Original Article

The endoplasmic reticulum stress-autophagy pathway is involved in apelin-13-induced cardiomyocyte hypertrophy *in vitro*

Feng XIE^{1,2, #}, Di WU^{1, #}, Shi-fang HUANG^{1, #}, Jian-gang CAO¹, He-ning LI¹, Lu HE¹, Mei-qing LIU¹, Lan-fang LI^{1, *}, Lin-xi CHEN^{1, *}

¹Institute of Pharmacy and Pharmacology, Learning Key Laboratory for Pharmacoproteomics, University of South China, Hengyang 421001, China; ²Nanshan District Maternity & Child Healthcare Hospital of Shenzhen, Shenzhen 518052, China

Abstract

Apelin is the endogenous ligand for the G protein-coupled receptor APJ, and plays important roles in the cardiovascular system. Our previous studies showed that apelin-13 promotes the hypertrophy of H9c2 rat cardiomyocytes through the PI3K-autophagy pathway. The aim of this study was to explore what roles ER stress and autophagy played in apelin-13-induced hypertrophy of cardiomyocytes *in vitro*. Treatment of H9c2 cells with apelin-13 (0.001–2 μ mol/L) dose-dependently increased the production of ROS and the expression levels of NADPH oxidase 4 (NOX4). Knockdown of Nox4 with siRNAs effectively prevented the reduction of GSH/GSSG ratio in apelin-13-treated cells. Furthermore, apelin-13 treatment dose-dependently increased the expression of Bip and CHOP, two ER stress markers, in the cells. Knockdown of APJ or Nox4 with the corresponding siRNAs, or application of NADPH inhibitor DPI blocked apelin-13-induced increases in Bip and CHOP expression. Moreover, apelin-13 treatment increased the formation of autophagosome and ER fragments and the LC3 puncta in the ER of the cells. Knockdown of APJ, Nox4, Bip or CHOP with the corresponding siRNAs, or application of DPI or salubrinal attenuated apelin-13-induced overexpression of LC3-II/I and beclin 1. Finally, knockdown of Nox4, Bip or CHOP with the corresponding siRNAs, or application of salubrinal significantly suppressed apelin-13-induced increases in the cell diameter, volume and protein contents. Our results demonstrate that ER stress-autophagy is involved in apelin-13-induced H9c2 cell hypertrophy.

Keywords: myocardial hypertrophy; apelin; APJ receptor; ROS; NOX4; ER stress; autophagy; reticulophagy

Acta Pharmacologica Sinica (2017) 38: 1589–1600; doi: 10.1038/aps.2017.97; published online 27 Jul 2017

Introduction

Apelin is a novel endogenous peptide ligand for the human APJ receptor and plays an important role in the cardiovascular system^[1]. The interactions between apelin and APJ have been observed to change, and these changes play a protective role in myocardial hypertrophy^[2]. However, the role of apelin/APJ in myocardial hypertrophy is currently controversial because APJ-null animals were found to be resistant to the pathological hypertrophic response to transaortic constriction (TAC)^[3]. Our previous studies showed that the apelin/APJ system is involved in the process of myocardial hypertrophy^[4] and the associated increases in the rat ventricular diastolic pressure ratio and ventricular wall^[5]. In addition, apelin-13 promotes increases in the myocardial cell diameter, volume and pro-

tein content through the PI3K-Akt-ERK1/2-p70S6K signaling pathways^[6]. Furthermore, APJ may be a pressure sensor that responds to external stimuli in myocardial hypertrophy^[4, 6]. All of these results suggest that apelin/APJ may be involved in myocardial hypertrophy. However, the mechanism by which apelin induces myocardial hypertrophy has not been fully investigated.

Reactive oxygen species (ROS) production plays a critical role in myocardial hypertrophy. NADPH oxidase (NOX), an enzyme that generates the superoxide anion, is a major pathway for the generation of ROS. The distribution of the NOX enzymes is tissue-specific. NADPH oxidase 2 (NOX2) and NADPH oxidase 4 (NOX4) are the main forms present in myocardial cells. Recent reports suggest that apelin/APJ is associated with ROS production and may be involved in the myocardial hypertrophy induced by apelin.

ER stress is a general term for the pathway by which various stimuli affect ER functions. Clinical studies have shown that endoplasmic reticulum stress is observed during the process of myocardial hypertrophy and plays an important role in

[#] These authors contributed equally to this work.

^{*} To whom correspondence should be addressed.

E-mail lxchen6@126.com (Lin-xi CHEN);

llanfang6@126.com (Lan-fang LI)

Received 2016-10-26 Accepted 2017-03-12

maintaining cell homeostasis^[7]. Moreover, ER stress may be linked to the autophagic response, which plays a key role in a cell's response to various stressors^[8]. However, it is unknown whether APJ can activate ER stress in the process of myocardial hypertrophy.

Autophagy is a bulk degradation process that is involved in the recycling of nutrients, the clearance of aggregates, and responses to various stresses. Increasing evidence from clinical and epidemiology studies suggests that an imbalance in autophagy is an important cause of myocardial hypertrophy^[9]. However, it is unknown whether APJ can activate autophagy in the process of myocardial hypertrophy.

In this pilot study, we examined the role of apelin-13 in the regulation of ER stress and autophagy. Subsequently, we also investigated the association of ER stress and autophagy with cell diameter and cell volume increases after exposure to apelin-13.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from HyClone (Grand Island, NY, USA). The H9c2 cell line was from ATCC (Manassas, VA, USA). Apelin-13 was purchased from Phoenix Pharmaceuticals (USA). Diphenyleneiodonium chloride and tunicamycin were purchased from Sigma-Aldrich (USA). Salubrinol was purchased from BioVision. The DCFH-DA detection kit and GSH/GSSG assay kit were purchased from Beyotime (Haimen, China). Bip siRNA, CHOP siRNA, NOX4 siRNAs, and a negative siRNA were designed by GenePharma (Shanghai, China). The Bip (GRP78) antibody, the CHOP (L63F7) mouse mAb, the LC3A/B rabbit mAb, and the SQSTM1/p62 antibody were from Cell Signaling (Beverly, MA, USA). The APJ antibody and Beclin 1 antibody were from Santa Cruz Biotechnology (Shanghai) Co, Ltd (Shanghai, China).

Cell culture

The H9c2 cells were cultured in DMEM containing 10% FBS in a humidified incubator with 5% CO₂ in air at 37 °C.

Determination of the cell diameter and volume

Cardiomyocyte hypertrophy was assessed on the basis of increases in the cell diameter, volume, and protein content. Briefly, 1×10⁴ cells/well were seeded in six-well cell culture plates and were incubated with or without treatment. After washing, the cells were resuspended and appropriately diluted in PBS to a concentration of approximately 5×10⁴–1×10⁵ cells/mL, and the single-cell suspensions were placed in 2-mL microcentrifuge tubes. The diameter and volume of the H9c2 rat cardiomyocytes were determined using a Scepter™ Handheld Automated Cell Counter (Millipore, Billerica, USA). The protein content was determined using a bicinchoninic acid (BCA) protein assay kit (HyClone Pierce).

Small interfering RNA (siRNA) transfection

The siRNA targeting Bip or CHOP (GenePharma, Shanghai,

China), NOX4 siRNA (GenePharma, Shanghai, China) or the negative siRNA were transiently transfected into cells using Attractene transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The sequences of the siRNAs were as follows:

Bip siRNA: Sense: 5'-GGAGCGCAUUGAUACUAGATT-3';

Anti-sense: 5'-UCUAGUAUCAAUGCGCUCCTT-3'.

CHOP siRNA: Sense: 5'-CUGGGAAACAGCGCAUGAA-3';

Anti-sense: 5'-UUCAUGCGCUGUUUCCCAG-3'.

Nox4-rat-200 siRNA: Sense: 5'-GGCCUAGGAUUGUGUUU-GATT-3';

Anti-sense: 5'-UCAAACACAAUCCUAGGCCTT-3'.

Nox4-rat-678 siRNA: Sense: 5'-GGCUUGUUGAAGUAU-CAAATT-3';

Anti-sense: 5'-UUUGAUACUUAACAAGCCTT-3'.

Nox4-rat-1237 siRNA: Sense: 5'-GCCCCUUAUCAAUCU-AGATT-3';

Anti-sense: 5'-UCUAGAUUGAAUGAAGGGCTT-3'.

Negative siRNA: Sense: 5'-UUCUCCGAACGUGUCAC-GUTT-3';

Anti-sense: 5'-ACGUGACACGUUCGGAGAATT-3'.

ROS determination

Reactive oxygen species generation was determined using a detection kit based on the fluorescence probe DCFH-DA. Briefly, the cells were incubated with an appropriate volume of diluted DCFH-DA (10 mol/L) for 20 min. Then, the cells were washed with serum-free cell culture medium and examined using fluorescence microscopy.

Reduced and oxidized glutathione determination

The cells were collected and subsequently incubated with a lysis buffer at 30 °C for 30 min. The cells were then clarified by centrifugation (9000×g, 5 min, 4 °C), and the supernatants were used for the determination of the total glutathione using the GSH and GSSG Assay Kit (Beyotime, China).

Reticulophagy detection

Reticulophagy was detected by evaluating the ER fragments using transmission electron microscopy and the number of LC3 puncta using fluorescence microscopy. Briefly, 1×10⁴ cells/well were seeded in six-well cell culture plates and transfected with a plasmid that expressed GFP-LC3 using the Attractene Transfection Reagent for 24 h at 37 °C. Then, the cells were treated with or without apelin-13 for 12 h. In addition, following washing with Hanks' Balanced Salt Solution, the cells were immediately stained with ER-Tracker Red dye for 30 min at 37 °C, and the expression of GFP-LC3 and the ER were visualized using fluorescence microscopy.

Western blotting

Total protein was extracted with a RIPA lysis buffer containing phenylmethanesulfonyl fluoride (PMSF) (9:1) and was measured using a BCA protein assay kit. Aliquots containing 30 µg of protein were subjected to SDS-PAGE, and the proteins were transferred to polyvinylidene difluoride membranes

(Millipore Corp, Bedford, MA, USA). The membranes were incubated for 2 h with primary antibodies diluted in blocking solution and then with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG as the secondary antibody.

Statistical analysis

The experiments were repeated a minimum of three times. All values are presented as the mean $\pm$ SEM. A one-way analysis of variance (ANOVA) was used to determine the statistical significance of the differences. A value of $P<0.05$ or $P<0.01$ was considered to be statistically significant.

Results

Apelin-13 increased reactive oxygen species (ROS) production in H9c2 cells

ROS production always appears in the development of cardiovascular diseases^[10]. Controlled ROS production has been recognized to be an integral part of cellular signaling in cardiovascular diseases, include cardiac hypertrophy^[11]. To bet-

ter understand the role of apelin-13 in the production of ROS, we employed fluorescence microscopy to analyze the production of ROS in H9c2 cells after they were treated with various concentrations of apelin-13 (0.001, 0.01, 0.1, 1, or 2 μ mol/L) for various lengths of time (3, 6, 12, or 24 h). When cells were treated with the various concentrations of apelin-13 for 12 h, the production of ROS was observed in the whole field of vision and increased with the various concentrations of apelin-13, especially in the cells treated with 1 and 2 μ mol/L apelin-13 (Figure 1A and 1B).

Apelin-13 upregulated the expression of NADPH oxidase 4

NADPH oxidase (NOX) plays a key role in ROS generation^[12], and the isoforms of NOX expressed in cardiomyocytes are mainly NOX4 and NOX2^[11]. To better understand the generation of ROS in H9c2 cells, we tested the effect of apelin-13 on the expression of NOX4. After treatment with various concentrations of apelin-13 (0.001, 0.01, 0.1, 1, or 2 μ mol/L) for 12 h, the expression of NOX4 in the H9c2 cells was significantly

Figure 1. Apelin-13 promotes the production of ROS by up-regulating the expression of NOX 4 in H9c2 cells. We stimulated cells with different concentrations of apelin-13 (control, 0.001, 0.01, 0.1, 1, and 2 μ mol/L) for different time (3, 6, 12, 24 h), fluorescence microscope analyze showed that apelin-13 increase the production of ROS (A and B). And the Western blot results showed that apelin-13 increase the expression of NOX4 (C and D). Three distinct, sequence-specific NOX4 siRNAs (Nox4-rat-200; Nox4-rat-678; Nox4-rat-1237) and a negative siRNA were designed, and the Nox4-rat-1237 has the best interference efficiency of NOX4 (E). These results suggest that apelin-13 increase the production of ROS by NOX4. The data are the mean $\pm$ SD, $n=4$, * $P<0.05$ vs the control group; ** $P<0.05$ vs the apelin-13 treated group.

increased in a manner related to the concentration of apelin-13 (Figure 1C). In addition, the expression of NOX4 was also significantly increased as a function of the apelin-13 treatment times (3, 6, 12, or 24 h) (Figure 1D). These findings suggest that apelin-13 increased the expression of NOX4 in dose- and time-dependent manners.

NOX4 siRNA reversed the decrease of the [GSH]/[GSSG] ratio induced by apelin-13

Oxidative stress (OS), which is due to an excess of pro-oxidant species [reactive oxygen species (ROS)] that is not counter-balanced by endogenous antioxidant molecules [eg, reduced glutathione (GSH)], is involved in the pathogenesis of cardiovascular diseases. We designed three distinct, sequence-specific NOX4 siRNAs (Nox4-rat-200, Nox4-rat-678, and Nox4-rat-1237), of which Nox4-rat-1237 was the most efficient at interfering with the expression of NOX4 (Figure 1E). Next, we analyzed the levels of the antioxidant markers in the cells. The GSSG levels were significantly higher and the GSH levels were significantly lower in the apelin-13-treated cells, and these effects were reversed in the cells treated with apelin-13+Nox4-rat-1237 (Table 1). The GSH/GSSG ratio was significantly lower in the apelin-13-treated cells, but this was reversed in the apelin-13+Nox4-rat-1237-treated cells (Table 1).

Apelin-13 induced ER stress through ROS

Given the central importance of the ER stress in the process of myocardial hypertrophy, we determined whether apelin-13 activates ER stress in H9c2 cells. After treatment with various concentrations of apelin-13 (0.001, 0.01, 0.1, 1, or 2 $\mu\text{mol/L}$) for 12 h or with apelin-13 at 1 $\mu\text{mol/L}$ for various times (3, 6, 12, or 24 h), Bip and CHOP were significantly increased as functions of the apelin-13 treatment concentration and time in the H9c2 cells (Figure 2A and 2B). In addition, four distinct, sequence-specific shRNAs that targeted the APJ mRNA and a negative shRNA were designed and cloned into the pGPU6/Neo vector (pGPU6/Neo-rat-531; pGPU6/Neo-rat-759; pGPU6/Neo-rat-1071; pGPU6/Neo-rat-399; and pGPU6/neo-shNC), and one of shRNAs was chosen based on its ability to interfere in the expression of APJ. The increases in Bip and CHOP were blocked when the cells were treated with the APJ shRNA (Figure 2C and 2D). Moreover, similar results were

observed when the cells were treated with the NOX inhibitor diphenyleneiodonium (DPI), Nox4-rat-1237 or NAC (Figure 2E-2G), which suggested that ROS play an important role in the activation of the ER stress induced by apelin-13.

Apelin-13 triggers reticulophagy

Autophagy is a pathway that evolved to degrade bulk cytoplasmic material. This pathway provides an important function in the clearance of aggregated or misfolded proteins in the ER. Reticulophagy acts as a selective form of autophagy that removes damaged or redundant endoplasmic reticulum fragments and has an important association with endoplasmic reticulum stress^[13]. Given the central importance of autophagy in ER stress, we monitored the morphological features of autophagy and ER stress using transmission electron microscopy (TEM). We detected autophagosomes, autolysosomes and endoplasmic reticulum fragments in the apelin-13 (1 $\mu\text{mol/L}$, 12 h)-treated group; these were not visible in the untreated cells (Figure 3A). To further extend our results, we examined the delivery of GFP-LC3 to the ER by transfecting the cells with a plasmid that expressed GFP-LC3 and staining with ER-tracker Red. In the untreated cells, the peripheral endoplasmic reticulum was visible as short membrane segments underneath the plasma membrane as indicated by the endoplasmic reticulum-tracker, and no GFP-LC3 expression was present. When the cells were treated with apelin-13 for 12 h, the peripheral ER appeared as long stretches of cortical membrane with prominent cytoplasmic extensions, and GFP-LC3 expression was observed in the endoplasmic reticulum (Figure 3B). These data confirmed that apelin-13 triggered reticulophagy in the cells.

ROS participated in the process of the autophagy induced by apelin-13

In our previous results, apelin-13 increased the expression of the autophagy markers beclin 1 and LC3-II/I. Here, we used APJ shRNA to interfere with the expression of APJ and found that the increases in beclin 1 and LC3-II/I induced by apelin-13 were prevented when the cells were treated with the APJ shRNA (Figure 4A). Moreover, the increases in beclin 1 and LC3-II/I were also reversed when the cells were treated with DPI, Nox4-rat-1237 or NAC (Figure 4B-4D). We also

Table 1. Nox4 siRNA blocked the increased of GSH/GSSG induced by apelin-13.

Group	GSSG	GSH	GSH/GSSG
Control	11.01±0.805	122.4±4.63	11.16±0.9918
Apelin-13	46.85±3.580*	107±29.06*	2.322±0.7103*
Apelin-13+Nox4-rat-1237	27.60±4.181 [#]	146.5±27.89 [#]	6.736±1.212 [#]
Nox4-rat-1237	12.41±0.860	171.4±54.28*	13.75±3.823
Apelin-13+negative control	25.09±3.379 [#]	108.1±32.93	4.468±1.880 [#]
Negative control	24.41±6.386*	305.7±75.80*	12.77±3.386

Apelin-13: 2 $\mu\text{mol/L}$; time: 48 h; Nox4-rat-1237: Nox4 siRNA; NC: negative control. Data are shown as the mean±SD of 3 independent experiments.

* $P<0.05$ vs the control group. [#] $P<0.05$ vs the apelin-13 group.

Figure 2. Apelin-13 induced the ER stress by ROS. We chosen the expression of Bip and CHOP as the marker of ER stress, Western blot results found that apelin-13 increased the expression of Bip and CHOP as dose and time dependent manners, especially at 1 $\mu\text{mol/L}$, 24 h (A and B). To further demonstrate it, we designed and synthesized 4 APJ shRNA to transfect the H9c2 cells, and pGPU6/Neo-rat-399 has the best interferent efficiency (C). APJ shRNA pGPU6/Neo-rat-399 abolished the increase of Bip and CHOP induced by apelin-13 (D). The antioxidants NAC, NADPH inhibitor DPI or Nox4-rat-1237 also crippled the increase of ER stress marker Bip and CHOP induced by apelin-13 (E-G). The data are the mean $\pm$ SD, $n=4$, $*P<0.05$ vs the control group; $\#P<0.05$ vs the apelin-13 treated group.

found that DPI, Nox4-rat-1237 or NAC inhibited the decrease in the autophagy substrate protein p62 (Figure 4E-4G), which suggested that ROS were involved in the autophagy induced by apelin-13.

ER stress regulated autophagy induced by apelin-13

It has been reported that ER stress regulates autophagy through 3 signaling pathways: PERK-eIF-2 α , IRE-1 and Ga²⁺[8]. We used tunicamycin to increase abnormal protein accumulation in the ER, salubrinal to inhibit eIF-2 α phosphatase, and Bip and CHOP siRNA to interfere with the expression of Bip and CHOP to determine whether ER stress regulated the cellular autophagy that was induced by apelin-13. We found that salubrinal blocked the increases in the autophagy

markers beclin 1 and LC3-II/I induced by apelin-13 and also inhibited the decrease of the autophagy substrate protein p62. By contrast, tunicamycin has no effect on the expression of beclin 1, LC3-II/I or p62 (Figure 5A and 5B). The Bip and CHOP siRNA also reversed the increases in beclin 1, LC3-II/I induced by apelin-13 and the decrease in p62 (Figure 5C and 5D).

ROS and ER stress were involved in the process of myocardial hypertrophy induced by apelin-13

We have reported that apelin-13 promotes cellular hypertrophy through autophagy. Here, we used Nox4-rat-1237 to interfere with the ROS production and found that Nox4-rat-1237 decreased the expression of BNP that was induced

Figure 3. Apelin-13 triggers reticulophagy. We monitored the morphological features of cells by transmission electron microscopy (TEM), and we clearly observed that apelin-13 (1 $\mu\text{mol/L}$; 12 h) increased the formation of autophagosome and ER fragment compared with control group (A). We also observed the expression of LC3 in ER by transfecting with a plasmid expressing GFP-LC3 and staining with endoplasmic reticulum (ER)-tracker Red. We found that the peripheral ER appeared as long stretches of cortical membrane with prominent cytoplasmic extension, and the GFP-LC3 expression observed in endoplasmic reticulum, while are not appeared in the untreated cells (B). $n=4$.

by apelin-13 and suppressed the apelin-13-induced increases in the cell diameter (Figure 6A), volume and protein content (Table 2). The same results were found when we used salubrinal to inhibit eIF-2 α phosphatase or tunicamycin to increase abnormal protein accumulation in the ER (Figure 6B, Table 3). Bip siRNA and CHOP siRNA also blocked the apelin-13-induced increases in the cell diameter, volume and protein content (Table 4). All of these results suggested that ROS and ER stress were involved in the process of hypertrophy induced by apelin-13.

Discussion

APJ is a G protein-coupled receptor that was discovered by O'Dowd in 1993. It was named the angiotensin II receptor-like

1 receptor because it is a homolog of the angiotensin II AT1 receptor. Apelin, which was discovered in 1998, is the endogenous peptide ligand for the human APJ receptor. This peptide has 77 amino acid residues and can be fragmented into various sizes of active peptides, including apelin-36, apelin-31, apelin-28, apelin-19, apelin-13, and apelin-12. Apelin-13 is considered to be the main subtype in the human heart and plays important roles in the cardiovascular system, such as in positive inotropic responses^[14], vascular smooth muscle proliferation^[15] and endothelial cell adhesion^[16]. Our laboratory has been committed to elucidating the role of apelin in the cardiovascular system and has discovered that apelin-13 promotes smooth muscle cell proliferation through the PI3K/Akt^[17], 14-3-3^[18], the Jagged-1/Notch3^[19] and NOX4 signaling path-

Figure 4. ROS involved in the autophagy induced by apelin-13. We used APJ shRNA to interfere the expression of APJ and we found that APJ shRNA blocked the increase of autophagy marker beclin 1 and LC3-II/I induced by apelin-13 (A). We used antioxidants NAC to eliminate the ROS, NADPH oxide inhibitor DPI or Nox4-rat-1237 to block the production of ROS. And we found that NAC, DPI or Nox4-rat-1237 all blocked the increase of autophagy marker beclin 1 and LC3-II/I induced by apelin-13 (B–D). In addition, NAC, DPI or Nox4-rat-1237 all abolished the decrease of p62 induced by apelin-13 (E–G). These suggest that ROS involved in the autophagy induced by apelin-13. The data are the mean±SD, $n=4$, * $P<0.05$ vs the control group; # $P<0.05$ vs the apelin-13 treated group.

ways^[20]. Apelin-13 regulates blood pressure through 14-3-3 signaling and promotes mononuclear cell adhesion to human umbilical vein endothelial cells^[21].

Myocardial hypertrophy is an adaptive response of the heart to hypertension or cardiovascular disease and is characterized by increases in the myocardial cell volume, protein synthesis, etc. In cardiomyocytes, high expression of APJ/apelin has been observed^[22]. Apelin has a positive inotropic effect on cardiac contractility^[23] and induces a long-term functional improvement in cardiac contraction^[5]. However, in addition to its protective role, increasing numbers of recent studies have indicated that apelin promotes the generation of myocardial hypertrophy. For example, apelin-13 increases the rat ven-

tricular diastolic pressure ratio and ventricular wall^[5]. APJ-knockout mice have reduced susceptibility to the myocardial hypertrophy that results from a pressure load^[3]. APJ mediates the pressure response by acting as a pressure sensor^[4]. Furthermore, apelin-13 promotes increases in the diameter, volume and protein content of myocardial cells^[6]. The PI3K-Akt-ERK1/2-p70S6K and PI3K-autophagy signaling pathways are involved in the myocyte hypertrophy induced by apelin-13^[6]. In the present study, we found that apelin increased the production of ROS and induced ER stress. Salubrinal, Bip siRNA and CHOP siRNA blocked the increases in the myocardial cell diameter, volume and the protein content that were induced by apelin-13, which suggested that ER stress was involved

Figure 5. ER stress involved in the autophagy induced by apelin-13. We used the salubrinol to inhibit eIF-2 α phosphatase, Tunicamycin to increase the abnormal protein accumulation in ER. And we found that salubrinol blocked the increase of autophagy marker beclin 1 and LC3-II/I induced by apelin-13 (A); and also inhibit the decrease of the autophagy substrate protein p62 (C); while Tunicamycin has no effect on the expression of beclin 1, LC3-II/I and p62 (A and C). We used Bip siRNA and CHOP siRNA to interfere the expression of Bip and CHOP, and to discuss if ER stress regulated cells autophagy induced by apelin-13. Bip siRNA and CHOP siRNA were also blocked the increase of beclin 1 and LC3-II/I (B), the decrease of the autophagy substrate protein p62 induced by apelin-13 (D). The data are the mean $\pm$ SD, $n=4$, * $P<0.05$ vs the control group; # $P<0.05$ vs the apelin-13 treated group.

in the process of myocyte hypertrophy that was induced by apelin-13.

Dysfunction of the ER, known as ER stress, is a pathological process induced by various stimuli that is characterized by an

accumulation of unfolded/misfolded proteins and calcium dysregulation. An appropriate endoplasmic reticulum stress response results in the degradation of the agglomerated proteins to restore the steady state in the endoplasmic reticulum

Figure 6. ER stress and autophagy blocked the increase of BNP induced by apelin-13. We used Nox4-rat-1237 to interfere the ROS production, and found that Nox4-rat-1237 decreases the expression of BNP induced by apelin-13 (A). The same results were found when we used the salubrinal to inhibit eIF-2 α phosphatase (B); while there is no effect on the expression of BNP when we used Tunicamycin to increase the abnormal protein accumulation in ER (B). The data are the mean $\pm$ SD, $n=4$, * $P<0.05$ vs the control group; # $P<0.05$ vs the apelin-13 treated group.

Table 2. Nox4 siRNA blocked the cardiomyocytes hypertrophy induced by apelin-13.

Group	Diameter ($\mu\text{m}/\text{cell}$)	Volume ($\times 10^3 \mu\text{m}^3/\text{cell}$)	Protein (pg/cell)
Control	15.04 $\pm$ 0.66	1.79 $\pm$ 0.24	441.7 $\pm$ 33.7
Apelin-13	17.36 $\pm$ 0.28***	2.69 $\pm$ 0.05**	780.7 $\pm$ 13.3***
apelin-13+Nox4-rat-1237	16.04 $\pm$ 0.05#	2.04 $\pm$ 0.13#	405.7 $\pm$ 10.3###
Nox4-rat-1237	15.61 $\pm$ 0.09##	1.97 $\pm$ 0.11#	372.7 $\pm$ 2.1###
Apelin-13+NC	17.45 $\pm$ 0.08***	2.70 $\pm$ 0.504**	663.3 $\pm$ 65.2***
NC	14.66 $\pm$ 0.14	1.61 $\pm$ 0.11	416.0 $\pm$ 1.7

Apelin-13: 2 $\mu\text{mol}/\text{L}$; time: 48 h; Nox4-rat-1237: Nox4 siRNA; NC: negative control. Data are shown as the mean $\pm$ SD of 3 independent experiments. * $P<0.05$ vs the control group. # $P<0.05$ vs the apelin-13 group.

Table 3. Salubrinal and tunicamycin blocked the cardiomyocytes hypertrophy induced by apelin-13.

Group	Diameter ($\mu\text{m}/\text{cell}$)	Volume ($\times 10^3 \mu\text{m}^3/\text{cell}$)	Protein (pg/cell)
Control	10.115 $\pm$ 0.46	0.544 $\pm$ 0.076	677 $\pm$ 139
Apelin-13 (2 $\mu\text{mol}/\text{L}$)	10.91 $\pm$ 0.49*	0.684 $\pm$ 0.095*	942 $\pm$ 202*
Apelin-13+tunicamycin	12.57 $\pm$ 0.30#	1.042 $\pm$ 0.075 #	1429 $\pm$ 217#
Apelin-13+salubrinal	9.654 $\pm$ 1.29#	0.488 $\pm$ 0.176#	606 $\pm$ 125#
Tunicamycin	11.6 $\pm$ 2.1*	0.870 $\pm$ 0.047*	1098 $\pm$ 48*
Salubrinal	8.79 $\pm$ 0.42*	0.357 $\pm$ 0.050*	363 $\pm$ 152*
DMSO	10.19 $\pm$ 1.03	0.61 $\pm$ 0.221	725 $\pm$ 109

Apelin-13: 2 $\mu\text{mol}/\text{L}$; time: 48 h; salubrinal: eIF-2 α phosphatase inhibitor; tunicamycin: increase the abnormal protein accumulation in ER. Data are mean $\pm$ SD of 3 independent experiments. * $P<0.05$ vs control group; # $P<0.05$ vs apelin-13 group.

by activating the unfolded protein response (UPR). However, when the stress exceeds the adaptive capability of the body, the apoptosis-related protein C/EBP homologous protein

(CHOP) increases and triggers a series of pathological reactions including cell apoptosis, adipogenesis and inflammatory pathways. Studies have reported that endoplasmic reticulum

Table 4. Bip siRNA and CHOP siRNA blocked the cardiomyocytes hypertrophy induced by apelin-13.

Group	Diameter ($\mu\text{m}/\text{cell}$)	Volume ($\times 10^3 \mu\text{m}^3/\text{cell}$)	Protein (pg/cell)
Control	10.115 $\pm$ 0.46	0.544 $\pm$ 0.076	677 $\pm$ 139
Apelin-13 (2 $\mu\text{mol}/\text{L}$)	10.91 $\pm$ 0.49*	0.684 $\pm$ 0.095*	942 $\pm$ 202*
Apelin-13+bip siRNA	8.616 $\pm$ 0.28#	0.335 $\pm$ 0.033#	457 $\pm$ 327#
Apelin-13+CHOP siRNA	8.490 $\pm$ 0.12#	0.320 $\pm$ 0.013#	302 $\pm$ 154#
Apelin-13+negative siRNA	10.389 $\pm$ 0.75	0.594 $\pm$ 0.124	819 $\pm$ 215
Bip siRNA	7.937 $\pm$ 0.40*	0.263 $\pm$ 0.039*	168 $\pm$ 138*
CHOP siRNA	8.181 $\pm$ 0.33*	0.287 $\pm$ 0.034*	368 $\pm$ 45*
Negative siRNA	10.112 $\pm$ 2.48	0.61 $\pm$ 0.460	638 $\pm$ 144

Apelin-13: 2 $\mu\text{mol}/\text{L}$; time: 48 h; Data are mean $\pm$ SD of 3 independent experiments. * P <0.05 vs control group; # P <0.05 vs apelin-13 group.

stress is important in the regulation of myocardial hypertrophy^[12] and that this process is inhibited by beta adrenergic receptor blockers in myocardial hypertrophy^[24]. Our results showed that apelin increased the expression of Bip and CHOP in H9c2 cells, and the endoplasmic reticulum fragment was observed by TEM in the apelin-13 (1 $\mu\text{mol}/\text{L}$, 12 h)-treated group. Therefore, we speculated that apelin-13 was likely to trigger the endoplasmic reticulum stress.

Autophagy is a cellular response to stimulation that transfers the damaged proteins to the cytolysosomes for degradation^[25]. According to the selective degradation of the substrates, "selective autophagy" has been described and may play an important role in maintaining cell homeostasis^[26]. Endoplasmic reticulum autophagy (reticulophagy), one of the forms of selective autophagy, is an important major degradation process that maintains the steady state of the endoplasmic reticulum^[27]. It is not clear how to detect reticulophagy, nor is the regulatory mechanism of reticulophagy understood. The presence of endoplasmic reticulum fragments in the autophagosomes or of autophagy marker proteins in the endoplasmic reticulum may be associated with the phenomenon of reticulophagy^[28]. In our results, endoplasmic reticulum fragments were observed in the autophagosomes and autolysosomes of the apelin-13 (1 $\mu\text{mol}/\text{L}$, 12 h)-treated cells. Expression of GFP-LC3 in the endoplasmic reticulum also appeared in apelin-13 (1 $\mu\text{mol}/\text{L}$, 12 h)-treated cells. These observations may be consistent with reticulophagy. However, in itself, the observation of endoplasmic reticulum fragments in the autophagosome is not sufficient evidence for reticulophagy because the ER/cytoplasm membranes may be directly imported into the vacuoles during autophagy^[29]. Further studies are needed to confirm that reticulophagy occurs in apelin-13-treated cells.

Several lines of evidence suggest that there is communication between ER stress and autophagy. ER stress triggers cellular autophagy through the UPR^[30, 31]. This process involves the ER molecular chaperone GRP78/BiP and three sensor proteins: PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE-1), and activating transcription factor 6 (ATF6)^[32]. Endoplasmic reticulum stress and autophagy have been reported to control and correct the protein quality in diabetic cardiomyopathy^[33] and ER stress-induced cardiac anomalies^[34]. In

addition, ER stress acts through the PERK/eIF2 alpha pathway to activate CHOP gene transcription and to regulate the apoptosis induced by triggering autophagy^[35]. Knocking out the PERK-eIF2 alpha gene inhibited the autophagic reaction that is induced by ER stress^[36]. Our results showed that the ER stress inhibitors salubrinal, Bip siRNA and CHOP siRNA blocked the increases in the autophagy markers beclin 1 and LC3-II/I that were induced by apelin-13 and inhibited the decrease in the autophagy substrate protein p62, which suggested that ER stress was involved in the process of autophagy induced by apelin-13. However, it was interesting that the ER stress inducer tunicamycin had no effect on the expression of beclin 1, LC3-II/I or p62 induced by apelin-13. Tunicamycin, an ER stress inducer, increases abnormal protein accumulation in the ER by inhibiting the N-glycosylation of proteins^[37, 38]. We suspect that an increased accumulation of abnormal proteins may not be the reason for the autophagy induced by apelin-13.

Endoplasmic reticulum stress can be caused by many factors, including the production of ROS and altered protein glycosylation (tunicamycin). The regulation of autophagy is also very complicated, and the production of reactive oxygen species by NOX is an important factor that can cause autophagy^[39, 40]. It has been reported that NOX-driven reactive oxygen species play an important role in the process of recruiting LC3^[41]. Another study found that NOX4-derived reactive oxygen species induce cardiomyocyte autophagy by activating the PERK/eIF-2 alpha/ATF4 pathway. Our results showed that the increased concentrations of apelin-13 are associated with increased ROS production. Apelin-13 also increases the expression of NOX4. DPI or Nox4 siRNA not only inhibited the increases in Bip and CHOP that were induced by apelin-13 but also blocked the increases in the autophagy marker proteins LC3-II/I and beclin 1 that were induced by apelin-13. Together, these results suggested that apelin-13 regulated the NADPH oxidase-derived reactive oxygen species. Furthermore, ROS regulated the cellular ER stress and autophagy that are induced by apelin-13.

However, a limitation of our study was that it did not provide sufficient evidence to define a cause-effect relationship between ER stress, ROS and autophagy. There was also not sufficient evidence to conclude that reticulophagy was pres-

ent in the apelin-induced process of cell hypertrophy. In terms of future research, it would be interesting to investigate how to regulate ROS, ER stress and autophagy to protect the myocardium. It would also be useful to explore the causal relationship between ROS, ER stress and autophagy in cardiac hypertrophy, especially in primary myocardial cells or even in animals. Another valuable area for future research would be to determine the most appropriate ways to detect reticulophagy in the cardiac hypertrophy induced by apelin and to determine how to trigger it.

Conclusion

Overall, our data indicate that ER stress triggers autophagy in cardiomyocyte hypertrophy and plays an important role in the cardiomyocyte hypertrophy that is induced by apelin-13. In particular, we provided an important new piece of information that reticulophagy, a new form of selective autophagy, was involved in the process of apelin-13-induced cardiomyocyte hypertrophy, which has never before been reported. Our results suggest that ER stress-autophagy is involved in the H9c2 rat cardiomyocyte hypertrophy induced by apelin-13.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (81503074, 81270420, 81470434, and 81670265), Hunan Provincial Natural Science Foundation (14JJ3102; 2017JJ2227), China Postdoctoral Science Foundation (2014M560647). We especially thank Bing-bing WANG (Assistant Professor, Perinatal Biology Laboratory, Division of Maternal-Fetal Medicine, Rutgers University-Robert Wood Johnson Medical School, USA) for the English language correction.

Author contribution

Lin-xi CHEN, Lan-fang LI, and Feng XIE conceived and designed the experiments; Feng XIE, Di WU, Shi-fang HUANG, Jian-gang CAO, He-ning LI, Lu HE, and Mei-qing LIU performed the experiments; Lin-xi CHEN, Lan-fang LI, Feng XIE, Di WU, and Shi-fang HUANG analyzed the data; Lin-xi CHEN and Lan-fang LI modified the language; Lin-xi CHEN and Lan-fang LI contributed reagents/materials/analysis tools; and Feng XIE wrote the paper.

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