Original Paper

Pterostilbene Reduces Acetaminophen-Induced Liver Injury by Activating the Nrf2 Antioxidative Defense System via the AMPK/Akt/GSK3 β Pathway

Xiaoye Fan^{a,b} Lidong Wang^a Jingbo Huang^a Hongming Lv^{a,b} Xuming Deng^{a,b}
Xinxin Ci^a

^aInstitute of Translational Medicine, The First Hospital, Jilin University, Changchun, ^bKey Laboratory of Zoonosis, Ministry of Education, College of Veterinary Medicine, Jilin University, Changchun, China

Key Words

Pterostilbene • Acetaminophen • Hepatotoxicity • Nrf2 • AMPK/Akt/GSK3 β pathway

Abstract

Background/Aims: Pterostilbene (Pts), a natural dimethylated analog of resveratrol from blueberries, exerts antioxidative and anti-apoptotic effects in various diseases. This study aims to investigate the protective effects and mechanism of Pts against acetaminophen (APAP)-induced hepatotoxicity *in vivo*. **Methods:** C57BL/6 mice were treated with APAP or APAP+Pts. HepG2 cells were used to further explore the underlying mechanisms *in vitro*. The effects of Pts on hepatotoxicity were measured by commercial kits, Hematoxylin and Eosin (H&E) staining, TUNEL assay, Western blot analysis, and Flow cytometry assay. **Results:** *In vivo*, Pts treatment effectively protected against APAP-induced severe liver injury by decreasing the lethality rate, the serum alanine transaminase (ALT) and aspartate aminotransferase (AST) levels, liver histological injury, liver malondialdehyde (MDA) formation and myeloperoxidase (MPO) levels and by increasing liver glutathione (GSH) and superoxide dismutase (SOD) levels. Moreover, in Pts-treated mice, the nuclear factor-erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) pathway was activated; however, APAP-induced c-Jun NH2-terminal kinase (JNK) activation, mitochondrial Bcl-2 Associated X Protein (Bax) translocation, apoptosis-inducing factor (AIF) levels and cytochrome c release were attenuated. *In vitro*, Pts was found to reverse hydrogen peroxide (H₂O₂)-induced cytotoxicity, reactive oxygen species (ROS) production and apoptosis that depended on Nrf2 activation. Moreover, Pts induced a dose-dependent increase in the phosphorylation of AMP-activated protein kinase (AMPK), serine/threonine kinase (Akt), and glycogen synthase kinase 3 β (GSK3 β) in HepG2 cells. Moreover, Pts protect against APAP or H₂O₂-induced toxicity were effectively attenuated or abolished in HepG2 Nrf2^{-/-} cells and Nrf2^{-/-} mice. **Conclusion:** Our data suggest that Pts protects against APAP-induced toxicity by activating Nrf2 via the AMPK/Akt/GSK3 β pathway.

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Published by S. Karger AG, Basel

Xinxin Ci

Institute of Translational Medicine, The First Hospital, Jilin University
Changchun, 130001 (China)
Tel. +86 431 88783044, E-Mail cixinxin@jlu.edu.cn

Introduction

Drug-induced liver injury (DILI) has been reported to be one of the main reasons for the withdrawal of many clinically used drugs and the most common cause of acute liver failure [1], which represents a major challenge in designing potential therapies [2]. Acetaminophen (APAP), a widely used analgesic and antipyretic drug worldwide, has been reported to be the main reason for DILI in the United States and the United Kingdom [3, 4]. Despite great efforts over last 40 years, the mechanism of APAP-induced liver injury has not been completely elucidated.

It is widely accepted that APAP can be metabolized by the cytochrome P450 system to generate the highly reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). When NAPQI is not properly detoxified, it forms protein adducts in the liver and induces toxicity by depleting glutathione (GSH) [5]. Moreover, it has been recognized that an APAP overdose causes mitochondrial dysfunction [6]. MAP kinases are activated by oxidative stress, eventually causing c-jun N-terminal kinase (JNK) phosphorylation and mitochondrial translocation [7]; the collapse of the mitochondrial membrane potential ultimately decreases B-cell lymphoma 2 (Bcl-2) protein levels, which inhibits cell death by preventing depolarization and Bcl-2-associated X (Bax) mitochondrial translocation. This accelerates cell death by inducing depolarization; the activation of caspase-3 and caspase-9, and apoptosis-inducing factor (AIF); and the release of mitochondrial cytochrome C (Cyt-C) [8], which further amplifies mitochondrial oxidative stress [9]. The amplification of mitochondrial oxidative stress triggers the opening of the mitochondrial permeability transition (MPT) pore, hepatocyte necrosis and subsequent liver injury [10]. The commonly used antidote for APAP detoxification is the antioxidant N-acetylcysteine (NAC), a precursor of cellular GSH synthesis. Although NAC attenuates APAP-induced hepatotoxicity, some cases of APAP-induced hepatotoxicity are not diagnosed or seen early enough, so liver injury may still develop despite the administration of the recommended dosage [11, 12]. Therefore, more effective and safe drugs for APAP detoxification are needed.

Nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor in the cap 'n' collar family, regulates the constitutive and inducible expression of a variety of genes involved in drug metabolism, detoxification, and antioxidative defenses [13]. It also plays an important role in cellular redox homeostasis through inducing more than 250 genes, including heme oxygenase 1 (HO-1), NAD(P)H: quinine oxidoreductase 1 (NQO1), and the γ -glutamyl cysteine synthetase catalytic subunit (GCLC) and modifier subunit (GCLM) [14]. Moreover, animals deficient in Nrf2 are extremely susceptible to toxic stimuli such as acetaminophen (APAP), benzo [a]pyrene, diesel and other oxidative stressors induced organ damage due to reduced antioxidant protection [15, 16]. Thus, the Nrf2 signaling pathway is a potential target for APAP-induced liver injury.

In addition to the Keap1 cysteine groups, which is directly oxidized or covalently modified, the signal of Nrf2/ARE can be regulated by the post-transcriptional modification of Nrf2 by kinases such as phosphatidylinositol 3-kinase (PI3K), PKC, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated protein kinase (ERK). Moreover, AMP-activated protein kinase (AMPK)-mediated inactivation of glycogen synthase kinase 3 β (GSK3 β) can increase the nuclear accumulation of Nrf2 [17]. AMPK is a conserved serine/threonine kinase that serves as a critical sensor in the regulation of cellular energy homeostasis and metabolic stress, including neurodegeneration, inflammation, and oxidative stress [18]. Emerging evidence indicates that AMPK is a negative modulator of oxidative stress responses that plays a protective role in an APAP-induced mouse model of DILI [19]. Since Nrf2 target genes can detoxify highly active intermediates, chemical activators of Nrf2 might be used to protect hepatocytes from the toxic effects of APAP or other drugs [20].

To date, abundant reports have revealed that natural products have made a huge contribution to the discovery of drug in the past few decades. Several studies have proved the benefits of natural products that counteract oxidative stress by modulating the Nrf2/ARE pathway [21]. Pterostilbene (Pts), a natural product extracted from blueberries and grapes,

has been shown to possess multiple biological effects in both *in vitro* and *in vivo* models, such as antioxidative, anti-apoptotic, anti-inflammatory, anti-neoplastic, and anti-aging, etc [22]. Resveratrol, an analog of Pts, has three hydroxyl groups, whereas pterostilbene has only one, which presumably increases its transport into the cell and its metabolic stability [23]. When comparing the antioxidant activity of resveratrol and pterostilbene, Mikstacka et al. found that pterostilbene was more effective in protecting the erythrocyte membrane against lipid peroxidation [24]. Furthermore, pterostilbene has a better pharmacokinetic profile than resveratrol, which ultimately makes pterostilbene as the better drug candidate [23]. Therefore, we speculated that Pts might exert protective effects against APAP-induced hepatotoxicity and acute liver injury. In this study, we investigated the Pts-induced protection against APAP-induced oxidative liver injury and the involvement of the AMPK/Akt/GSK3 β signaling pathway.

Materials and Methods

Animal experiments

Wild-type (WT) and Nrf2^{-/-} (knockout) C57BL/6 mice, 18-20 g, were purchased from Liaoning Changsheng Technology Industrial Co., LTD (Certificate SCXK2010-0001; Liaoning, China) and The Jackson Laboratory (Bar Harbor, ME, USA), respectively. All of the animals were raised under SPF-condition after feeding for several days. The mice were allowed free access to water and laboratory chow. All studies were in accordance with the International Guiding Principles for Biomedical Research Involving Animals, which was published by the Council for the International Organizations of Medical Sciences. All of the studies were conducted in accordance with the experimental practices and standards approved by the Animal Welfare and Research Ethics Committee in Jilin University. To induce APAP hepatotoxicity, WT mice were randomly divided into five groups (n=15/group for protocol 1 and n=5/group for protocol 2): control (saline), Pts only (50 mg/kg dissolved in 0.5% DMSO), APAP only (900 mg/kg or 400 mg/kg dissolved in saline), APAP (900 mg/kg or 400 mg/kg) + DMSO (0.05 ml/kg) or APAP (900 mg/kg or 400 mg/kg) + Pts (50 mg/kg). In addition, Nrf2^{-/-} mice were randomly divided into three groups: control (saline), APAP only (900 mg/kg or 400 mg/kg dissolved in saline) or APAP (900 mg/kg or 400 mg/kg) + Pts (50 mg/kg). In briefly, the mice were treated with Pts (50 mg/kg) i.p. two times for 12 hours each time. One hour after the last dose of Pts, the mice were exposed to a lethal dose of APAP (900 mg/kg) in protocol 1 to observe mortality rate. Protocol 2 mice were treated with APAP (400 mg/kg) for 3 h, blood and liver tissue samples were taken for blood biochemical and histopathological analyses respectively.

Blood chemistry and Histopathological evaluation

The blood was collected and centrifuged to obtain serum. The serum ALT and AST were measured using an assay kit according to the manufacturer's instructions. Portions of mouse livers were stored in 4% paraformaldehyde and fixed for 48 h, dehydrated in 70% ethanol, then sectioned, embedded in paraffin and cut into 3 μ m sections. Formalin-fixed paraffin-embedded liver sections were stained using Hematoxylin and Eosin (H&E) for pathological analysis. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed for DNA strand break assessment with the Trans Detect In Situ Fluorescein TUNEL Cell Apoptosis Detection Kit, following manufacturer's instructions.

Measurement of MPO, MDA, SOD and GSH levels in liver tissues

The mice liver tissues were homogenized and dissolved in extraction buffer to measure the MPO, MDA, GSH and SOD levels upon the manufacturer's instructions. To examine the accumulation of neutrophils and level of lipid peroxidation in the liver tissue, MPO and MDA levels were assessed using commercial assay kits according to the respective manufacturer's instructions. Moreover, to further detect the antioxidative enzyme activities in the liver tissues, GSH and SOD levels were measured following the manufacturer's instructions respectively.

Isolation of mitochondria

Fresh liver tissues were homogenized in ice-cold isolation buffer (pH 7.4, containing 1 mM ethylene glycol tetraacetic acid, 22 mM mannitol, 70 mM sucrose, 10 mM EDTA, 2.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 0.1% bovine serum albumin) on ice. Homogenates were centrifuged at 2500 g for 10 min. The supernatant was then centrifuged at 20,000 g for 10 min, the pellet was mitochondria, and the supernatant was preserved as the cytosolic fraction.

Preparation of nuclear and cytosolic fractions

The nuclear and the cytoplasmic extracts, on ice or at 4 °C, were isolated using an NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's instructions.

Cell culture and Cell viability assay

HepG2 cell line was purchased from the China Cell Line Bank (Beijing China). The cells were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), containing 100 U/ml of penicillin, 100 U/ml streptomycin and 3 mM glutamine at 37 °C with 5% CO₂. In all experiments, cells were allowed to acclimate for 24 h before any treatments. The cells (1 × 10⁴ cells/well) were plated in 96-well plates for 24 h. Then, cells were pretreated with different concentrations of Pts (7.5, 15 and 30 μM) for 1 h, followed by the addition of H₂O₂ (300 μM) in the presence or absence for 24 h stimulation. MTT solution (5 mg/ml) was added to the cells for 4 h incubation, the supernatant was removed, and DMSO was added to each well to lyse the cells, and absorbance was measured at 570 nm.

CRISPR/Cas9 knockout

HepG2 cells were cultured in a 12-well plate (2.5 × 10⁵ cells/well) for 24 h incubation, then co-transfected with the plasmids of expressing Cas9 with Nrf2-sgRNA and puromycin resistant gene using Viafect transfection reagent (Promega). 2 mg/ml puromycin was added to select cells until 48h after transfection. Two days later, cells were cultured in 96-well plates at a density of 1 cell per well. The level of gene editing efficiency after the clonal expansion was determined by western blotting analysis with Nrf2 antibody. To verify the edited genes, DNA sequencing was employed.

Detection of ROS levels

To detect intracellular ROS production, HepG2 cells (1 × 10⁴ cells/well) were grown in 96-well plates for 24 h. The cells were treated with different concentrations of Pts (7.5, 15 and 30 μM) for 18 h before H₂O₂ (300 μM) was added to each well. After 18 h, the cells were stained with 50 μM of DCFH-DA for 40 min. Fluorescence was immediately read in a multi detection reader with excitation at 488 nm and emission at 535 nm.

Flow cytometry assay

HepG2 cells (2.5 × 10⁵ cells/well) were cultured in 12-well plates for 24 h and were then treated with Pts for 1 h. Subsequently, H₂O₂ (300 μM) was added for an additional 24 h. Next, the cells were washed twice with ice-cold PBS and pelleted by centrifugation at 1500 rpm/min for 5 min at 4 °C. The percentages of apoptosis and necrosis were monitored by flow cytometry (LSR II Flow Cytometer; BD Biosciences, San Jose, CA, USA).

Western blot analysis

To extract protein, liver samples or cells were lysed in RIPA with protease and phosphatase inhibitors for 30 min. The BCA protein assay kit (Beyotime, China) was used to measure protein concentrations, and equal amounts of protein (40 μg) was separated by 10% SDS-polyacrylamide gel and then electrophoretically transferred to PVDF membrane. The membrane was blocked with blocking solution (5% (w/v) nonfat dry milk) for 40 min, followed by an overnight incubation at 4 °C with a specific primary antibody. Then the membrane was incubated 1 h with HRP-conjugated secondary antibody (1:5000 dilution) at room temperature after being thoroughly washed three times with TBST. Protein bands were detected using ECL (Amersham Pharmacia Biotech, Piscataway, NJ) and band intensities were quantified using Image J gel analysis software. All experiments were performed in triplicate for each experimental condition.

Statistical analysis

Experimental data are expressed as mean $\pm$ S.E.M. Analysis was performed using SPSS19.0 (IBM). One-way analysis of variance (ANOVA) procedures were used to assess the comparisons between experimental groups. Statistical significance was accepted when * $p < 0.05$ or ** $p < 0.01$.

Results

Pts treatment protected mice against APAP-induced mortality and liver injury

As shown in Fig. 1A and B, the mortality rate of mice treated with APAP (900 mg/kg) was much higher than the mice treated with APAP (900 mg/kg) + Pts (50 mg/kg). Serum AST and ALT levels were used as well-established marker of hepatic injury. Mice treated with 400 mg/kg APAP developed severe liver injury at 3 h, 6 h and 12 h post-APAP, as indicated by increased plasma ALT and AST activities. The results showed that the serum ALT and AST levels were significantly increased by APAP (400 mg/kg) administration, whereas Pts (50 mg/kg) reduced these increases (Fig. 1D and E). Next, we examined the degree of liver damage by histopathology to verify the protective effect of Pts. Mice treated with APAP (400 mg/kg) exhibited severe intrahepatic hemorrhage and necrosis, but treatment with Pts (50 mg/kg) improved this phenomenon, indicating that Pts effectively blocked APAP-induced hepatic toxicity (Fig. 1F).

Pts treatment alleviated APAP-induced oxidant stress in mice

Due to oxidative damage plays a crucial role in APAP-induced mice, we tested whether Pts pretreatment could protect against hepatotoxicity by inhibiting oxidative stress. Indeed, as shown in Fig. 2, pretreatment with Pts reduced the MPO and MDA levels (Fig. 2A and B) and obviously increased the SOD and GSH levels (Fig. 2C and D). These data suggested that Pts played an important role in preventing APAP-induced oxidant stress.

Increased Nrf2 expression and AMPK, Akt and GSK3 β phosphorylation by Pts in APAP-induced mice

In order to study the protective mechanism of Pts in APAP-induced mice, western blotting was used to detect the activation of Nrf2, AMPK, Akt and GSK3 β , which are responsible for alleviation of liver damage. As shown in Fig. 2E-G, Pts increased Nrf2 expression and nuclear translocation as well as AMPK, Akt and GSK3 β phosphorylation in liver tissue, indicating that the protective of Pts treatment on APAP-induced liver injury may be associated with enhancing phosphorylation of AMPK, Akt and GSK3 β as well as upregulation of Nrf2.

Pts alleviated mitochondrial dysfunction in APAP-induced mice

As shown in Fig. 3, APAP overdose induced JNK activation (phosphorylation) in the cytosol, and activated P-JNK translocation to the mitochondria as early as 3 h. Next, we observed Bax translocation to mitochondria after APAP and Pts almost completely prevented this change (Fig. 3C). As a result, the release of mitochondrial intermembrane proteins AIF and cytochrome c were also completely eliminated by Pts (Fig. 3D). To show the effect of Pts on protein regulators of apoptosis following AILI, the expression of Bcl2 were detected by Western Blot, which showed Pts significantly increased the expression of Bcl2 protein (Fig. 3E). The corresponding DNA fragmentation as indicated by the TUNEL assay (Fig. 1F) is caused by release of mitochondrial endonucleases. Consistent with the elimination of mitochondrial intermembrane protein release, Pts treatment also prevented DNA fragmentation as indicated by the elimination of TUNEL positive cells.

Fig. 1. Pts protected against APAP-induced fulminant hepatic failure. (A) Mice were treated with Pts (50 mg/kg) i.p. twice at an interval of 12 hours. The survival rate of mice that were treated with APAP (900 mg/kg) at a lethal dose 1 h after the last dose of Pts (50 mg/kg). The survival rates of the mice were observed within 48 h after APAP treatment. The data are shown as the percentage of surviving mice at each time point. n=10 in each group. (B) Mice were treated with APAP (900 mg/kg) for 1h, followed by treatment with Pts (50 mg/kg), to determine the mortality rate. The data are shown as the percentage of surviving mice at each time point. n=10 in each group. (C) One hour after the last dose of Pts, APAP (400 mg/kg) was administered (n=5/group). Blood was harvested at 3, 6 or 12h post-APAP. A serum alanine transaminase (ALT) activity. (D, E) One hour after the last dose of Pts, APAP (400 mg/kg) was administered for 3 h (n=5/group). We harvested serum for an analysis of alanine transaminase (ALT) and aspartate aminotransferase (AST). (F) Representative liver histological sections were stained with hematoxylin and eosin (H&E) (magnification 400×) and TUNEL (40×). The data represent the average of three independent experiments. All data are presented as the mean ± SEM (n=5 in each group). **p<0.01 versus the control group; #p<0.05 versus the APAP group.

Fig. 2. Effect of Pts treatment on the levels of APAP-mediated oxidative stress as well as Nrf2 activation and AMPK, Akt and GSK3β phosphorylation in mice. Mice were treated with Pts (50 mg/kg) i.p. twice. One hour after the last dose of Pts, mice received APAP (400 mg/kg). Liver tissues were obtained from the mice 3 h after the APAP challenge for the measurement of myeloperoxidase (MPO) and malondialdehyde (MDA) formation and of superoxide dismutase (SOD) and glutathione (GSH) activities. Furthermore, Protein samples were extracted from liver tissue homogenates and analyzed by Western blot. (A, B) Effects of Pts on APAP-induced MDA and MPO content in liver. (C, D) Effects of Pts on APAP-induced GSH and SOD content in liver. (E, G) Effects of Pts on APAP-induced the expression and nuclear translocation of Nrf2 and HO-1 protein expression in liver. F) Effects of Pts on APAP-induced the AMPK, Akt and GSK-3β phosphorylation in liver. The data represent the average of three independent experiments. Data presented as the mean ± SEM (n=5 in each group). *p<0.05 and **p<0.01 versus the control group; #p<0.05 and ##p<0.01 versus the APAP group.

Fig. 3. Effect of Pts protected against mitochondrial dysfunction. Mice were treated with Pts (50 mg/kg) i.p. twice. One hour after the last dose of Pts, the mice received APAP (400 mg/kg). (A, B) Western blot detected the phosphorylated JNK levels in mitochondrial and cytosolic liver fractions at 3 h post-APAP. (C, D) Mitochondria Bax and cytosolic Bax, AIF and cytochrome c measured by immunoblot analysis at 3 h post-APAP injection. (E) Bcl2 in liver tissue lysates were assayed by Western blot. The results shown the average of three independent experiments. * $p < 0.05$ and ** $p < 0.01$ versus the control group; # $p < 0.05$ and ## $p < 0.01$ versus the APAP group.

Pts attenuated H_2O_2 -induced cytotoxicity and oxidant stress injury in HepG2 cells

To determine the effects of different concentrations of Pts on H_2O_2 -mediated cell viability, we performed MTT assay. The results showed that H_2O_2 (300 μM) caused severe cytotoxicity, which was suppressed by Pts (30 μM) pretreatment (Fig. 4A). In our study, to further assess the protective effect of Pts against oxidant stress, we employed H_2O_2 , which can induce various types of cellular injury as a type of ROS. Pts exposure markedly decreased H_2O_2 -induced ROS generation and apoptosis in a dose-dependent manner (Fig. 4B, C).

Pts treatment upregulated the Nrf2 expression and nuclear translocation in HepG2 cells

As shown in Fig. 5A and B, Pts increased the expression of Nrf2 and HO-1 protein in a time-and-dose-dependent manner in HepG2 cells. Based on this observation, we examined whether Pts could enhance Nrf2 nuclear translocation. Pts treatment resulted in an increase in the accumulation of Nrf2 protein in the nucleus and a concomitant decrease in the cytoplasmic levels (Fig. 5C). Next, we used HepG2 Nrf2^{-/-} cells to investigate whether the up-regulation of HO-1 expression was mediated by Nrf2. We observed a significant reduction in Nrf2 and HO-1 protein levels induced by Pts in HepG2 Nrf2^{-/-} cells (Fig. 5D and E). Furthermore, our results showed that Pts protected H_2O_2 -induced cell death in the HepG2 WT cells was higher than in the HepG2 Nrf2^{-/-} cells (Fig. 5F).

Fig. 4. Effect of Pts exposure on H₂O₂-induced cytotoxicity, oxidative stress and apoptosis in HepG2 cells. (A) HepG2 cells were administrated with various concentrations of Pts (7.5, 15 and 30 μM) for 1 h, the cells received H₂O₂ (300 μM) for 24 h, and cell viability was evaluated using an MTT assay. (B) HepG2 cells were administrated with Pts (7.5, 15 and 30 μM) for 18 h, the cells received H₂O₂ (300 μM) for 5 min, and then stained with 50 μM DCFH-DA for 40 min. The fluorescence was immediately read in a multi-detection reader with excitation at 488 nm and emission at 535 nm. (C) HepG2 cells were administrated with various concentrations of Pts (7.5, 15 and 30 μM) for 1 h, the cells received H₂O₂ (300 μM) for 24 h, the percentage of apoptotic and necrotic cells were determined by Flow cytometry. The results shown the average of three independent experiments. *p<0.05 and **p<0.01 versus the control group; #p<0.05 and ##p<0.01 versus the H₂O₂ group.

Fig. 5. Effect of Pts treatment on Nrf2 expression and nuclear translocation and Nrf2-mediated defense against H₂O₂-stimulated cytotoxicity in HepG2 cells. (A-C) HepG2 cells were administrated with Pts for the demonstrative time, and Western blot analysis determine the protein levels. (D-E) HepG2 WT and HepG2 Nrf2^{-/-} cells were grown with or without Pts (30 μM) for 18 h; Nrf2 and HO-1 expression were detected by a Western blot analysis. (F) HepG2 WT and Nrf2^{-/-} cells were administrated with Pts (30 μM) for 1 h before H₂O₂ (300 μM) treatment for 24 h; cell viability was determined by an MTT assay. All the data represent the average of three independent experiments. **p<0.01 versus WT control group; ##p<0.01 versus WT H₂O₂ group; **p<0.01 versus WT H₂O₂ plus Pts group.

Pts treatment upregulated the Nrf2 expression due to AKT-mediated phosphorylation of GSK3β

To identify the upstream effector of Pts on Nrf2 activation, we further investigated the role of AKT-mediated GSK3β inhibitory phosphorylation. As shown in Fig. 6A and B, Pts significantly increased phosphorylation of AKT and GSK3β. In addition, preincubation with LY294002 (AKT inhibition, 20 μM) blocked Pts ability to increase Nrf2 nuclear translocation, GSK3β phosphorylation and cytoprotection (Fig. 6C and D).

Akt/GSK3β-mediated Nrf2 nuclear translocation is involved in AMPK activation by Pts

Recent reports have suggested that AMPK activated the PI3K/AKT signaling pathway. Furthermore, AMPK regulates inactivation of GSK-3β to induce nuclear translocation of Nrf2. HepG2 AMPK^{-/-} cells were used to confirm the relationship between AMPK, Akt/GSK3β and Nrf2 nuclear translocation by Pts. The result showed that Pts significantly induced AMPK phosphorylation in a dose-dependent manner in HepG2 cells (Fig. 7A). Whereas in Fig. 7B, HepG2 AMPK^{-/-} cells observably blocked AMPK, Akt and GSK3β phosphorylation and Nrf2

Fig. 6. Effect of Pts exposure on AKT-mediated GSK3β phosphorylation and its critical role in the Pts-induced protection of H₂O₂-induced cytotoxicity in HepG2 cells. (A, B) HepG2 cells were incubated for 18 h with Pts; the levels of AKT and GSK3β phosphorylation were determined by Western blot. (C) HepG2 cells were pretreated with LY294002 (20 μM) for 18 h and then treated with Pts for 18 h; Western blot analysis determine the levels of AKT and GSK3β and Nrf2 nuclear translocation. (D) The cells were pretreated with LY294002 (20 μM) for 1 h before treatment with Pts. After 1 h, the cells were received H₂O₂ for 24 h, and cell viability was measured by MTT assay. The results shown the average of three independent experiments. **p<0.01 versus the control group; ##p<0.01 versus the H₂O₂ group; ++p<0.01 versus H₂O₂ plus Pts group.

Fig. 7. Pts-induced AMPK phosphorylation is essential for AKT/GSK3β-mediated Nrf2 activity and cytoprotection in HepG2 cells. (A) HepG2 cells were cultured with Pts for 18 h. Western blot was used to detect the expression of p-AMPK and AMPK. (B) HepG2 WT and AMPK^{-/-} cells were incubated with or without Pts (30 μM) for 18 h; Western blot was used to detect the phosphorylation of AMPK, Akt, GSK3β and Nrf2. (C) HepG2 WT and AMPK^{-/-} cells were pretreated with 30 μM Pts for 1 h before H₂O₂ (300 μM) treatment for 24 h; cell viability was determined by an MTT assay. All the data presented as the average of three independent experiments. **p<0.01 versus the control group; #p<0.05 vs. the H₂O₂ group; ++p<0.01 vs. H₂O₂ plus Pts group.

nuclear translocation. And, HepG2 AMPK^{-/-} cells partially inhibited the protective effect of Pts (Fig. 7C). Thus, we suggested that Pts induced Nrf2 nuclear translocation via the activation of AMPK/Akt/GSK3 β signaling pathway to prevent H₂O₂ toxicity in HepG2 cells.

Protective effects of Pts-mediated Nrf2 on APAP-induced mice

To ascertain whether the protective effect of Pts-displayed against APAP-induced AILI is dependent upon Nrf2 activation, WT mice and Nrf2^{-/-} mice were conducted. As shown in Fig. 8, Pts treatment markedly protected survival rate in WT mice, but significantly blocked in Nrf2^{-/-} mice. Moreover, Pts treatment effectively attenuated severe histopathological changes in WT mice were obviously abrogated in Nrf2^{-/-} mice. Importantly, Pts treatment mediated increases of Nrf2 and HO1 protein expressions in WT mice were significantly inhibited in Nrf2^{-/-} mice. Taken together, our experimental results revealed that Pts plays an important role in the attenuation of APAP-induced oxidative stress, which may be dependent upon upregulation of Nrf2.

Fig. 8. Protective effects of Pts-mediated regulation of Nrf2 on APAP-induced AILI in mice. WT and Nrf2^{-/-} mice were intraperitoneally injected with Pts (50 mg/kg) twice at an interval of 12 hours. (A) Survival rate of WT and Nrf2^{-/-} mice that received a lethal dose of APAP (900 mg/kg) 1 h after the last dose of Pts (50 mg/kg). The data are represented the percentage of surviving mice at each time point. n=15 in each group. (B) One hour after the last dose of Pts and 3 h after the APAP (400 mg/kg) challenge, livers (n=5) from each experimental group were processed for gross examination. (C) Representative histological sections of the liver were stained with hematoxylin and eosin (H & E staining). (D) Effects of Pts on the protein expression of Nrf2 and HO-1 protein. All data are represented the mean $\pm$ SEM (n=5 in each group). ##p<0.01 vs the control group; *p<0.05 and **p<0.01 vs the APAP group.

Discussion

APAP-induced liver injury (AILI) is the main reason for DILI. Excessive oxidative stress and/or overwhelming mitochondrial dysfunction are thought to play essential roles in the pathogenesis of AILI [25]. To counteract oxidative stress, cells have developed adaptive, dynamic programs to maintain the cellular redox homeostasis and attenuate oxidative damage through a series of antioxidant molecules and detoxifying enzymes. The Nrf2 pathway, which plays an critical role in cellular redox homeostasis, is one of the main defense mechanisms against oxidative stress [26]. Pts, a natural activator of Nrf2, attenuates the intracellular ROS level, conferring neuroprotection depending on its antioxidative stress activity [27]. In the current study, we found that the therapeutic modulation of the Nrf2 pathway by Pts may be an effective strategy for treating AILI via the AMPK/Akt/GSK3 β pathway.

In the current study, we found that Pts is effective in preventing APAP-induced liver damage and dysfunction in mice. To test the protective effect of Pts, we examined mortality, biochemical markers, and histological sections. The result showed that Pts decreased APAP-induced mortality and serum ALT and AST activity, and further liver histological evaluation also confirmed that Pts has a protective effect on mice Liver injury. Next, we tested whether Pts blocked the hepatotoxicity by reducing oxidative stress. Our data suggested that Pts pretreatment reduced the MPO and MDA content and obviously enhanced the SOD and GSH levels, which play a crucial role in preventing APAP-induced oxidative stress. In addition, mitochondrial oxidative stress has been suggested to be critical in the progression of APAP-induced liver injury [28], and JNK activation and its translocation to mitochondria have been involved in APAP-induced hepatic toxicity. Our results indicated that an APAP overdose caused JNK phosphorylation in cytoplasm and triggered its translocation to mitochondria, but Pts pretreatment can obviously suppress this phenomenon. JNK has also been shown to regulate members of the bcl2 family such as Bax, which translocate to mitochondria, as well as to accelerate cytochrome *c* release from mitochondria. In line with previous reports, APAP caused Bax mitochondrial translocation, cytochrome *c* and AIF loss from mitochondria. But, Pts almost completely prevented such phenomena. The above results demonstrated *in vivo* that the liver protective effects of Pts involve the suppression of oxidative stress and the restoration of mitochondrial function. Another issue to consider is the difference in the time course of APAP hepatotoxicity in mice. In mice, the injury normally begins at 3 h and peaks around 12 h post-APAP [29]. Since Pts is still effective in mice when given at 3 h post-APAP, Pts could be regarded as a promising therapeutic agent for late-presenting patients in the clinic.

Since oxidative stress plays a key role in the progression of APAP-induced hepatotoxicity, the strict control of oxidative stress by antioxidants and detoxification enzymes is a strategy to alleviate hepatotoxicity. Nrf2, a transcription factor, adjust and control cytophyllaxis by inducing the expression of various detoxification and antioxidative genes, e.g., HO-1 [30]. Importantly, Pts has been identified as a potent Nrf2 activator [31] that protects β -cells against oxidative stress [22]. In this study, our data suggested the induction of Nrf2 target genes, including HO-1, by Pts in mice. Indeed, Pts promoted translocation of Nrf2 into the nucleus, which closely related to the increase of nuclear Nrf2 content and Nrf2-target gene transactivation. It has been reported that AMPK induced PI3K/Akt signaling pathway activation [32]. Furthermore, AMPK suppresses the phosphorylation of GSK3 β [33], which contributes to mitochondrial defend against iron-induced oxidative stress [33]. Our data show that Pts treatment dramatically increases the phosphorylation of AMPK, Akt and GSK3 β in liver tissue.

In view of the above results, our studies further explored the hepatoprotective effects and mechanism of Pts *in vitro*. Our findings showed that Pts significantly reduced H₂O₂-induced cytotoxicity, ROS production and apoptosis in HepG2 cells. The Nrf2 pathway is thought to be the most important cellular pathway that protects against oxidative stress. Moreover, Numerous studies have shown that some natural products inhibit APAP-induced hepatotoxicity by increasing Nrf2 transcription and the downstream expression

of antioxidant genes, including HO-1 [20, 34, 35]. As shown in Fig. 6, Pts enhanced Nrf2 expression and nuclear translocation, as well as HO-1 expression, in HepG2 cells, while expression and cytoprotection of HO-1 by Pts were weakened or abolished in HepG2 Nrf2^{-/-} cells, demonstrating that the Nrf2-mediated up-regulation of antioxidant genes is induced by Pts to defend against H₂O₂-induced hepatotoxicity. The present findings obtained from *in vivo* assays were confirmed *in vitro*, where Pts caused a time- and dose-dependent increase in the phosphorylation of AMPK, Akt and GSK3 β . These observations suggest that the phosphorylation and activation of AMPK, Akt and GSK3 β upon Pts treatment may account for the beneficial effects of Pts on AILI. Finally, by using HepG2 AMPK^{-/-} cells, we further confirmed that AMPK is upstream of Akt, GSK3 β and Nrf2. Meanwhile, AMPK also played a key role in the cytoprotection by Pts. In short, Pts not only induced the phosphorylation of AMPK but also caused the phosphorylation of Akt and GSK-3 β in an AMPK-dependent manner. Thus, these results indicate that the AMPK/Akt/GSK3 β /Nrf2 pathway is responsible for the pharmacological action of Pts.

Given all these results, to further illuminate whether the Pts-induced antioxidant activity in APAP-induced AILI is directly associated with Nrf2 activation, Nrf2-deficient mice were used as a tool to investigate the presence of an underlying connection. Our data showed that Pts prevented the mortality associated with an APAP challenge in WT mice; however, Nrf2-knockout mice were more susceptible to APAP-induced liver injury than WT mice. Additionally, the Pts-attenuated severe histopathological changes in WT mice were effectively abrogated in Nrf2^{-/-} mice. Moreover, the Pts-mediated increases in Nrf2 and HO-1 protein expression in WT mice were significantly inhibited in Nrf2^{-/-} mice. In summary, our results support the possibility that the ability of Pts to inhibit APAP-induced toxicity may be mediated by the activation of Nrf2.

Conclusion

In the end, the findings of this study suggest that Pts plays a crucial role in liver protection by suppressing APAP-induced oxidative stress. The underlying mechanisms may be closely related to Nrf2 activation through the AMPK/Akt/GSK3 β pathway. Consequently, the study provides significant information about the pharmacological mechanism and potential application of Pts for the prevention and/or treatment of APAP-induced liver toxicity.

Acknowledgements

This work was in part supported by the National Science Foundation of China (Grant No. 81603174) and the General Financial Grant from the China Postdoctoral Science Foundation (Grant No.168847).

Disclosure Statement

The authors declare no conflicts of interest.

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