concentration of DMSO was 1%, which had no effect on cell viability, and the final concentrations of LY294002 and U0126 were 10 μM (12). Drug solutions were freshly prepared prior to each experiment.

A pathologist blinded to the study reviewed 10 sections per culture dish. All images were obtained using an Olympus LCX100 Imaging system.

MTT assay of cell proliferation. Cell proliferation was evaluated by MTT assay (13). After synchronization for 24 h by serum starvation, cells were treated with APL-13 (50, 100 or 200 nM) for 24 h, following which 5 mg/ml MTT was added and cells were incubated for 4 h at 37˚C. Subsequently, the supernatant was removed from each well. The coloured formazan crystal produced from MTT was dissolved in 150 μl DMSO and the absorbance was measured at a wavelength of 490 nm using a microplate reader (Model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Percentage viability was calculated as the optical density (OD) of drug-treated sample/OD of control sample x100%, assuming that the absorbance of control sample was 100%.

Western blotting. Western blotting was used to determine the protein expression levels of p-Akt and p-ERK, as described previously (12). Briefly, H9c2 cells were seeded into 6-well culture plates. After a 24-h drug incubation, cells were collected and lysed for 30 min on ice in lysis buffer. Radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Jiangsu, China) was used to extract total protein from cultured cells. The quantity of protein extracted from the cells was measured using a Bicinchoninic Acid protein assay reagent kit (Pierce; Thermo Fisher Scientific, Inc.). An equal amount of total protein (80 μg per lane) from each sample was separated by 5-10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% non-fat dry milk in PBS with 0.05% Tween 20 and incubated overnight at 4˚C with primary antibodies followed by a 2 h incubation with HRP-conjugated goat anti-rabbit IgG (7074S; 1:5,000; Cell Signaling Technology, Inc., Danvers, MA, USA). The blots were developed using an enhanced chemiluminescence detection kit (EMD Millipore, Billerica, MA, USA) and visualized using a FluroChem E Imager (ProteinSimple; Bio-Techne, Minneapolis, MN, USA). Measurements to determine the relative densities were normalized to that of β-actin using Image J software (version 1.38x; National Institutes of Health, Bethesda, MD, USA) (14).

Statistical analysis. The data are presented as the mean ± standard deviation. Unpaired Student's t-test was used to compare values between two groups, and one-way analysis of variance was used to compare differences between more than two groups, followed by a Newman-Keuls post hoc test. Analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

APL-13 alone can enhance H9c2 proliferation. To determine whether APL-13 itself promotes H9c2 proliferation in the absence of other stimuli, H9c2 growth was assessed in response to various physiologically relevant concentrations of APL-13 (0, 5, 25, 50, 100, 200. 400, 600, 800 and 1,000 nM) by MTT assay. APL-13 treatment alone increased the percentage cell viability compared with the baseline constitutive release over a range of physiological extracellular concentrations from 50 to 600 nM, with a maximal effect at 200 nM (Fig. 1A). Microscopic examination confirmed these results (Fig. 1B).

APL-13 upregulates the protein expression levels of p-Akt and p-ERK. The Akt and ERK signaling pathways have been suggested to serve an important role in cell proliferation, differentiation, survival and apoptosis (15). To determine whether Akt and ERKs are implicated in APL-13-mediated proliferative activity, changes in the levels of p-Akt and p-ERK expression in H9c2 cells in the presence or absence of APL-13 were examined. As presented in Fig. 2, upregulation of p-Akt was observed after APL-13 treatment alone (50, 100 and 200 nM) in a dose-dependent manner when incubated for 24 h. In addition, APL-13 treatment increased the levels of p-ERK expression in a similar manner (Fig. 3).

APL-13-mediated H9c2 proliferation requires Akt and ERK signaling pathway activation. To further investigate

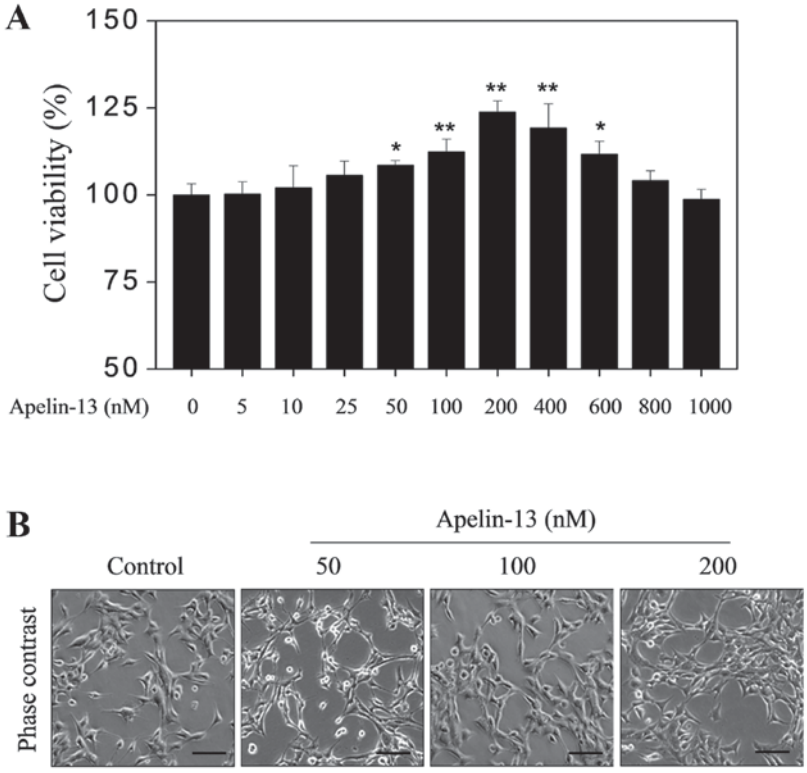

Figure 1. APL-13 promotes H9c2 cell proliferation. H9c2 cells were seeded into 96-well plate and treated with APL-13 for 24 h. (A) Quantification and (B) representative images of cell viability, as detected by MTT assay. Magnification, x40; scale bar=50 μm. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. control group (0 nM). APL, apelin.

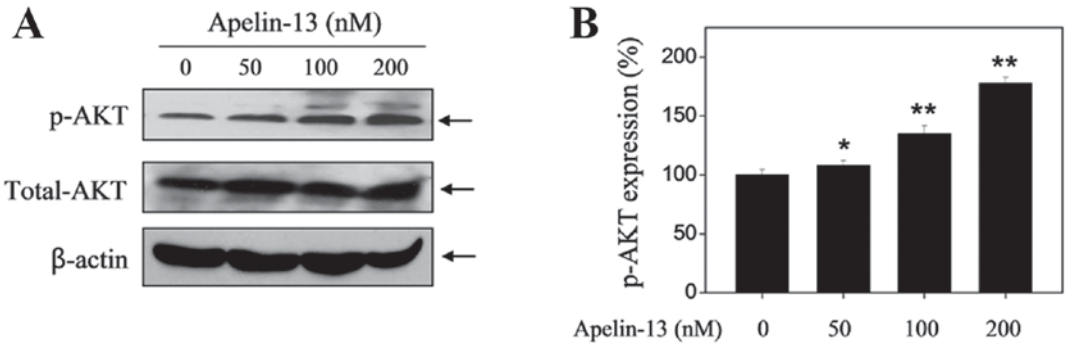

Figure 2. Apelin-13 triggers Akt phosphorylation in H9c2 cells. (A) Representative western blot images and (B) quantification of protein expression levels of p-Akt and Akt, as assessed by western blotting. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. control group (0 nM). Akt, protein kinase B; p, phosphorylated.

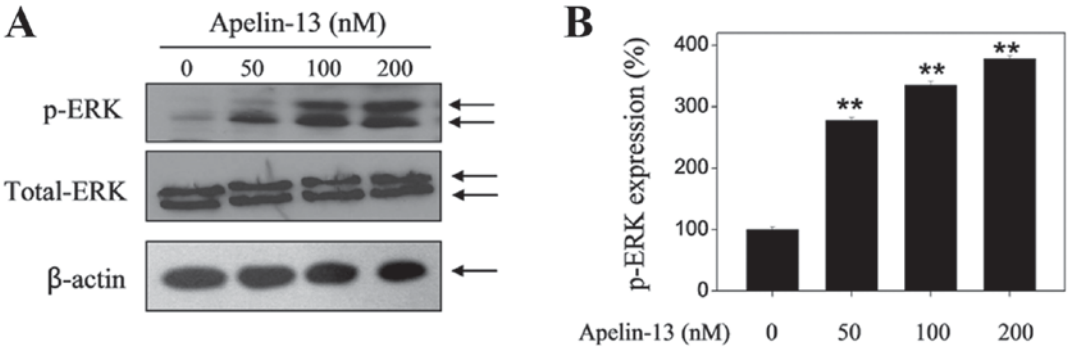

Figure 3. Apelin-13 triggers ERK phosphorylation in H9c2 cells. (A) Representative western blot images and (B) quantification of protein expression levels of p-ERK and ERK, as assessed by western blotting. Data are presented as the mean ± standard deviation. **P<0.01 vs. control group (0 nM). ERK, extracellular signal-regulated kinase; p, phosphorylated.

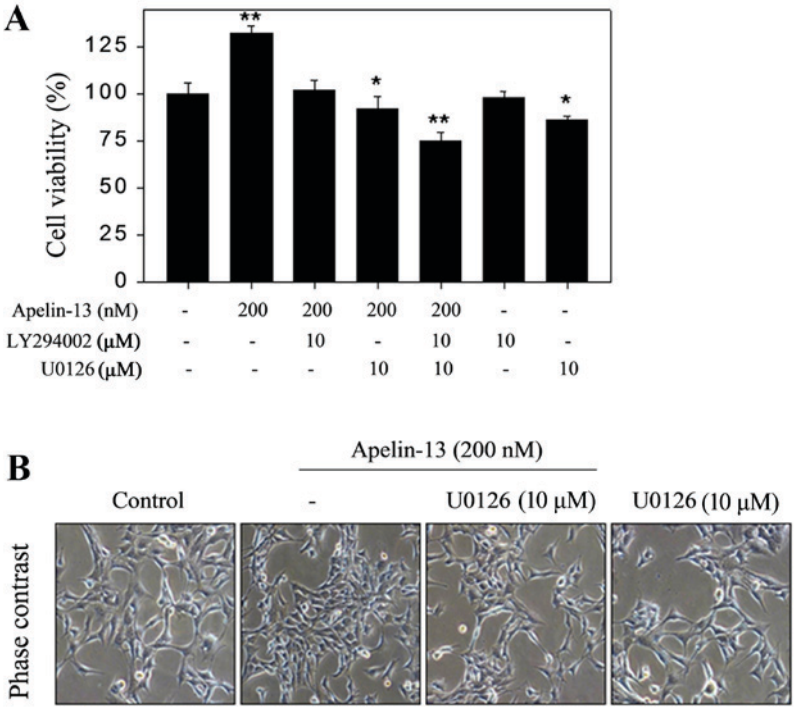

Figure 4. Role of ERK and AKT in APL-13-induced proliferation in H9c2 cells. H9c2 cells were pretreated with 10 μM LY294002 (Akt inhibitor) or 10 μM U0126 (ERK inhibitor) for 2 h, and then co-treated with APL-13 for 24 h. (A) Quantification and (B) representative images of cell viability, as detected by MTT assay. Magnification, x40; scale bar=50 μm. Data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01.

the molecular mechanism by which APL-13 induces H9c2 proliferation, H9c2 cells were pretreated with inhibitors of Akt (LY294002; 10 μM) and ERK1/ERK2 (U0126; 10 μM) 2 h before stimulation with APL-13 (200 nM) to determine whether ERK1/2 and Akt are involved in APL-13-induced cell proliferation. LY294002 and U0126 co-treatment markedly abolished APL-13 induced proliferation as determined by the MTT assay, while LY294002 or U0126 alone had no effect on H9c2 proliferation in cells not treated with APL-13. However, LY294002 reversed the pro-proliferative H9c2 activity of APL-13. Treatment with U0126 reversed APL-13-induced cell proliferation (Fig. 4). Taken together, these results suggest that the Akt and MAPK signaling pathways are involved in the mechanisms underlying the proliferative effects of apelin-13. Collectively, these data suggested that APL-13 mediates H9c2 growth via activation of the Akt and ERK1/2 signaling pathways.

Discussion

To the best of our knowledge, the present study demonstrated for the first time that APL-13 alone can enhance H9c2 cell proliferation, which is mediated by the ERK1/2 and Akt signaling pathway. APL has been reported to activate multiple protective mechanisms to prevent heart, brain, liver and kidney injury, thus rising to be a promising therapeutic target for ischemic and other associated diseases (16). APL-13 appears to be the predominant isopeptide in the human myocardium and plasma (17), and it has greater biological activity than APL-36 or -17, measured as the extracellular acidification rate in cultured cells expressing the APJ receptor (18). In particular, APL-13 serves a fundamental role in the occurrence and development of cardiovascular diseases.

A biological rationale for the potential use of APL as a therapeutic agent is confirmed by its low level in patients with acute coronary syndromes and established coronary artery disease. Thus, plasma APL concentration is considerably reduced in patients with acute MI in comparison with the control group, and this low level is maintained over time (19,20). Consistent with this, upregulation of myocardial APL mRNA expression during ischaemic insult is reverses in response to reperfusion in a rat model of MI (21). Its promise is heightened further by the observation that, unlike other and more established cardio-protective pathways, it appears to be downregulated in heart failure, suggesting that augmentation of this axis may significantly impact HF (17). Therefore, downregulation of APL may contribute to myocardial apoptosis in MI and HF, and supports the use of exogenous APL peptides for the treatment of HF. However, APL synthesis appears to occur predominantly in the endothelium, despite the broad distribution of APJ receptors (17). Therefore, it was hypothesized that prolonged exogenous supplementation of APL may encourage myocardial survival by combining with the APJ receptor in established HF. Based on the protective mechanism of APL-13 in response to various injury model and stimuli, the present study aimed to elucidate the key role of exogenous APL-13 in H9c2 cells. It was demonstrated that when H9c2 cells were incubated with various concentrations of APL-13, cell proliferation was significantly increased at concentrations from 50 to 600 nM, as determined by MTT assay, which is supported by the fact that defects in APL in mice induces age-dependent progressive cardiac dysfunction (22).

Subsequently, the cellular and molecular mechanisms underlying APL-13-induced H9c2 proliferation were examined. The PI3K/Akt and ERK1/2 signaling pathways serve an important role in cell proliferation, differentiation, survival and apoptosis, especially under various pathological states (12,23). In addition, APL has been reported to promote the phosphorylation of ERK and Akt in umbilical endothelial cells and vascular smooth muscular cells (6,7). Consist with this, the present study revealed that treatment of APL-13 alone stimulated ERK activation. Furthermore, APL-13 upregulated p-Akt and p-ERK1/2, indicating a potential involvement of Akt and ERK signaling pathways in APL-13-mediated H9c2 proliferation. To strengthen the potential conclusion, the present study further tested the effects of the specific inhibitors LY294002 and U0126 on the proliferative effects of APL-13. The results demonstrated that pretreatment of LY294002 or U0126 blocked APL-13-mediated H9c2 proliferation, further indicating that APL-13 exerts a neuroprotective activity via activating PI3K/Akt and MAPK signaling pathways.

Further studies are required to evaluate the functional effect of these novel analogs *in vivo*, with a Pressure-Volume curve device, and to further test their comparative cardio-protective potential in established experimental HF model. In addition, determining the therapeutic potential of augmenting APL signaling in patients with heart failure.

In conclusion, APL-13 upregulated p-Akt and p-ERK1/2 levels and increased H9c2 cell proliferation under physiological states, suggesting that Akt and ERK1/2 signals are involved in the mechanism of the proliferative role of APL. The present study expanded on current knowledge of APL-13 as an endogenous surviving signal, and implicate it as a potential treatment option for cardiovascular disease.

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