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Original Article

# Paeoniflorin protects cells from GalN/TNF-α-induced apoptosis via ER stress and mitochondria-dependent pathways in human L02 hepatocytes

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**Paeoniflorin (PF) is one of the main effective components extracted from the root of *Paeonia lactiflora*, which has been used clinically to treat hepatitis in traditional Chinese medicine, but the details of the underlying mechanism remain unknown. The present study was designed to investigate the mechanism of protective effect of PF on D-galactosamine (GalN) and tumor necrosis factor-α (TNF-α)-induced cell apoptosis using human L02 hepatocytes. Our results confirmed that PF could attenuate GalN/TNF-α-induced apoptotic cell death in a dose-dependent manner. The disruption of mitochondrial membrane potential and the disturbance of intracellular $Ca^{2+}$ concentration were also recovered by PF. Western blot analysis revealed that GalN/TNF-α induced the activation of a number of signature endoplasmic reticulum (ER) stress and mitochondrial markers, while PF pre-treatment had a marked dose-dependent suppression on them. Additionally, the anti-apoptotic effect of PF was further evidenced by the inhibition of caspase-3/9 activities in L02 cells. These findings suggest that PF can effectively inhibit hepatocyte apoptosis and the underlying mechanism is related to the regulating mediators in ER stress and mitochondria-dependent pathways.**

*Keywords* paeoniflorin; GalN/TNF-α; L02 human hepatocytes; endoplasmic reticulum stress; mitochondria; apoptosis

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## Introduction

Endoplasmic reticulum (ER) is the primary site for new protein synthesis, folding, and processing as well as intracellular $Ca^{2+}$ regulation [1,2]. Various conditions that impair the ER function, collectively designated 'ER stress', have been reported to cause hepatic cell death in a number of experimental or naturally occurring models of liver disease [3]. ER stress-induced cell death is a new exciting apoptotic response which is mainly mediated by the protein kinase-like ER-resident kinase (PERK) signaling pathway. Regulation of the signature markers in this pathway includes phosphorylation of PERK and eukaryotic initiation factor 2 alpha (eIF2α), glucose-regulated protein (GRP)-78 expression, and translational up-regulation of the activating transcription factor (ATF)-4 [4–7]. Another downstream molecule, CAAT/enhancer-binding protein homologous protein or growth arrest DNA damage inducible gene 153 (CHOP/GADD153), is also up-regulated during prolonged ER stress [8]. Furthermore, it has been proposed that human caspase-4, which is localized on the ER membrane, is activated specifically by ER stress, suggesting an important role in this caspase [9].

Besides ER, mitochondria also play an important role during apoptosis. Previous studies have linked ER stress with apoptosis mediated by mitochondrial mechanisms involving the Bcl-2 family of proteins. It has been suggested that one of the anti-apoptotic actions of Bcl-2 is to antagonize ER stress-induced cell damage [10]. Recently, Bcl-2 proteins were thought to mainly regulate the mitochondria-mediated apoptotic pathway [11,12]. Indeed, the ratio of Bax to Bcl-2 (Bax/Bcl-2) is a major checkpoint in the mitochondrial pathway of apoptosis [13]. Regulation of this pathway also includes depolarization of mitochondrial inner membrane and release of cytochrome c [14], which lead to caspase activation and ultimately programmed cell death [15,16].

The dried root of *Paeonia lactiflora* has been used for over 1500 years in traditional Chinese medicine as a remedy for rheumatoid arthritis and hepatitis problems. Paeoniflorin (PF; **Fig. 1**), one of the main effective glucosides extracted from the root of *P. lactiflora* [17,18], has many bioactivities, such as anti-thrombosis, anti-oxidative, anti-hepatic injury, and immunoregulatory activities [19–22]. Recently, it has been shown to be a potent therapeutic agent against LPS-induced liver inflammation and immunological liver injury [23,18]. But the molecular mechanisms by which the glucoside mediates its effects have not been defined. Here,

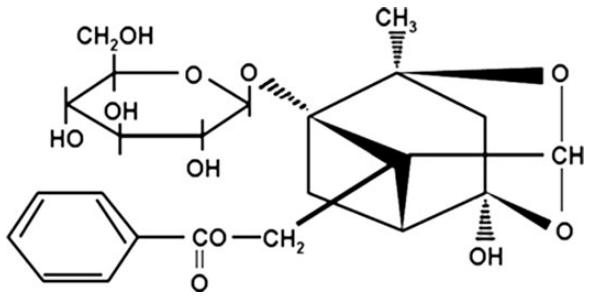

**Figure 1. Chemical structure of PF [18]**

we explored the inhibitory effects of PF on hepatocyte apoptosis and its possible mechanism, focusing on the possible signaling pathway within L02 cells.

## Materials and Methods

### Reagents

PF was provided by Shanghai Yousi Biotechnology Co. Ltd. (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Co. (New York, USA). D-Galactosamine (GalN), Fluo-3 AM, and Rhodamine 123 (Rh-123) were obtained from Sigma (St Louis, USA). Tumor necrosis factor-α (TNF-α) was obtained from PeproTech, Inc. (Rocky Hill, USA). Cell counting kit-8 (CCK-8) and the Annexin V-FITC apoptosis detection kit were purchased from Dojin Laboratory (Kumamoto, Japan) and Invitrogen Life Technologies (Carlsbad, USA), respectively. Caspase-3 and caspase-9 activity kits were obtained from the Beyotime Institute of Biotechnology (HaiMen, China). All other chemicals used were of the commercially available grade, and were provided by Nanjing Ronghua Reagent Co. (Nanjing, China).

### Cell culture and drug treatment

Human hepatocyte cell line L02 was obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were cultured in DMEM supplemented with 10% FBS at 37°C in a humidified 5% $CO_2$ incubator. In the subsequent experiments, the cells were divided into five groups: normal control group, GalN/TNF-α group, and three concentrations of PF (1, 10, and 100 μM) groups. The normal control group was incubated with medium only; the GalN/TNF-α group was pre-incubated with vehicle (PBS) for 12 h and then were treated with GalN (44 μg/ml) and TNF-α (100 ng/ml) for another 12 h; the PF groups were pre-incubated with PF at 1, 10, and 100 μM for 12 h, and then apoptosis was induced by GalN (44 μg/ml) and TNF-α (100 ng/ml) for another 12 h.

### Measurement of cell viability

L02 cells were plated to each well of a 96-well plate at a density of $8 \times 10^3$ cells/well in the culture medium. At 80% confluency, the cells of different groups were treated with various designated treatments as described previously. The viability of L02 cells was measured by the CCK-8 assay described by Yao *et al*. [24]. Before terminating the cell culture, 5 mg/ml 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8, Dojindo, Kumamoto, Japan) was added to each well and incubated at 37°C for 1 h. Then the optical density was measured at 450 nm using an enzyme-immunoassay instrument (BioRad, Richmond, USA). Cell viability rate = $(OD_{PF}/OD_{control}) \times 100\%$.

### Apoptosis detection by HCS analysis

High-content screening (HCS) has emerged as a powerful platform technology which is integrated into all aspects of contemporary drug discovery. HCS is employed by creating systems cell biology profiles for the cellular properties of interest, including early and late apoptosis, intracellular free $Ca^{2+}$, as well as inner mitochondrial membrane potential [25,26]. In this experiment, L02 cells were plated to each well of a 96-well plate at a density of $8 \times 10^3$ cells/well in the culture medium. At 80% confluency, the cells of different groups were treated with various designated treatments as described previously. To differentiate between apoptotic and necrotic cells, we performed double staining with an Annexin V-FITC Apoptosis Detection kit. Briefly, the cultured cells were collected, suspended in 200 μl of binding buffer, and mixed with 100 μl of Hoechst, 5 μl of Annexin V-FITC, and 1 μl of 100 μg/ml PI working solution. After 15 min of incubation in the dark, the cells were scanned with the HCS Kinetic Scan Reader (Thermo Fisher Scientific-Cellomics, Pittsburgh, USA) to acquire images using the ×10 objective. Images were analyzed with the HCS software and the percentage of apoptotic and necrotic cells was calculated, respectively.

### Intracellular free $Ca^{2+}$ detection

Fluo-3 AM, a fluorescent probe which can specifically combine with $Ca^{2+}$, was used to measure the level of intracellular free $Ca^{2+}$ in L02 cells. Briefly, after various designated treatments as described previously, L02 cells were harvested and washed twice with PBS, then loaded with Fluo-3 AM (5 μM) for 30 min at 37°C. Detection of intracellular $Ca^{2+}$ was done immediately with the HCS Kinetic Scan Reader to acquire images using the ×10 objective. Images were analyzed with the HCS software and the percentage of $Ca^{2+}$ fluctuation was calculated.

### Mitochondrial membrane potential detection

Mitochondrial membrane potential can be monitored by the fluorescent dye, Rh-123, which preferentially enters into mitochondria in a transmembrane potential-dependent manner. After various designated treatments as described previously, L02 cells were harvested and washed twice in

cold PBS, then resuspended in Rh-123 (2 μM) for 30 min in the dark at 37°C. Fluorescence was then measured by the HCS Kinetic Scan system.

### Western blot analysis

For western blot analysis, cells were lysed with PBS containing 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, and 10 mg/ml leupeptin, followed by centrifugation at 13,400 *g* for 10 min. The protein concentration of each supernatant was determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, USA). About 30 μg of protein per lane was then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. These membranes were subsequently blocked overnight with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST). After three times washing with TBST, the membranes were incubated with primary antibodies in TBST overnight at 4°C. The following primary antibodies were used in this study: anti-Bcl-2 (N-19), anti-Bax (B-9), anti-cytochrome c, anti-GRP78, anti-ATF4, and anti-CHOP/GADD153 from Santa Cruz Biotechnology (Santa Cruz, USA); anti-phospho-PERK, anti-phospho-eIF2α, and anti-cleaved caspase-4 from Cell Signaling Technology (Beverly, USA). Polyclonal anti-β-actin antibody (Sigma) was used to normalize protein loading. Thereafter, the blots were washed three times with TBST, and then further incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 1 h. The signals were detected using an enhanced chemiluminescence system (Pierce, Rockford, USA).

### Caspase-3 and caspase-9 activity assay

The activity of caspase-3 or caspase-9 was measured using a specific chromogenic enzymatic assay according to the manufacturer's instructions (Beyotime Institute of Biotechnology). Briefly, cell lysates were prepared after their respective treatment. Assays were performed on 96-well microtiter plates by incubating 10 μl cell lysate protein per sample in 80-μl reaction buffer [1% NP-40, 20 mM Tris-HCl (pH 7.5), 137 mM nicotinamide adenine dinucleotide (NAD) and 10% glycerol] containing 10 μl caspase-3 substrate (Ac-DEVDpNA, 2 mM) or caspase-9 substrate (Ac-LEHD-pNA, 2 mM). After incubation for 4 h at 37°C, $A_{405}$ readings were taken using an enzyme-immunoassay instrument.

### Statistical analysis

All data were presented as mean ± standard deviation. Data were analyzed using a one-way analysis of variance and Student's *t*-test to determine the levels of significance. $P < 0.05$ was considered statistically significant.

## Results

### Effect of PF on GalN/TNF-α-induced cytotoxicity in human L02 hepatocytes

As shown in **Fig. 2**, GalN/TNF-α markedly decreased the viability of L02 cells compared with normal control group ($P < 0.01$), while PF at 10 and 100 μM significantly relived the cell damage induced by GalN/TNF-α ($P < 0.05$, $P < 0.01$, respectively). In addition, PF pre-treatment alone exhibited no effect on cell viability in L02 hepatocytes (data not shown).

### Effect of PF on GalN/TNF-α-induced apoptosis in L02 hepatocytes

To explore the exact effects of PF on hepatocyte apoptosis, an HCS assay was employed for the quantification of healthy cells (Annexin V-FITC negative/PI negative), early apoptotic cells (Annexin V-FITC positive/PI negative), late apoptotic/necrotic cells (Annexin V-FITC positive/PI positive), and dead cells (Annexin V-FITC negative/PI positive). Data derived from these images were analyzed with the HCS software. The results shown in **Fig. 3** suggested that GalN/TNF-α induced apoptotic cell death in L02 hepatocytes. Compared with the normal control group, obvious decreases in nuclear size were detected ($P < 0.01$) in cells exposed to GalN/TNF-α. PF at 10 and 100 μM, however, effectively protected cells from apoptosis and the average nucleus size was increased ($P < 0.01$, $P < 0.05$, respectively) (**Fig. 3B**). In addition, the early apoptosis rate, as well as the percentage of late apoptosis and dead cells in PF-pretreated cells, was considerably lower compared with those of GalN/TNF-α-treated cells ($P < 0.05$) (**Fig. 3C**,**D**).

### Effect of PF on intracellular $Ca^{2+}$ mobilizations induced by GalN/TNF-α

An increased intracellular $Ca^{2+}$ level has been reported to be the key factor for cell death and cell injury [27,28]. With this

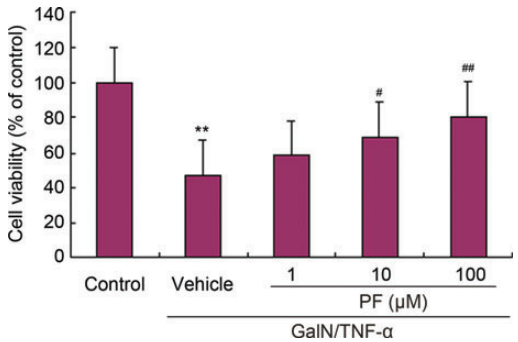


**Figure 2. Effect of PF on cell viability in GalN/TNF-α-treated L02 cells** L02 cells were incubated with GalN (44 μg/ml) and TNF-α (100 ng/ml), and different doses of PF (1, 10, and 100 μM). Data are presented as means ± SD of values from triplicate samples. $^{**}P < 0.01$, compared with the normal control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the GalN/TNF-α group.

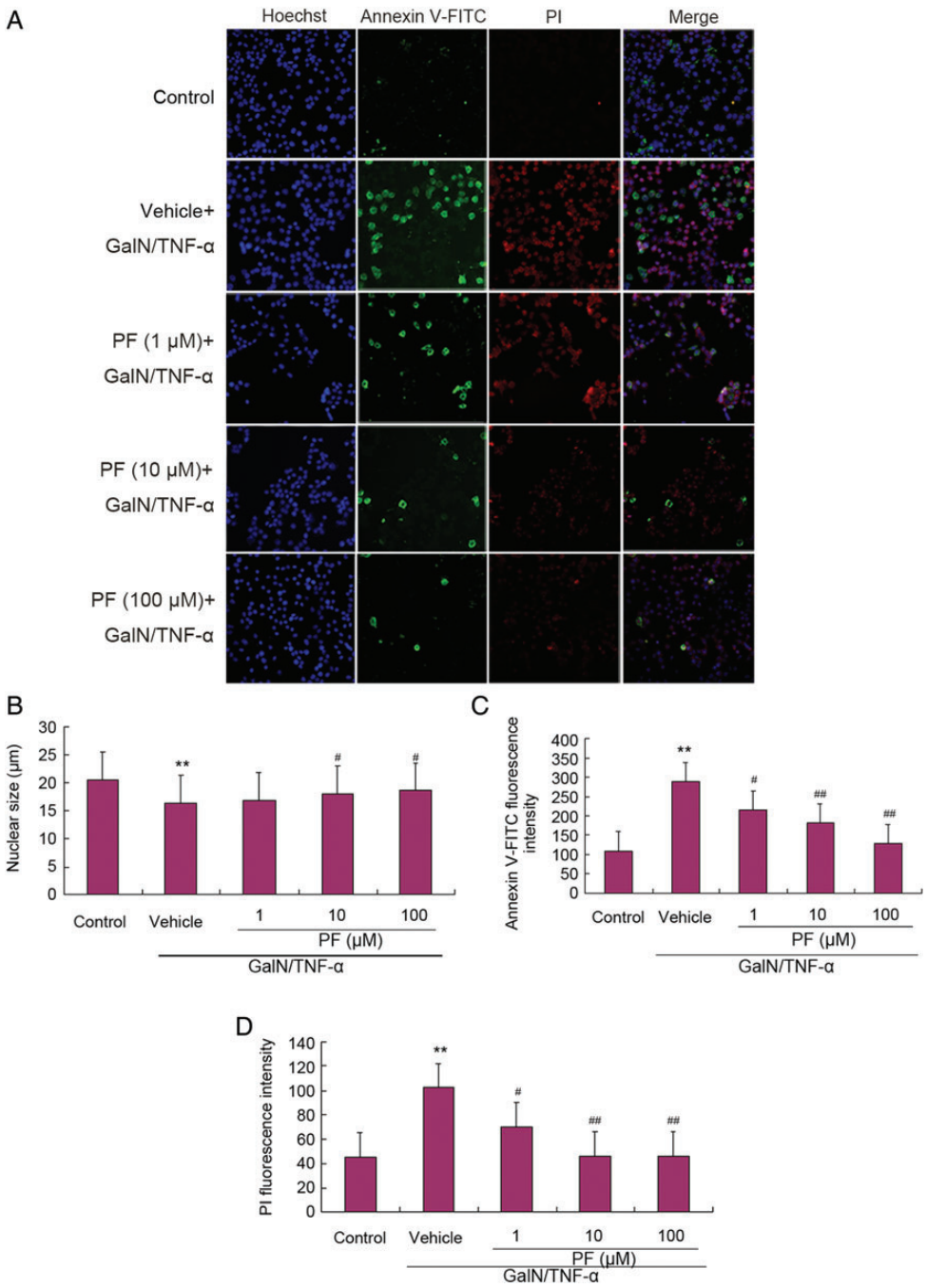


**Figure 3. Effect of PF on GalN/TNF-α-induced apoptosis in L02 hepatocytes evaluated by HCS analysis** (A) Images of cells were taken by the HCS KineticScan Reader (×10). The samples are analyzed for blue fluorescence (Hoechst), green fluorescence (FITC), and red fluorescence (PI). (B) The average cell nuclear size was quantified and analyzed by the HCS KineticScan software. (C) The early apoptotic cells were reflected by the average intensity of fluorescent dyes for Annexin V-FITC. (D) The dead cells were reflected by the average intensity of fluorescent dyes for PI. Data are presented as means ± SD of values from triplicate samples. $^{**}P < 0.01$, compared with the normal control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the GalN/TNF-α group.

in mind, we used Fluo-3 AM to examine the effect of PF on intracellular $Ca^{2+}$ mobilizations induced by GalN/TNF-α in L02 cells. It was shown that treatment of L02 cells with GalN/TNF-α caused the release of calcium into the cytoplasm from ER stores (**Fig. 4**). The results indicated that the increase of intracellular $Ca^{2+}$ was associated with GalN/TNF-α-induced L02 cell apoptosis. As the PF concentration increased from 1 to 100 μM, intracellular free $Ca^{2+}$ fluorescence decreased dramatically ($P < 0.01$) (**Fig. 4**), which was in accordance with the tendencies of cell apoptosis.

### Effect of PF on the expression of ER stress-related proteins after treatment with GalN/TNF-α

To determine whether PF protects L02 cells from ER stress-induced apoptosis, the expressions of two signature ER stress markers, phospho-PERK and phospho-eIF2α,

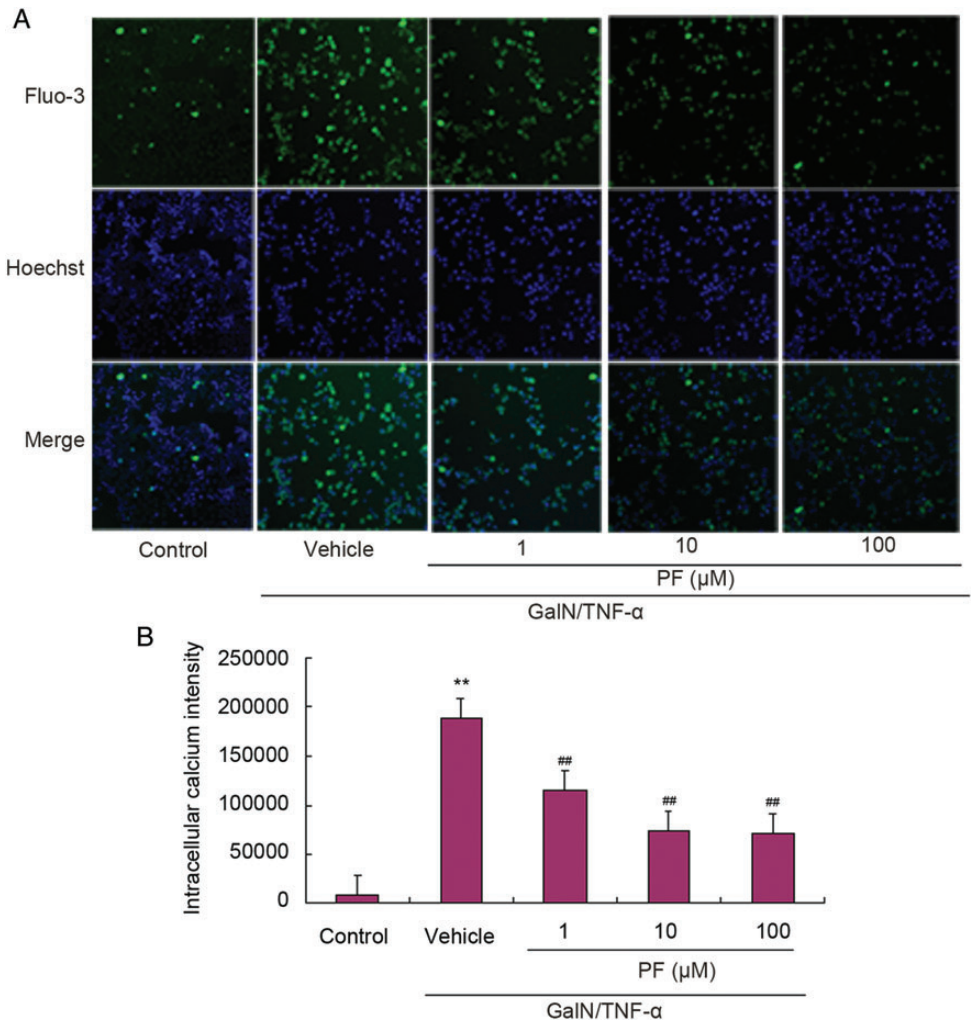


**Figure 4. Effect of PF on intracellular free $Ca^{2+}$ in GalN/TNF-α-induced L02 hepatocytes** (A) Images of cells were taken by the HCS KineticScan Reader (×10). The intracellular $Ca^{2+}$ concentrations of the samples were analyzed for green fluorescence (Fluo-3). All nuclei were stained with Hoechst (blue fluorescence). (B) The intracellular free $Ca^{2+}$ was quantified and analyzed by the HCS KineticScan software. Results are expressed as relative fluorescent intensity (means ± SD from triplicate samples). $^{**}P < 0.01$, compared with the normal control group; $^{\#\#}P < 0.01$, compared with the GalN/TNF-α group.

were examined. Increases in phosphorylation of PERK and its substrate, eIF2α, were significant after 6-h treatment with GalN/TNF-α, while total ERK and eIF2α were not changed (**Fig. 5A**,**B**). The rapid changes suggest that ER stress is an immediate response attributable to direct stimulation by GalN/TNF-α. We next examined the expression of GRP78, which serves as a gatekeeper to the activation of ER stress transducers [29]. The data in **Fig. 5B** demonstrated that the expressions of GRP78 by GalN/TNF-α were dramatically increased after 6-h treatment ($P < 0.01$). All three ER stress markers were significantly inhibited by PF in a concentration-dependent manner as shown in **Fig. 5B**.

We further examined the expression of ER stress-response proteins, ATF4 and CHOP/GADD153, which are in the downstream of PERK/eIF2α [30]. Results are shown in **Fig. 6**, ATF4 and CHOP/GADD153 were also over-expressed after exposure to GalN/TNF-α in L02 cells compared with control, while PF pre-treatment decreased their expression dose-dependently.

It has been suggested that caspase-4 plays a key role in ER stress-induced apoptosis in human cells [9]. Thus, we also examined whether GalN/TNF-α could induce caspase-4 activation in human L02 cells. As shown in **Fig. 6**, an obvious increase of the cleavage product of caspase-4 was detected in L02 cells after 6 h of GalN/TNF-α treatment. However, its activation was inhibited by PF in a dose-dependent manner (**Fig. 6B**). Together, these results support the involvement of ER stress in the inhibition of apoptosis by PF in GalN/TNF-α treated L02 cells.

### Effect of PF on disruption of mitochondrial membrane potential induced by GalN/TNF-α

It was reported that reduction of mitochondrial membrane potential irreversibly leads to cell apoptosis [31,32]. In order

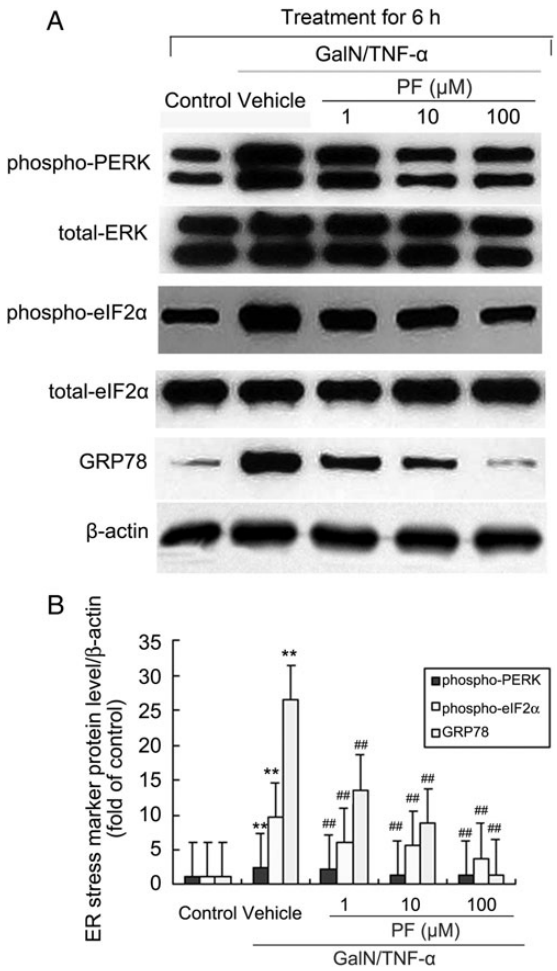


**Figure 5. Effect of PF on the expression of ER stress marker proteins after treatment with GalN/TNF-α** (A) The phosphorylation of ERK, eIF2α and the expression of GRP78 were measured by western blot. (B) The band densities of the proteins were measured by the Quantity One 1D analysis software program. Data are presented as means ± SD of values from triplicate samples. $^{**}P < 0.01$, compared with the normal control group; $^{\#\#}P < 0.01$, compared with the GalN/TNF-α group.

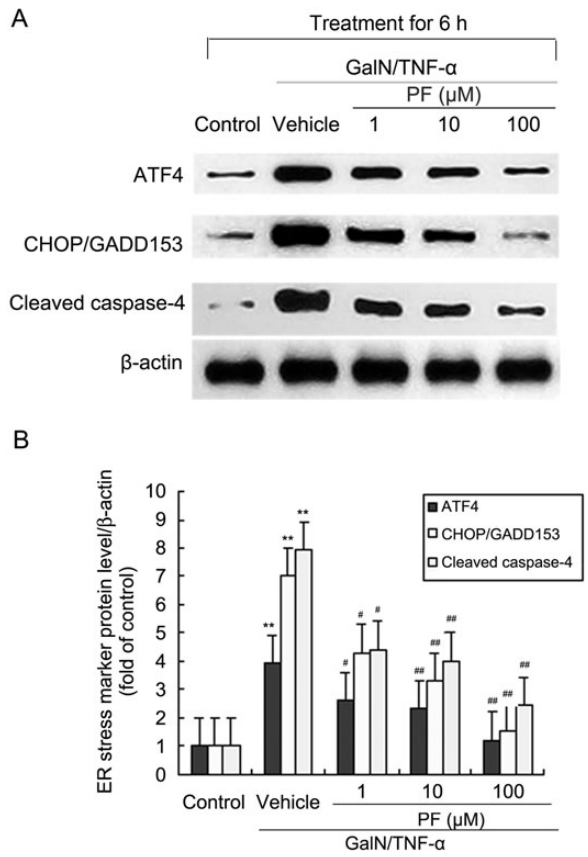


**Figure 6. Effect of PF on the expression of ER stress-response proteins after treatment with GalN/TNF-α** (A) The expressions of ATF4, GADD153/CHOP, and cleavage of caspase-4 were measured by western blotting. (B) The band densities of the proteins were measured by the Quantity One 1D analysis software program. Data are presented as means ± SD of values from triplicate samples. $^{**}P < 0.01$, compared with the normal control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the GalN/TNF-α group.

to gain a better understanding of the mechanism of PF on GalN/TNF-α-induced L02 cell apoptosis, we used Rh-123 to measure the mitochondrial membrane potential by examining its fluorescent intensity. As shown in **Fig. 7**, the fluorescent intensity was significantly decreased with the treatment of GalN/TNF-α. While there was a concentration-dependent increase of Rh-123 fluorescence after treatment with PF, compared with the GalN/TNF-α group. This result indicated that disruption of mitochondrial membrane potential was recovered by PF in L02 cells, suggesting the involvement of mitochondria in GalN/TNF-α-induced hepatocyte apoptosis.

### Effect of PF on the expression of mitochondria-related proteins after treatment with GalN/TNF-α

To better understand the molecular mechanisms by which PF exerts its anti-apoptotic effects, we followed the protein expression of mediators in mitochondrial signaling pathways. First, we determined whether GalN/TNF-α stimulated the release of cytochrome c into the cytosolic fraction in L02 hepatocytes. As expected, after 12 h of GalN/TNF-α treatment, the release of cytochrome c from the mitochondria into the cytosol was significantly increased ($P < 0.01$), while PF pretreatment at 1, 10, and 100 μM effectively inhibited its release, reflected by a dose-dependent increase of cytochrome c expression in the mitochondria ($P < 0.05$, $P < 0.01$, $P < 0.01$, respectively) (**Fig. 8B**,**D**).

Since the Bcl-2 family proteins play a critical role in regulating the release of cytochrome c, we then investigated the possible involvement of Bax and Bcl-2 in the process of GalN/TNF-α-mediated hepatocyte apoptosis. Compared with normal control cells, induction of apoptosis by GalN/TNF-α was accompanied by an obvious increase in pro-apoptotic Bax expression ($P < 0.01$), and a sharp decrease in anti-apoptotic Bcl-2 expression ($P < 0.01$), while PF pretreatment decreased Bax expression and increased Bcl-2 expression dose-dependently (**Fig. 8A**,**C**).

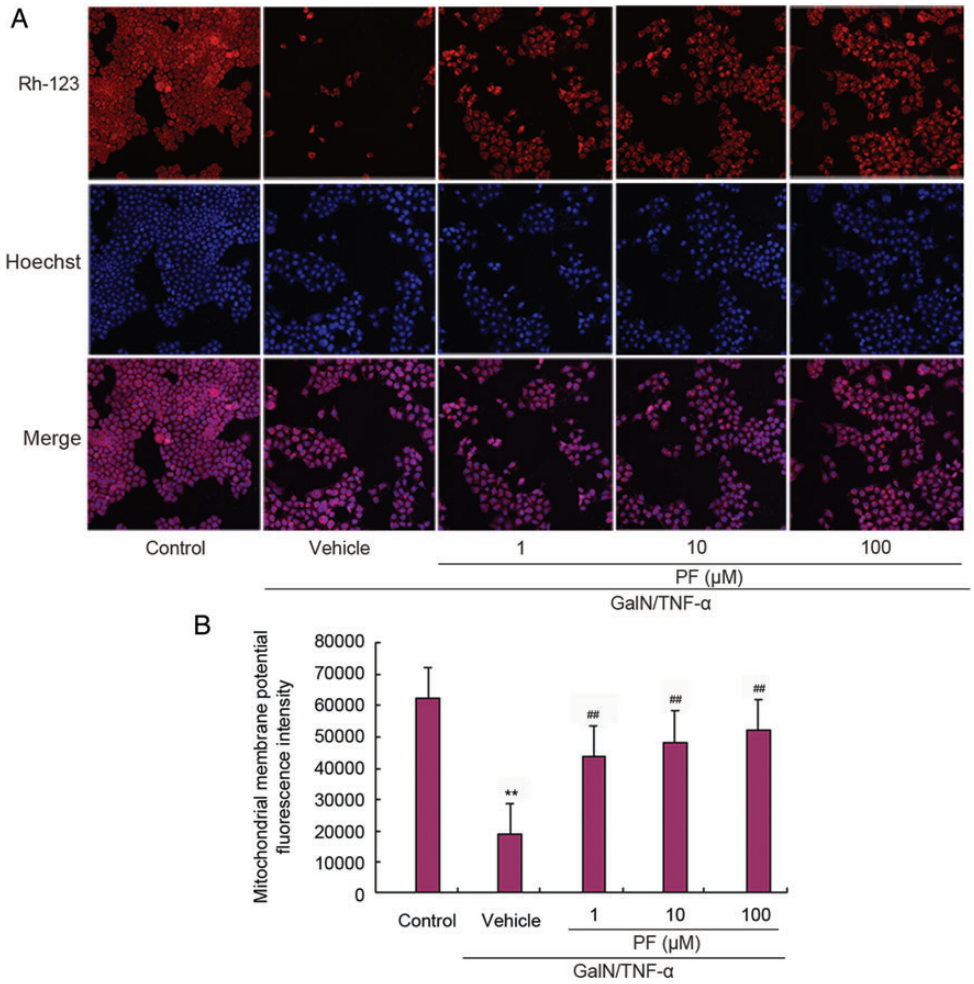


**Figure 7. Effect of PF on mitochondrial membrane potential in apoptotic L02 cells** (A) Images of cells were taken by the HCS KineticScan Reader (×10). The mitochondrial membrane potential of the samples was analyzed for red fluorescence (Rh-123). All nuclei were stained with Hoechst (blue fluorescence). (B) The mitochondrial membrane potential was quantified and analyzed by the HCS KineticScan software. Results are expressed as relative fluorescent intensity (means ± SD from triplicate samples). $^{**}P < 0.01$, compared with the normal control group; $^{\#\#}P < 0.01$, compared with the GalN/TNF-α group.

### Effect of PF on GalN/TNF-α-induced caspase-3 and caspase-9 activation

Regardless of varying conditions under which apoptotic cell death can occur, caspase activation is considered an important step in the execution phase of apoptosis. Both caspase-3 and caspase-9 have been demonstrated to be the main effectors of caspases [33]. As expected, we detected increased activity of caspase-3 ($P < 0.01$) and caspase-9 ($P < 0.01$) when injured by GalN/TNF-α, and incubation of the cells with PF (1, 10, and 100 μM) significantly prevented the increase ($P < 0.05$, $P < 0.01$, $P < 0.01$, respectively) (**Fig. 9**). The results suggest that PF had a direct suppressive effect on the activation of caspases.

## Discussion

Currently, many investigations have focused on the specific mechanisms involved in hepatocyte apoptosis in order to develop new therapeutic agents for liver diseases. PF is a monoterpenoid glycoside compound ($C_{23}H_{28}O_{11}$, MW 480) extracted from the dried root of *P. lactiflora*. It has been reported that PF administration to rats can markedly ameliorate LPS-induced liver dysfunction and organ damage [23]. The most common anti-hepatic injury mode upon PF treatment seems to be related to inhibition of apoptosis. The present study was carried out to investigate the mechanism by which PF elicited the suppressive effect on hepatocyte apoptosis.

Initially, we assessed the effect of PF on hepatocyte apoptosis using a human hepatocyte cell line L02 stimulated by GalN/TNF-α. The administration of GalN/TNF-α has been used to trigger hepatocyte apoptosis both *in vivo* and *in vitro* [34]. GalN is an amino sugar selectively metabolized by the hepatocyte, which induces a depletion of the uridine triphosphate pool and thereby an inhibition of RNA and protein synthesis [35]. The broad impact of GalN on hepatocyte

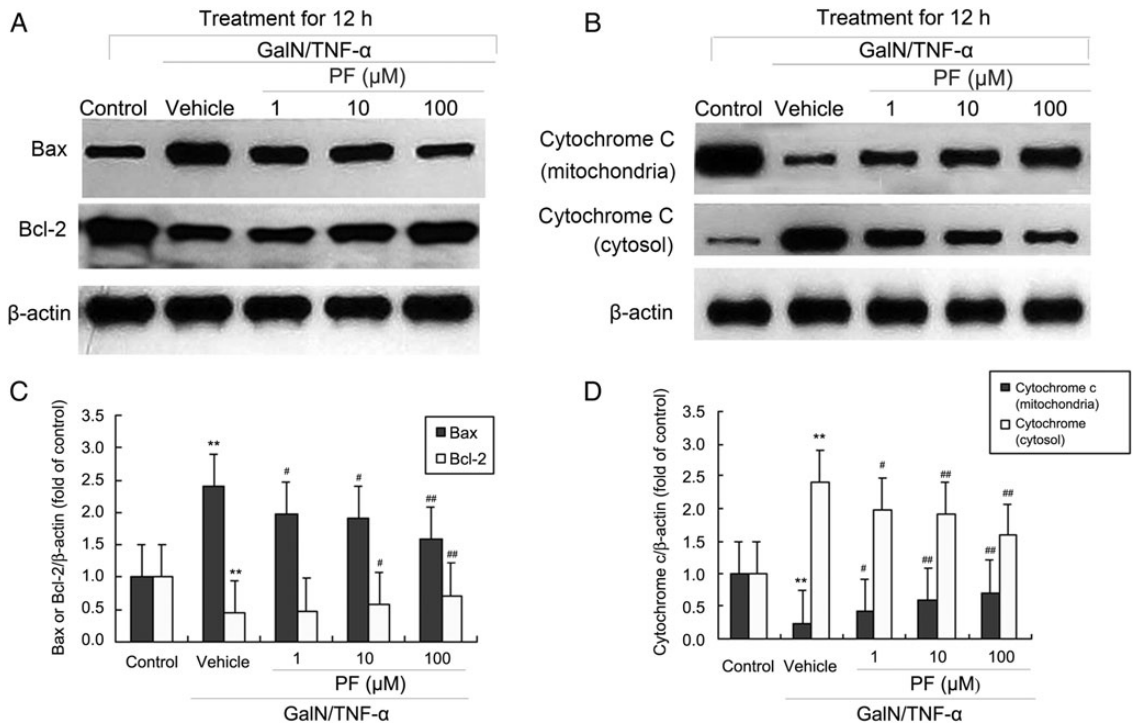


**Figure 8. Regulation of mediators in the mitochondrial pathway in apoptotic L02 cells by PF** (A) PF inhibited Bax expression and increased Bcl-2 expression as detected by immunoblotting. (B) PF decreased the release of cytochrome *c* from the mitochondria into the cytosol. (C and D) The band densities of the proteins were measured by the Quantity One 1D analysis software program. Data are presented as means ± SD of values from triplicate samples. $^{**}P < 0.01$, compared with the normal control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the GalN/TNF-α group.

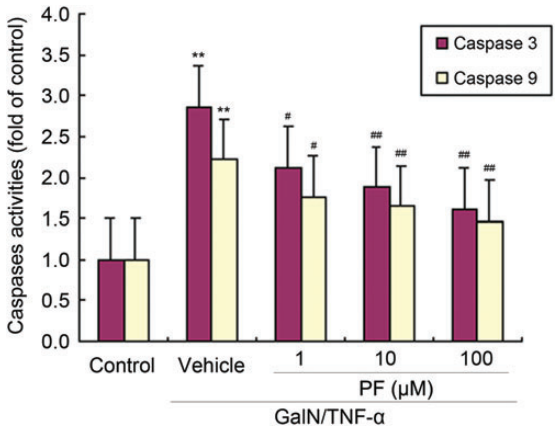


**Figure 9. Effect of PF on GalN/TNF-α-induced caspase-3 and caspase-9 activation** Values were expressed as the ratio of the caspase-3 and caspase-9 activation levels to the control level, and the value of normal control was set to 1. Data are presented as means ± SD of values from triplicate samples. $^{**}P < 0.01$, compared with the normal control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the GalN/TNF-α group.

RNA and protein synthesis leads to sensitization to TNF-α, a single pro-inflammatory cytokine [36]. TNF-α-induced hepatocellular cell death is known to be related to various types of hepatic diseases, such as chronic and viral hepatitis, fibrosing liver disease, liver regeneration, and hepatocarcinogenesis [34–38]. However, apoptosis cannot be induced by TNF-α alone in normal human hepatocytes [39]. GalN sensitization is fundamentally a means for amplifying the hepatotoxic effects of TNF-α [36]. According to our previous experiments, after 12-h combined stimulation of GalN (44 μg/ml) and TNF-α (100 ng/ml), apparent apoptosis could be induced in L02 cells (data not shown). Thus, the model of hepatocellular apoptosis characterized by the addition of GalN/TNF-α was established in this study.

As expected, our study showed that PF pre-treatment dose-dependently protected L02 cells from GalN/TNF-α-induced cytotoxicity (**Fig. 2**). The PF-induced cytoprotection was associated with the inhibition of GalN/TNF-α-induced hepatocyte apoptosis (**Fig. 3**). The PF-induced cytoprotection was also related to the inhibition of GalN/TNF-α-induced increase in intracellular calcium (**Fig. 4**). Maintenance of $Ca^{2+}$ homeostasis within the ER is responsible for controlling numerous cellular processes, such as protein folding, modification, transport, and signal transduction [40,41]. It was found that depletion of ER $Ca^{2+}$ stores resulted in the development of ER stress response and mitochondrial dysfunction, which in turn contributed to hepatocyte apoptosis and subsequent liver dysfunction [8,42]. Since the inhibition of GalN/TNF-α-induced $Ca^{2+}$ loss from the ER could prevent both elevation of cytoplasmic $Ca^{2+}$ and depletion of ER $Ca^{2+}$, treatments that inhibit ER $Ca^{2+}$ release would be cytoprotective. So PF treatment is likely to be protective against GalN/TNF-α-induced apoptosis for this reason.

In the current research, ER stress signaling, which is an adaptive cellular response to the loss of ER $Ca^{2+}$ homeostasis, was the direct reason for cell apoptosis. It has been known that ER stress induces apoptotic response in various types of cells [29,43], by the up-regulation of protein translation mediated through the PERK-eIF2α-ATF4 pathway [1,5]. For example, upon ER stress, the ER chaperone GRP78 dissociates from the PERK, initiates transphosphorylation, and subsequent activation of the kinase [44]. The activated PERK then leads to phosphorylation of the translation initiation factor eIF2α and subsequent ATF4 protein expression, which is essential for ER stress-induced apoptosis [7]. Misra and Pizzo [45] reported that insulin treatment caused ER stress, as evidenced by an increased expression of GRP78, phosphorylated PERK (p-PERK), and phosphorylated eIF2α (p-eIF2α) in murine peritoneal macrophages exposed to insulin. Fribley *et al*. [46] showed that p-PERK activation induced by proteasome inhibitor PS-341 led to an up-regulation of ATF4 in head and neck squamous cell carcinoma cells, through the induction of ER stress-reactive oxygen species. In our study, treatment with GalN/TNF-α induced the activation of a number of signature ER stress markers in human L02 hepatocytes. It was found that GalN/TNF-α did cause ER stress, as evidenced by the elevation in the phosphorylation of PERK and eIF2α, as well as the increased expression of GRP78 and ATF4, while PF treatment was able to reverse this process (**Figs. 5** and **6**).

On the other hand, it has been proposed that ER-associated pro-apoptotic molecules, such as caspase-4 and CHOP/GADD153, are critical executioners of ER stress [9,47,48]. Pae *et al*. [49] found that the activations of CHOP/GADD153 and caspase-4 were both involved in curcumin-induced pro-apoptotic ER stress in human leukemia HL-60 cells. Choi *et al*. [50] reported that suppression of CHOP expression and inhibition of caspase-4 activity significantly reduced withaferin A-induced apoptosis mediated by ER stress in human renal carcinoma cells. In our study, as expected, GalN/TNF-α induced the elevation in the expression of CHOP/GADD153 and cleavage of procaspase-4, while PF treatment significantly inhibited the expression of CHOP/GADD153 and blocked the activation of caspase-4 in human L02 hepatocytes (**Fig. 6**). These observations further suggest that the anti-apoptotic activity of PF could be associated with the ER stress signaling cascade. ER stress which has been linked to the development of several disorders, including Alzheimer's disease, cardiovascular diseases, Type-1 diabetes mellitus, and glomerulonephritis, has been studied for a long time [51–55]. As far as we are aware, this is the first report describing the effect of a hepatoprotective agent on ER stress.

We further observed that GalN/TNF-α-treated L02 hepatocytes underwent an abnormal loss of mitochondrial membrane potential, which is direct evidence for mitochondrial dysfunction in hepatocyte apoptosis. But PF showed obvious protective effects on cells, reflected by a dose-dependent increase in mitochondrial membrane potential, indicating that it may protect mitochondrial function directly. On the other hand, mitochondrial dysfunction such as the change in the mitochondrial membrane potential often accompanies with the release of several mitochondrial proteins, such as cytochrome c, into the cytoplasm, which appears to be important for the progression of the apoptotic pathway. In our study, GalN/TNF-α was found to induce cytochrome c release from the mitochondria into the cytosol in human L02 hepatocytes, while PF treatment was able to inhibit this process (**Fig. 7**). These results suggest that PF may attenuate GalN/TNF-α-induced hepatocyte apoptosis via a cytochrome c-dependent mitochondrial pathway.

It has been well documented that Bcl-2 family members are essential for regulating the mitochondrial pathway [13,56]. As the mitochondrial membrane proteins Bcl-2 and Bax reside upstream of irreversible cellular damage and act directly on the mitochondria, they function as key regulators of apoptosis [57,58]. For example, Bcl-2 can bind to the mitochondrial membrane to stabilize its membrane potential, reduce oxidative stress, and suppress cytochrome c release [59]. ER stress-induced apoptosis, similar to mitochondria-mediated apoptosis, is also regulated by the Bcl-2 family of proteins [60,61]. It was reported that over-expression of Bcl-2, or deficiency of Bax, a pro-apoptotic homolog of Bcl-2, conferred protection against lethal ER stress and thus inhibited apoptosis [62]. One of the mechanisms of the cytoprotective effects of Bcl-2 is thought to stem from its ability to inhibit ER calcium depletion [10,63]. Also, Bcl-2 targeted to the ER membrane may preserve ER membrane integrity under ER stress, by maintaining pro-death Bax in its inactive conformations [61,64]. In our study, we demonstrated that PF inhibited GalN/TNF-α-induced down-regulation of Bcl-2 (**Fig. 8**), the major anti-apoptotic protein on membranes of the ER, mitochondria, and nucleus. It also prevented the activation of Bax in GalN/TNF-α-treated L02 hepatocytes, resulting in a dramatic decrease in the Bax/Bcl-2 protein ratio, a response that would be protective against hepatocyte apoptosis. Therefore, our results suggest that different levels of proteins of the Bcl-2 family are involved in PF-induced anti-apoptotic action in the human L02 hepatocyte cell line.

It is now well established that all upstream signals in the mitochondria-mediated apoptotic pathway, such as regulation of Bcl-2 family members and release of cytochrome c from the intermembrane space, can lead to caspase activation and subsequent cell death [65,66]. In most cell types, it was noted that the release of cytochrome c from the mitochondria into the cytosol initiates a downstream caspase cascade, activating caspase-9, with subsequent activation of the executioner caspase, caspase-3 [33]. In this study, as expected, we

found that PF pre-treatment significantly inhibited the GalN/TNF-α-induced activation of caspase-3 and caspase-9 (**Fig. 9**). Srivastava *et al*. [67] proposed a mechanism whereby the depletion of ER $Ca^{2+}$ induces apoptosis in Jurkat T cells through a pathway involving an increase in intracellular $Ca^{2+}$ levels, followed by a reduction in mitochondrial membrane potential, release of mitochondrial cytochrome c, and activation of caspase-3. These observations suggest that PF inhibited L02 cell apoptosis by decreasing caspase-3/9 activity, which could be due to upstream events in the intrinsic pathways. However, the exact mechanisms in the genetic and signal transduction pathways remain to be elucidated.

In conclusion, our present work demonstrated that PF could effectively suppress apoptosis in human hepatocytes. The possible mechanistic explanations are associated not only with the inhibition of intracellular calcium increase, the alterations in mitochondrial dysfunctions, the inhibition of caspase activities, but also more importantly with the regulation of apoptotic mediators in the ER stress and mitochondria-dependent pathways. Overall, two apoptotic processes may proceed in parallel and in most cases, ER stress-induced apoptosis is dependent on the mitochondrial pathway. These findings not only provide an integrated, unified mechanism linking the cellular dysfunction to apoptosis, but also suggest that the hepatoprotective activity of PF might be useful as an adjunctive therapy in the treatment of apoptosis-related liver diseases.

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