Original Article
Polyenylphosphatidylcholine alleviates palmitic acid-induced apoptosis in HepG2 cells via inhibiting endoplasmic reticulum stress

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Abstract: Polyenylphosphatidylcholine (PPC) can reduce hyperlipidemia, relieve arteriosclerosis and decrease triglyceride. The aim of the present study was to investigate whether polyenylphosphatidylcholine (PPC) could alleviate apoptosis induced by palmitic acid (PA), and explore the possible molecular mechanisms in HepG2 cells. In this study, MTT assay was performed to identify the cell viability after PA (50, 100, 150 and 200 μmol/L) treatment for 24 h in HepG2 cells. The cell viability was significantly decreased by PA treatment in a dose dependent manner. Flow cytometry confirmed that co-incubation of PPC reduced PA-induced apoptosis. Moreover, the secretion level of tumor necrosis factor-α (TNF-α) was conspicuously reduced after PPC treatment using enzyme-linked immunosorbent assay (ELISA). We observed that treatment of the cells with PA resulted in activation of Endoplasmic Reticulum Stress (ERS) associated proteins including glucose-regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP). Western blot and real-time polymerase chain reaction (RT-PCR) confirmed that the expression levels of Bcl-2 associated X protein (Bax) and B cell lymphoma-2 (Bcl-2) were significantly regulated and the expression levels of GRP78 and CHOP were dramatically decreased by co-incubation of PPC. These results suggested that PPC could alleviate cell apoptosis and reduce the ERS-related proteins expressions after cells pre-treatment with PA, which showed that ERS might play an important role in cell apoptosis caused by PA.

Keywords: Polyenylphosphatidylcholine (PPC), palmitic acid (PA), endoplasmic reticulum stress (ERS), apoptosis, HepG2

Introduction
Non-alcoholic fatty liver disease (NAFLD) is, a metabolic stress-induced liver damage, closely related with insulin resistance (IR) and genetic susceptibility. NAFLD is considered to be benign lesions that could be treated. Otherwise, it could further develop into the end-stage liver disease, such as hepatic cirrhosis and liver cancer. Therefore, NAFLD is regarded as one of the major causes of liver failure [1-3]. With the improvement of living standards and the aging of population, obesity and metabolic syndrome have become an increasingly serious health problem among NAFLD patients [4]. Obesity can increase the content of free fat acid (FFA) in blood. The study found that free fat acid (FFA) could promote the formation of triglycerides, which led to lipid accumulation in liver [5, 6]. PA is a central component of lipid in the body and a common high level of saturated fatty acid in food [7]. Some studies confirmed that PA could induce apoptosis in various cells [7-13]. Therefore, HepG2 cells were treated with PA to establish hyperlipidemia model in vitro in this study.

According to the level and stage of disease in NAFLD, it can be classified into non-alcoholic simple fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), related hepatic cirrhosis and hepatocellular carcinoma [5]. NASH is a critical pathological phase, during which NAFLD may progress to the late-stage liver disease [7]. Hepatocyte lipoapoptosis participates in the entire pathogenesis and is considered as an
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important step in onset and progression of NASH [12, 13].

PPC from soybean is a highly concentrated phospholipids and an essential component of cell membranes and sub cellular ones. The main active ingredient of PPC is poly phosphatidylcholine diacylglycerol or polyketides lecithin [14]. Numerous of animal experiments have manifested that PPC could reduce cell apoptosis, inhibit stress reaction, relieve and inflammation [15, 16]. Stimulation from various external environments can cause the imbalance of homeostasis in ER that results in ERS ultimately [17, 18]. ERS can cause cell programmed death via up-regulating the expression levels of related genes including GRP78 and CHOP [18]. This study aimed at determining the anti-apoptosis effect of PPC on HepG2 cells treated with PA and exploring underlying molecular mechanisms.

Materials and methods

Cell culture

HepG2 cells were obtained from cell bank of Shanghai Branch, Chinese Academy of Science (China) and incubated in Dulbecco’s modified Eagle’s medium (DMEM) (Hangzhou Sijiqing Co., Ltd, Zhejiang, China), 10% Fetal Bovine Serum (FBS) (Sijiqing), 100 U/ml penicillin and 100 μg/mL streptomycin (Solarbio, Beijing, China) at 37°C. The medium changed every other day. HepG2 cells were digested by 0.25% trypsin (Beyotime Biotechnology Co., Ltd, Shanghai, China) for sub-culturing when cells confluence reached 80%. HepG2 cells in exponential growth phase were chosen to do the experiment.

MTT assay

PA (Sigma, USA) was dissolved in 0.1 M NaOH in 70°C water bath to prepare 50 mM PA stock solution. HepG2 cells seeded in a 24-well plate at the density of 1×10⁶ cells/well with 100 mL culture medium. After being cultured for 24 h, PA was added to the medium to the final concentrations (50, 100, 150 and 200 μmol/L PA) and cultured for another 12, 24 and 48 h. Then 100 μL of MTT solution (Ameresco, USA) was added to each well and incubated in 5% CO₂ at 37°C for 4 h. The optical density (OD) values were read at 492 nm by a microplate reader (Thermo, USA). A Value of each experimental group was divided by that of control group, and multiplied by 100%, the resultant value was collected to access cell viability in each experimental group.

Flow cytometry assay

HepG2 cells was pre-treated with PPC (100 and 200 μmol/L) (Sanofi-Aventis Pharmaceutical Co., Ltd, Beijing, China) for 6 h, and 200 μmol/L of PA was added into the culture medium for 24 h. The apoptotic rate was analyzed by flow cytometer using an Annexin V-FITC apoptosis detection Kit (Beyotime Biotechnology Co., Ltd, Shanghai, China) according to the manufacturer’s instructions. In brief, cells were harvested after being cultured for 24 h and washed with ice-cold PBS (Beyotime), resuspended in 500 μL of binding buffer, followed by addition of 5 μL of Annexin V stock solution and incubation for 10 min at 4°C. Propidium iodide (PI) (5 μL) was added to the cells, and they were immediately analyzed using a flow cytometer (BD, San Jose, CA).

ELISA testing

HepG2 cells was pre-treated with PPC (100 and 200 μmol/L) (Sanofi-Aventis Pharmaceutical Co., Ltd, Beijing, China) for 6 h, and 200 μmol/L of PA was added into the culture medium for 24 h, cell supernatant was collected and centrifuged at 3000 rpm with low temperature. The content of TNF-α was tested according to the manual of ELISA kit (Beyotime Biotechnology Co., Ltd, Shanghai, China).

Reverse transcription and RT-PCR

HepG2 cells were seeded at a density of 1×10⁵ cells/well in 6-well plates, cultured overnight and then pre-treated with PPC (100 and 200 μmol/L) (Sanofi-Aventis Pharmaceutical Co., Ltd, Beijing, China) for 6 h, and 200 μmol/L of PA was added into the culture medium for 24 h. Total RNA from treated cells was isolated using TRIzol reagent (Invitrogen). Reverse transcription reaction was performed using 2 μg of total RNA with the first strand cDNA kit (Sigma, Munich, Germany), according to the manufacturer’s instructions. PCR amplification was performed for 10 min at 95°C, followed by 40 cycles at 95°C for 15 s, annealing/extension at 60°C for 45 s in ABI 7300 Thermocycler.
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(Applied Biosystems, Foster City, CA, USA), using the SYBR Premix Ex Taq kit (Takara). The specific primer sequences for each gene were listed as follows: 5’ CTCAACATGGATCTGTTCCG 3’ and 5’ CCAGTTGCTGAATCTTTGGA 3’ for GRP78 (product: 125 bp); 5’ CGCATGAAGGAGAAAACA 3’ and 5’ CACCATTCGGTCAATCAGAG 3’ for CHOP (product: 125 bp) and 5’ TGGCACCGACAATGAA 3’ and 5’ CTAAGTCATAGTCCGCTAGAAGCA 3’ for actin (product: 125 bp). Data analysis was done using the $2^{-\Delta\Delta CT}$ method for relative quantification, and all samples were normalized to actin, which was used as an endogenous control.

Western blot

HepG2 cells were seeded at a density of $1 \times 10^5$ cells/well in 6-well plates, cultured overnight and then pre-treated with PPC (100 and 200 μmol/L) for 4 h, and PPC (100 and 200 μmol/L) was added into the culture medium, and incubated for 24 h. Flow cytometry was performed to detect cell apoptosis. E: Cells were pre-treated with 200 μmol/L of PA for 4 h, and PPC (100 and 200 μmol/L) was added into the culture medium, and incubated for 24 h. ELISA was performed to detect the level of TNF-α. Data were presented as mean ± SD, n=3, *P < 0.05 and **P < 0.01 vs. control; *P < 0.05 and **P < 0.01 vs. PA.
μmol/L) (Sanofi-Aventis Pharmaceutical Co., Ltd, Beijing, China) for 6 h, and 200 μmol/L of PA was added into the culture medium for 24 h. Cells were harvested and lysed in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Equal amounts of protein were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), and the bands were transferred to polyvinylidene fluoride membrane (Millipore, USA). The membrane was blocked and then incubated with antibodies against Bax, Bcl-2 (Abcam), GRP78, CHOP (Beyotim) and actin (Santa). After washing, the membranes were incubated with peroxidase-conjugated secondary antibodies (Zhongshan, Beijing, China) for 1 h. Blots were visualized using enhanced chemiluminescence (ECL, Thermo Scientific, Shanghai, China). Relative protein levels in each sample were normalized to actin to standardize the loading variations.

Statistical analysis

Data were expressed as the Mean ± SEM from at least three independent experiments. The differences between groups were analyzed using a Student t test when only 2 groups or 1-way analysis of variance in more than 2 groups were compared. All tests performed were 2-sided. *P < 0.05 was taken as statistical significance.

Results

**PPC improve cell viability in HepG2 cells which suppressed by PA**

In order to identify the effect of PA on the proliferation of HepG2 cells, MTT assay was employed for cell viability analysis. After treatment of PA (50, 100, 150 and 200 μmol/L) for 12, 24 and 48 h, PA over the dose of 50 μmol/L obviously suppressed the cell viability of HepG2 cells (Figure 1A). All the effects were in a time and dose dependent manner. In addition, PA inhibited the cell proliferation in a dose dependent manner (Figure 1A). The inhibitive effect of 200 μmol/L PA was the most significant at 24 h, therefore, 200 μmol/L of PA was determined for the subsequent experiment. After PA/PPC co-treatment, the cell viability was increased by 100 and 200 μmol/L of PPC (Figure 1B).
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liferation in a dose dependent manner (Figure 1B).

PPC reduces PA-induced apoptosis and the level of TNF-α in HepG2 cells

In Figure 1, PA significantly promoted the apoptosis of HepG2 cells compared with the control group, while PPC inhibited the apoptosis caused by PA in HepG2 cells. The apoptosis rate of HepG2 cells was 3.8 ± 0.28%, 18.7 ± 1.51%, 10.4 ± 0.97% and 5.1 ± 0.6%, respectively (Figure 1C and 1D). The level of TNF-α was significantly increased in PA treatment group compared with the control group and that was dramatically decreased in PA/PPC co-treatment group in a dose dependent manner (Figure 1E). Compared with control group, the expression level of Bax was increased and that of Bcl-2 was decreased after incubating with PA or/and PPC for 24 h (Figure 2). Bax expression in PA/PPC co-treatment group was remarkably less than PA treatment group, while Bcl-2 expression in PA/PPC co-treatment group was remarkably higher than PA treatment group. In addition, Bcl-2/Bax ratio in mRNA and protein levels of PA group was significantly decreased, compared to that of PA group, and Bcl-2/Bax ratio in mRNA and protein levels of PA/PPC co-treatment group was significantly increased, compared to that of PA group (Figure 2C and 2F). The expression levels of Bax and Bcl-2 were significantly regulated by PA or/and PPC treatment.

PPC reduces the expression levels of ERS-related genes in HepG2 cells

The GRP78 and CHOP, as ER-associated genes, were assayed by RT-PCR and Western blot. 200 μmol/L of PA were used as induction concentrations, and 100 and 200 μmol/L of PPC were used as intervention concentration. The expression of target genes were detected after incubating with PA or/and PPC for 24 h. As shown in Figure 3, GRP78 and CHOP genes were evidently up-regulated with the induction of 200 μmol/L PA (P < 0.05). Compared with PA group, the expression levels of GRP78 and CHOP were evidently downregulated by PPC in a dose dependent manner.

Discussion

NASH is a pivotal pathological stage during which NAFLD may progress to the late-stage liver disease [19, 20]. Intra-hepatic fatty acids and triglyceride accumulation are the pathogenesis basis of NASH progression. Nevertheless, epidemiological research manifested that NAFLD arrested the phase of simple fatty liver in majority of patients, and only small parts of patients suffered from NASH. This pathogenesis was still unclear [17, 18]. It was reported that elevated plasma FFAs concentration, fatty lesions and apoptosis of hepatocytes were significant features in NASH [21]. TNF-α plays an important role in cell apoptosis and can activate Caspase. In addition, TNF-α can transmit death signal into cell. The highly expressed TNF-α promotes cell apoptosis. The increased
FFAs can cause ectopic accumulation of lipid in the liver to release excessive TNF-α by activating ERS [22]. These inflammatory factors interfere with glucose and lipid metabolisms by binding cytokine receptors and pattern recognition receptors in adipose tissue and other histocytes. Meanwhile, the sensitivity of hepatocytes to inflammation responses and various damage elements is also enhanced. Moreover, lipotoxicity can lead to hepatocytes dysfunction or death, and lipoapoptosis can lead to cell apoptosis [23]. PA is an important fatty acid which constitutes liver TG for healthy people and NAFLD patients [24]. In our results, excessive PA inhibited the cell viability, caused cell apoptosis, increased the TNF-α level and ERS marker GRP78 and CHOP expressions (Figures 1 and 2). It showed that PA caused cell apoptosis via up-regulating the ERS-related proteins expressions to activate ERS.

As one of physiological phospholipids, PPC has been reported its protective effects on liver [25, 26]. Firstly, PPC can inhibit lipid per-oxidation in hepatocytes induced by carbon tetrachloride and arachidonic acid, as well as inhibit stress reaction and lipid per-oxidation caused by ethanol. A study showed that on one hand, PPC could suppress ethanol and LPS-induced TNF-α production and mitochondrial apoptosis in Kupffer cells [15]. On the other hand, PPC inhibited HSC activation and protects against liver fibrosis [27]. Therefore, PPC can alleviate PA-induced hepatocytes ERS and lipoapoptosis. In this study, we detected the cell viability, cell apoptosis, TNF-α level and related genes expressions in PA/PPC co-treatment groups. Obviously, PPC increased the cell viability, reduced the apoptosis and decreased the TNF-α level. It was a kind of clue for further researches on anti-apoptotic molecular mechanism. Above results have confirmed that PA induced ERS in cells (Figure 2). Bax and Bcl-2 as important members of Bcl-2 protein family, can regulate cell apoptosis [28].

The component proportion ratio of the member of Bcl-2 protein family is one of the key mechanisms for death receptor signaling pathway and mitochondria signaling pathway [29]. Especially, Bcl-2/Bax ratio directly determines cell survival. In addition, the increased CHOP can change the expression levels of Bax and Bcl-2 to regulate cell apoptosis. CHOP is a highly expression in the process of ERS and can up-regulate the expression levels of Bax and down-regulate the expression levels of Bcl-2 [30-32]. In our results, the expression levels of GRP78 and CHOP were decreased by PPC treatment, and that of Bax was decreased and Bcl-2 was increased by PPC treatment. Bcl-2/Bax ratio was significantly decreased during PA-induced apoptosis in HepG2 cells. However, PPC pre-treatment could up-regulate Bcl-2/Bax ratio (Figures 2 and 3). It showed that PPC inhibited ERS and regulated Bax and Bcl-2 expressions.

In conclusion, PA could reduce significantly cell viability and promote cell apoptosis. ERS-related proteins were up-regulated significantly. It showed that ERS might be activated by PA. Therefore, PPC could alleviate PA-induced apoptosis in HepG2 cells via inhibiting ERS and regulating apoptosis related genes including Bax and Bcl-2, however, ER stress-related pathways still need further studies.

Disclosure of conflict of interest

None.

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