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World J Gastroenterol 2017 October 28; 23(40): 7201-7346



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Volume 23 Number 40 October 28, 2017

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NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

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PUBLICATION DATE
October 28, 2017

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Basic Study

Prostaglandin E1 protects hepatocytes against endoplasmic reticulum stress-induced apoptosis *via* protein kinase A-dependent induction of glucose-regulated protein 78 expression

Fang-Wan Yang, Yu Fu, Ying Li, Yi-Huai He, Mao-Yuan Mu, Qi-Chuan Liu, Jun Long, Shi-De Lin

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Supported by the National Natural Science Foundation of China, No. 81160067 and No. 814600124.

Institutional review board statement: This study was approved by the Institutional Review Board of Affiliated Hospital of Zunyi Medical College, Guizhou Province, China.

Conflict-of-interest statement: The authors declare that they have no conflicts interest related to this study.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at linshade6@hotmail.com.

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Manuscript source: Unsolicited manuscript

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Received: May 11, 2017

Peer-review started: May 13, 2017

First decision: June 22, 2017

Revised: July 24, 2017

Accepted: August 25, 2017

Article in press: August 25, 2017

Published online: October 28, 2017

Abstract

AIM

To investigate the protective effect of prostaglandin E1 (PGE1) against endoplasmic reticulum (ER) stress-induced hepatocyte apoptosis, and to explore its underlying mechanisms.

METHODS

Thapsigargin (TG) was used to induce ER stress in the human hepatic cell line L02 and hepatocarcinoma-derived cell line HepG2. To evaluate the effects of PGE1 on TG-induced apoptosis, PGE1 was used an hour prior to TG treatment. Activation of unfolded protein response signaling pathways were detected by western blotting and quantitative real-time RT-PCR. Apoptotic index and cell viability of L02 cells and HepG2 cells were determined with flow cytometry and MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-

tetrazolium] assay.

RESULTS

Pretreatment with 1 μ mol/L PGE1 protected against TG-induced apoptosis in both L02 cells and HepG2 cells. PGE1 enhanced the TG-induced expression of C/EBP homologous protein (CHOP), glucose-regulated protein (GRP) 78 and spliced X box-binding protein 1 at 6 h. However, it attenuated their expressions after 24 h. PGE1 alone induced protein and mRNA expressions of GRP78; PGE1 also induced protein expression of DNA damage-inducible gene 34 and inhibited the expressions of phospho-PKR-like ER kinase, phospho-eukaryotic initiation factor 2 α and CHOP. Treatment with protein kinase A (PKA)-inhibitor H89 or KT5720 blocked PGE1-induced up-regulation of GRP78. Further, the cytoprotective effect of PGE1 on hepatocytes was not observed after blockade of GRP78 expression by H89 or small interfering RNA specifically targeted against human GRP78.

CONCLUSION

Our study demonstrates that PGE1 protects against ER stress-induced hepatocyte apoptosis *via* PKA pathway-dependent induction of GRP78 expression.

Key words: Hepatocytes; Endoplasmic reticulum stress; Thapsigargin; Glucose-regulated protein 78; Protein kinase A; Apoptosis

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Core tip: The mechanism underlying the hepatoprotective effect of prostaglandin E1 (PGE1) remains unclear. In this study, we found that pretreatment with PGE1 protected hepatocytes against thapsigargin-induced apoptosis. PGE1 alone induced protein and mRNA expressions of glucose-regulated protein (GRP)78. Treatment with protein kinase A (PKA)-inhibitor H89, KT5720 or small interfering (si)RNA specifically targeted against human GRP78 blocked PGE1-induced up-regulation of GRP78. The hepatoprotective effect of PGE1 was lost by blocking GRP78 expression with either H89 or siRNA. Our study demonstrates for the first time that PGE1 protects against endoplasmic reticulum stress-induced hepatocyte apoptosis *via* PKA pathway-dependent induction of GRP78 expression.

Yang FW, Fu Y, Li Y, He YH, Mu MY, Liu QC, Long J, Lin SD. Prostaglandin E1 protects hepatocytes against endoplasmic reticulum stress-induced apoptosis *via* protein kinase A-dependent induction of glucose-regulated protein 78 expression. *World J Gastroenterol* 2017; 23(40): 7253-7264 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i40/7253.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i40.7253>

INTRODUCTION

Hepatocyte apoptosis can be triggered by intra- or extracellular signals^[1]. The intracellular signals for hepatocyte apoptosis are induced by DNA damage, oxidative stress, growth factor deprivation, mitochondrial dysfunction, ATP depletion and endoplasmic reticulum (ER) stress^[2,3]. A complex interaction occurs among these intracellular apoptotic signaling pathways. ER stress is known to induce hepatocyte apoptosis under various pathological conditions^[4]. ER stress is implicated in the pathogenesis of various liver diseases, such as obesity-associated fatty liver disease^[5,6], viral hepatitis^[7], alcohol-induced liver injury^[8], drug-induced liver injury^[9] and ischemia/reperfusion injury of the liver^[10,11]. Devising a treatment strategy to protect hepatocytes from ER stress-induced apoptosis will benefit most patients with liver diseases.

ER is a multifunctional intracellular organelle responsible for the synthesis, processing and trafficking of proteins that are essential for cell growth and survival. ER also serves as a storage organelle for calcium^[12]. When the homeostasis of ER is disturbed under various pathophysiological conditions, ER stress is induced and the unfolded protein response (UPR) is activated. The UPR activates three ER transmembrane transducers: inositol-requiring enzyme (IRE) 1 α , PKR-like ER kinase (PERK), and activating transcription factor (ATF) 6 α ^[12-14]. Activation of these three UPR pathways enhances the ER's protein folding *via* up-regulation of the synthesis of glucose-regulated protein (GRP) 78. UPR signals also accelerate the degradation of misfolded proteins and reduce the synthesis of new proteins. Therefore, UPR during ER stress facilitates restoration of homeostasis. However, sustained or unresolved ER stress can activate a cascade of apoptotic signals that eventually result in cell death^[15,16].

In several experimental models of liver injury, prostaglandin (PG) E1 has been shown to protect against hepatocyte apoptosis^[17-19]. PGE1 is also effective in the treatment of patients with fulminant hepatitis and those with primary graft non-function after liver transplantation^[20,21]. Thus, PGE1 appears to protect hepatocytes against apoptosis through various mechanisms^[22-24]. However, the underlying mechanism of its hepatoprotective effect is not well understood.

In two recent studies, PGE1 was shown to induce expressions of heat-shock protein (HSP) and GRP78 in animal models of liver injury caused by ischemia reperfusion and hepatectomy^[25,26]. These findings suggest that modulation of UPR may mediate the hepatoprotective effects of PGE1. However, the role of PGE1 in ER stress-induced apoptosis of hepatocytes is largely unknown.

In this study, we evaluated the protective effect of PGE1 against ER stress-induced hepatocyte apoptosis in both the normal human hepatocyte cell line L02 and

the hepatocarcinoma-derived cell line HepG2^[27].

MATERIALS AND METHODS

Chemical reagents

RPMI 1640 was obtained from Thermo-Fisher Biochemical Products Co. Ltd (Beijing, China). Fetal bovine serum (FBS), thapsigargin (TG), protein kinase A (PKA) inhibitor H89 and KT5720, 4-phenylbutyric acid (PBA) and PGE1 were purchased from Sigma (St. Louis, MO, United States). Antibodies against GRP78, PERK, eukaryotic translation initiation factor-2 α (eIF-2 α), phospho-PERK (p-PERK) and phospho-eIF2 α (p-eIF2 α), C/EBP homologous protein (CHOP), spliced X box-binding protein 1 (sXBP1), growth arrest and DNA damage-inducible gene 34 (GADD34) and β -actin were purchased from Santa Cruz Biotechnology (Dallas, TX, United States). Annexin V-FITC/propidium iodide (PI) apoptosis detection kit was purchased from Dojindo Laboratories (Kumamoto, Japan). Small interfering (si)RNA scramble control and validated human GRP78-siRNA were purchased from Santa Cruz Biotechnology. All other chemicals and reagents were obtained from Sigma, unless stated otherwise.

Cell culture

The human hepatocyte cell line L02 and hepatocarcinoma-derived cell line HepG2 were obtained from the cell bank of the Type Culture Collection at the Chinese Academy of Sciences (Shanghai, China). The L02 and HepG2 cells were propagated at 37 °C in 5% CO₂ in RPMI 1640 medium containing 10% (v/v) FBS and 100 units/mL penicillin, and were passaged every 5-7 d. The L02 and HepG2 cells were cultured until they acquired 80%-100% confluence. Thereafter, the cells were rinsed three times with 10 mL of phosphate-buffered saline (PBS) and cultured in a medium lacking FBS for 24-36 h. To evaluate the effects of PGE1 or PBA on TG-induced apoptosis, PGE1 or PBA was used an hour prior to TG treatment and, thereafter, the medium was not changed. TG and H89 were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 40 μ mol/L and 20 μ mol/L, respectively, as stock solution. PGE1 was dissolved in ethanol at a concentration of 2.82 mmol/L as stock solution. PBA was dissolved in RPMI 1640 medium. All control conditions included corresponding vehicles at the appropriate concentrations (ethanol for PGE1 and DMSO for TG and H89).

Cell apoptosis analysis

Apoptosis was determined using the annexin V-FITC/PI apoptosis detection kit, according to the manufacturer's instructions. Briefly, 2×10^6 cells were harvested using 0.05% trypsin with 0.5% mmol/L EDTA. To analyze the whole apoptotic cell population, non-adherent

cells present in the culture medium were added to the harvested cells. The cells were then washed twice with pre-chilled PBS and resuspended in 500 μ L annexin binding buffer. Then, 5 μ L of annexin V-FITC and 5 μ L of PI were added to each sample and incubated in dark at room temperature for 10 min. Flow cytometry (Gallios; Beckman Coulter, Brea, CA, United States) was performed according to the manufacturer's specifications. The apoptotic index was calculated as the percentage of annexin V⁺ cells divided by the total number of cells in the gated region.

Cell viability assay

Cell viability was assessed with MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] method using the CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI, United States) according to the manufacturer's instructions. In brief, early passage of L02 or HepG2 cells were plated in triplicate on 96-well plates (10000 cells/well) and cultured in modified RPMI 1640 for 24 h. Thereafter, the cells were rinsed three times in 200 μ L of PBS and cultured in a medium lacking fetal calf serum. Cell viability was determined by replacing the medium with 20 μ L of MTS. After incubation of the cells at 37 °C for 3 h, the absorbance was measured at 490 nm using a microplate reader (Bio-Rad model 680; Bio-Rad, Hercules, CA, United States). Cell viability was normalized as a percentage of control. This experiment was performed five times.

Western blotting

Cell lysates containing 40 μ g of protein were resolved by SDS-PAGE using 4%-20% polyacrylamide gradient gel, and the fractioned proteins were subsequently transferred to nitrocellulose membranes. After blocking with Tris-based saline buffer containing 5% dry milk and 0.1% Tween 20 for 1 h, the membranes were blotted with the corresponding antibodies. The primary and secondary antibodies used were: rabbit anti-human GRP78 (1:500 dilution), PERK (1:1000 dilution), phospho (p)-PERK (1:500 dilution), eIF-2 α (1:500 dilution), p-eIF-2 α (1:250 dilution), sXBP1 (1:500 dilution), CHOP (1:1000 dilution), GADD34 (1:1000 dilution), mouse anti-human β -actin, goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP), and goat anti-mouse IgG conjugated with HRP. The membranes were developed using a chemiluminescence detection system and thereafter exposed to BioMax Light Film (Kodak, Rochester, NY, United States). The band intensity for each protein was measured using ImagePro Plus analysis software (MediaCybernetics, Silver Spring, MD, United States) and the expression normalized to that of β -actin.

Quantitative real-time RT-PCR

Total RNA was isolated from L02 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. RNase-free DNase I (TaKaRa, Dalian, China) was applied before cDNA synthesis to remove any genomic contamination. A total of 1 μ g RNA from each sample was used for cDNA synthesis with a reverse transcription kit (TaKaRa). Reverse transcription (final volume of 20 μ L) was performed at 42 $^{\circ}$ C for 10 min, followed by 75 $^{\circ}$ C for 5 min. The real-time PCR reaction (25 μ L) containing 12.5 μ L of 2 \times SYBR Premix ExTaq II (Tli RNaseH Plus; TaKaRa), 1 μ L of each 10 μ mol/L primers, 2 μ L of cDNA template, and 8.5 μ L RNase/DNase-free water was performed on a CFX96 PCR system (Bio-Rad). The reaction process was as follows: denaturation at 95 $^{\circ}$ C for 3 min, followed by 40 cycles of amplification (95 $^{\circ}$ C for 10 s and 60 $^{\circ}$ C for 30 s), ending with a melt curve ranging from 60 $^{\circ}$ C to 95 $^{\circ}$ C with a heating rate of 0.3 $^{\circ}$ C/15 s. All samples were run in triplicate. Relative expression of GRP78 was calculated using the delta-delta-Ct method with β -actin as the reference control.

Primers used in the PCR were: GRP78 forward, 5'-AAATAAGCCTCAGCGTTTCTT-3' and reverse, 5'-TCAAGTCTTGCCGTTCAGG-3'; β -actin forward, 5'-CGGGAAATCGTGCGTGAC-3' and reverse, 5'-CAGGAAGGAAGGCTGGAAG-3' (TaKaRa). Quantitative real-time PCR was performed according to the MIQE guidelines^[28].

GRP78-siRNA transfection

Briefly, cell culture plates containing 6-wells were seeded with 2 \times 10⁵ cells/well and cultured in RPMI 1640 medium containing 10% (v/v) FBS and 100 units/mL penicillin. siRNA scramble control and validated human GRP78-siRNA were used. GRP78 inhibition was performed using a commercially available siRNA kit (Santa Cruz Biotechnology). Knock-down of the target molecule, GRP78, was monitored by western blotting and real-time PCR.

Statistical analysis

Results of cell apoptosis and cell viability are expressed as mean \pm SD. One-way analysis of variance (ANOVA) with Bonferroni's post hoc analysis was performed to compare multiple groups. The Student's *t*-test was used to assess between-group differences. The level of significance was set at $P < 0.05$.

RESULTS

TG-induced ER stress and apoptosis in L02 cells and PGE1 protected L02 and HepG2 cells against ER stress-induced apoptosis

We confirmed TG-induced ER stress in L02 cells (Figure 1). At 48 h, TG (1 μ mol/L) caused significant

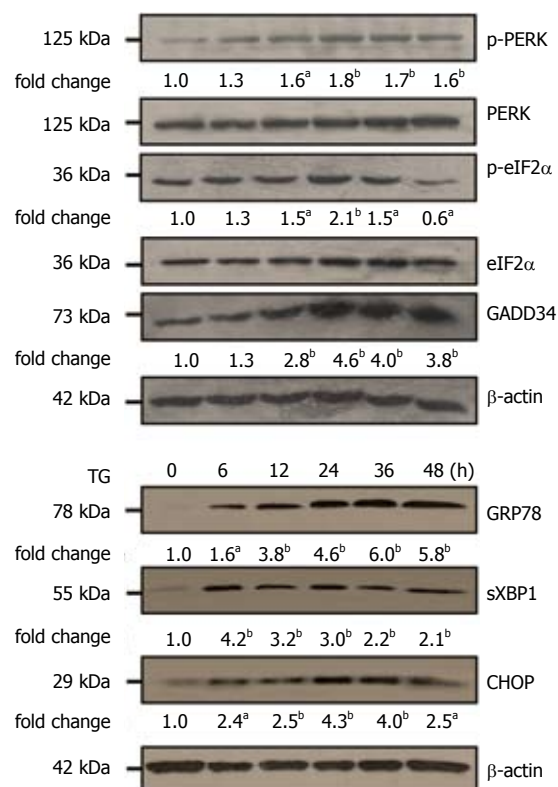


Figure 1 TG induced endoplasmic reticulum stress in L02 cells. L02 cells were treated with 1 μ mol/L TG for 6, 12, 24, 36 and 48 h. Expressions of p-PERK, p-eIF2 α , GADD34, GRP78, sXBP1 and CHOP were assessed by western blotting. Representative blots from three independent experiments are presented. The results of densitometric analysis are presented as a fold-change compared to that at 0 h (^a $P < 0.05$; ^b $P < 0.01$). CHOP: C/EBP homologous protein; ER: Endoplasmic reticulum; GADD34: Growth arrest and DNA damage-inducible gene34; GRP78: Glucose-regulated protein 78; p-eIF2 α : Phospho-eukaryotic translation initiation factor-2 α ; p-PERK: Phospho-PKR-like ER kinase; sXBP1: Spliced X box-binding protein1; TG: Thapsigargin.

enhancement in phosphorylation of PERK and GADD34 proteins, as compared to that observed at 6 h. Further, phosphorylation of eIF2 α at 24 h was also up-regulated, as compared to that at 6 h. TG also induced a significant increase in the expressions of GRP78, CHOP and sXBP1 proteins.

We also confirmed TG-induced apoptosis in both L02 and HepG2 cells by means of flow cytometry and MTS assay. As shown in Figure 2A, the apoptotic index of L02 cells after TG treatment was significantly higher than that of control from 6 h to 48 h ($P < 0.01$). At 48 h, the apoptotic index reached 32.66%. TG also showed a dose-dependent increase in apoptotic index with increase in the concentration of TG (1 μ mol/L, 2 μ mol/L and 3 μ mol/L; data not shown). Cell viability of both L02 and HepG2 cells significantly decreased from 24 h to 48 h after TG treatment (Figure 2B and C).

We assessed the effect of PGE1 on ER stress-induced apoptosis in L02 cells (Figure 2A). From 6 h to 48 h, 1 μ mol/L PGE1 pretreatment significantly decreased TG-induced apoptotic index. At 48 h, 0.5 μ mol/L and 1 μ mol/L PGE1 showed dose-dependent

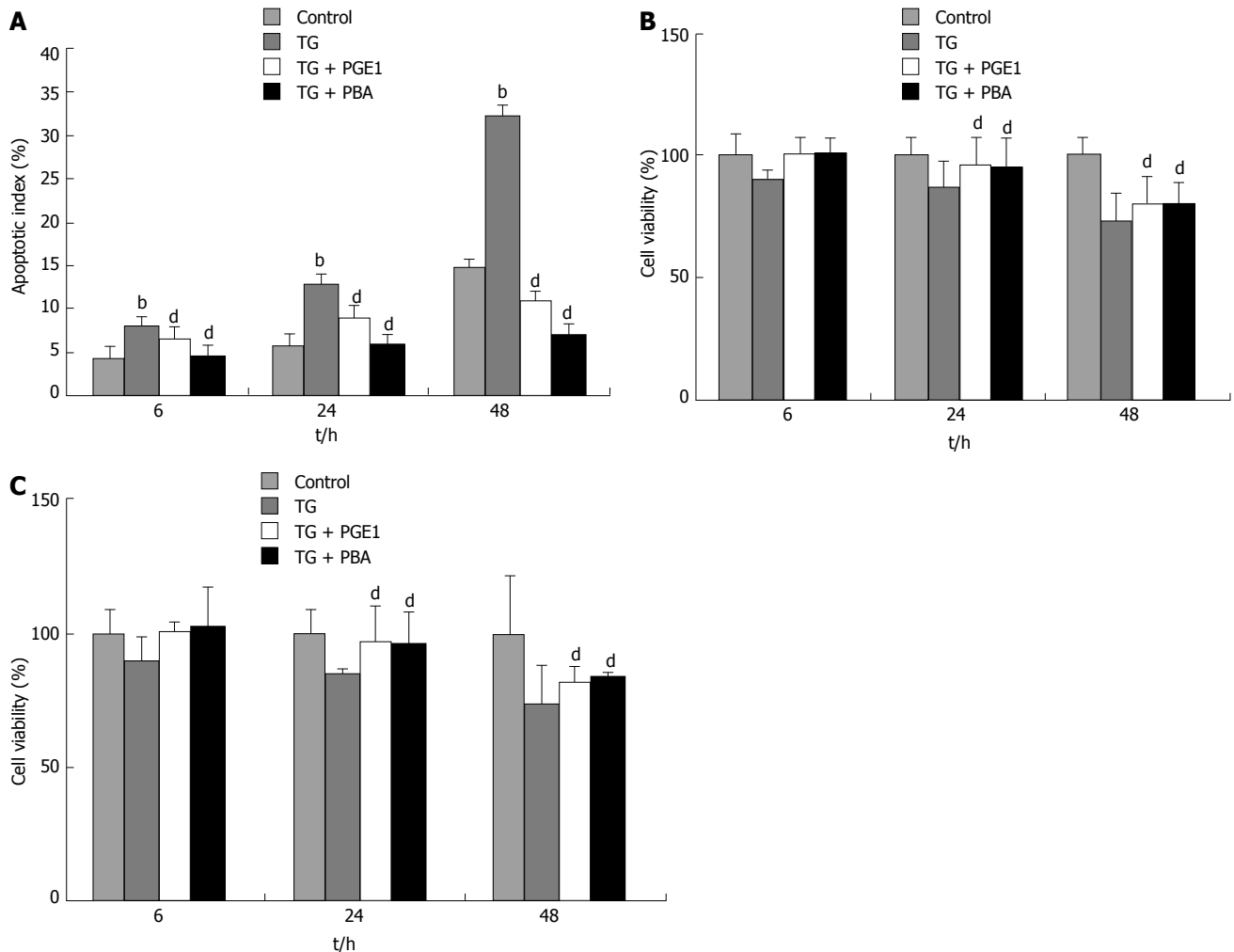


Figure 2 Prostaglandin E1 protected against thapsigargin-induced apoptosis in both L02 cells and HepG2 cells. L02 cells and HepG2 cells were pretreated with PGE1 or PBA for 1 h and treated with a final concentration of 1 μ mol/L TG for 6, 24 and 48 h. A: Apoptotic index of L02 cells was determined by flow cytometry. Histograms represent mean \pm SD of five separate experiments, each of which was performed in triplicate. ^b $P < 0.01$ vs control at the same time point; ^d $P < 0.01$ vs TG at the same time point; B and C: Cell viability of L02 cells and HepG2 cells was determined by MTS assay. The absorbance was measured at 490 nm and cell viability was normalized as a percentage of control. Histograms represent mean \pm SD of five separate experiments, each of which was performed in triplicate. ^d $P < 0.01$ vs TG at the same time point. MTS: [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]; PBA: 4-phenylbutyric acid; PGE1: Prostaglandin E1; TG: Thapsigargin.

protection against TG-induced apoptosis (data not shown). PBA is a low molecular weight chemical chaperone and an ER stress inhibitor. PBA at 10 nmol/L significantly inhibited TG induced apoptosis (Figure 2A). MTS assay revealed that PGE1 significantly increased the viability of TG-treated L02 cells and HepG2 cells (Figure 2B and C). These results demonstrate the protective effect of PGE1 on ER stress-induced apoptosis in L02 cells and in HepG2 cells.

PGE1 promoted rapid recovery of ER stress

After demonstration of the cytoprotective effect of PGE1, we investigated the effects of PGE1 on UPR signals induced by TG. We selected three indicators from UPR, GRP78, CHOP and sXBP1, as GRP78 is a common downstream target of the three UPR signal pathways. CHOP is the major protein involved in apoptotic signals induced by ER stress, and sXBP1 is activated through the oligomerization of IRE1a^[29].

As shown in Figure 3, 1 μ mol/L PGE1 appeared to increase TG-induced GRP78, CHOP and sXBP1 expressions till 6 h post-treatment. However, from 24 h to 48 h, PGE1 suppressed TG-induced GRP78 and CHOP expressions. These results indicate that although PGE1 increased the early expression of UPR signals, it promoted the rapid recovery of ER stress.

PGE1 induced GRP78 expression via the PKA pathway

The observation that PGE1 attenuated the ER stress-induced apoptosis and promoted the rapid recovery from ER stress prompted us to investigate the effect of PGE1 on GRP78, CHOP and GADD34 expressions. GRP78 is a critical chaperone which determines the outcome of ER stress. GADD34 is involved in both recovery and resumption of protein synthesis as well as in the ER stress-induced apoptosis^[14,30]. We also investigated the effects of PGE1 on phosphorylation of eIF2 α and PERK, since the activation of PERK and

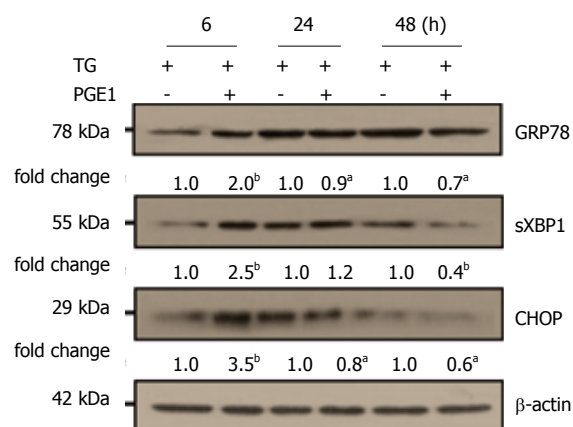


Figure 3 Prostaglandin E1 promoted rapid recovery of endoplasmic reticulum stress. L02 cells were pretreated with 1 $\mu\text{mol/L}$ PGE1 for 1 h and at a final concentration of 1 $\mu\text{mol/L}$ TG for 6, 24 and 48 h. Expressions of GRP78, CHOP and sXBP1 were assessed on western blotting. One representative blot each of three individual experiments is presented. The results of densitometric analysis are presented as a fold-change compared to those of TG at the same time point (^a $P < 0.05$, ^b $P < 0.01$). CHOP: C/EBP homologous protein; ER: Endoplasmic reticulum; GRP78: Glucose-regulated protein 78; PGE1: Prostaglandin E1; sXBP1: Spliced X box-binding protein 1; TG: Thapsigargin.

eIF2 α is known to induce CHOP and GADD34. From 3 h to 24 h after treatment with 1 $\mu\text{mol/L}$ PGE1, a significant increase in GRP78 expression was observed in both L02 cells and HepG2 cells (Figure 4A and B). PGE1 also decreased CHOP expression in L02 cells and induced the expression of GADD34; however, it suppressed the expression of p-PERK and p-eIF2 α proteins (Figure 4A). Results of quantitative real-time RT-PCR also demonstrated that PGE1 induced the mRNA expression of GRP78 from 3 h to 24 h (Figure 4C).

To explore the signal pathways that may mediate the effect of PGE1 on GRP78 expression, we inhibited the PKA pathway by H89 or KT5720. Treatment with 10 $\mu\text{mol/L}$ of H89 or 1 $\mu\text{mol/L}$ KT5720 appeared to counteract the effect of PGE1 on GRP78 expression (Figure 5A and B). These results indicate that PGE1 increased GRP78 expression *via* a PKA-dependent pathway.

PGE1 protected L02 and HepG2 cells against ER stress-induced apoptosis *via* PKA-dependent induction of GRP78 expression

H89 at 10 $\mu\text{mol/L}$ appeared to inhibit the protective effect of PGE1 on hepatocyte apoptosis, which suggests that the cytoprotective effect of PGE1 was mediated *via* the PKA signaling pathway (Figure 5C). To further test whether the protective effect of PGE1 was dependent on the induction of GRP78 expression, we inhibited GRP78 expression by transfection with siRNA specifically targeted against human GRP78. On blocking the PGE1-induced up-regulation of GRP78 expression by siRNA (Figure 6A-C), PGE1 appeared to lose its cytoprotective effect in both L02 cells and HepG2 cells (Figure 6D-G). These findings imply that

PGE1 protected against hepatocyte apoptosis *via* PKA-dependent induction of GRP78 expression.

DISCUSSION

The key findings of this study are that PGE1 protected hepatocytes from ER stress-induced apoptosis, that PGE1 enhanced the expression of GRP78 *via* the PKA pathway, and that the cytoprotective effect of PGE1 on hepatocytes was mediated *via* PKA-dependent GRP78 induction.

TG is known to induce ER stress by blocking ER Ca²⁺ uptake, which leads to depletion of ER Ca²⁺ stores^[31]. PGE1 can also increase intracellular Ca²⁺ level by promoting the influx of Ca²⁺ from the external medium as well as by mobilization of Ca²⁺ from intracellular stores^[32]. As intracellular Ca²⁺ level is an important factor in hepatocyte apoptosis and necrosis, cotreatment with TG and PGE1 may have modulated apoptotic signals of L02 cells, which eventually led to cell death by necrosis. Therefore, we assessed cell apoptosis and viability on flow cytometry and by MTS assay. Our results strongly suggest that PGE1 protects hepatocytes from ER stress-induced apoptosis.

PGE1 is known to have a direct vasodilator as well as an anti-platelet effect^[22,33]. Several *in vivo* studies have indicated that PGE1 protects against hepatocyte apoptosis and promotes hepatocyte proliferation *via* inducing down-regulation of proinflammatory cytokine levels^[24,26], suppression of tumor necrosis factor- α receptor and adhesion molecule expression^[23,24], and by up-regulating cyclin C and cyclin D1 expressions^[34]. PGE1 also inhibits oxidative stress and nitrosative stress-induced hepatocyte death by inhibiting production of superoxide anion, by enhancing nitric oxide synthase expression and by inhibiting nuclear factor- κ B activation *in vitro*^[19,35,36]. In this study, we demonstrated that PGE1 protected hepatocytes from ER stress-induced apoptosis. Our findings suggest that modulation of UPR may mediate the cytoprotective effect of PGE1 in various pathological conditions.

The mechanisms involved in ER stress-induced apoptosis are yet to be elucidated. The adaptive capacity of cells to ER stress is an important determinant of the outcomes of ER stress. Apoptosis results from sustained or strong ER stress when UPR fails to restore ER homeostasis^[37,38]. In the present study, PGE1 treatment enhanced TG-induced CHOP, GRP78 and sXBP1 expressions at 6 h, and significantly attenuated TG-induced CHOP, GRP78 and sXBP1 expressions, as assessed at 48 h. These results suggest that PGE1 boosted the initial expression of UPR and promoted restoration of ER homeostasis during ER stress.

During ER stress, UPR may have been activated to restore ER homeostasis. UPR is precisely regulated by three ER stress transducers and their downstream signals. GRP78 is considered as a master regulator of response to ER stress^[39,40]. GRP78 binds with all three major UPR sensors (PERK, IRE1a and ATF6) and

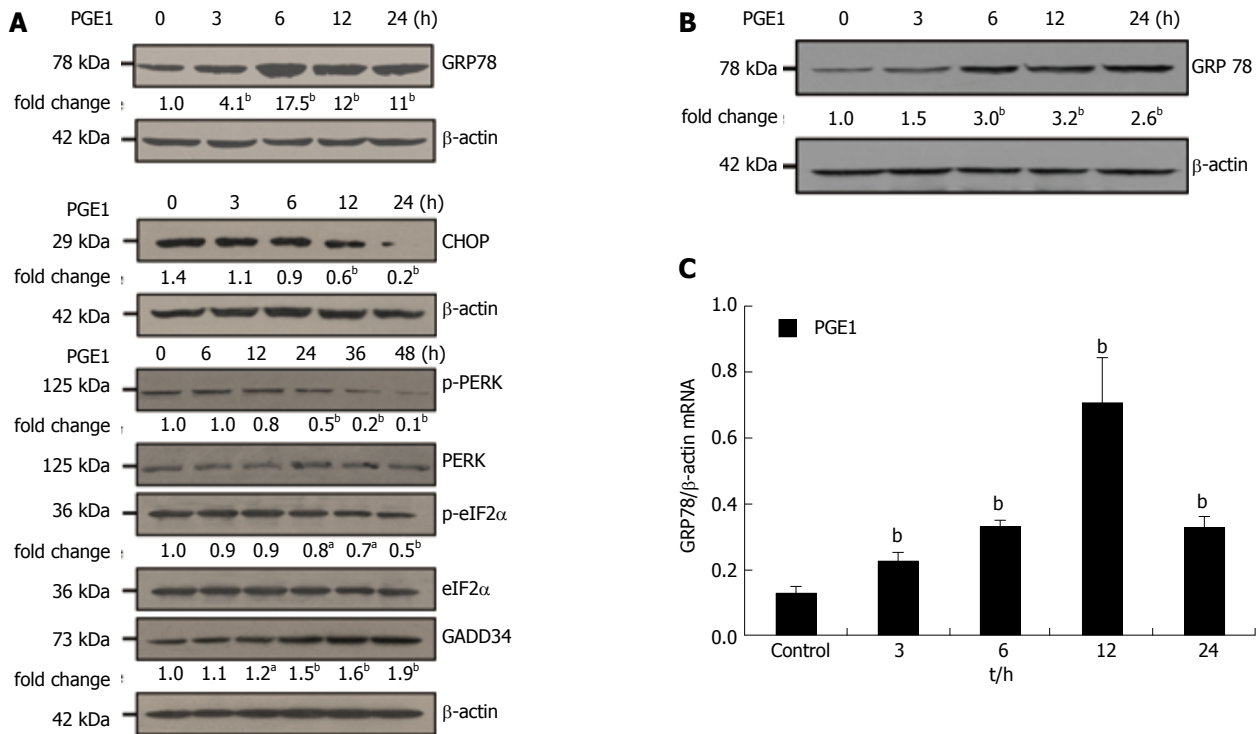


Figure 4 Prostaglandin E1 induced glucose-regulated protein 78 protein and mRNA expressions. To determine GRP78 and CHOP protein or mRNA expressions, L02 cells and HepG2 cells were treated with 1 μ mol/L PGE1 for 3, 6, 12 and 24 h; for detecting GADD34 and the p-PERK and p-eIF2 α , L02 cells were treated with 1 μ mol/L PGE1 for 6, 12, 24, 36 and 48 h. A: Expressions of GRP78, CHOP, GADD34, p-PERK and p-eIF2 α in L02 cells were assessed by western blotting. One representative blot each from the three individual experiments is presented. The results of densitometric analysis are presented as a fold-change compared to those at 0 h (^a $P < 0.05$, ^b $P < 0.01$); B: Expression of GRP78 in HepG2 cells was assessed by western blotting. One representative blot each from the three individual experiments is presented. The results of densitometric analysis are presented as a fold-change compared to those at 0 h (^a $P < 0.05$, ^b $P < 0.01$); C: mRNA expression of GRP78 in L02 cells was assessed by quantitative real-time PCR (E). Histograms represent mean \pm SD of three experiments (^a $P < 0.05$ vs those at 0 h). CHOP: C/EBP homologous protein; ER: Endoplasmic reticulum; GADD34: Growth arrest and DNA damage-inducible gene 34; GRP78: Glucose-regulated protein 78; p-eIF2 α : Phospho-eukaryotic translation initiation factor-2 α ; PGE1: Prostaglandin E1; p-PERK: Phospho-PKR-like ER kinase; TG: Thapsigargin.

keeps them inactivated while the homeostasis of ER is maintained. During ER stress, GRP78 dissociates from PERK, ATF6 and IRE1 α and binds to unfolded or misfolded proteins, promotes their proper folding or directs them to degradation. The dissociation of GRP78 from PERK, ATF6 and IRE1 α , triggers the UPR response and further enhances the expression of GRP78. Increased GRP78 augments the folding capacity of the ER, inactivates the three ER sensors and promotes restoration of ER homeostasis^[29].

The protective role of GRP78 against apoptosis during ER stress has been demonstrated both *in vivo* and *in vitro*^[41-44]. The insulin signaling pathway has been found to promote cell proliferation and improve cell survival *via* up-regulation of GRP78 expression^[45]. In this study, PGE1 significantly inhibited TG-induced apoptosis. PGE1 induced protein and mRNA expressions of GRP78. Further, inhibition of GRP78 expression *via* either H89 or siRNA hindered the protective role of PGE1 on TG-induced apoptosis. These results demonstrate that the cytoprotective effect of PGE1 on hepatocytes was mediated *via* induction of GRP78 during ER stress. The protein expression of GRP78 induced by PGE1 peaked at 6 h; however, mRNA expression of GRP78 induced by PGE1

peaked at 12 h. It is difficult to explain the difference in the time frame for attainment of peak levels of mRNA and protein expressions of GRP78. One explanation is that PGE1 regulated the GRP78 expression not only at the transcriptional level but also at the translational or posttranslational level. Whether PGE1 regulates the expression of GRP78 at the translational or posttranslational level warrants further studies.

The other important signal pathways in ER stress-induced apoptosis are mediated *via* induction of CHOP and GADD34. During UPR, phosphorylation of eIF2 α *via* the PERK pathway results in inhibition of mRNA translation and general protein synthesis. However, mRNA for activating transcription factor (ATF) 4 is selectively up-regulated. Activation of ATF4 results in induction of CHOP and GADD34 expressions. GADD34 and protein phosphatase 1 were shown to promote dephosphorylation of eIF2 α and allow protein synthesis^[46]. However, recent studies have shown that induction of GADD34 exacerbates the disturbance of ER homeostasis and leads to cell apoptosis by increasing oxidative stress^[47]. CHOP has been identified as one of the most important mediators of ER stress-induced apoptosis; it induces apoptosis through various signal pathways^[48].

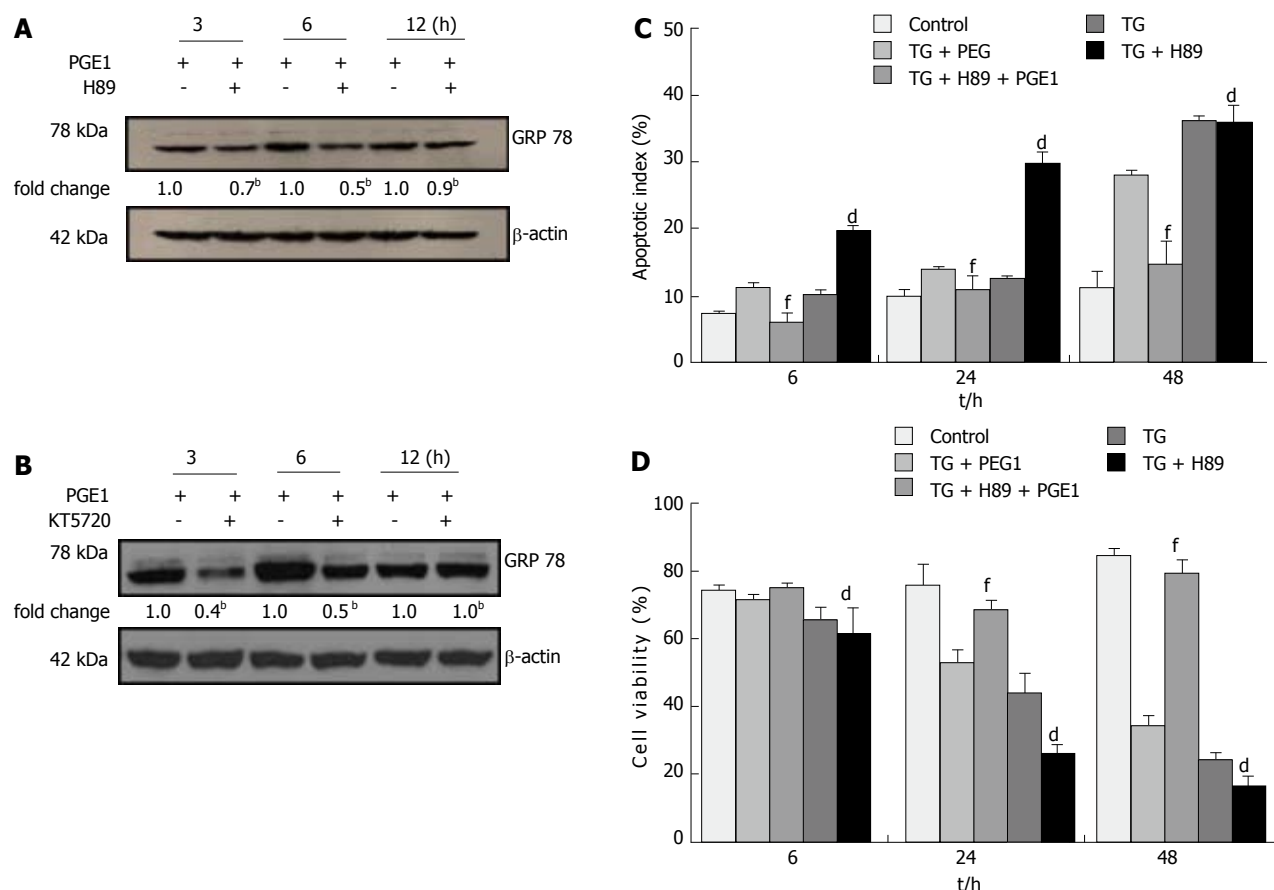


Figure 5 Prostaglandin E1 induced glucose-regulated protein 78 expression and protected L02 cells against endoplasmic reticulum stress-induced apoptosis via a protein kinase a-dependent pathway. A and B: L02 cells were pretreated with or without PKA inhibitor H89 (10 $\mu\text{mol/L}$) or KT5720 (1 $\mu\text{mol/L}$), and then 1 $\mu\text{mol/L}$ PGE1 for 3, 6 and 12 h. The expressions of GRP78 were detected by western blotting. One representative blot each from three independent experiments is presented. The results of densitometric analysis are presented as a fold-change compared to those of PGE1 at the same time points (^b $P < 0.01$); C: L02 cells were pretreated with or without 10 $\mu\text{mol/L}$ H89, and then 1 $\mu\text{mol/L}$ PGE1 and a final concentration of 1 $\mu\text{mol/L}$ TG for 6, 24 and 48 h. The apoptotic index was determined by flow cytometry. Histograms represent mean \pm SD of five separate experiments, each of which was performed in triplicate. (^d $P < 0.01$ vs those of TG + PGE1 at the same time point; ^f $P < 0.01$ vs those of TG at the same time point); D: L02 cells were pretreated with or without 10 $\mu\text{mol/L}$ H89, and then 1 $\mu\text{mol/L}$ PGE1 and a final concentration of 1 $\mu\text{mol/L}$ TG for 6, 24 and 48 h. Cell viability of L02 cells was determined by MTS assay. Histograms represent mean \pm SD of five separate experiments, each of which was performed in triplicate. (^d $P < 0.01$ vs those of TG + PGE1 at the same time point; ^f $P < 0.01$ vs those of TG at the same time point). GRP78: Glucose-regulated protein 78; MTS: [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]; PGE1: Prostaglandin E1; PKA: Protein kinase A; TG: Thapsigargin.

In the current study, we had anticipated inhibition of GADD34 by PGE1. However, we observed an enhanced expression of GADD34 and significant inhibition of p-PERK, CHOP and p-eIF2 α expressions in L02 cells. Our results indicate that the cytoprotective effect of PGE1 against hepatocyte apoptosis does not depend on the inhibition of GADD34. Since PGE1 lost its protective effects after inhibition of GRP78 expression in this study, it is possible that the increased expression of GADD34 in our study was the result of induction of GRP78 by PGE1 and that this represented restoration of ER homeostasis. Further study is needed to clarify the role of GADD34 in ER stress-induced apoptosis.

The biological effects of PGE result from its binding to its receptors, EP1 to EP4, and previous studies have shown that L02 cells express EP1 receptors^[49]. Binding of PGE1 with its receptors stimulates production of the second messenger cyclic 3, 5 adenosine monophosphate (cAMP). cAMP may act *via* distinct

intracellular signaling effectors, such as PKA and the exchange proteins activated by cAMP^[50]. A previous study has shown that cAMP has PKA-independent interaction with Ca²⁺ stored in lymphocytes^[51]. To test whether the hepatoprotective effect of PGE1 is mediated *via* PKA-independent interaction with Ca²⁺ stores in L02 cells, we used H89 to block the PKA pathway. The results showed that the protective effects of PGE1 were largely dependent on the PKA pathway. However, H89 inhibited GRP78 expression most effectively at 6 h, and then at 12 h the inhibitory effect was alleviated; the increased apoptotic index lasted from 6 h to 48 h. It is difficult to explain this result. One explanation is that H89 may also have inhibited other kinases in addition to PKA. H89 has been shown to inhibit at least 8 kinases beside PKA^[52]. The role of PKA and other kinases in ER stress-induced apoptosis remains to be studied in the future.

It is known that GRP78 expression is regulated at the transcriptional level by ER stress. Previous

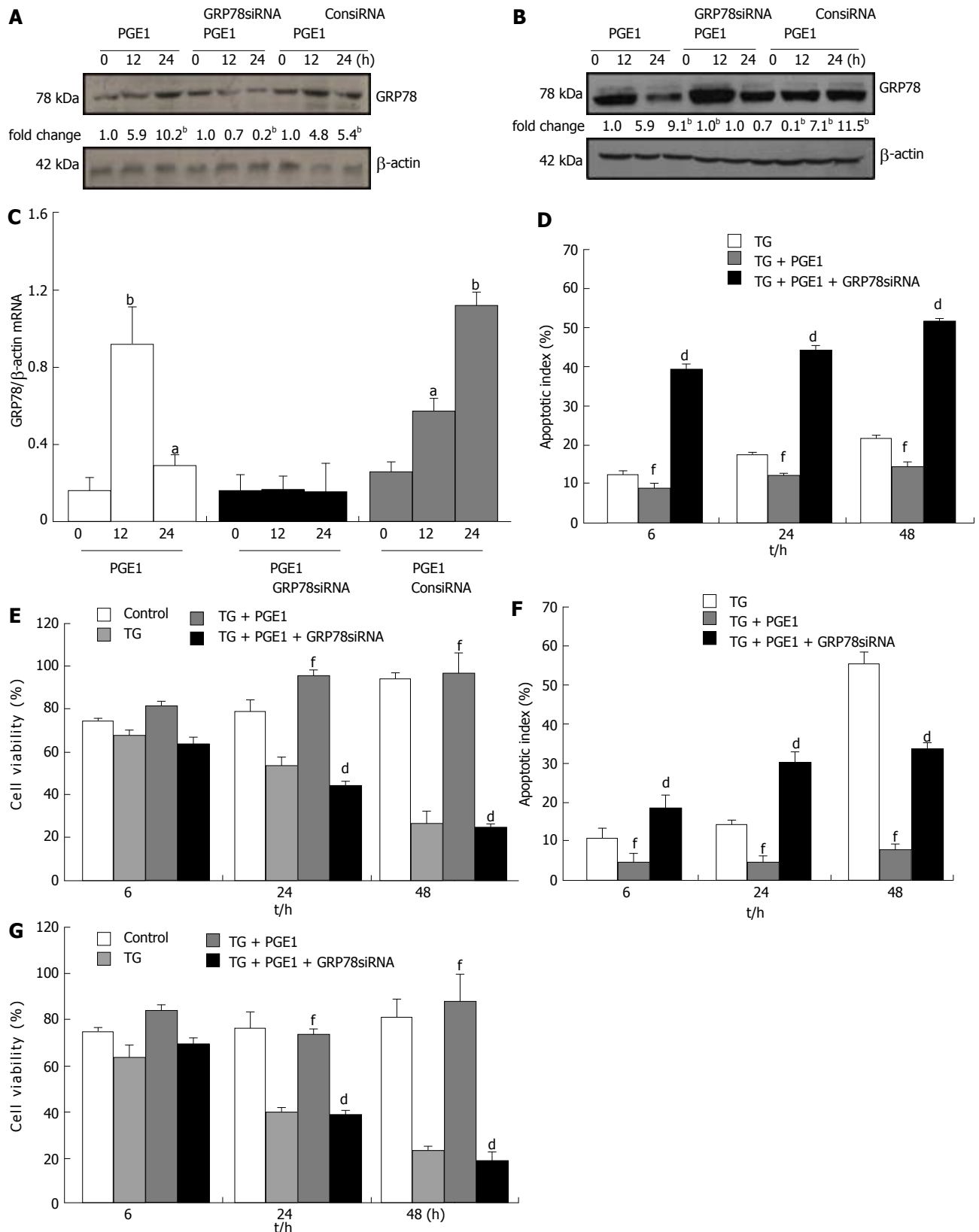


Figure 6 Prostaglandin E1 protected against endoplasmic reticulum stress-induced apoptosis via induction of glucose-regulated protein 78 expression in both L02 and HepG2 cells. L02 and HepG2 cells were transfected with either siRNA scramble control (ConsiRNA) or siRNA against human GRP78 (GRP78 siRNA) for 48 h. The cells were treated with 1 μ mol/L PGE1 for 12 h and 24 h. A and B: Expressions of GRP78 in L02 cells and in HepG2 cells were detected by western blotting. One representative blot each from the three individual experiments is presented. The results of densitometric analysis are presented as a fold change compared to those at 0 h ($^aP < 0.05$; $^bP < 0.01$ vs those at 0 h). C: mRNA expression of GRP78 in L02 cells was detected by real-time PCR. Histograms represent mean \pm SD of three experiments ($^aP < 0.05$; $^bP < 0.01$ vs those at 0 h). D and F: Apoptotic indices of L02 cells and HepG2 cells were determined by flow cytometry. Histograms represent mean \pm SD of five independent experiments, each of which was performed in triplicate ($^dP < 0.01$ vs those of TG + PGE1 at the same time point; $^fP < 0.01$ vs those of TG at the same time point). E and G: Cell viability of L02 cells and HepG2 cells was determined by MTS assay. Histograms represent mean \pm SD of five independent experiments, each of which was performed in triplicate ($^dP < 0.01$ vs those of TG + PGE1 and $^fP < 0.01$ vs those of TG at the same time point). GRP78: Glucose-regulated protein 78; PGE1: Prostaglandin E1; MTS: [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]; siRNA: Small interfering RNA; TG: Thapsigargin.

studies have shown that preconditioning to ER stress protects against cell death *via* induction of GRP78 or autophagy^[53]. In this study, although PGE1 pretreatment significantly induced GRP78 expression and enhanced TG-induced early CHOP and sXBP1 expressions, PGE1 alone inhibited the expressions of p-PERK and p-eIF2 α and CHOP. Therefore, whether PGE1 induced GRP78 expression *via* induction of ER stress is not known. In previous studies, leptin was shown to induce GRP78 expression in neuronal cells through the PI3K-mTOR pathway^[54]; further, oncostatin M was also shown to induce GRP78 expression without triggering ER stress^[55]. The mechanisms involved in the induction of GRP78 expression by PGE1 warrant further investigation.

In conclusion, this is the first study to demonstrate that the cytoprotective effect of PGE1 against ER stress-induced apoptosis is mediated *via* PKA-dependent induction of GRP78 expression in hepatocytes. Further studies are required for devising treatment strategies to protect hepatocytes against ER stress-induced apoptosis, which will be of much clinical relevance in the context of liver diseases.

COMMENTS

Background

Prostaglandin (PG) E1 has been shown to protect against hepatocyte apoptosis; however, the role of Prostaglandin E1 (PGE1) in endoplasmic reticulum (ER) stress-induced apoptosis of hepatocytes is largely unknown.

Research frontiers

ER stress has been implicated in the pathogenesis of various liver diseases. Understanding the mechanisms underlying ER stress-induced apoptosis and devising a treatment strategy to protect hepatocytes from ER stress-induced apoptosis will benefit most patients with liver diseases.

Innovations and breakthroughs

Pretreatment with PGE1 protected hepatocytes against thapsigargin-induced apoptosis. PGE1 alone induced protein and mRNA expressions of glucose-regulated protein (GRP) 78. Treatment with protein kinase A (PKA)-inhibitor H89, KT5720 or small interfering (si)RNA specifically targeted against human GRP78 blocked PGE1-induced up-regulation of GRP78. The hepatoprotective effect of PGE1 was lost by blocking GRP78 expression by either H89 or siRNA. Our study demonstrates, for the first time, that PGE1 protects against ER stress-induced hepatocyte apoptosis *via* PKA pathway-dependent induction of GRP78 expression.

Applications

PKA and GRP78 may be new targets for pharmacological treatment in patients with liver diseases.

Peer-review

The strengths of this study lie in the experimental design, methodology and that the authors describe the caveats and potential pitfalls in great detail. Overall, the study was well-designed and the paper is interesting and sound.

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P- Reviewer: Cubero FJ, McMillin MA **S- Editor:** Wei LJ
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ISSN 1007-9327



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