Punicalagin Inhibits Palmitate Acid-induced Lipoapoptosis through Inhibition of ER Stress, and Activation of SIRT1/Autophagy in HepG2 Cells

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Abstract Lipid metabolism balance plays a vital role in maintaining normal levels of sterols, triglycerides, and free fatty acids (FFA). Inhibiting FFA-induced lipotoxicity is considered a highly potential treatment for non-alcoholic fatty liver disease (NAFLD) and other lipid metabolism diseases. Punicalagin (PU), a pomegranate-derived polyphenol, has been found to effectively regulate lipid metabolism and prevent fatty liver, cardiovascular and other chronic diseases caused by lipid metabolism disorders. In the present study, we found that PU protects HepG2 cells from palmitic acid (PA)-induced lipapoptosis. Silent information regulator 1 (SIRT1) plays a key role in protecting against lipapoptosis in HepG2 cells. Knockdown of SIRT1 significantly diminished the protective effect of PU. Besides, PU could be able to reactivate weakened autophagy and inhibit PA-induced ER stress. Our findings supported that PU can effectively attenuate FFA-induced lipotoxicity via activating SIRT1/autophagy and inhibiting ER stress.

Keywords: punicalagin, palmitic acid, lipotoxicity, SIRT1, endoplasmic reticulum stress, autophagy

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1. Introduction

Lipid metabolism is a complex physiological process related to nutrient regulation, hormone regulation and homeostasis. The body has the ability to adjust lipid metabolism, but unhealthy lifestyles and chronic overnutrition can induce lipid metabolism disorders, leading to many diseases. When sustained lipid accumulation exceeds the processing capacity of hepatic cells, oxidative stress and ER stress are caused, which leads to mitochondrial dysfunction and eventually triggers lipoapoptosis (a type of programmed cell death related to excessive lipid accumulation). Recent studies have shown that lipid-induced ER stress is the main factor promoting liver steatosis.

Sirtuins are a class of NAD+ -dependent histone deacetylases that are highly conserved from archaeabacteria to humans. Among the seven sirtuins, SIRT1 is the most important in the regulation of metabolic pathways. In recent years, accumulating evidences indicate that SIRT1 plays beneficial roles in regulating hepatic lipid metabolism and controlling hepatic oxidative stress through deacetylation some transcriptional regulators [1,2]. When cells encounter nutritional stress, SIRT1 affects alternative pathways that provide energy, resulting in changes in lipid metabolism in various pathophysiological environments.

Additionally, resveratrol (RSV), a polyphenolic compound that mimics calorie restriction (CR), can activate SIRT1 to inhibit high-fat diet-induced steatosis [3]. A previous study showed that hepatic overexpression of SIRT1 attenuated ER stress and hepatic steatosis in diet-induced and genetically obese mice [4]. Mechanistically, SIRT1 can also activate the heat shock factor1 (HSF1) and reduce its binding to the promoter of heat shock protein (HSP) genes, thereby reducing ER stress-mediated lipoapoptosis [4]. Therefore, this reminds us of the potential connection between the SIRT1 pathway and ER stress-mediated lipoapoptosis.

In addition, SIRT1 can also regulate autophagy. Autophagy is a highly conservative degradation pathway mediated by lysosomes that is necessary to maintain normal internal environment and normal physiological functions in the body. Effective autophagy can prevent many diseases [5,6]. SIRT1 can regulate autophagy by deacetylating autophagy-related proteins, such as Atg5, Atg7, and Atg8 [7,8]. Moreover, not only can ER stress regulate autophagy, but basic levels of autophagy turnover also play an important role in regulating ER stress and maintaining ER homeostasis [9,10,11]. Moreover, although the function of autophagy in regulating lipid metabolism is still controversial, it has been demonstrated by our research group that activation of autophagy may be an effective way to regulate ER stress-mediated hepatic lipid metabolism [12]. Taken together, these studies indicate
that SIRT1 may be a prominent participant in the process of inducing autophagy under cellular stress, and this role is particularly important in maintaining the homeostasis of cellular lipids.

Pomegranate, the fruit of Punica granatum, has been widely reported to show brilliant antioxidant, anticancer and anti-inflammatory properties owing to its abundant bioactive phytochemicals including ellagicinsmins, gallo-tannins and flavonoids [13]. Punicalagin (PU) is an ellagatannin that is the most abundant in pomegranate polyphenols. Most of the previous researches are about PU regulating lipid metabolism through its antioxidant ability. PU can not only attenuate PA-induced lipotoxicity in HepG2 cells by activating the Keap1-Nrf2 antioxidant defense system and reducing the phosphorylation level of JNK and p38 [14], but also can inhibit high fat diet (HFD)-induced hyperlipidemia and liver lipid deposition by down-regulating uncoupling protein 2 (UCP2) and inhibiting the oxidation of mitochondrial protein [15]. However, whether and how PU protects hepatocyte from FFA-induced lipotoxicity through ER stress pathway remains unclear. The current study aims to investigate the protective effect of PU on SIRT1, autophagy and ER stress-mediated lipoapoptosis by PA.

2. Materials and Methods

2.1. Chemicals and Reagents

Punicalagin (PU purity>99%), palmitic acid (PA) (P0500), cycloheximide (CHX) (#C1988) and bafilomycin A1(BAF) (#B1793) were purchased from Sigma-Aldrich. Antibodies for GRP78 (#3183), c-PARP (#9548), p-PERK (#3192), p-eIF2α (#3597) were purchased from Cell Signaling Technology. LC-3 (#PM036), p62 (#PM045) were purchased from MBL International Corporation. Antibody for p-IRE1α (#ab48187) was purchased from Abcam. And β-actin antibody (#AT0001) was purchased from Action Biotech. Specific secondary antibodies were purchased from MBL International Corporation.

2.2. Cell Culture and Treatments

HepG2 cells were cultured with Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum. The cells (1×10^5 cells/well) were seeded in a 6-well plate, and the medium was changed and treated with corresponding agents after 12-24 h of plating.

2.3. Crystal Violet Staining

HepG2 cells are exposed to PA and/or PU at a specified time. After treatment, 1% glutaraldehyde solution was used to fix the cells. After fixing, the cells were stained with 0.02% crystal violet solution for 30 min, and then 70% ethanol was added to dissolve the stained cells. The OD value at 570 nm was measured by a microplate reader with a reference filter at 405 nm (Thermo).

2.4. Apoptosis Evaluation

Apoptosis was measured by Annexin V/PI staining of externalized phosphatidyl-serine (PS) in apoptotic cells by flow cytometry using a commercially available kit.

2.5. Western Blotting

HepG2 cells were lysed with ice-cold RIPA buffer. The proteins were separated by SDS-PAGE electrophoresis, and then transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was then incubated with the primary antibody and then recognized with the corresponding secondary antibody. Then use X-ray film to obtain immunoreacted bands. Use Image J software to quantitatively analyze Western blot images.

2.6. RNA Interference

siRNAs targeting SIRT1 (4427037) was obtained from Ambion. According to the manufacturer's instructions, the cells were transfected with SIRT1-siRNAs or negative control siRNAs using INTERFER in siRNA transfection reagent. After 24 h post-transfection, the cells were used for subsequent experiments.

2.7. Statistical Analysis

Data are presented as mean ± SD. The statistical analysis was evaluated by one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. p <0.05(*) could be considered statistically significant.

3. Results

3.1. PU prevents Lipid Accumulation and Inhibits Cytotoxicity Induced by PA in HepG2 Cells

Our previous study has shown that PA induced lipoapoptosis in hepatocyte cells [16]. In this study, we further evaluate the protective effect of PU on lipotoxicity induced by PA. HepG2 cells were treated with PU and PA alone or in combination for 48 h. Changes of cell viability was detected by the method of crystal violet staining. As shown in Figure 1A, exposure to 10 μM PU did not cause significant changes in cell viability. However, treatment with PA alone at the concentration of 200μM exerted a substantial reduction in the number of HepG2 cells. Next, we used Annexin V/PI staining to further detect the effect of PU on cell death of HepG2 cells induced by PA. Next, Annexin V/PI staining was used to further verify the effect of PU on PA-induced HepG2 cell death. Figure 1B shows that PA induced a dramatic increase in cell death, while PU pretreatment significantly abolished the toxicity of PA in HepG2 cells.

3.2. SIRT1 Plays a Key Role in Protecting against Cytotoxicity in HepG2 Cells

Our previous study has found that PU is able to up-regulate NER-related XPC expression and the deacetylation level of XPA in a SIRT1-dependent manner in human skin keratinocyte HaCaT [17]. Here, we further explore whether SIRT1 is involved in the
protective effects of PU on PA-induced lipotoxicity in hepatocyte cells. As shown in Figure 2A, it is found that PU increased the protein level of SIRT1 in HepG2 cells. Furthermore, PU compensates for the impaired SIRT1 induced by PA. Next, to confirm whether SIRT1 is essential for the protective effects of PU, we silenced SIRT1 in HepG2 cells (Figure 2B). It can be consistently demonstrated that PU blocked PA-induced cell death, while the protective effect of PU could be no longer observed in SIRT1-silenced cells. These data demonstrate that PU prevents hepatocyte cells from PA-induced cytotoxicity in a SIRT1-dependent pathway.

**Figure 1.** PU prevents lipid accumulation and inhibits PA-induced cytotoxicity in HepG2 cells: (A) Crystal violet staining was used to determine the inhibitory effect of PU and/or PA in HepG2 cells. (B) HepG2 cells were treated with PA, PU or their combination for 48 h and the cell death was measured by Annexin V/PI staining of externalized phosphatidylserine in apoptotic cells. Quantitative data are presented as mean ± SD based on biological repeats. *p < 0.01 compare with control group.

**Figure 2.** PU protects against cytotoxicity via SIRT1 activation in HepG2 cells: (A) Western blot was used to analyze the expression of SIRT1 in HepG2 cells. (B) HepG2 cells were transfected with siRNA against SIRT1 and then were treated with PU/PA, and the expression levels of cleaved-PARP and SIRT1 were analyzed by western blotting.
3.3. PU Re-activates the Impaired Autophagy

Studies have shown that autophagy is impaired in PA-treated hepatocyte models, MCD mouse models, and non-alcoholic fatty liver patients [18,19]. Earlier research by the research group on Glycyrrhizin Coumarin (GCM) showed that GCM can restore the autophagic flow blocked by PA [16], thereby inhibiting ER stress and lipid apoptosis, which indicates that activation of autophagy is an effective way to mediate lipid metabolism of liver cells. Based on the above researches, we asked if autophagy was also involved in hepatoprotective activity of PU. We first measured the autophagy response to PA and/or PU by analyzing the autophagy marker LC3. As shown in Figure 3A, we tested the increased conversion of LC3-I to LC3-II in PU treatment cells.

The activation of autophagy by PU was further confirmed by measuring the protein level of p62. In this study, PA increased p62 due to autophagy inhibition, while PU increased p62 as well (Figure 3A). To exclude the possibility of transcriptional regulation of P62 by PU, we used the protein synthesis inhibitor cycloheximide (CHX). As shown in Figure 3B, with the CHX treatment, PA still caused an increase of p62, while PU significantly blocked the P62 protein level, indicating autophagy activation by PU. These data indicate that PU increases p62 protein levels by activating transcription rather than inhibiting its protein degradation. In addition, reduced p62 levels were also found in cells treated with the combination of PA and PU, which further indicated that PA-mediated autophagy inhibition was recovered by PU. To further confirm the effect of PU on autophagy activation, we applied an inhibitor of autophagosome degradation, bafilomycin A1 (BAF), and monitored the autophagy flux.

Data in Figure 3C shows that BAF led to the accumulation of LC3-II, whereas PU treatment rescued the autophagy flux impaired by BAF. These data suggest that PU re-activates the impaired autophagy.

3.4. PU inhibits ER Stress-associated Cytotoxicity in Response to PA Exposure

ER stress induced by PA is negatively regulated by autophagy activation [16]. ER stress leads to a series of reactions such as lipid accumulation, inflammation, and apoptosis in liver cells [20]. Here, we explored the effect of PU on PA-induced ER stress. As shown in Figure 4, under PA treatment, the protein abundance of ER chaperone GRP78 in HepG2 cells increased significantly, and the phosphorylation levels of PERK and its downstream eIF2α also increased obviously. However, PU pretreatment significantly reduced the induction of these ER stress markers. It should be noted that unlike the
PERK pathway, the level of phosphorylated IRE1α seemed to be further increased by PU treatment. It may be due to the presence of PU so that the ER stress can be resolved, and the pro-apoptotic signal is counteracted by the regulated IRE1-dependent decay (RIDD) [21]. Consistently, PU reduced the increase of c-PARP expression induced by PA.

![Figure 4. PU inhibits ER stress-associated liptoxicity in response to PA exposure: In the presence or absence of PU, cells were exposed to PA for 24 h, and western blotting was used to analyze the key ER stress markers including GRP78, the phosphorylation of PERK, IRE1α and eIF2α, as well as the cleaved-PARP](Image)

4. Discussion

Lipid accumulation is the most important cause of initial toxic effects on hepatocytes, and the activation of ER stress is considered to be a critical event for FFA-induced cytotoxicity. PU is a natural polyphenol compound isolated from pomegranate. Previous researches on the mechanism of PU regulating lipid metabolism mainly focused on its antioxidant function. In this study, we clarified the underlying mechanism of PU as a chemopreventive agent to protect hepatocyte lipoapoptosis. We found that PU significantly re-activates autophagy, thereby relieving the impairment from ER stress. The rescue for PA-induced lipoapoptosis is depending on PU-mediated SIRT1 activation. These data enable us to better understand the role of PU in regulating lipid metabolism.

Endoplasmic reticulum (ER) stress is of great significance for maintaining the homeostasis of cells and normal physiological functions. ER stress will further lead to a series of responses such as lipid accumulation and inflammation, and its induction is considered to be the key mechanisms underlying hepatocyte lipoapoptosis [20]. In addition, the coordination between the two branches of IRE1α and PERK determines the apoptotic cell fate [22]. In this study, our results demonstrated that both PERK and IRE1α signaling pathways were activated by PA exposure. PU pretreatment leads to protective effect on hepatocyte lipoapoptosis by down-regulating PA-induced p-PERK and p-eIF2α (Figure 4), which in turn inhibiting JNK and CHOP [23]. However, the expression of p-IRE1α seemed to continue to be up-regulated under PA and PU combination group (Figure 4), which is different from the previously published report [24], suggesting that the protective effect of PU through the PERK pathway may be related to the type of cell. It is reported that IRE1 counteracts apoptotic signaling by degrading DR5 mRNA through IRE1-dependent decay (RIDD) [21]. In our study, PA initially activated the UPR, and the addition of PU caused the pro-apoptotic signal to be offset by regulating RIDD, thereby down-regulating p-PERK and continuing to up-regulate p-IRE1α to increase cell protection. The function of IRE1 in PU-regulated PERK branch should be further investigated.

In the Sirtuins family, SIRT1 is an important regulator of lipid homeostasis whose activation could inhibit steatosis induced by high-fat diet [3]. It suggests that Sirtuins regulators have certain potential in preventing diseases related to lipid metabolism. According to the former research, PA induced decreases of SIRT1 [25]. In the present study, our data show that PU protects against cytotoxicity via SIRT1 activation in HepG2 cells (Figure 2A), and silencing SIRT1 significantly increased PA-induced cell death (Figure 2B), which has not been reported in current studies on liver cells. It has been reported that SIRT1 can activate HSF1 and increase the expression of HSP, thereby reducing ER stress-mediated lipid apoptosis [26,27]. Therefore, we speculate that the regulation of ER stress-mediated lipid apoptosis by PU is dependent on SIRT1. In other words, PU can compensate for the protein expression of SIRT1 inhibited by PA, which might contribute to the inhibition of PERK pathway and activation of the IRE1 pathway.

Autophagy is a highly conserved degradation pathway mediated by lysosomes, and it can be induced by multiple forms of cellular stress, such as oxidative stress, ER stress, protein aggregates, damaged organelles and lipogenic challenge [28,29,30]. On the one hand, a large number of studies have shown that ER stress can regulate cell autophagy [31,32]. On the other hand, basal autophagy has also been shown to play an important role in regulating ER stress and maintaining ER homeostasis [9,10,33,34]. A previous study by our research group have shown that glycycoumarin (GCM) can restore PA-blocked autophagic flow, thereby inhibiting ER stress and lipoapoptosis, which indicates that activating autophagy is an effective way to inhibit ER stress-mediated hepatocyte lipoapoptosis. Our current data showed PU activates autophagy through relieving PA-inhibited autophagy flux (Figure 3A), which was further verified by using an inhibitor of autophagosome degradation, BAF (Figure 3C). PA-impaired autophagy was reactivated by PU with the reducing of LC3II/LC3I (Figure 3A) and p62 (Figure 3B). It is worth noting that there have been reports that in the PA-treated cell model, autophagy is also regulated by SIRT1 [35,36], which reminds us of the possibility that SIRT1 might act as a key role in PU-regulating autophagy and ER stress. The detailed mechanisms involved in the function of SIRT1-mediated autophagy under PU treatment need to be further investigated.

In summary, our present study has identified that PU could exert protective role in inhibiting PA-induced lipoapoptosis depending on SIRT1. Besides, PU is able to reduce PA-induced cytotoxicity through suppressing ER
stress and re-activating impaired autophagy in HepG2 cells.

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