

# Palmitate induces myocardial lipotoxic injury via the endoplasmic reticulum stress-mediated apoptosis pathway

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**Abstract.** Increased free fatty acids in cardiomyocytes induce myocardial lipotoxic injury, but the underlying mechanisms are not well understood. Therefore, the aim of the present study was to explore the role of the endoplasmic reticulum (ER) stress-mediated apoptosis pathway in palmitate (PA)-induced cardiomyocyte lipotoxicity. H9c2 cells were treated with various doses (100, 200 and 400  $\mu$ M) of PA to mimic cardiomyocyte lipotoxicity *in vitro*. Oil Red O staining was used to determine the accumulation of intracellular lipids. An MTT assay was used to determine the cell viability. Lactate dehydrogenase (LDH) activity was used to measure the injury of H9c2 cells. Flow cytometry analysis was used to detect apoptosis. Western blotting was used to evaluate the expression change of ER stress-mediated apoptosis pathway proteins, including 78 kDa glucose-regulated protein (GRP78), eukaryotic initiation factor 2  $\alpha$  (eIF2 $\alpha$ ), protein kinase R-like endoplasmic reticulum kinase (PERK), C/EBP homologous protein (CHOP) and cleaved caspase-12. The results demonstrated that various doses of PA promoted excessive lipid deposition in cardiomyocytes and resulted in decreased cell viability, and increased the LDH activity and apoptosis rate in a dose-dependent manner. Furthermore, the expression of GRP78, a marker of ER stress, and the phosphorylation of eIF2 $\alpha$  and PERK were increased following treatment with PA. Notably, the levels of CHOP and cleaved caspase-12, critical regulators of ER stress-mediated apoptosis pathway, were also elevated, and this effect was reversed by a specific ER stress inhibitor (4-phenyl butyric acid). In conclusion, the results of the current study demonstrated that PA induces myocardial lipotoxic injury by triggering ER stress and the ER stress-mediated apoptosis pathway.

## Introduction

Lipotoxic cardiomyopathy often occurs in patients with diabetes and obesity, and is attributed to excessive accumulation of lipids and their intermediate products in cardiomyocytes (1). Diabetes and obesity usually result in the disordered lipid metabolism and elevation of free fatty acids (FFAs) (2). Previous studies have demonstrated that increased FFAs, particularly saturated FFAs, induce myocardial injury, and ultimately result in heart dysfunction to some extent (3,4). However, the underlying mechanisms responsible for lipotoxic cardiomyopathy remain unknown.

The endoplasmic reticulum (ER) is a fundamental organelle that has a key role in the modification, folding and oligomerization of the majority synthesized structural and secreted proteins (5). It has been established that multiple physiological and pathological conditions, including hypoxia, hyperglycemia and acidosis, can result in ER homeostasis breakdown and an elevated accumulation of unfolded/misfolded proteins within the ER lumen, which is commonly termed 'ER stress' (6). In order to cope with ER stress, cells initially induce the unfolded protein response (UPR) signaling to adapt to stress conditions and maintain the balance of ER homeostasis again. However, prolonged or excessive ER stress will trigger ER stress-mediated apoptosis pathway, which is an apoptosis signal pathway independent of the death-receptor and mitochondria mediated-apoptosis pathways (7).

Palmitate (PA), a type of saturated FFA, induces cardiomyocyte apoptosis (8), and thus, is used to mimic cardiomyocytes lipotoxicity *in vitro* (9). Several previous studies have reported that PA induces ER stress-mediated apoptosis in hepatocytes (10), pancreatic  $\beta$  cells (11) and mature adipocytes (12), but whether PA induces ER stress-mediated apoptosis in cardiomyocytes remains unknown. Therefore, the aim of the present study was to explore the role of ER stress-mediated apoptosis pathway in PA-induced cardiomyocyte lipotoxicity.

## Materials and methods

**Cell culture and PA treatment.** The H9c2 rat cardiomyocyte cell line, obtained from the Shanghai Institutes for Biological Sciences (Shanghai, China), was routinely cultured in Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal

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bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>. When the confluence of H9c2 cells was ~80%, PA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at doses of 100, 200 and 400  $\mu$ M was added to the medium and incubated for 12 h. An ER stress inhibitor, 4-phenyl butyric acid (4-PBA; 5 mM), was administrated 90 min before 400  $\mu$ M PA treatment.

**Oil Red O staining.** Oil Red O staining was used to measure the accumulation of intracellular lipids. Briefly, 150 mg Oil Red O powder (Sigma-Aldrich; Merck KGaA) was dissolved in 50 ml 60% isopropanol to prepare the Oil Red O reserve solution (3 mg/ml) and further diluted by distilled water (3:2) to prepare a working solution. H9c2 cells were fixed in 4% paraformaldehyde for 30 min at room temperature, washed with PBS three times, then followed by incubation with the Oil Red O working solution for 30 min at room temperature. After washing with PBS three times, nuclei were counterstained with 50% hematoxylin for 2 min at room temperature. Finally, the result of Oil Red O staining was observed under an optical microscope (Olympus Corporation, Tokyo, Japan).

**Measurement of cell viability.** MTT assay was used to determine the cell viability following PA treatment. Briefly, H9c2 cells were seeded in a 96-well plate and treated with various concentrations of PA as described above. After 12 h, the medium was removed and replaced with 0.5 mg/ml MTT solution (Sigma-Aldrich; Merck KGaA), 200  $\mu$ l per well. After incubation at 37°C for 4 h, the MTT solution was removed and each well was washed with PBS three times. The precipitated formazan in each well was solubilized by dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) and the optical density value was detected by an automated microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 490 nm.

**Measurement of cell injury.** Lactate dehydrogenase (LDH) activity in the culture medium was detected to determine the injury of H9c2 cells using a commercial LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols.

**Measurement of apoptosis.** Apoptosis was detected by flow cytometry analysis using the PE-labeled Annexin V/7-aminoactinomycin D apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocols.

**Western blotting analysis.** Cells were harvested after PA treatment for 12 h and mixed with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Nanjing, China) and protease inhibitor phenylmethanesulfonyl fluoride on ice for 20 min. Proteins were extracted from cells following 5 min of centrifugation of 13,000  $\times$  g at 4°C. The concentration of protein was measured using Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology). For western blot analysis, 50  $\mu$ g protein denatured by heating was subjected to 10% SDS-polyacrylamide gel electrophoresis for separation and then transferred to polyvinylidene difluoride membranes. The

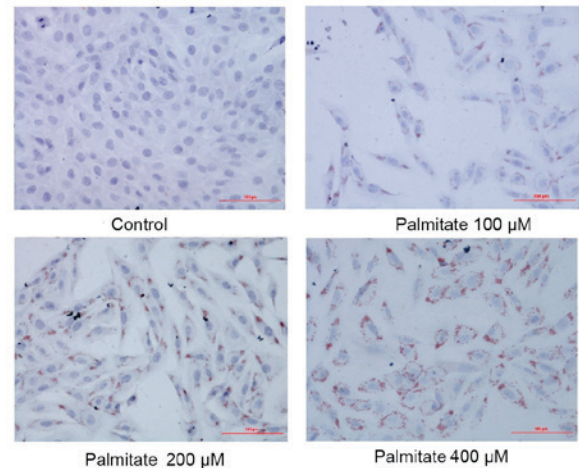


Figure 1. Palmitate-induced intracellular accumulation of lipids in H9c2 cells. Lipid droplets in cardiomyocytes were stained red by Oil Red O. Cells were treated with palmitate for 12 h. Scale bar, 100  $\mu$ m.

membrane was blocked using 2% bovine serum albumin (BSA) for 1 h at room temperature and then incubated overnight at 4°C with the specific primary antibodies including monoclonal anti-78 kDa glucose-regulated protein (GRP78; cat no. 3183; 1:1,000), anti-protein kinase R-like endoplasmic reticulum kinase (PERK; cat no. 3192; 1:1,000), anti-phospho (p)-PERK (cat no. 3179; 1:1,000), anti-eukaryotic initiation factor 2  $\alpha$  (eIF2 $\alpha$ ; cat no. 5324; 1:1,000), anti-p-eIF2 $\alpha$  (cat no. 3398; 1:1,000) (all from Cell Signaling Technology, Inc., Danvers, MA, USA), anti-cleaved caspase-12 (cat no. ab18766; 1:1,000), anti-C/EBP homologous protein (CHOP; 1:1,000, cat no. ab11419) (both from Abcam, Cambridge, UK), and anti- $\beta$ -actin (TA-09; 1:2,000; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit (cat no. sc-2004; 1:2,000) or rabbit anti-mouse immunoglobulin G (cat no. sc-358914; 1:2,000) (both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 37°C for 2 h. Detection of protein band was performed using an enhanced chemiluminescence kit (Santa Cruz Biotechnology, Inc.) according to the manufacturer's protocols. The levels of phosphorylated proteins were normalized to their corresponding total protein levels. Relative densitometry was calculated using ImageJ software version 2x (National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis.** The data were expressed as the mean  $\pm$  standard deviation. Statistical analysis was performed by software SPSS 17.0 version (SPSS, Inc., Chicago, IL, USA). Differences between groups were initially evaluated using one-way analysis of variance, and if the differences were significant, multiple comparison analysis was further performed using Fisher's least significant difference test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Effect of PA on cell lipid accumulation.** The result of Oil Red O staining demonstrated a gradual increase in the extent of lipid accumulation in H9c2 cells following treatment with increasing concentrations of PA (0, 100, 200 and

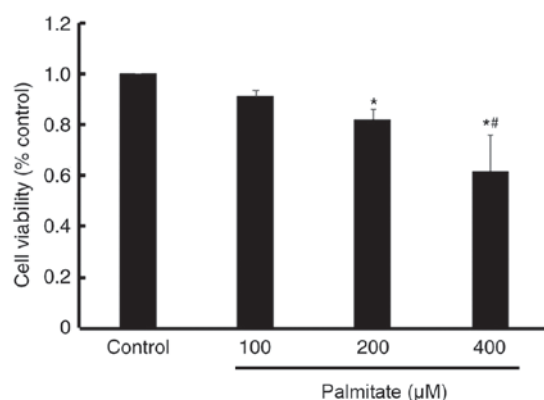


Figure 2. H9c2 cells viability was measured by MTT assay following treatment with various doses of palmitate for 12 h. \*P<0.05 vs. control; \*\*P<0.05 vs. 200 μM.

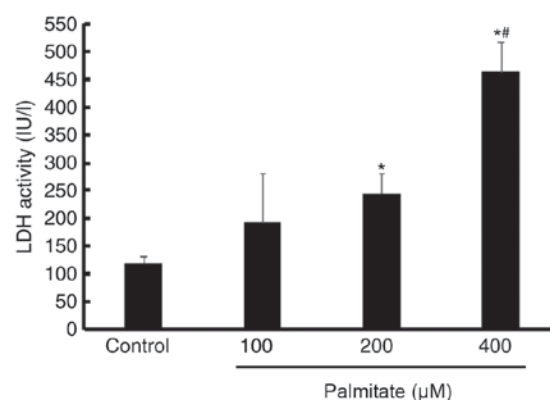


Figure 3. LDH activity was measured following treatment with various doses of palmitate for 12 h. \*P<0.05 vs. control; \*\*P<0.05 vs. 200 μM. LDH, lactate dehydrogenase.

400 μM; Fig. 1), suggesting PA induced excessive lipid accumulation in H9c2 cells.

**Effect of PA on cell viability.** An MTT assay demonstrated a significant decrease in cell viability following treatment with 200 and 400 μM PA compared with the control. Furthermore, PA reduced cell viability in a dose-dependent manner (Fig. 2).

**Effect of PA on cell injury.** An LDH assay indicated that LDH activity was significantly increased by 200 and 400 μM PA compared with the control. Furthermore, LDH activity in the 400 μM PA treatment group was higher than in the 200 μM PA group, which suggested that PA induced H9c2 cell injury in a dose-dependent manner (Fig. 3).

**Effect of PA on cell apoptosis.** PA (200 and 400 μM) significantly increased H9c2 cell apoptosis compared with the

control, demonstrated by increased apoptosis rate. In addition, apoptosis induced by PA was also displayed in a dose-dependent manner (Fig. 4).

**Effect of PA on the expression of ER stress markers.** As presented in Fig. 5, the expression of GRP78, a well-established ER stress marker, was increased by various dose of PA in a dose-dependent manner. Furthermore, the phosphorylation of eIF2α and PERK were also increased in a dose-dependent manner (Fig. 5), which indicated that PERK/eIF2α signaling may be activated by PA.

**Effect of PA on the ER stress-mediated apoptosis pathway.** Various doses of PA significantly increased the expression of CHOP compared with the control; however, no significant differences in the expression of CHOP were detected among the different PA-treated groups. Furthermore, various dose of

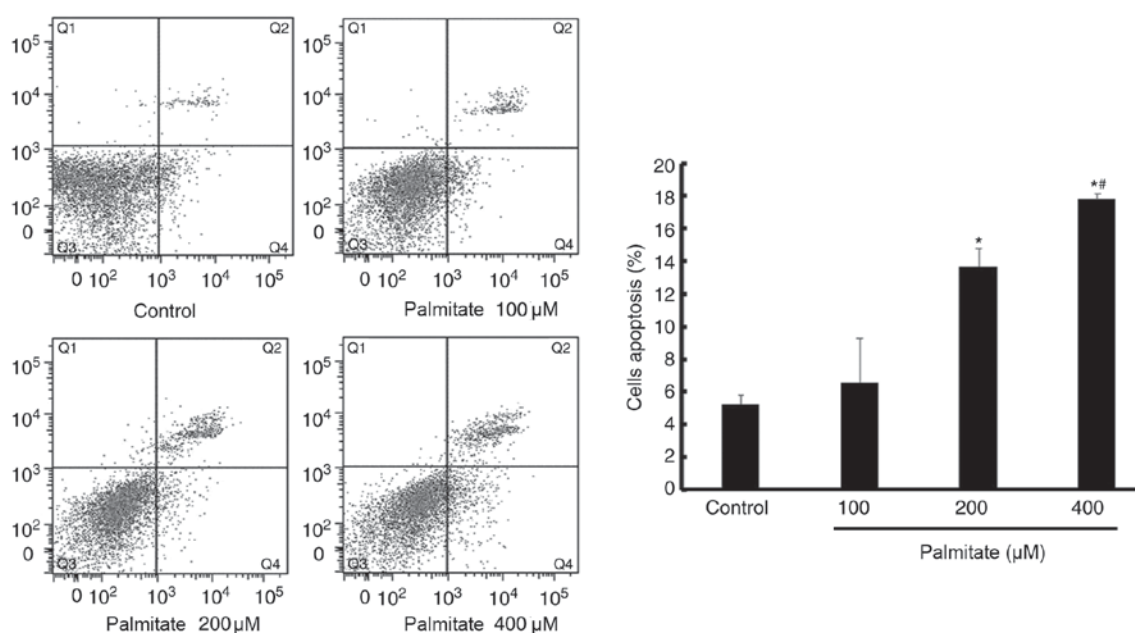


Figure 4. H9c2 cell apoptosis were measured by flow cytometry analysis following treatment with various doses of palmitate for 12 h. The X-axis represents PE labeled-annexin V and the Y-axis represents 7-aminoactinomycin D. The sum of Q2 and Q4 quadrants was used to show apoptosis. \*P<0.05 vs. control; \*\*P<0.05 vs. 200 μM. LDH, lactate dehydrogenase.

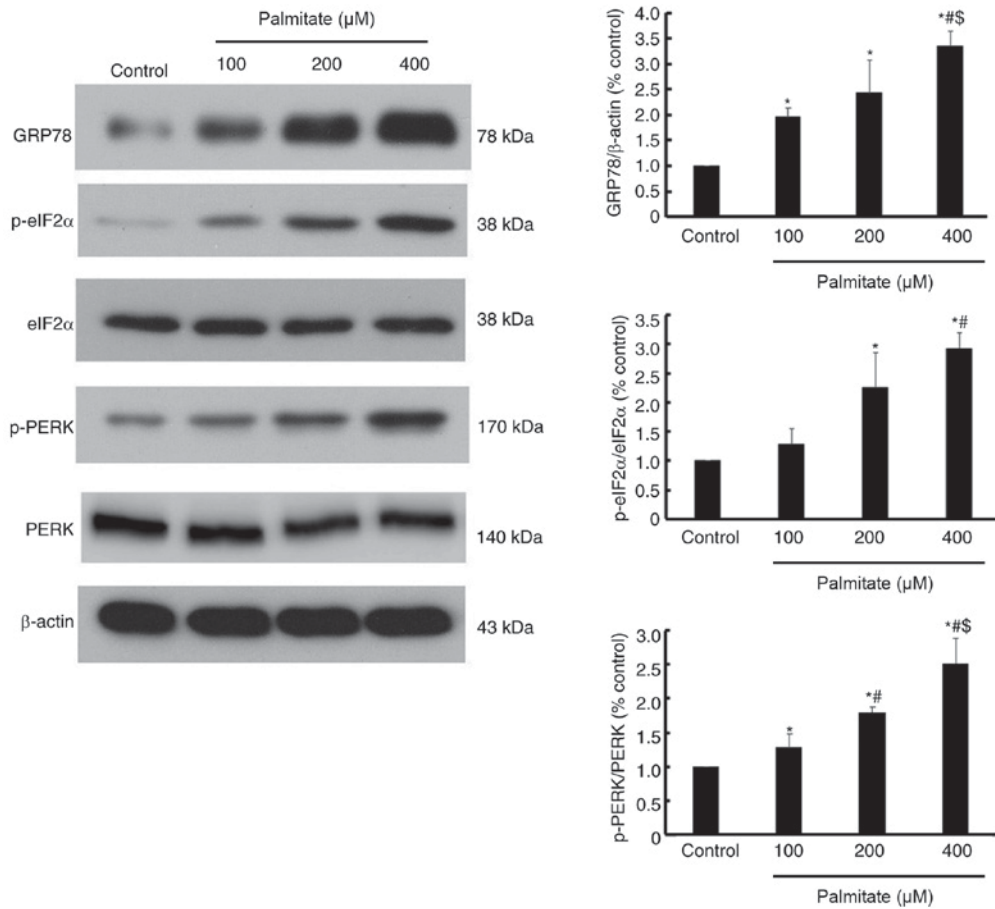


Figure 5. Effect of palmitate on the expression of endoplasmic reticulum stress markers measured by western blotting. Relative GRP78, p-eIF2α and p-PERK levels were determined following treatment with various doses of palmitate for 12 h. \*P<0.05 vs. control; #P<0.05 vs. 100 μM; S P<0.05 vs. 200 μM. GRP78, 78 kDa glucose-regulated protein; p-, phospho-; eIF2α, eukaryotic initiation factor 2 α; PERK, protein kinase R-like endoplasmic reticulum kinase.

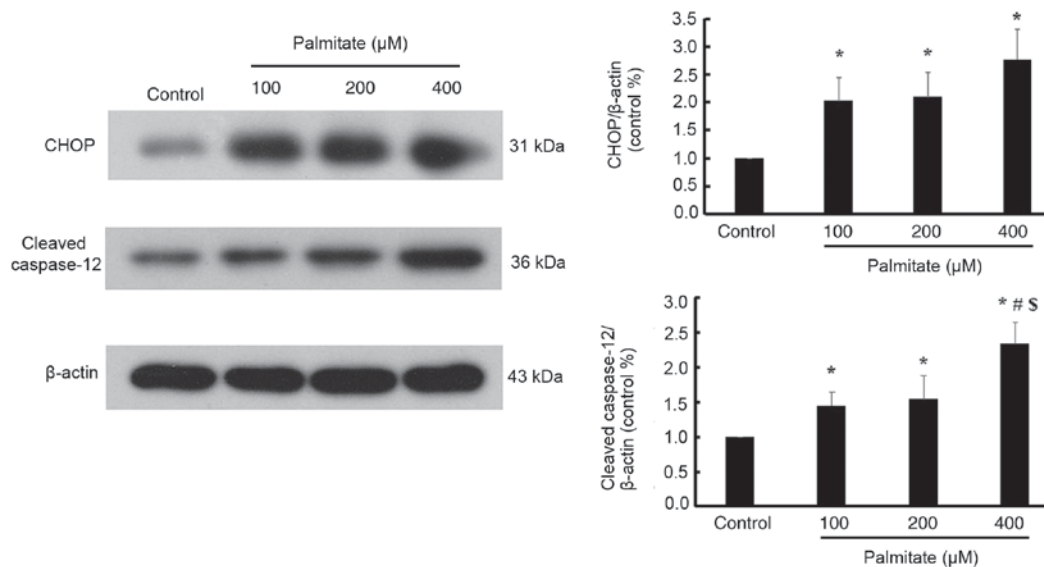


Figure 6. Effect of palmitate on the endoplasmic reticulum stress-mediated apoptosis pathway measured by western blotting. Relative CHOP and cleaved caspase-12 protein expression were determined following treatment with various doses of palmitate for 12 h. \*P<0.05 vs. control; #P<0.05 vs. 100 μM; S P<0.05 vs. 200 μM. CHOP, C/EBP homologous protein.

PA significantly increased the expression of cleaved caspase-12 in a dose-dependent manner (Fig. 6).

*Effect of 4-PBA on PA-induced apoptosis.* In order to reconfirm the role of ER stress in PA induced apoptosis, 4-PBA, a



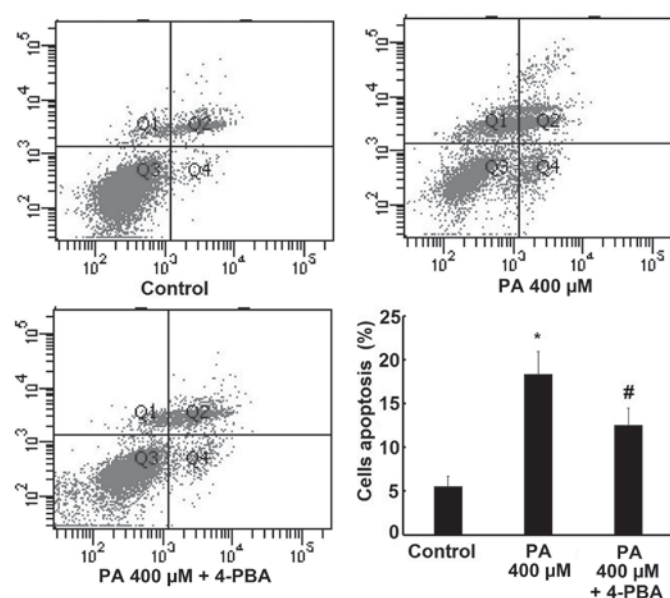


Figure 7. Effect of 4-PBA on cell apoptosis induced by PA measured by flow cytometry analysis. H9c2 cells were treated by 400  $\mu$ M PA for 12 h and 5 mM 4-PBA, an ER stress inhibitor, 90 min before PA treatment. \* $P$ <0.05 vs. control; # $P$ <0.05 vs. PA 400  $\mu$ M. PA, palmitate; 4-PBA, 4-phenyl butyric acid.

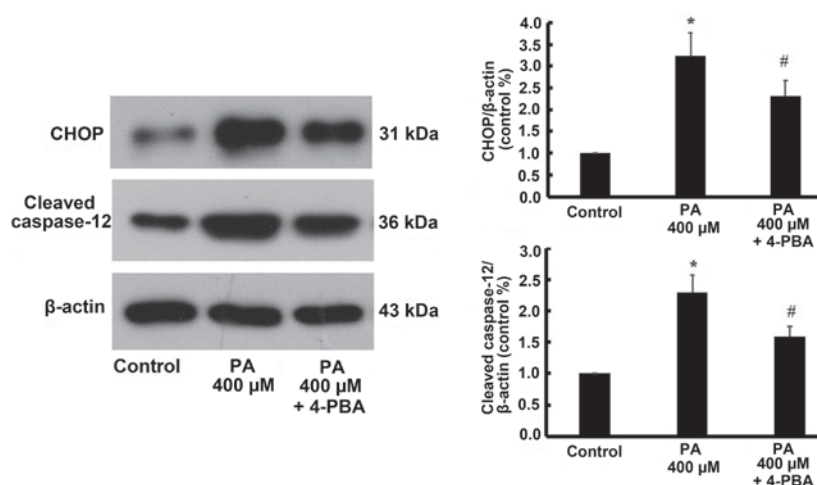


Figure 8. Effect of 4-PBA on the level of ER stress-mediated apoptosis pathway induced by PA measured by western blotting. Relative CHOP and cleaved caspase-12 protein expression were determined following treatment with 400  $\mu$ M PA for 12 h and 5 mM 4-PBA, an ER stress inhibitor, 90 min before PA treatment. \* $P$ <0.05 vs. control; # $P$ <0.05 vs. PA 400  $\mu$ M. CHOP, C/EBP homologous protein; PA, palmitate; 4-PBA, 4-phenyl butyric acid.

specific ER stress inhibitor, was administered to examine the changes in cell apoptosis rate and the expression of CHOP and cleaved caspase-12 following ER stress inhibition. The results revealed that 400  $\mu$ M PA significantly increased H9c2 cells apoptosis rate, but this effect was reversed by treatment with 4-PBA (Fig. 7). Similarly, the expression of CHOP and cleaved caspase-12 were increased by 400  $\mu$ M treatment, but were also reversed by 4-PBA treatment (Fig. 8).

## Discussion

Myocardial damage caused by hyperlipemia predominantly occurs by two mechanisms: In most cases, hyperlipemia, a well-known independent risk factor of coronary heart disease, accelerates the development of coronary atherosclerosis and results in myocardial ischemia, and even myocardial

necrosis, eventually (13); additionally, sustained and excessive hyperlipemia can also directly result in myocardial damage, termed 'myocardial lipotoxic injury' during severe metabolic disorders, including diabetes and severe obesity (14,15). The present study demonstrated that PA promoted excessive lipid deposition in cardiomyocytes and resulted in decreased cell viability, increased LDH activity and apoptosis rate in a dose-dependent manner, which is consistent with the findings of Wei *et al* (4,16). Therefore, the present study reconfirmed that PA could induce myocardial lipotoxic injury *in vitro*.

ER stress, an important adaptive response in eukaryotic cells, is often activated under the conditions of various pathophysiological procedures, including anoxia (17), poisoning (18) and infection (19). In particular, previous studies have demonstrated that ER stress is involved in different cardiovascular diseases including atherosclerosis (20), hypertension (21)

and heart failure (22). The current study demonstrated that GRP78, a marker of ER stress, was elevated in cardiomyocytes following PA treatment. Furthermore, PERK/eIF2 $\alpha$ , part of a well-established ER stress-associated pathway, were activated by PA treatment, as demonstrated by increased phosphorylation of PERK and eIF2 $\alpha$ . The current results were similar to previous reports indicating that ER stress is activated in response to chronically elevated free fatty acids in hepatocytes (10) and pancreatic  $\beta$  cells (11).

However, ER stress is a double-edged sword, in that prolonged or excessive ER stress will trigger an ER stress-mediated apoptosis pathway (7). Unlike the death receptor- and mitochondria-mediated apoptosis pathways, specific ER stress-induced apoptosis proteins, including CHOP and caspase-12, were activated (23). One of the notable novel findings of the current study is that myocardial lipotoxic injury induced by various doses of PA were involved in the activation of ER stress-mediated apoptosis pathway. Although, Park *et al* (24) demonstrated that ER stress-mediated autophagy had an important role in regulating myocardial lipotoxic injury induced by PA, whether ER stress-mediated apoptosis is implicated in the onset of myocardial lipotoxic injury remains unknown. The present study demonstrated that CHOP and cleaved caspase-12 were significantly up-regulated in cardiomyocytes when treated with different dose of PA, but the effect reversed following ER inhibition using 4-PBA. This indicated that myocardial lipotoxic injury induced by PA may be involved in the activation of the ER stress-mediated apoptosis pathway.

In conclusion, the current study demonstrated that PA induces myocardial lipotoxic injury, potentially by triggering ER stress and the ER stress-mediated apoptosis pathway.

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