Panax quinquefolium saponin attenuates cardiomyocyte apoptosis induced by thapsigargin through inhibition of endoplasmic reticulum stress

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Abstract

Background Endoplasmic reticulum (ER) stress-related apoptosis is involved in the pathophysiology of many cardiovascular diseases, and Panax quinquefolium saponin (PQS) is able to inhibit excessive ER stress-related apoptosis of cardiomyocytes following hypoxia/reoxygenation and myocardial infarction. However, the pathway by which PQS inhibits the ER stress-related apoptosis is not well understood. To further investigate the protective effect of PQS against ER stress-related apoptosis, primary cultured cardiomyocytes were stimulated with thapsigargin (TG), which is widely used to model cellular ER stress, and it could induce apoptotic cell death in sufficient concentration.

Methods Primary cultured cardiomyocytes from neonatal rats were exposed to TG (1 μmol/L) treatment for 24 h, following PQS pre-treatment (160 μg/mL) for 24 h or pre-treatment with small interfering RNA directed against protein kinase-like endoplasmic reticulum kinase (Si-PERK) for 6 h. The viability and apoptosis rate of cardiomyocytes were detected by cell counting kit-8 and flow cytometry respectively. ER stress-related protein expression, such as glucose-regulated protein 78 (GRP78), calreticulin, PERK, eukaryotic translation initiation factor 2α (eIF2α), activating transcription factor 4 (ATF4), and C/EBP homologous protein (CHOP) were assayed by western blotting.

Results Both PQS pre-treatment and PERK knockdown remarkably inhibited the cardiomyocyte apoptosis induced by TG, increased cell viability, decreased phosphorylation of both PERK and eIF2α, and decreased protein levels of both ATF4 and CHOP. There was no statistically significant difference between PQS pre-treatment and PERK knockdown in the cardioprotective effect.

Conclusions Our data indicate that the PERK-eIF2α-ATF4-CHOP pathway of ER stress is involved in the apoptosis induced by TG, and PQS might prevent TG-induced cardiomyocyte apoptosis through a mechanism involving the suppression of this pathway. These findings provide novel data regarding the molecular mechanisms by which PQS inhibits cardiomyocyte apoptosis.

Keywords: Cardiomyocyte apoptosis; Endoplasmic reticulum stress; Panax quinquefolium saponin; Thapsigargin

1 Introduction

Cardiomyocyte apoptosis plays an important role in the myocardial injury of ischemic heart disease.[1,2] The endoplasmic reticulum (ER) stress-related apoptotic pathway has been proposed for more than a decade as a mechanism that can be activated by ischemia,[3,4] hypoxia/reoxygenation,[5] and pressure overload.[6] In mammals, the ER stress response is mediated by three transmembrane sensor proteins in the endoplasmic reticulum, namely, inositol requiring enzyme 1 (IRE1), protein kinase-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6). Moderate ER stress plays a favorable part in reducing the levels of unfolded proteins and restoring cell homeostasis, whereas, following prolonged or severe ER stress, cells deteriorate and may enter apoptosis.[7] When the ER chaperone glucose-regulated protein 78 (GRP78) is dissociated from IRE1, PERK and ATF6, these sensors will be then activated. PERK phosphorylates the α-subunit of the translation initiation factor eukaryotic translation initiation factor-2 (eIF2α), resulting in attenuation of global translation initiation. When eIF2α phosphorylation is induced by PERK, the expression of ATF4 and its key downstream target C/EBP homologous protein (CHOP) increases. CHOP induces apoptosis mainly by suppressing the pro-survival protein Bcl-2. The ATF6 branch also induces CHOP expression transcriptionally,[7] Calreticulin (CRT), a 46 kDa Ca2+-binding chaperone protein located mainly in the ER, is
also a crucial factor responsible for regulating ER stress.[8] Our previous studies showed that the CHOP pathway is involved in apoptosis of non-infarcted cardiomyocytes of rats following acute myocardial infarction.[3]

Thapsigargin (TG), a sesquiterpene alkaloid, is a highly selective inhibitor of sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA) pumps. By inhibiting SERCAs, TG suppresses Ca\textsuperscript{2+} transport into the ER lumen and subsequently increases the Ca\textsuperscript{2+} concentration within the cytosol.[9–11] Disturbances of luminal ER Ca\textsuperscript{2+} concentration lead to protein unfolding because of the Ca\textsuperscript{2+}-dependent ER chaperones, such as GRP78, GRP94 and CRT.[12] Accumulation of unfolded proteins triggers the ER stress, and if it persists, this will eventually cause ER stress-related apoptosis.[3] Despite the accumulated knowledge, the pathway by which PQS inhibits the ER stress-related apoptosis is not well understood. In addition, there is no published report directly examining the relationship of PQS and ER stress-related apoptosis induced by TG. Therefore, in the present experiment, the possible mechanisms of the ER stress-related apoptosis induced by TG were studied through RNA interference-based gene silencing of PERK, and the effects and its underlying mechanism of PQS on cultured neonatal rat cardiomyocytes treated by TG were investigated.

2 Methods

2.1 Materials

Panax quinquefolium saponin (PQS) prevents hypoxia/reoxygenation injury in rat neonatal cardiomyocytes by inhibiting excessive ER stress-related apoptosis.[13] PQS prevents ventricular remodeling after acute myocardial infarction (AMI) by inhibiting CHOP-mediated ER stress-related apoptosis.[3] Despite the accumulated knowledge, the pathway by which PQS inhibits the ER stress-related apoptosis is not well understood. In addition, there is no published report directly examining the relationship of PQS and ER stress-related apoptosis induced by TG. Therefore, in the present experiment, the possible mechanisms of the ER stress-related apoptosis induced by TG were studied through RNA interference-based gene silencing of PERK, and the effects and its underlying mechanism of PQS on cultured neonatal rat cardiomyocytes treated by TG were investigated.

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located to six groups: (a) normal control group cells were treated with 0.1% dimethylsulfoxide (DMSO, vehicle control); (b) TG group cells were treated with 1 μmol/L TG (stock solution was 1 mmol/L in DMSO) for 24 h; (c) PQS 160 μg/mL + TG group cells were pre-treated with 160 μg/mL PQS for 24 h prior to exposed to 1 μmol/L TG for 24 h. A PQS stock solution was prepared at 16 mg/mL in phosphate-buffered saline, filtered and stored at 4°C. For application, these stock solutions were diluted 100-fold and added to culture medium. The concentration tested was based on our previous experiments.[5] (d) PERK knockdown + TG group (Si-PERK + TG) cells were transfected with 50 nmol/L siRNA against rat PERK (NC_005103.3) using Lipofectamine®2000 according to the manufacturer’s instructions. The sequence of the rat PERK siRNA was 5'-AAGUAGAAGAGACCAUGCCUC-3'. Six hours after transfection, the cells were transferred to complete medium, followed by the addition of TG for 24 h. (e) Randomized double-stranded RNA transfection control + TG group (Mock + TG) cells were transfected as above with randomly synthesized double-stranded stealth siRNA for 6 h, and the cells were then transferred to complete medium and treated with TG for 24 h.

2.4 Cell counting Kit-8 (CCK-8) assay

Neonatal rats’ cardiomyocytes were seeded in a 96-well plate at a density of 1×10⁴ cells/well. Cells were then pre-treated with PQS or vehicle for 24 h, followed by treatment with 1 μmol/L TG at 37°C in a CO₂ incubator (95% air, 5% CO₂). Finally, ten microliters WST-8 mixture was added to each well and incubated at 37°C in 5% CO₂ for 3 h. The absorbance of each well was then measured on a microplate reader at 450 nm. All assays were performed in triplicate and repeated at least three times.

2.5 Annexin V and propidium iodide double-staining assay

Cells were labeled with Annexin V-FITC and propidium iodide (PI) according to the manufacturer’s instructions. In brief, 1 × 10⁵ cells were washed with cold phosphate-buffered saline. After resuspending in 500 μL binding buffer, Annexin V-FITC and PI were added to the cell preparations and incubated for 25 min in the dark, then analyzed by flow cytometry.

2.6 Western blot analyses

Cardiomyocytes were plated in T-25 flasks at a cell density of 1×10⁵ cells/flask. After treatment, proteins were extracted from cardiomyocytes in each group and 80 μg/lane (determined by the Bicinchoninic Acid method according to the manufacturer’s instructions) was separated by electrophoresis in 12% sodium dodecyl sulfate-polyacrylamide gels and subsequently transferred electrophoretically to nitrocellulose membranes. Membranes were blocked with 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) at room temperature for 40 min, and then probed with primary antibodies against GRP78, CRT, PERK, p-eIF2α, CHOP, or GAPDH at a 1: 500 dilution; p-PERK or ATF4 at a 1: 200 dilution; p-PERK or ATF4 at a 1: 200 dilution, at 4°C overnight. The primary antibody solution was removed, then membranes were rinsed in TBS-T and incubated with a secondary antibody solution consisting of either a 1: 1,000 dilution of HRP-conjugated goat anti-mouse IgG (for CHOP) or a 1: 1,000 dilution of HRP-conjugated goat anti-rabbit IgG (for GRP78, CRT, PERK, p-eIF2α, p-eIF2α, ATF4 and GAPDH) at 25°C for 2 h, before enhanced chemiluminescence detection of immunoreactive proteins. The optical density of bands (as measured in arbitrary densitometry units) was determined using Image-Pro Plus, and normalized to GAPDH signals.

2.7 Statistical analysis

Data were expressed as the mean ± SD. Each assay was performed in triplicate. For the comparison between two groups, the Student’s t-test was used. A one-way ANOVA followed by the LSD post hoc comparison test was used for multiple comparisons. Statistical analyses were performed using SPSS 17.0 statistical software (SPSS, Chicago, IL, USA). Values of P < 0.05 was considered significant.

3 Results

3.1 Apoptosis and viability of cardiomyocytes

To confirm the cardiotoxicity of TG and investigate the effects of PQS on cardiomyocytes apoptosis induced by TG, we determined the cardiomyocytes apoptosis and viability using flow cytometry and cell viability assay respectively. The proportion of cardiomyocytes in apoptosis was significantly greater after TG treatment compared with the normal control group (26.7% vs. 3.1%, P < 0.05). Pre-treatment with 160 μg/mL PQS for 24 h decreased the apoptosis rate by 36.2% and increased the cell viability by 53.2% as compared with TG treatment (P < 0.05); knockdown of PERK reduced the apoptosis by 45.2% and increased the cell viability by 72.3% (P < 0.05 vs. TG treatment). There was no significant difference between the ability of PQS + TG or the Si-PERK + TG treatments to reduce apoptosis and increase cell viability (Figure 1). These results showed that PQS could protect rat cardiomyocytes against TG induced injury.
Figure 1. The effect of PQS and PERK knockdown on the apoptosis rate and cell viability. (A): The apoptotic cells stained with Annexin V-FITC and PI were detected by flow cytometry. Representative images are shown. In the 2D coordinates, the X-axis shows the intensity of FITC signal, and the Y-axis shows the intensity of PI signal. AnnexinV-FITC+/PI+: apoptosis cells (Quadrant I); Annexin V-FITC+/PI−: harm cells (Quadrant II); Annexin V-FITC−/PI+: normal cells (Quadrant III); Annexin V-FITC−/PI−: early apoptosis cells (Quadrant IV). (B): quantification of flow cytometric counts of apoptotic cells. (C): cardiomyocyte viability was measured by CCK-8 assay. *P < 0.05 vs. control; #P < 0.05 vs. TG; △P < 0.05 vs. Mock + TG; n = 3. CCK-8: cell counting kit-8; ER: endoplasmic reticulum; FITC: fluorescein isothiocyanate; PERK: protein kinase-like ER kinase; PI: propidium iodide; PQS: panax quinquefolium saponin; TG: thapsigargin.

3.2 Expression of ER stress-relevant molecules

Based on reports showing that TG is associated with the induction of ER stress-related apoptosis, and that PQS inhibits the ER stress-related apoptosis, this study examined whether PQS plays a role in inhibiting the ER stress-related
apoptosis induced by TG. Western blotting showed that TG treatment increased the protein expression of GRP78 and CRT by 4.9-fold and 4.2-fold (Figure 2A, 2B), increased the protein expression of ATF4 and CHOP by a factor of 8.7 and 3.1, and increased the phosphorylation of PERK and eIF2α by 96.9% and 82.9%, respectively, as compared with the control group (all \( P < 0.05 \)), (Figure 2A, 2D). Compared with the TG group, pre-treatment with PQS decreased the protein levels of GRP78 and CRT by 75.5% and 78.4%, respectively (\( P < 0.05 \)) (Figures 2A, 2B); decreased the phosphorylation of PERK and eIF2α by 36.4% and 27.6%, respectively (\( P < 0.05 \)) (Figures 2A, 2C); and decreased the protein levels of ATF4 and CHOP by 75.1% and 60.6%, respectively (\( P < 0.05 \)) (Figures 2A, 2D). These results suggested that PQS inhibited the apoptosis induced by TG via inhibiting ER stress.

To further explore the role of the PERK/eIF2α pathway in ER stress-related cardiomyocyte apoptosis induced by TG treatment, transfection of cultured neonatal rat cardiomyocytes with specific siRNA against PERK gene was performed. After pre-incubation with PERK siRNA and then stimulation with 1 μmol/L TG for 24 h, the expression of PERK, ATF4 and CHOP at protein levels were all significantly down-regulated compared with TG treatment alone (\( P < 0.05 \)) (Figures 2A, 2D). Furthermore, the phosphorylation of PERK and eIF2α were also decreased significantly compared with TG group (\( P < 0.05 \)) (Figures 2A, 2C). However, the protein levels of GRP78 and CRT exhibited no significant changes (\( P > 0.05 \)) (Figures 2A, 2B). These results provided evidence that the PERK-eIF2α-ATF4-CHOP pathway was involved in TG-induced cardiomyocyte apoptosis.

There were no significant differences between Mock + TG group and TG group in the phosphorylation of PERK or eIF2α, or the protein levels of GRP78, CRT, ATF4 and CHOP. It indicated that the transfection operation itself had no influence. And the efficacy was similar between PQS pre-treatment and PERK knockdown in reducing the phosphorylation of PERK and eIF2α, and in reducing the protein levels of ATF4 and CHOP (\( P > 0.05 \)) (Figure 2A and 2B). These results suggested that PQS might inhibit the apoptosis induced by TG via the PERK-eIF2α-ATF4-CHOP pathway.

**Figure 2.** The effect of PQS pre-treatment and PERK knockdown on ER stress protein levels and phosphorylation status. (A): Protein levels were determined by Western blotting; (B): the levels of GRP78 and CRT proteins relative to GAPDH; (C): the ratio of p-PERK to PERK and p-eIF2α to eIF2α; and (D): the levels of ATF4 and CHOP proteins relative to GAPDH. \(^* P < 0.01 \) vs. control group; \(^* P < 0.05 \) vs. TG group; \(^\triangle P < 0.05 \) vs. Mock + TG group; \( n = 3 \). ATF4: activating transcription factor-4; CHOP: C/EBP-homologous protein; CRT: calreticulin; ER: endoplasmic reticulum; eIF2α: eukaryotic translation initiation factor-2; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GRP78: glucose-regulated protein 78; PERK: protein kinase-like ER kinase; PI: propidium iodide; PQS: *panax quinquefolium* saponin; TG: thapsigargin.
4 Discussion

Our previous studies demonstrated that PQS has some beneficial effects on cardiovascular disease in rats via inhibition of ER stress-related apoptosis. However, the signaling pathways involved in PQS inhibiting ER stress-mediated apoptosis are not fully understood. In this study, the results indicated that the PERK-eIF2α-ATF4-CHOP pathway of ER stress was involved in TG-induced apoptosis, and demonstrated that PQS could protect the cardiomyocytes against apoptosis induced by TG, and suggested that suppressing the PERK-eIF2α-ATF4-CHOP pathway might contribute to the mechanism of the cardioprotection of PQS.

The activation of PERK, induced by the accumulation of unfolded proteins in the ER, represents an essential branch of ER stress. Sustained and severe ER stress leads to expression of transcription factor ATF4 through the PERK-eIF2α pathway, and then ATF4 induces expression of CHOP. CHOP-mediated ER stress-related apoptosis plays an important part in various cardiovascular diseases. Zhang, et al. found that abdominal aortic constriction in rats induced a significant up-regulation in CRT protein expression at an early stage post-surgery, indicating that ER stress contributes to suppression of myocardial hypertrophy induced by abdominal aortic constriction; whereas prolonged and severe ER stress leads to CHOP-mediated cardiomyocyte apoptosis, which has an adverse effect on myocardial hypertrophy. PERK activation is associated with an inhibition of protein translation and triggers activation of two transcriptional programs. One is directed by ATF4, which is translated more efficiently following phosphorylation of eIF2α. The other, is directed by NF-E2 (nuclear factor erythroid-derived 2)-related factor 2 (Nrfa), a direct PERK substrate. To study the pathways involved in TG-induced ER stress-related apoptosis, we transfected cardiomyocytes with siRNA against PERK. Our results showed that PERK knockdown reduced TG-induced cardiomyocyte apoptosis, increased cell viability, decreased both PERK and eIF2α phosphorylation and reduced the expression of both ATF4 and CHOP. However, PERK knockdown did not affect the levels of the ER chaperones GRP78 and CRT; this result is consistent with the study of Chitnis, which also indicated that GRP78 and CRT are not regulated by PERK.

ER stress induces apoptosis through various different signals, including the activation of transcription factors, such as CHOP, ER-resident caspases, and Bcl-2 family proteins. Among all the reported death pathways of ER stress-related apoptosis, the PERK-ATF4-CHOP pathway is a dominant pro-apoptotic mechanism triggered by prolonged ER stress, and is the most extensively studied pathway. In our study, the PERK-eIF2α-ATF4-CHOP pathway proteins were examined, which is similar to the study of Hsieh. Whether the other pathways of ER stress participate in the toxic effects of TG on the cultured neonatal rat cardiomyocytes needs to be further evaluated.

The present study showed PQS pre-treatment remarkably reduced cardiomyocyte apoptosis induced by TG and increased cell viability, accompanied by decreased phosphorylation of both PERK and eIF2α, and reduced protein levels of both ATF4 and CHOP. And there was no significant difference in the above effects between PQS pre-treatment and PERK knockdown. Our results indicated that PQS attenuated the cardiomyocyte apoptosis induced by TG, and the possible mechanism might be associated with suppressing the PERK-eIF2α-ATF4-CHOP pathway of ER stress. However, our experiment did not identify whether the effects of PQS against ER stress-related apoptosis could be completely attributed to PERK, or also directly to its downstream molecules eIF2α, ATF4 and CHOP, and so further studies are needed to confirm this.

In conclusion, TG induced apoptosis via triggering the PERK-eIF2α-ATF4-CHOP pathway of ER stress in cultured neonatal rat cardiomyocytes; PQS pre-treatment increased cardiomyocyte viability and decreased cardiomyocyte apoptosis induced by TG; a possible mechanism of the protective effects of PQS against TG induced apoptosis might be associated with inhibiting the PERK-eIF2α-ATF4-CHOP pathway.

Acknowledgements

This work was supported by International Science and Technology Cooperation Project (2010DFA31690), National Natural Science Foundation of China (81030063 and 81170140) and China Postdoctoral Science Foundation (2014M562608). The authors declare no conflict of interests regarding the publication of this paper.

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