Acta Biochim Biophys Sin, 2015, 47(12), 969–980
doi: 10.1093/abbs/gmv111
Original Article

Original Article

Apelin-13 promotes cardiomyocyte hypertrophy via PI3K-Akt-ERK1/2-p70S6K and PI3K-induced autophagy

Feng Xie[1,†], Wei Liu[1,2,†], Fen Feng[1,†], Xin Li[1], Lu He[1], Deguan Lv[1], Xuping Qin[1], Lifang Li[3], Lanfang Li[1,*], and Linxi Chen[1,*]

[1]Institute of Pharmacy and Pharmacology, Learning Key Laboratory for Pharmacoproteomics, University of South China, Hengyang 421001, China, [2]Department of Pharmacy, The Third Xiangya Hospital of Central South University, Changsha 410013, China, and [3]Departments of Microbiology and Immunology, University of South China, Hengyang 421001, China

[†]These authors contributed equally to this work.
*Correspondence address. Tel: +86-734-8281587; Fax: +86-734-8281239; E-mail: lxchen6@126.com (L.C.)/Wjliu829@yahoo.com.cn (L.L.)

Received 15 May 2015; Accepted 12 August 2015

Abstract

Apelin is highly expressed in rat left ventricular hypertrophy Sprague Dawley rat models, and it plays a crucial role in the cardiovascular system. The aim this study was to clarify whether apelin-13 promotes hypertrophy in H9c2 rat cardiomyocytes and to investigate its underlying mechanism. The cardiomyocyte hypertrophy was observed by measuring the diameter, volume, and protein content of H9c2 cells. The activation of autophagy was evaluated by observing the morphology of autophagosomes by transmission electron microscopy, observing the subcellular localization of LC3 by light microscopy, and detecting the membrane-associated form of LC3 by western blot analysis. The phosphatidylinositol 3-kinase (PI3K) signaling pathway was identified and the proteins expression was detected using western blot analysis. The results revealed that apelin-13 increased the diameter, volume, and protein content of H9c2 cells and promoted the phosphorylation of PI3K, Akt, ERK1/2, and p70S6K. Apelin-13 activated the PI3K-Akt-ERK1/2-p70S6K pathway. PI3K inhibitor LY294002, Akt inhibitor 1701-1, ERK1/2 inhibitor PD98059 attenuated the increase of the cell diameter, volume, protein content induced by apelin-13. Apelin-13 increased the autophagosomes and up-regulated the expressions of beclin 1 and LC3-II/I both transiently and stably. The autophagy inhibitor 3MA ameliorated the increase of cell diameter, volume, and protein content that were induced by apelin-13. These results suggested that apelin-13 promotes H9c2 rat cardiomyocyte hypertrophy via PI3K-Akt-ERK1/2-p70S6K and PI3K-induced autophagy.

Key words: apelin, APJ, cardiomyocyte hypertrophy, PI3K, autophagy

Introduction

Apelin is the endogenous ligand for APJ (putative receptor protein related to the angiotensin receptor AT1) and plays a crucial role in the cardiovascular system [1]. Several apelin peptides, such as apelin-77, apelin-36, apelin-17, apelin-13, and apelin-12, have been shown to be present *in vivo*, all of which have different effects on the APJ receptor due to their different sizes [2]. Apelin-13 that acts as a mature apelin peptide, is the most commonly studied and has comparable affinity and agonist activity with the native apelin receptor in human tissues [3]. Apelin-13 is the predominant apelin isoform in human cardiac

tissue [4] and plays a protective role in myocardial contraction, blood pressure regulation [5,6], myocardial injury [7], and so on. In addition to its protective role [8–10], apelin-13 promotes vascular smooth muscle cell (VSMC) proliferation [11–13] and endothelial cell (EC) adhesion [14–16]. In myocardial hypertrophy, an altered balance of apelin/APJ is observed [17], but the regulatory mechanisms for the apelin system in the hypertrophic cardiac system have not been fully investigated. Moreover, apelin has been reported to prevent cardiac hypertrophy induced by oxidative stress [18] or high-fat diets [19]. APJ-null mice displayed resistance to chronic pressure overload by markedly reducing myocardial hypertrophy and heart failure [20–22], indicating that the apelin/APJ system plays a complex role in myocardial hypertrophy. Thus, it is important to explore the function and regulatory mechanisms of apelin in myocardial hypertrophy.

At cellular and molecular levels, intracellular signal transduction pathways play an important role in the development of myocardial hypertrophy. Phosphatidylinositol 3-kinase (PI3K), Akt, ERK1/2, and p70S6K are signal transduction proteins that are crucial for many aspects of cell growth, survival and apoptosis, and are involved in some pathological processes [23]. In the myocardial context, PI3K/Akt indirectly regulates calcium channels [24], cardiac muscle contraction, and myocardial hypertrophy [25,26]. It also mediates cell growth and proliferation [27], protein synthesis [28], and apoptosis [29].

Autophagy is a degradation pathway involving lysosomes and is essential for survival, differentiation, development, and homeostasis. Autophagy not only possesses these physiological functions but also plays a role in the initiation and prevention of human diseases [30,31]. In the heart, the level of autophagy is altered in response not only to stress, such as ischemia/reperfusion [32–34], but also to stress triggered by cardiovascular diseases, such as cardiac hypertrophy [35] and heart failure [30,36]. The PI3K/Akt and mTOR/p70S6 kinase pathways contribute to survival signaling by inducing cell autophagy. ERK1/2 and p70S6k also regulate autophagy [37–39]. In myocardial hypertrophy, whether autophagy can be regulated by PI3K, Akt, or p70S6k and how they regulate the process of myocardial hypertrophy are still unknown.

In the present study, it is hypothesized that apelin-13 promotes H9c2 rat cardiomyocyte hypertrophy via the PI3K-Akt-ERk1/2-p70S6k and PI3K-induced autophagy pathways. By using H9c2 cells that are cardiac myoblasts from rat [40], we demonstrated that apelin-13 increased the diameter, volume and protein content of H9c2 cells and promoted the apoptosis of H9c2 cells. Apelin-13 activated the PI3K-Akt-ERk1/2-p70S6k pathway, and the PI3K inhibitor LY294002, the Akt inhibitor 1701-1, and the ERK inhibitor PD98059 significantly inhibited the increase of the diameter, volume, and protein content of cells induced by apelin-13. The PI3K/Akt pathway regulated autophagy induced by apelin-13 over a long period of time, and class III phosphoinositide-3 kinases (PI3Ks) transiently regulated autophagy induced by apelin-13. The autophagy inhibitor 3MA blocked the hypertrophy induced by apelin-13, suggesting that apelin-13 promotes hypertrophy in H9c2 rat cardiomyocytes via the PI3K-Akt-ERK1/2-p70S6k and PI3K-induced autophagy pathways.

Materials and Methods

Cell culture

H9c2 cells (passage numbers 16–21) were purchased from the American Type Culture Collection (ATCC, Manassas, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, USA) supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 humidified atmosphere incubator.

Cardiomyocyte hypertrophy detection

The morphology of H9c2 cells was observed using an inverted microscope, and cardiomyocyte hypertrophy was evaluated by measuring the diameter, volume, and protein content of the H9c2 cells. The diameter and volume of the H9c2 cells were determined by a Scepter^{TM} Handheld Automated Cell Counter (Millipore, Billerica, USA), and the protein content was measured by using a BCA protein assay kit (Hyclone, Rockford, USA).

The detail method has been described previously [22]. Briefly, 1×10^4 cells per well were seeded in 6-well cell culture plates. After drug treatment, the cells were digested and collected by centrifugation at 100 *g* for 5 min. Then, the cells were resuspended and appropriately diluted in phosphate buffered saline (PBS) so that the concentration was approximately 5×10^4–1×10^5 cells/ml, and the single-cell suspension was placed in a 2-ml microcentrifuge tube. The diameter and volume of the H9c2 cells were detected using a ScepterTM Handheld Automated Cell Counter.

Transmission electron microscopy (TEM) analysis

The cells were collected and fixed with 5% glutaraldehyde in 0.1 M phosphate buffer for 2 h and then washed three times with 0.1 M PBS, followed by post-fixation with 1% osmic acid for 2 h. Then cells were dehydrated with graded alcohol and embedded in acetone-embedding liquid (1:1) for 12 h and then in embedding liquid for 12 h. Ultrathin sections were then cut and stained with 3% uranyl acetate and lead nitrate. The images were captured and analyzed using an FEI Tecnai G2 Spirit transmission electron microscope (FEI, Hillsboro, USA).

Immunofluorescence detection of the cell number with punctate LC3

About 2×10^5 cells per well were seeded into 6-well culture plates. When the cells grew to 80% confluence, they were washed twice with PBS, fixed with 4% polyformaldehyde for 20 min, and then blocked with bovine serum albumin (BSA) for 1 h at room temperature. The cells were then incubated with the anti-LC3 primary antibody (1:100; Epitomics, Burlingame, USA) for 2 h, washed 3 times with PBS, and incubated with fluorescein-conjugated anti-rabbit IgG secondary antibody (1:1000; Invitrogen, Carlsbad, USA). The number of punctate LC3 structures per cell was then determined using immunofluorescence to accurately measure the autophagosome number.

Delivery of GFP-LC3 into lysosome

Cells were transfected with a plasmid expressing GFP-LC3 using Attractene Transfection Reagent (Qiagen, Hilden, Germany) for 24 h at 37°C. Then, the cells were treated with different drugs (apelin-13; LY294002; apelin-13+LY294002; and DMSO) for 24 h. After being washed with Hanks' Balanced Salt Solution, the cells were immediately stained with LysoTracker Red dye (Beyotime Biotechnology, Shanghai, China) for 30 min at 37°C, and the expression of GFP-LC3 in cells was visualized using fluorescence microscopy.

LC3 turnover assays

About 2×10^5 cells per well were seeded in 6-well culture plates. When the cells grew to 80% confluence, they were treated with different drugs (apelin-13; LY294002; apelin-13+LY294002; and DMSO) for 24 h and then incubated with or without the lysosomal inhibitor hydroxychloroquine for 30 min, fixed with 4% polyformaldehyde for 20 min, and then blocked with BSA for 1 h at room temperature.

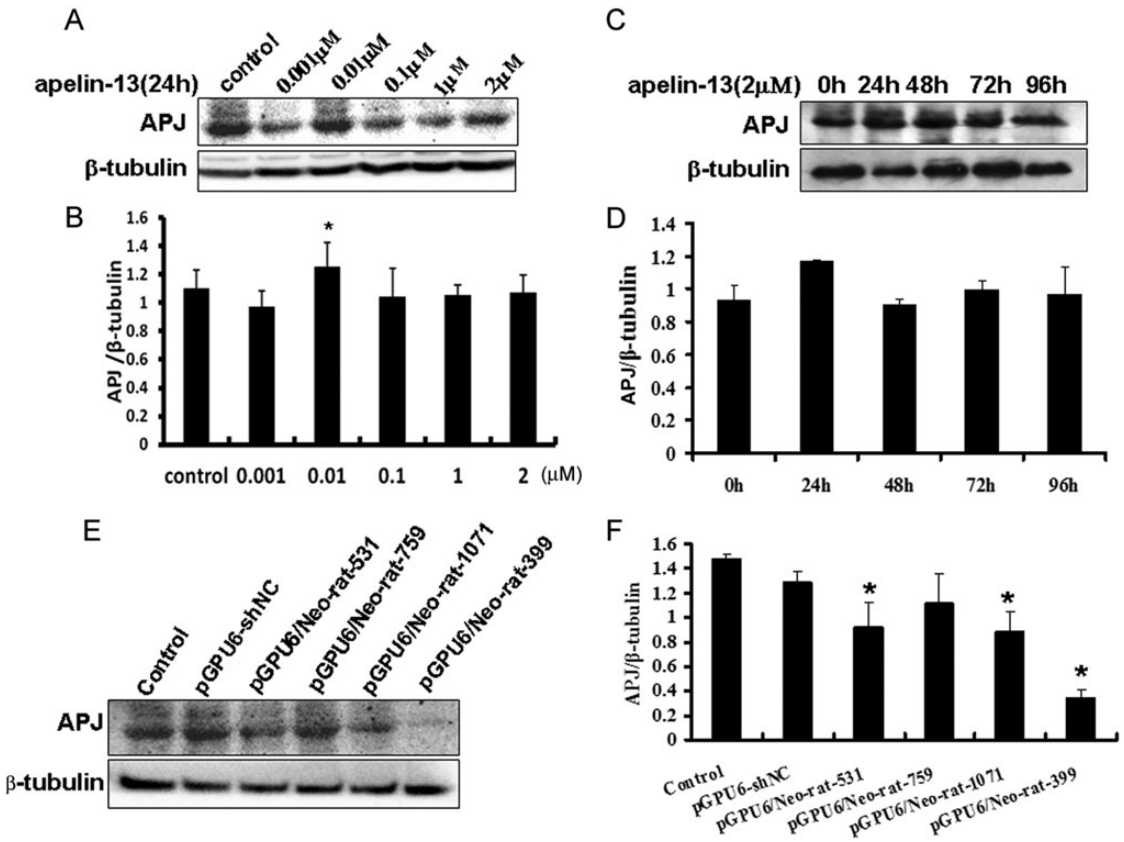

Figure 1. Apelin-13 has no effect on the expression of APJ The expression of APJ is up-regulated at 0.01 and 2 μM when cells were treated with different concentrations of apelin-13 (A,B). The expression of APJ was not changed when cells were treated for different time (C,D). Four APJ shRNAs were designed and synthesized to knock down the expression of APJ, and the most efficient shRNA, pGPU6/Neo-rat-399, was chosen for further study (E,F). The data are shown as the mean ± SD, $n = 4$. $^*P < 0.05$ vs. the control group.

Next, the cells were incubated with the anti-LC3 primary antibody (1:100) for 2 h, washed 3 times with PBS, and then incubated with Alexa 488-conjugated anti-rabbit IgG secondary antibody (Invitrogen). LC3 expression in cells was then detected by fluorescence microscopy.

Western blot analysis

Cells were washed twice with ice-cold PBS and lysed with RIPA lysis buffer containing phenylmethanesulfonyl fluoride (PMSF) (9:1). After centrifugation at 1613 *g* for 30 min, the supernatants were collected, and their protein concentrations were determined using a BCA protein assay kit (Hyclone). Aliquots containing 30 μg of protein were electrophoresed in 12% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). The membranes were then blocked with BSA for 2 h at room temperature. The proteins were detected with primary antibodies against the following proteins: APLNR (C-17) (Santa Cruz Biotechnology, Santa Cruz, USA), PI3K (Epitomics), p-PI3K (Bioworld Technology, St Louis, USA), Akt1 (Epitomics), p-Akt(S473) (Bioworld Technology), ERK1/2 (Abzoom Biolabs, Dallas, USA), p-ERK1/2 (Santa Cruz Biotechnology), p70S6Kinase (N-term) (Epitomics), p-p70S6 kinase α (A-6) (Santa Cruz Biotechnology), LC3A/B (Epitomics), beclin-1 (H-300) (Santa Cruz Biotechnology), and SQSTM1/p62 (Cell Signaling Technology, Beverly, USA). The membranes were incubated with primary antibody for 2 h and then with the corresponding horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody. The densitometric analyses of the proteins were carried out using analysis software (AlphaImager 2200, San Leandro, USA), and the protein levels were quantified by calculating the ratio of their expression levels to that of β-tubulin.

Table 1. Apelin-13 promotes cardiomyocyte hypertrophy by APJ

Group	Diameter (μm/cell)	Volume (10^3 μm^3/cell)	Protein (pg/cell)
Control	13.87 ± 1.24	1.42 ± 0.39	676 ± 23
Apelin-13	16.47 ± 1.41*	2.37 ± 0.63*	944 ± 76*
Apelin-13+pGPU6/ Neo-rat-399	14.30 ± 1.00$^\#$	1.43 ± 0.35$^\#$	487 ± 57$^\#$
Apelin-13+pCDNA-APJ	17.20 ± 0.90$^\#$	2.67 ± 0.39$^\#$	996 ± 84$^\#$
Apelin-13+pGPU6-shNC	16.70 ± 0.20	1.85 ± 0.09	941 ± 60
Apelin+pCDNA	16.15 ± 1.60	2.21 ± 0.47	943 ± 74
pGPU6/Neo-rat-399	12.80 ± 0.90*	1.11 ± 0.22*	391 ± 69*
pCDNA-APJ	15.70 ± 0.33*	2.08 ± 0.23*	832 ± 54*
pGPU6-shNC	13.10 ± 0.30	1.17 ± 0.08*	1074 ± 138*
pCDNA	14.62 ± 0.44	1.64 ± 0.15	477 ± 146

Apelin-13: 2 μM; time: 96 h; pGPU6/Neo-rat-399: APJ shRNA plasmid; and pGPU6-shNC: negative-shRNA plasmid. The data are expressed as the mean ± SD of six independent experiments. $^*P < 0.05$ vs. control group; $^\#P < 0.05$ vs. apelin-13 group.

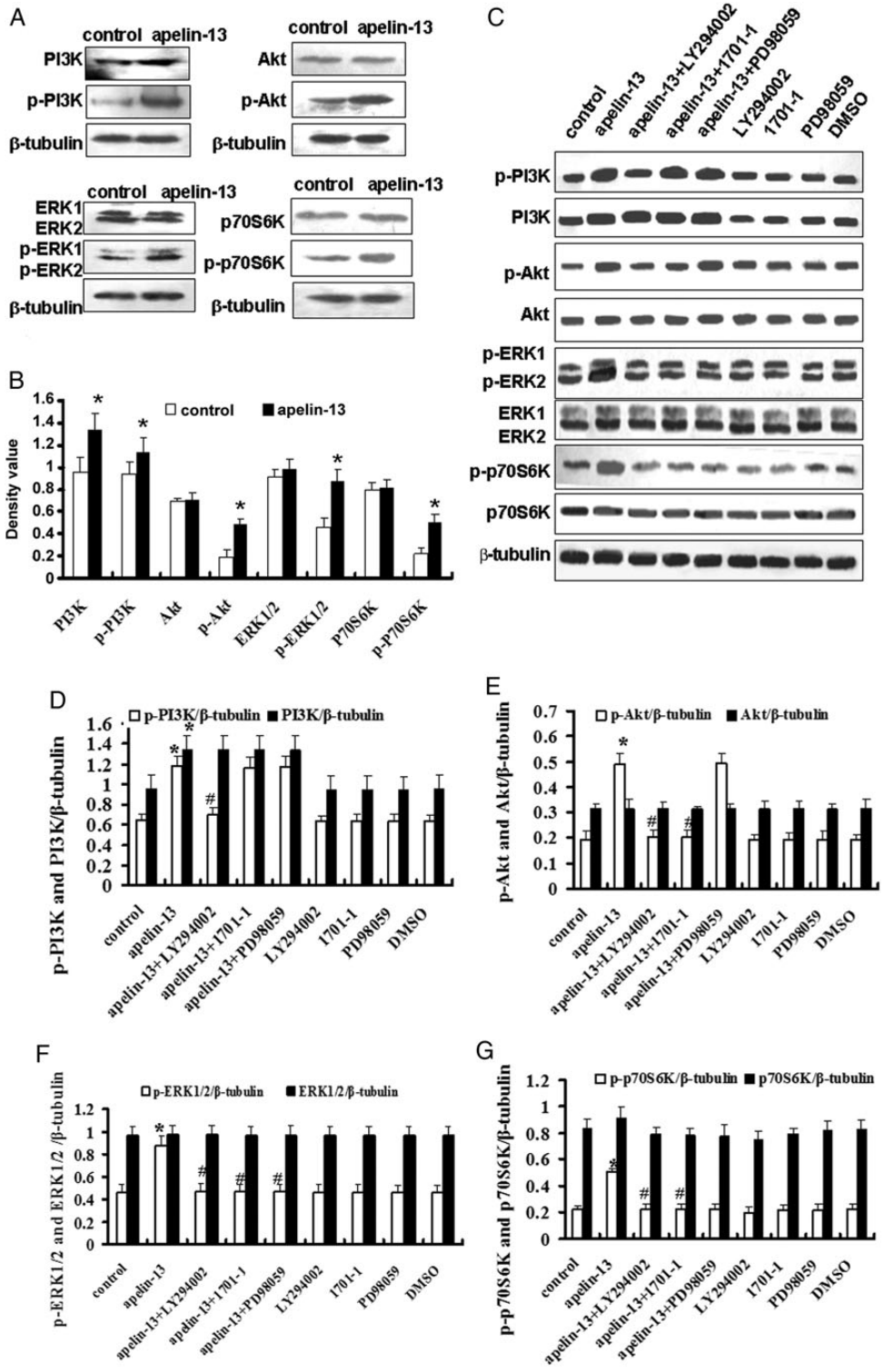

Figure 2. Apelin-13 activates the PI3K-Akt-ERK1/2-p70S6K pathway in H9c2 rat cardiomyocytes Apelin-13 up-regulated PI3K and enhanced the phosphorylation of PI3K (p-PI3K), the phosphorylation of Akt (p-Akt), the phosphorylation of ERK1/2 (p-ERK1/2), and the phosphorylation of p70S6K (p-p70S6K) (A). LY294002 (25 μM) decreased the enhancement of p-PI3K, p-Akt, p-ERK1/2, and p-p70S6K that were induced by apelin-13 (B–G). 1701-1 (10 μM) significantly blocked the up-regulation of p-Akt, p-ERK1/2, and p-p70S6K that were induced by apelin-13. PD98059 (10 μM) significantly attenuated the high levels of p-ERK1/2 and p-p70S6K that were induced by apelin-13. The data are shown as the mean ± SD, $n = 4$. $^{*}P < 0.05$ vs. the control group; $^{\#}P < 0.05$ vs. the apelin-13-treated group.

Table 2. The PI3K inhibitor LY294002, Akt inhibitor 1701-1, and ERK1/2 inhibitor PD98059 block the increases in the diameter, volume, and protein content induced by apelin-13

Group	Diameter (μm/cell)	Volume (10^3 μm^3/cell)	Protein (pg/cell)
Control	14.2 ± 1.3	1.52 ± 0.38	720 ± 39
Apelin-13	16.7 ± 1.5*	2.47 ± 0.55*	895 ± 67*
Apelin-13+LY294002	14.7 ± 1.1#	1.68 ± 0.34#	741 ± 43#
Apelin-13+1701-1	14.4 ± 1.6#	1.58 ± 0.32#	751 ± 45#
Apelin-13+PD98059	15.0 ± 0.9#	1.77 ± 0.23#	764 ± 52#
LY294002	13.8 ± 1.4	1.38 ± 0.19	698 ± 34
1701-1	14.0 ± 1.5	1.45 ± 0.24	710 ± 36
PD98059	13.9 ± 1.2	1.42 ± 0.28	716 ± 31
DMSO	14.1 ± 1.1	1.48 ± 0.22	719 ± 40

Apelin-13: 2 μM; time: 96 h; LY294002: PI3K inhibitor; 1701-1: Akt inhibitor; PD98059: ERK1/2 inhibitor. The data are expressed as the mean ± SD of six independent experiments. *$P < 0.05$ vs. control group; #$P < 0.05$ vs. apelin-13 group.

Statistical analysis

Data are presented as the mean ± SD of the indicated number of measurements. A one-way ANOVA test was used to determine significance. $P < 0.05$ was considered to be statistically significant.

Results

Apelin-13 has no effect on the expression of APJ in cardiomyocytes

Apelin-13 acts as the ligand of the APJ receptor and may regulate the expression of APJ. To detect the change of APJ expression in cells treated with apelin-13, cells were treated with different concentrations of apelin-13 (0, 0.001, 0.01, 0.1, 1, and 2 μM) for 24 h. Western blot analysis results showed that apelin-13 up-regulated the expression of APJ at 0.01 and 2 μM (**Fig. 1A,B**). Then cells were stimulated with apelin-13 (2 μM) for different time (0, 24, 48, 72, and 96 h), and western blot analysis results showed that the APJ expression did not change (**Fig. 1C,D**). These results suggested that apelin-13 does not affect the expression of APJ (Fig. 1).

Apelin-13 (2 μM) was used to stimulate H9c2 cells for 96 h and cardiomyocyte hypertrophy was detected by measuring the diameter, volume, and the protein content of cells (Table 1). The results showed that 2 μM of apelin-13 alone significantly increased the cell diameter, volume, and protein content compared with the control group. To further explore the role of APJ in heart induced by apelin-13, four APJ shRNAs were designed and synthesized to knock down the expression of APJ. The most efficient APJ shRNA, pGPU6/Neo-rat-399, was chosen for further study (**Fig. 1E,F**). The results showed that APJ shRNA blocked the increase of the cell diameter, volume, and protein content that was induced by apelin-13, while APJ shRNA treatment alone had little effect on these factors compared with the control group (Table 1). Thus, the role of APJ in the cardiomyocyte hypertrophy is controversial.

Apelin-13 promotes cardiomyocyte hypertrophy via the PI3K-Akt-ERK1/2-p70S6K pathway

PI3k, Akt, ERK1/2, and p70S6K are crucial to many aspects of cell growth, survival, and apoptosis, and are involved in some processes that contribute to pathology [23], especially myocardial hypertrophy. A specific PI3K inhibitor LY294002, a specific Akt inhibitor 1701-1, and a specific ERK1/2 inhibitor PD98059 were used to stimulate H9c2 cells and then their effects on the expressions of PI3K, p-PI3K, Akt, p-Akt, ERK1/2, p-ERK1/2, p70S6K, and p-p70S6K in H9c2 rat cardiomyocytes induced by apelin-13 were detected. Western blot analysis results showed that apelin-13 up-regulated PI3K expressing and enhanced the phosphorylation of PI3K, Akt, ERK1/2, and p70S6K (**Fig. 2A**). The PI3K-specifiic inhibitor LY294002 (25 μM) inhibited the PI3K, Akt, ERK1/2, and p70S6K phosphorylation enhancement induced by apelin-13 (**Fig. 2C–G**). The Akt-specific inhibitor 1701-1 (10 μM) significantly blocked the up-regulation of p-Akt, p-ERK1/2, and p-p70S6K expressions induced by apelin-13 (**Fig. 2B–G**). The ERK inhibitor PD98059 (10 μM) significantly attenuated the high level of p-ERK1/2 and p-p70S6K induced by apelin-13 (**Fig. 2C–G**). These data suggested that apelin-13 can activate the PI3K-Akt-ERK1/2-p70S6K pathway in H9c2 rat cardiomyocytes.

The PI3K-Akt-ERK1/2-P70S6K pathway is involved in H9c2 rat cardiomyocyte hypertrophy induced by apelin-13

LY294002 (25 μM), 1701-1 (10 μM), and PD98059 (10 μM) were used to stimulate H9c2 rat cardiomyocytes, and the changes of the diameter, volume, and protein content of the H9c2 rat cardiomyocytes induced by apelin-13 (2 μM, 96 h) were detected. The results showed that 2 μM of apelin-13 alone significantly increased the cell diameter, volume, and protein content, compared with the control group ($P < 0.05$). Compared with the apelin-13 (2 μM)-alone group, the groups that were pre-incubated with 25 μM of LY294002, 10 μM of 1701-1, or 10 of μM PD98059 prior to the addition of 2 μM apelin-13 displayed a significant reduction in cell diameter, volume, and protein content (Table 2). These results showed that LY294002, 1701-1, and PD98059 all blocked the increase of diameter, volume, and protein content of H9c2 rat cardiomyocytes induced by apelin-13.

Apelin-13 induces cardiomyocyte autophagy via the PI3K pathway

Autophagy is a process by which cells recycle cytoplasm and defective organelles during stress situations such as nutrient starvation. Autophagy is not only a physiological process but also a pathological process, and it has been reported that autophagy is altered in cardiac hypertrophy [35]. Autophagy may be related to the process of cardiac hypertrophy that is induced by apelin-13. Different concentrations of apelin-13 (control, 0.001, 0.01, 0.1, 1, and 2 μM) were used to simulate H9c2 rat cardiomyocytes for 24 h, and western blot analysis results showed that the expressions of the autophagy markers LC3-II/I and beclin-1 were increased and that the level of the substrate protein p62 was decreased in a concentration-dependent manner (**Fig. 3A,B**). siRNA was used to knock down the APJ receptor and results showed that APJ siRNA reversed the increase of LC3-II/I and beclin-1 that was induced by apelin-13 (**Fig. 3C,D**). Apelin-13 was then used to stimulate cells for different time (0, 3, 6, 12, and 24 h) and results showed that apelin-13 up-regulated the expressions of LC3-II/I and beclin-1 and reduced the level of p62 at 12 and 24 h (**Fig. 3E,F**). These results suggested that apelin-13 induces cardiomyocyte autophagy for an extended time. Interestingly, the expression of beclin-1 exhibited an early increase at 3 h. So there may be another way to increase the expression of beclin-1 transiently. Then apelin-13 was used to stimulate cells for a short time (0, 15 min, 30 min, 1, 2, and 4 h). Results showed that apelin-13 increased the expression of beclin-1 at 30 min and decreased the expression of p62 at 15 min, but increased the expression of

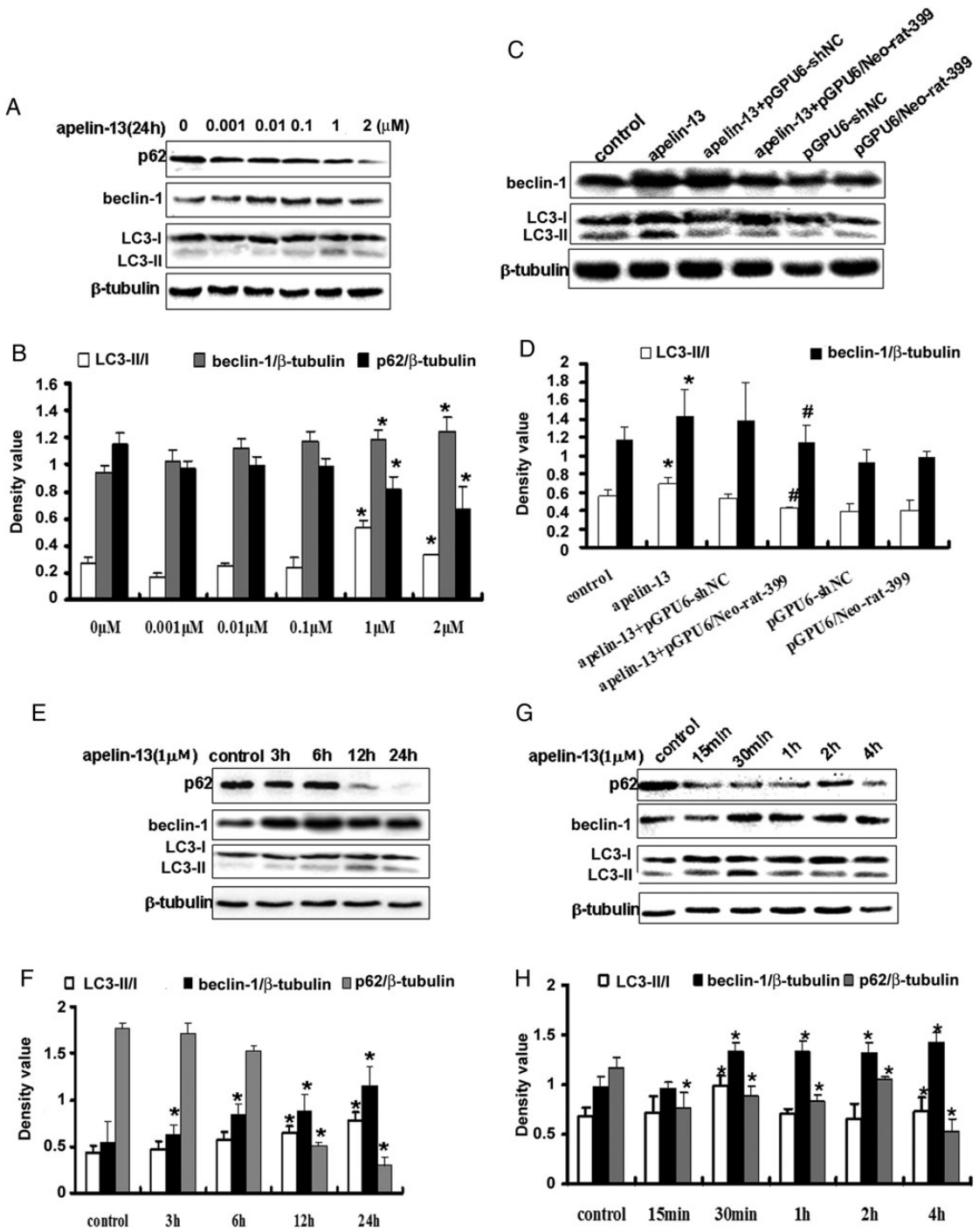

Figure 3. Apelin-13 stably and transiently induces cardiomyocyte autophagy The expression of autophagy markers LC3-II/I and beclin-1 was increased, while the expression of the substrate protein p62 was decreased in a concentration-dependent manner (A,B). The APJ shRNA ameliorated the increases in LC3-II/I and beclin-1 levels that were induced by apelin-13 (C,D). The expression of LC3-II/I was up-regulated, and the level of p62 was reduced at 12 or 24 h, while beclin-1 expression was increased at earlier time beginning at approximately 3 h, when cells were treated with apelin-13 for various time (0, 3, 6, 12, 24 h) (E,F). The expression of beclin-1 was increased beginning at 30 min and the expression of p62 was decreased beginning at 15 min, while the expression of LC3-II/I was increased only at 30 min (G,H) when cells were treated with apelin-13 for different time (0, 15 min, 30 min, 1 h, 2 h, 4 h), suggesting that apelin-13 also transiently induces cardiomyocyte autophagy, especially at 30 min. The data are shown as the mean ± SD, $n = 4$. $^{*}P < 0.05$ vs. the control group; $^{\#}P < 0.05$ vs. the apelin-13-treated group.

LC3-II/I only at 30 min (**Fig. 3G,H**), suggesting that apelin-13 also transiently induces cardiomyocyte autophagy, especially at 30 min. Therefore, it was believed that apelin both stably and transiently induces cardiomyocyte autophagy.

Apelin-13 stably induces cardiomyocyte autophagy through the PI3K/Akt pathway

Phosphoinositide-3 kinases (PI3Ks) are proteins that are coupled to a variety of cell surface receptors and play a key role in the signal

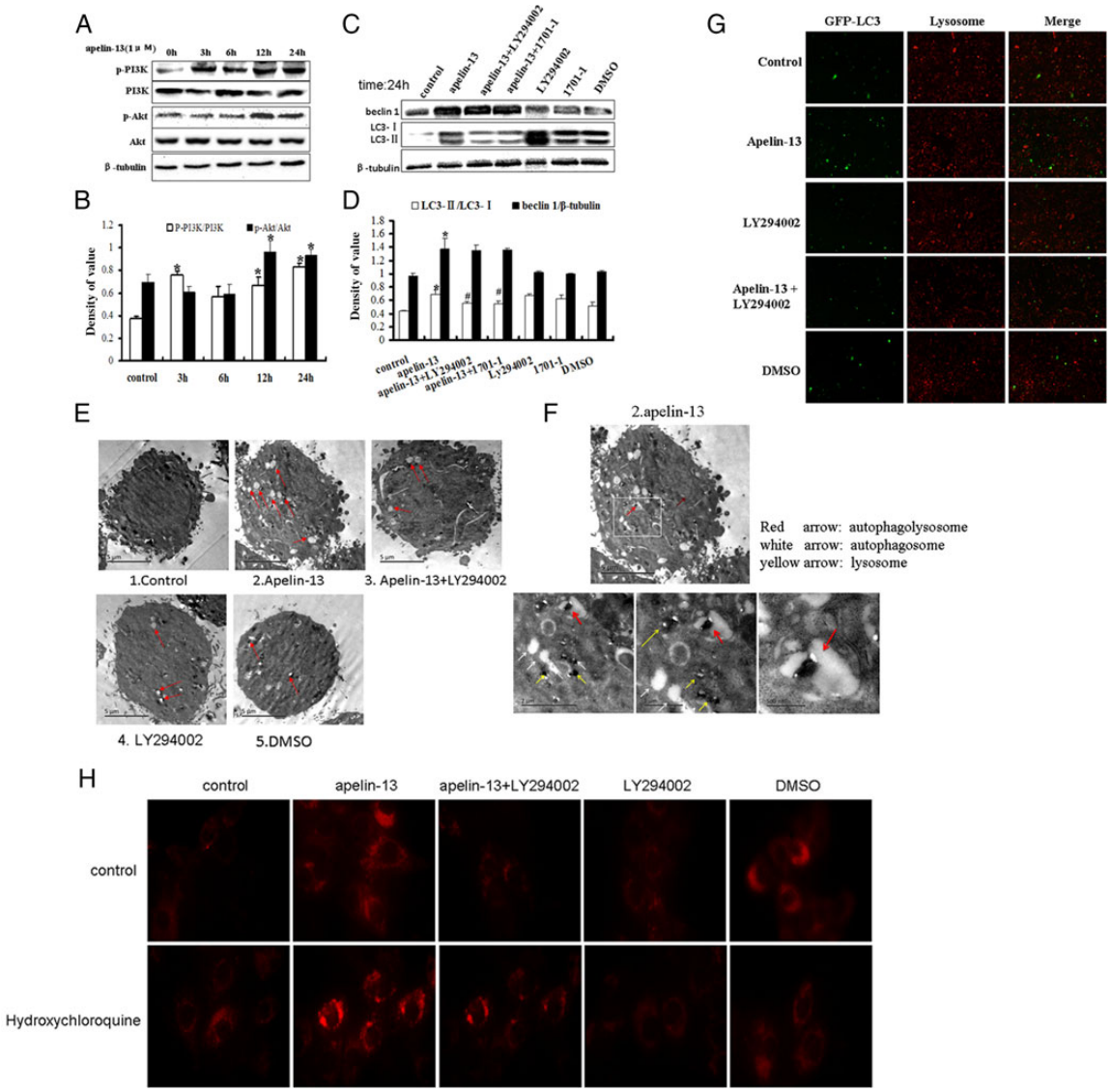

Figure 4. Apelin-13 stably induces cardiomyocyte autophagy through the PI3K/Akt pathway The phosphorylation of PI3K and Akt was increased 12 or 24 h after induction by apelin-13, and PI3K phosphorylation exhibited an early increase at 3 h (A,B). LY294002 and 1701-1 reversed the increase in LC3-II/I expression that was induced by apelin-13, but had no effect on the increase in beclin-1 expression (C,D). The generation of autophagosomes and autolysosomes were observed in cells treated with apelin-13 (1 μM; 12 h), while that was barely observed in the other groups of cells (control; apelin-13+LY29402; LY294002; DMSO) (E,F). The expression of GFP-LC3 was also observed in apelin-13- and apelin-13+1701-1-treated cells, while little expression was observed in other groups of cells (control; apelin-13 +LY294002; LY294002; 1701-1; DMSO) (G). The same results were obtained when hydroxychloroquine was used to inhibit degradation by autophagosome (H). These data suggested that LY294002, but not 1701-1, is involved in the autophagy induced by apelin-13 over an extended time (12 h). The data are shown as the mean ± SD, $n = 4$. $^{*}P < 0.05$ vs. the control group; $^{\#}P < 0.05$ vs. the apelin-13-treated group.

transduction cascade regulating fundamental cellular functions such as transcription, proliferation, and survival [41]. PI3Ks have been categorized into three classes: I, II, and III. The class I PI3K, together with its main downstream signaling molecule Akt, has been found to regulate autophagy [42,43]. In contrast, the class III PI3K, also known as human vacuolar protein sorting 34 (hVps34), plays a different role in the regulation of autophagy from that of the class I PI3K, as shown by its co-immunoprecipitation with the beclin-1 protein, a major determinant in the initiation of autophagy [44–46]. Our results showed that the phosphorylation of PI3K and Akt is increased within 12–24 h after 12 h of apelin-13 (1 μM) induction. PI3K phosphorylation is also increased at 3 h (**Fig. 4A,B**). LY294002 and 1701-1 reversed the increase of LC3-II/I levels that was induced by apelin-13, while these inhibitors had no effect on the increase of beclin-1 levels that was induced by apelin-13 (**Fig. 4C,D**). Autophagosomes were found in apelin-13-treated cells by TEM, but not in untreated cells (**Fig. 4E,F**). Autophagy acts as a dynamic process, and the autophagosome is an intermediate structure in this dynamic process. The accumulation of autophagosomes may represent either autophagosomes generation-induction or autophagosomes degradation-suppression. Therefore, a plasmid expressing GFP-LC3 was transfected into cells, and LysoTracker Red was used to stain the lysosomes to visualize the delivery of GFP-LC3 using fluorescence microscopy. The results showed that LY294002 blocked the expression of GFP-LC3 that was induced by apelin-13 (**Fig. 4G**). Autophagic flux can be monitored by analyzing LC3 turnover, which is based on the observation that

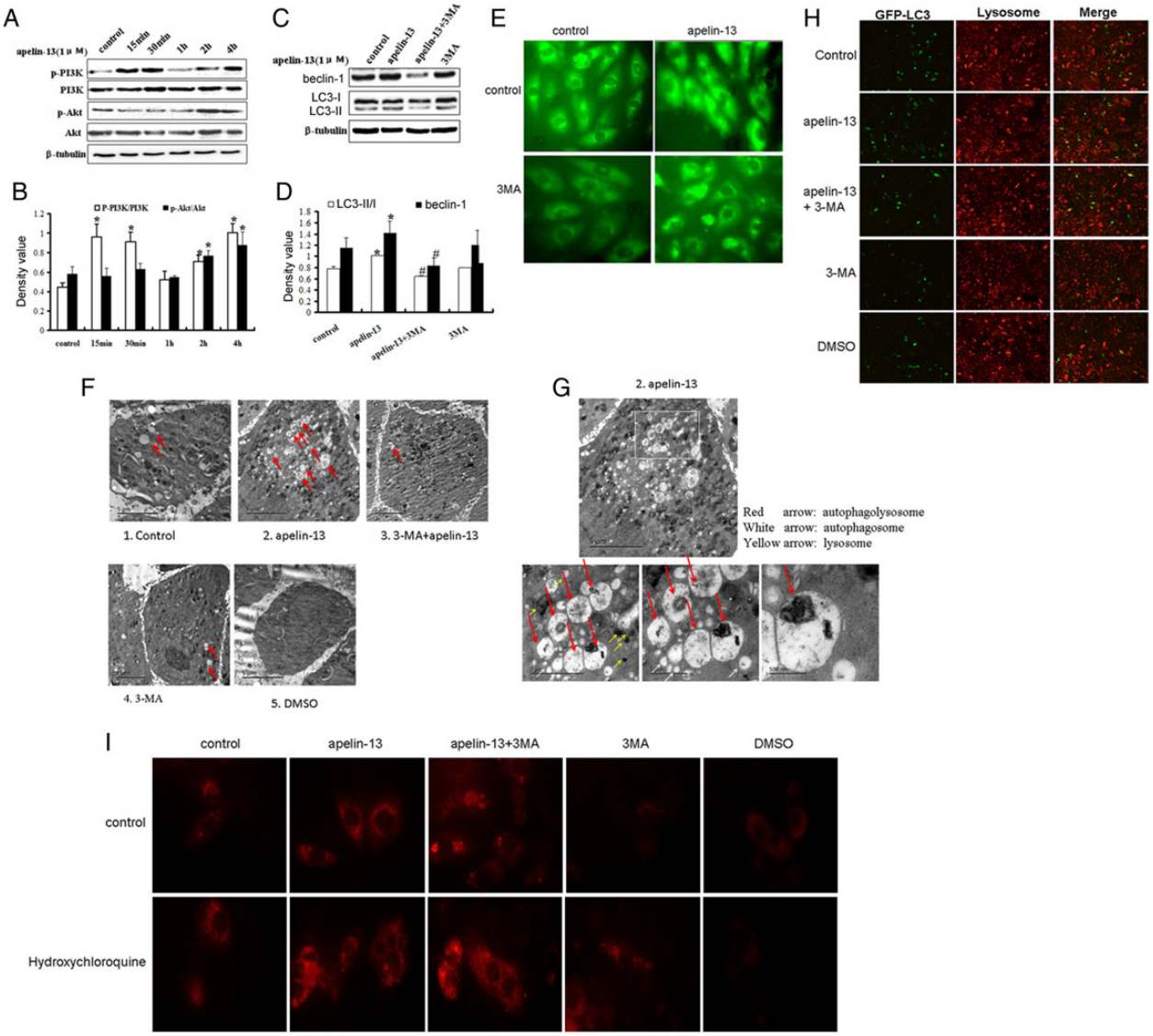

Figure 5. Apelin-13 transiently regulates autophagy via the Class III PI3K pathway The increase of PI3K phosphorylation was observed at 15 min, 30 min, 2h, and 4 h. But Akt phosphorylation was observed only at 2 and 4 h, no activation of Akt was observed at the 15 or 30 min (A,B). 3MA (class III PI3K inhibitor) significantly blocked the increase of LC3-II/I and beclin-1 expression that were induced by apelin-13 (C,D). Apelin-13 treatment (1 μM; 30 min) increased the formation of autophagosomes compared with the control group and that 3MA blocked the formation of autophagosome induced by apelin-13 (1 μM; 30 min) (E,F). Apelin-13 also promoted LC3 conjugation to the cytoplasmic membrane, and the class III PI3K inhibitor 3MA significantly blocked this effect not only when cells were incubated with an LC3 primary antibody and an Alexa 488-conjugated secondary antibody (G), but also when cells were transfected with the MAP1LC3B GFP-tagged ORF clone (H). The same results were obtained when hydroxychloroquine was used to inhibit degradation by autophagosomes (I). These data suggest that 3MA transiently (30 min) regulates autophagy induced by apelin-13. The data are shown as the mean ± SD, $n = 4$. $^{*}P < 0.05$ vs. the control group; $^{\#}P < 0.05$ vs. the apelin-13-treated group.

LC3-II is degraded in autolysosomes. Therefore, cells were incubated with or without the lysosomal inhibitor hydroxychloroquine for 30 min, and the expression of LC3 was observed by fluorescence microscopy. The expression of LC3 was increased after the degradation of autophagosome was inhibited by hydroxychloroquine treatment. LY294002 blocked the increase of LC3 levels induced by apelin-13, even in hydroxychloroquine-treated cells (**Fig. 4H**). These results suggested that the PI3K/Akt pathway regulates autophagy induced by apelin-13.

Apelin-13 transiently regulates autophagy via the Class III PI3K pathway. Interestingly, our results showed that the expression of beclin-1 exhibited an early increase at 3 h (**Fig. 3E,F**) and that PI3K phosphorylation was also increased at 3 h (**Fig. 4A,B**). However, LY294002 and 1701-1 did not affect the expression of beclin-1 induced by apelin-13 (**Fig. 4C,D**). These results implied that short-term regulation of beclin-1 exists in cells undergoing autophagy induced by apelin-13. To analyze this short-term regulation, cells were treated with apelin-13 (1 μM) for different time (0, 15 min, 30 min, 1, 2, and 4 h), and results showed that apelin-13 up-regulated the expression of PI3K phosphorylation at 15 min, 30 min, 2 and 4 h, but the increase of Akt phosphorylation was observed only at 2 and 4 h, not at 15 and 30 min (**Fig. 5A,B**). The increase of PI3K phosphorylation was similar to the changes of LC3-II/I, beclin-1 and p62 expressions (**Fig. 3G,H**). Consequently, it was suggested that it may be the

class III PI3K, not the class I PI3K that transiently regulates autophagy induced by apelin-13. To confirm this, cells were treated with the class III PI3K inhibitor 3-methyladenine (3MA) for 30 min and results showed that 3MA (0.1 μM) significantly blocked the increase of the LC3-II/I and beclin-1 levels that were induced by apelin-13 (**Fig. 5C, D**). TEM observation also showed that apelin-13 treatment (1 μM; 30 min) promoted the generation of autophagosomes (**Fig. 5F,G**), and immunofluorescence detection showed that apelin-13 treatment increased the expression of LC3 and this effect was blocked by 3MA (**Fig. 5E**). The same results were obtained when the GFP-LC3 plasmid was transfected into cells (**Fig. 5H**) and when the degradation of autophagosomes was inhibited by hydroxychloroquine (**Fig. 5I**).

Table 3. The autophagy inhibitor 3MA blocks the increases in the diameter, volume, and protein content induced by apelin-13

Group	Diameter (μm/cell)	Volume (10^3 μm^3/cell)	Protein (pg/cell)
Control	13.1 ± 0.2	1.19 ± 0.06	633 ± 23
Apelin-13	16.4 ± 1.0*	2.35 ± 0.39*	1123 ± 97*
Apelin-13+3MA	14.8 ± 1.3$^{\#}$	1.91 ± 0.24$^{\#\#}$	688 ± 41$^{\#\#}$
Apelin-13+ rapamycin	17.0 ± 0.5	2.58 ± 0.53	1184 ± 14
Rapamycin	15.6 ± 1.0*	2.01 ± 0.4*	940 ± 11*
3MA	14.1 ± 1.2	1.59 ± 0.25	677 ± 39
DMSO	12.8 ± 0.9	1.28 ± 0.22	837 ± 75

Apelin-13: 2 μM; time: 96 h; 3MA (5 mM): autophagy inhibitor; rapamycin (0.1 μM): autophagy revulsive; The data are expressed as the mean ± SD of six independent experiments. $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. apelin-13 group; $^{\#\#}P < 0.01$ vs. apelin-13 group.

These data supported our assumption that the class III PI3K transiently regulates autophagosome formation induced by apelin-13.

Apelin-13 induces cardiomyocyte hypertrophy by autophagy

3MA (typically used at 5 mM) is a known inhibitor of autophagy (lysosomal self-degradation) that functions through blocking autophagosome formation via the inhibition of class III PI3K. Cells were stimulated with 3MA (5 mM) and results showed that this treatment reversed the increases of the cell diameter, volume, and protein content induced by apelin-13. Meanwhile, rapamycin, the autophagy activation agent, increased cell hypertrophy when used alone, but this effect was not significant in the cardiomyocyte hypertrophy induced by apelin-13 (**Table 3**). These results suggested that autophagy is involved in cardiomyocyte hypertrophy induced by apelin-13 (**Fig. 6**).

Discussion

Cardiac hypertrophy represents a typical feature of various cardiomyopathies, including ischemic heart disease and hypertension. Its main features are the increase of the protein synthesis and the size of cells. At the early stage of cardiomyopathy, myocardial hypertrophy is a compensatory function, but ultimately, this can lead to heart failure and has become a significant independent risk factor of cardiovascular disease [47]. At present, myocardial hypertrophy occurs mainly due to mechanical load [1–3] and neurohumoral factors [4–6]. The earliest hemodynamic effect in the pathogenesis of myocardial hypertrophy has been identified. It has been reported that, in addition to the renin angiotensin aldosterone system (RASS), norepinephrine

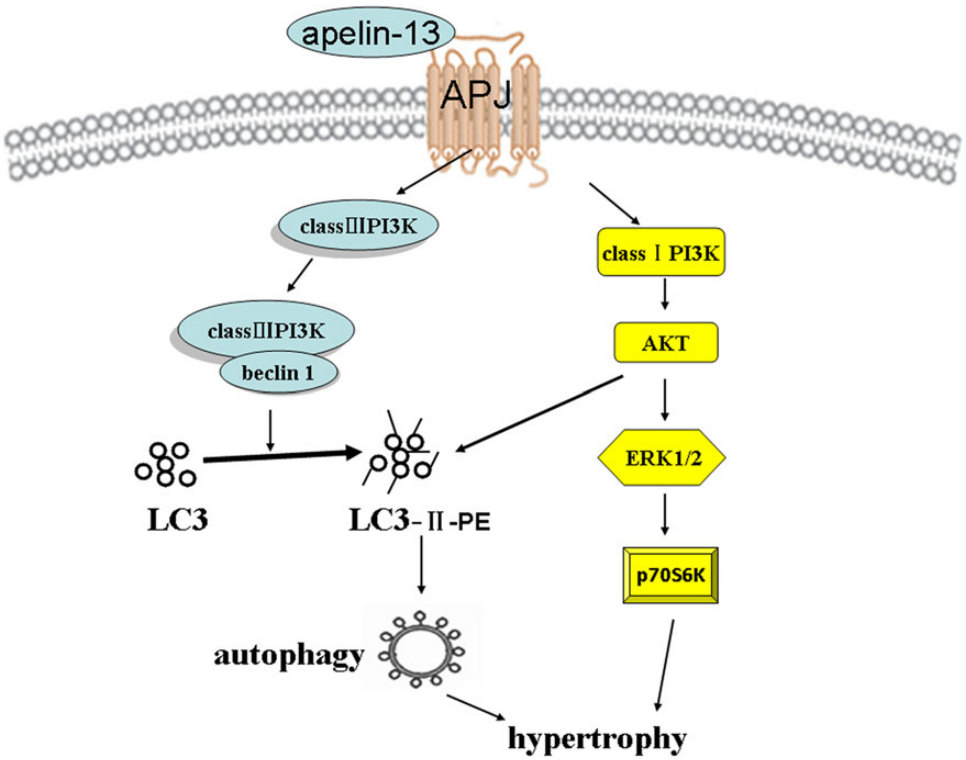

Figure 6. Apelin-13 promotes cardiomyocyte hypertrophy via the PI3K-Akt-ERK1/2-p70S6K and autophagy pathway Apelin-13 activates the PI3K-Akt-ERK1/2-p70S6K pathway to promote cardiomyocyte hypertrophy. Apelin-13 transiently induces autophagy through the PI3K/Akt pathway for an extended time via class III PI3K. Therefore, autophagy is involved in cardiomyocyte hypertrophy induced by apelin-13.

[48–50] and angiotensin II (Ang II) [51–53] induce myocardial cells to undergo hypertrophy.

In this study, a high concentration of apelin-13 (2 μM) was used to stimulate H9c2 rat cardiomyocytes for 4 days and results showed that apelin-13 increased the cell diameter, volume, and protein content, suggesting that apelin-13 promotes cardiomyocyte hypertrophy. These results differ from a recent report that apelin prevented myocardial hypertrophy caused by Ang II and 5-HT [18]. In that study, the influence of treatment with low concentrations of apelin (0.1–100 nM) for 48 h on rat myocardial hypertrophy induced by 5-HT (10 μM) was investigated, but there was no mention of the effect of higher concentrations of apelin or the effect of subtypes of apelin. In this study, cells were treated with a high dose of apelin-13 (2 μM) for an extended time (4 days), and it was demonstrated that apelin-13 did not act as a protective factor for hypertrophy, but instead act as a risk factor that induces hypertrophy. Additional reports also support our results. For example, apelin-13 was found to significantly increase the mouse heart-to-weight ratio and heart end-diastolic pressure [10], and APJ was proved to be essential for the cardiomyocyte response to pressure [20]. However, the expression of APJ was not changed when cells were treated with apelin-13 for different time in this study. It was believed that apelin-13 may only affect the function of APJ and have little effect on its expression. The relationship between the activation and function of APJ needs to be determined in future studies.

In addition, our results showed that apelin-13 activated the PI3K-Akt-ERK1/2-p70S6K pathway in H9c2 rat cardiomyocytes. LY294002, 1701-1, and PD98059 separately inhibited the increases of cell diameter, volume, and protein content induced by apelin-13, suggesting that the PI3K-Akt-ERK1/2-p70S6K pathway is involved in the promotion of H9c2 rat cardiomyocyte hypertrophy induced by apelin-13.

Autophagy is a process by which cells deliver cytoplasmic material and organelles to lysosomes for degradation [54]. The autophagic process includes two multiprotein components and two ubiquitin-like modification systems: a protein kinase Apg1 complex [55,56] and a beclin–phosphatidylinositol 3-kinase (class III PI3K) complex [57,58], which are responsible for the conjugation of Apg12 to Apg5 [59] and the conjugation of LC3 to membrane phospholipid(s) [60], respectively. The autophagy-specific class III PI3K complex appears to be essential for recruitment of Atg12-Atg5 conjugates [61] and LC3/Apg8 conjugates to the pre-autophagosomal structure [44]. These two systems promote the elongation of the isolation membrane and the formation of autophagosomes. In this study, our results showed that apelin-13 induced the expressions of the autophagy markers LC3-II/I and beclin-1 and reduced the level of the autophagy substrate protein p62, suggesting that apelin-13 induces H9c2 rat cardiomyocyte autophagy. Our results also demonstrated that beclin-1 levels were increased at the earlier stage and accompanied by an increase of p-PI3K levels. Therefore, we propose that the PI3K/Akt pathway only affects the expression of LC3-II/I over the long term, while class III PI3K transiently regulates both LC3-II/I and beclin-1 and promotes the elongation of the isolation membrane and the formation of the autophagosome. The immunofluorescence and monodansylcadaverine results supported our inference that apelin promotes LC3 conjugation to the cytoplasmic membrane and induces the formation of autophagosomes. The class III PI3K inhibitor 3MA significantly blocked the conjugation of LC3 to the cytoplasmic membrane and the formation of autophagosomes. It has been reported that PI3K/Akt negatively regulates autophagy by the activation of mTOR, while our results showed that PI3K/Akt positively regulates the expression of LC3-II/I. The mechanism of this regulation is currently unknown, and it is believed that the PI3K/Akt pathway may have alternative methods for inducing autophagy independent of mTOR.

In the heart, the levels of autophagy are lower than one under normal conditions, and autophagy is essential for cellular homeostasis. However, there is evidence that autophagy is enhanced in various pathological conditions, including cardiac hypertrophy [35], cardiomyopathy [62], and heart failure. Our results revealed that 3MA reversed the increase of the diameter, volume, and protein content of H9c2 cells induced by apelin-13. Rapamycin, an autophagy inhibitor, increased cell hypertrophy when administered alone, but its effect on apelin-13-induced cardiomyocyte hypertrophy was not significant (**Table 3**). Therefore, it was concluded that autophagy is involved in cardiomyocyte hypertrophy induced by apelin-13.

In summary, our data indicated that apelin-13 promotes H9c2 rat cardiomyocyte hypertrophy by activating the PI3K-Akt-ERK1/2-p70S6k and PI3K-autophagy pathways (**Fig. 6**).

Acknowledgements

We would like to thank Assistant Professor Bingbing Wang of the Perinatal Biology Laboratory, Division of Maternal-Fetal Medicine, Rutgers University-Robert Wood Johnson Medical School for the English language proofreading.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (Nos. 81270420, 81470434, and 81503074), the Hunan Province Cooperative innovation Center for Molecular Target New Drug Study, Hunan Provincial Education Department (Approval number: 2014-405), the Hunan Provincial Natural Science Foundation (No. 14JJ3102), and the China Postdoctoral Science Foundation (Nos. 2014M560647 and 2015T80875).

References

1. Xie F, Lv D, Chen L. ELABELA: a novel hormone in cardiac development acting as a new endogenous ligand for the APJ receptor. *Acta Biochim Biophys Sin* 2014, 46: 620–622.
2. Mesmin C, Fenaille F, Becher F, Tabet JC, Ezan E. Identification and characterization of apelin peptides in bovine colostrum and milk by liquid chromatography-mass spectrometry. *J Proteome Res* 2011, 10: 5222–5231.
3. Fan X, Zhou N, Zhang X, Mukhtar M, Lu Z, Fang J, DuBois GC, *et al*. Structural and functional study of the apelin-13 peptide, an endogenous ligand of the HIV-1 coreceptor, APJ. *Biochemistry* 2003, 42: 10163–10168.
4. Maguire JJ, Kleinz MJ, Pitkin SL, Davenport AP. [Pyr1]apelin-13 identified as the predominant apelin isoform in the human heart: vasoactive mechanisms and inotropic action in disease. *Hypertension* 2009, 54: 598–604.
5. Lee DK, Saldivia VR, Nguyen T, Cheng R, George SR, O'Dowd BF. Modification of the terminal residue of apelin-13 antagonizes its hypotensive action. *Endocrinology* 2005, 146: 231–236.
6. Wu D, He L, Chen L. Apelin/APJ system: a promising therapy target for hypertension. *Mol Biol Rep* 2014, 41: 6691–6703.
7. Azizi Y, Faghihi M, Imani A, Roghani M, Nazari A. Post-infarct treatment with [Pyr1]-apelin-13 reduces myocardial damage through reduction of oxidative injury and nitric oxide enhancement in the rat model of myocardial infarction. *Peptides* 2013, 46: 76–82.
8. Koguchi W, Kobayashi N, Takeshima H, Ishikawa M, Sugiyama F, Ishimitsu T. Cardioprotective effect of apelin-13 on cardiac performance and remodeling in end-stage heart failure. *Circ J* 2012, 76: 137–144.
9. Tao J, Zhu W, Li Y, Xin P, Li J, Liu M, Li J, *et al*. Apelin-13 protects the heart against ischemia-reperfusion injury through inhibition of ER-

dependent apoptotic pathways in a time-dependent fashion. *Am J Physiol Heart Circ Physiol* 2011, 301: H1471–H1486.
10. Li L, Zeng H, Chen JX. Apelin-13 increases myocardial progenitor cells and improves repair postmyocardial infarction. *Am J Physiol Heart Circ Physiol* 2012, 303: H605–H618.
11. Li F, Li L, Qin X, Pan W, Feng F, Chen F, Zhu B, *et al*. Apelin-induced vascular smooth muscle cell proliferation: the regulation of cyclin D1. *Front Biosci* 2008, 13: 3786–3792.
12. Li L, Li L, Xie F, Zhang Z, Guo Y, Tang G, Lv D, *et al*. Jagged-1/Notch3 signaling transduction pathway is involved in apelin-13-induced vascular smooth muscle cells proliferation. *Acta Biochim Biophys Sin* 2013, 45: 875–881.
13. Liu C, Su T, Li F, Li L, Qin X, Pan W, Feng F, *et al*. PI3K/Akt signaling transduction pathway is involved in rat vascular smooth muscle cell proliferation induced by apelin-13. *Acta Biochim Biophys Sin* 2010, 42: 396–402.
14. Li X, Zhang X, Li F, Chen L, Li L, Qin X, Gao J, *et al*. 14-3-3 mediates apelin-13-induced enhancement of adhesion of monocytes to human umbilical vein endothelial cells. *Acta Biochim Biophys Sin* 2010, 42: 403–409.
15. Mao X, Su T, Zhang X, Li F, Qin X, Li X, Liao D, *et al*. Apelin-13 promote monocytes adhesion to HUVECs via PI3K signaling. *Prog Biochen Biophys* 2011, 38: 1162–1170.
16. Lv D, Li H, Chen L. Apelin and APJ, a novel critical factor and therapeutic target for atherosclerosis. *Acta Biochim Biophys Sin* 2013, 45: 527–533.
17. Falcao-Pires I, Goncalves N, Gavina C, Pinho S, Teixeira T, Moura C, Amorim MJ, *et al*. Correlation between plasma levels of apelin and myocardial hypertrophy in rats and humans: possible target for treatment? *Expert Opin Ther Targets* 2010, 14: 231–241.
18. Foussal C, Lairez O, Calise D, Pathak A, Guilbeau-Frugier C, Valet P, Parini A, *et al*. Activation of catalase by apelin prevents oxidative stress-linked cardiac hypertrophy. *FEBS Lett* 2010, 584: 2363–2370.
19. Ceylan-Isik AF, Kandadi MR, Xu X, Hua Y, Chicco AJ, Ren J, Nair S. Apelin administration ameliorates high fat diet-induced cardiac hypertrophy and contractile dysfunction. *J Mol Cell Cardiol* 2013, 63: 4–13.
20. Scimia MC, Hurtado C, Ray S, Metzler S, Wei K, Wang J, Woods CE, *et al*. APJ acts as a dual receptor in cardiac hypertrophy. *Nature* 2012, 488: 394–398.
21. Xie F, Li LF, Chen LX. APJ acts as a reponse for pressure overload to induce myocardial hypertrophy. *Prog Biochen Biophys* 2013, 40: 33–36.
22. Xie F, Liu W, Feng F, Li X, Yang L, Lv D, Qin X, *et al*. A static pressure sensitive receptor APJ promote H9c2 cardiomyocyte hypertrophy via PI3K-autophagy pathway. *Acta Biochim Biophys Sin* 2014, 46: 699–708.
23. Martelli AM, Nyakern M, Tabellini G, Bortul R, Tazzari PL, Evangelisti C, Cocco L. Phosphoinositide 3-kinase/Akt signaling pathway and its therapeutical implications for human acute myeloid leukemia. *Leukemia* 2006, 20: 911–928.
24. Viard P, Butcher AJ, Halet G, Davies A, Nurnberg B, Heblich F, Dolphin AC. PI3K promotes voltage-dependent calcium channel trafficking to the plasma membrane. *Nat Neurosci* 2004, 7: 939–946.
25. Samuelsson AM, Bollano E, Mobini R, Larsson BM, Omerovic E, Fu M, Waagstein F, *et al*. Hyperinsulinemia: effect on cardiac mass/function, angiotensin II receptor expression, and insulin signaling pathways. *Am J Physiol Heart Circ Physiol* 2006, 291: H787–H796.
26. Wohlschlaeger J, Schmitz KJ, Palatty J, Takeda A, Takeda N, Vahlhaus C, Levkau B, *et al*. Roles of cyclooxygenase-2 and phosphorylated Akt (Thr308) in cardiac hypertrophy regression mediated by left-ventricular unloading. *J Thorac Cardiovasc Surg* 2007, 133: 37–43.
27. Tabe Y, Jin L, Konopleva M, Shikami M, Kimura S, Andreeff M, Raffeld M, *et al*. Class IA PI3K inhibition inhibits cell growth and proliferation in mantle cell lymphoma. *Acta Haematol* 2014, 131: 59–69.
28. Perez-Perez A, Gambino Y, Maymo J, Goberna R, Fabiani F, Varone C, Sanchez-Margalet V. MAPK and PI3K activities are required for leptin stimulation of protein synthesis in human trophoblastic cells. *Biochem Biophys Res Commun* 2010, 396: 956–960.
29. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* 2002, 2: 489–501.
30. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell* 2008, 132: 27–42.
31. Xie F, Li L, Chen L. Autophagy, a new target for disease treatment. *Sci China Life Sci* 2013, 56: 856–860.
32. Hamacher-Brady A, Brady NR, Gottlieb RA. Enhancing macroautophagy protects against ischemia/reperfusion injury in cardiac myocytes. *J Bio Chem* 2006, 281: 29776–29787.
33. Matsui Y, Takagi H, Qu X, Abdellatif M, Sakoda H, Asano T, Levine B, *et al*. Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. *Circ Res* 2007, 100: 914–922.
34. Huang C, Yitzhaki S, Perry CN, Liu W, Giricz Z, Mentzer RM Jr, Gottlieb RA. Autophagy induced by ischemic preconditioning is essential for cardioprotection. *J Cardiovasc Transl Res* 2010, 3: 365–373.
35. Dammrich J, Pfeifer U. Cardiac hypertrophy in rats after supravalvular aortic constriction. II. Inhibition of cellular autophagy in hypertrophying cardiomyocytes. *Virchows Arch B Cell Pathol Incl Mol Pathol* 1983, 43: 287–307.
36. Nishino I, Fu J, Tanji K, Yamada T, Shimojo S, Koori T, Mora M, *et al*. Primary LAMP-2 deficiency causes X-linked vacuolar cardiomyopathy and myopathy (Danon disease). *Nature* 2000, 406: 906–910.
37. Vergne I, Deretic V. The role of PI3P phosphatases in the regulation of autophagy. *FEBS Lett* 2010, 584: 1313–1318.
38. Hua Y, Zhang Y, Ceylan-Isik AF, Wold LE, Nunn JM, Ren J. Chronic Akt activation accentuates aging-induced cardiac hypertrophy and myocardial contractile dysfunction: role of autophagy. *Basic Res Cardiol* 2011, 106: 1173–1191.
39. Faghiri Z, Bazan NG. PI3K/Akt and mTOR/p70S6 K pathways mediate neuroprotectin D1-induced retinal pigment epithelial cell survival during oxidative stress-induced apoptosis. *Exp Eye Res* 2010, 90: 718–725.
40. Kimes BW, Brandt BL. Properties of a clonal muscle cell line from rat heart. *Exp Cell Res* 1976, 98: 367–381.
41. Chen K, Iribarren P, Gong W, Wang JM. The essential role of phosphoinositide 3-kinases (PI3Ks) in regulating pro-inflammatory responses and the progression of cancer. *Cell Mol Immunol* 2005, 2: 241–252.
42. Wu YT, Tan HL, Huang Q, Ong CN, Shen HM. Activation of the PI3K-Akt-mTOR signaling pathway promotes necrotic cell death via suppression of autophagy. *Autophagy* 2009, 5: 824–834.
43. Saiki S, Sasazawa Y, Imamichi Y, Kawajiri S, Fujimaki T, Tanida I, Kobayashi H, *et al*. Caffeine induces apoptosis by enhancement of autophagy via PI3K/Akt/mTOR/p70S6 K inhibition. *Autophagy* 2011, 7: 176–187.
44. Tassa A, Roux MP, Attaix D, Bechet DM. Class III phosphoinositide 3-kinase--Beclin1 complex mediates the amino acid-dependent regulation of autophagy in C2C12 myotubes. *Biochem J* 2003, 376: 577–586.
45. Castino R, Bellio N, Follo C, Murphy D, Isidoro C. Inhibition of PI3k class III-dependent autophagy prevents apoptosis and necrosis by oxidative stress in dopaminergic neuroblastoma cells. *Toxicol Sci* 2010, 117: 152–162.
46. Farre JC, Mathewson RD, Manjithaya R, Subramani S. Roles of Pichia pastoris Uvrag in vacuolar protein sorting and the phosphatidylinositol 3-kinase complex in phagophore elongation in autophagy pathways. *Autophagy* 2010, 6: 86–99.
47. Gaasch WH, Delorey DE, St John Sutton MG, Zile MR. Patterns of structural and functional remodeling of the left ventricle in chronic heart failure. *Am J Cardiol* 2008, 102: 459–462.
48. Gupta MK, Neelakantan TV, Sanghamitra M, Tyagi RK, Dinda A, Maulik S, Mukhopadhyay CK, *et al*. An assessment of the role of reactive oxygen species and redox signaling in norepinephrine-induced apoptosis and hypertrophy of H9c2 cardiac myoblasts. *Antioxid Redox Signal* 2006, 8: 1081–1093.
49. Koshman YE, Piano MR, Russell B, Schwertz DW. Signaling responses after exposure to 5 alpha-dihydrotestosterone or 17 beta-estradiol in norepinephrine-induced hypertrophy of neonatal rat ventricular myocytes. *J Appl Physiol* 2010, 108: 686–696.
50. Tsoporis JN, Marks A, Kahn HJ, Butany JW, Liu PP, O'Hanlon D, Parker TG. Inhibition of norepinephrine-induced cardiac hypertrophy in s100beta transgenic mice. *J Clin Invest* 1998, 102: 1609–1616.
51. Zou XJ, Yang L, Yao SL. Propofol depresses angiotensin II-induced cardiomyocyte hypertrophy *in vitro*. *Exp Biol Med (Maywood)* 2008, 233: 200–208.

52. Vanamala SK, Gopinath S, Gondi CS, Rao JS. Effect of human umbilical cord blood cells on Ang-II-induced hypertrophy in mice. *Biochem Biophys Res Commun* 2009, 386: 386–391.
53. Yang Y, Ago T, Zhai P, Abdellatif M, Sadoshima J. Thioredoxin 1 negatively regulates angiotensin II-induced cardiac hypertrophy through upregulation of miR-98/let-7. *Circ Res* 2011, 108: 305–313.
54. Eskelinen EL, Saftig P. Autophagy: a lysosomal degradation pathway with a central role in health and disease. *Biochim Biophys Acta* 2009, 1793: 664–673.
55. Kamada Y, Funakoshi T, Shintani T, Nagano K, Ohsumi M, Ohsumi Y. Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J Cell Biol* 2000, 150: 1507–1513.
56. Abeliovich H, Zhang C, Dunn WA Jr, Shokat KM, Klionsky DJ. Chemical genetic analysis of Apg1 reveals a non-kinase role in the induction of autophagy. *Mol Biol Cell* 2003, 14: 477–490.
57. Kihara A, Kabeya Y, Ohsumi Y, Yoshimori T. Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. *EMBO Rep* 2001, 2: 330–335.
58. Kihara A, Noda T, Ishihara N, Ohsumi Y. Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in Saccharomyces cerevisiae. *J Cell Biol* 2001, 152: 519–530.
59. Mizushima N, Kuma A, Kobayashi Y, Yamamoto A, Matsubae M, Takao T, Natsume T, *et al*. Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. *J Cell Sci* 2003, 116: 1679–1688.
60. Otomo C, Metlagel Z, Takaesu G, Otomo T. Structure of the human ATG12~ATG5 conjugate required for LC3 lipidation in autophagy. *Nat Struct Mol Biol* 2013, 20: 59–66.
61. Suzuki K, Kirisako T, Kamada Y, Mizushima N, Noda T, Ohsumi Y. The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *EMBO J* 2001, 20: 5971–5981.
62. Zaglia T, Milan G, Ruhs A, Franzoso M, Bertaggia E, Pianca N, Carpi A, *et al*. Atrogin-1 deficiency promotes cardiomyopathy and premature death via impaired autophagy. *J Clin Invest* 2014, 124: 2410–2424.