

Adaptive unfolded protein response promotes cell survival in rifampicin-treated L02 cells

WEIPING ZHANG^{1,2} and JIANMING XU¹

¹Department of Gastroenterology, The First Affiliated Hospital of Anhui Medical University, Key Laboratory of Gastroenterology of Anhui Province; ²The First Affiliated Hospital of AUTCM, Hefei, Anhui 230022, P.R. China

Received July 26, 2016; Accepted January 23, 2018

DOI: 10.3892/ijmm.2018.3438

Abstract. An important concept in drug-induced liver injury (DILI) is adaptation, which means the injury reverses with the continuation of the drug. The mechanism of adaptation of drugs remains enigmatic, adaptive unfolded protein response (UPR) is possibly involved. We once observed adaptation phenomenon of rifampicin (RFP) in animal models, in this study, we investigate the effects of RFP on adaptive UPR in L02 cells, and after inhibiting UPR by using 4-phenylbutyrate (4-PBA), the change of cell viability and cell apoptosis in RFP-treated cells. We found that with the concentration of RFP increased and the treatment time was prolonged, the glucose-regulated protein 78 (GRP78), a hallmark of the UPR, was upregulated, and was dose- and time-dependent. RFP also activates the p-eukaryotic initiation factor 2 α (eIF2 α) protein expression. 4-PBA decreased GRP78 and p-eIF2 α protein expression levels. Moreover, FCA showed that cell apoptosis rate obviously increased, and MTT assay showed that cell survival rate obviously decreased, this indicates that after inhibiting the UPR, the cell damage increased, which shows that the UPR is an adaptation mechanism to protect cells against injury induced by RFP. This also proves that when the degree of UPR induced by RFP is relatively mild, adaptive UPR is helpful for cell survival.

Introduction

The endoplasmic reticulum (ER) plays a major role in folding, transportation, post-translational modification, and quality control of newly synthesized proteins (1). ER via expanding its protein folding ability adapts to increasing physiological demands for protein synthesis (2). Abnormalities such as the accumulation of unfolded proteins can interfere with the func-

tion of ER, resulting in a dysfunctional situation described as ER stress (3). The ER stress response constitutes a series of cellular processes, the unfolded protein response (UPR), which aims to clear unfolded proteins and restore ER homeostasis, is a very important adaptive mechanism (4). Accumulation of unfolded proteins activates signaling pathways in the cytosol via the three stress sensor proteins: inositol-requiring kinase 1 (IRE1), PKR-like ER kinase (PERK) and activating transcription factor 6 (ATF6), which combine with glucose-regulated protein 78 (GRP78)/binding immunoglobulin protein (BIP) under normal circumstances. Activation of the UPR results in reduced protein synthesis and transcriptional activation of genes regulated by spliced X-box binding protein-1S (XBP-1S), activating transcription factor 4 (ATF4), and ATF6. Consequently, UPR activation increases ER chaperone and folding enzyme concentrations to minimize misfolding and aggregation (5). Activation of the UPR is emerging as a common theme in protein-misfolding diseases, with relevant markers observed in patient tissue and mouse models (6). Several recent studies have shown that UPR may actually be beneficial to cells to fulfill specific requirements in unique cell types (7-10).

Rifampicin (RFP) is commonly used to treat tuberculosis worldwide, and it is well-known to induce drug-induced liver injury (DILI). RFP affects the expression of many of the cytochrome P450 drug metabolism genes, and alters the expression of many of the clinically relevant hepatic drug transporters, which may contribute to differences in drug exposure and possibly effect (11). In previous studies we found that RFP increased multidrug resistance protein 2 (Mrp2) and bile salt export pump (BSEP) expression in rats (12). RFP has been shown to cause hepatic toxicity in animal studies and clinical settings, but we also found that RFP could induce adaptive injury in mice (13). Mice were orally administered with a single dose of rifampicin (200 mg/kg), serum ALT, AST, ALP, total bilirubin (TB), conjugated bilirubin (CB), unconjugated bilirubin (UCB) and bile acids (BA) almost all started to rise from 0.5 h, reached the peak at 6 h and then declined. The adaptation phenomenon plays an important role in understanding the toxic effects of drugs and clinical drug use. Adaptation is a situation in which the injury reverses with the continuation of the drug. A series of responses could mediate adaptation, including adaptive UPR induced by ER stress to regulate stress (14).

Correspondence to: Professor Jianming Xu, Department of Gastroenterology, The First Affiliated Hospital of Anhui Medical University, Key Laboratory of Gastroenterology of Anhui Province, Hefei, Anhui 230022, P.R. China
E-mail: xujm1017@163.com

Key words: unfolded protein response, rifampicin, 4-phenylbutyrate, L02 cells, and cell survival

In this study, we hypothesized that RFP would induce adaptive UPR in L02 cells. We used tunicamycin (TM) as control drug, and we used 4-phenylbutyrate (4-PBA) to inhibit UPR. TM is a nucleoside antibiotic, it prevents N-linked glycosylation and has both antibiotic and antiviral properties. It is known to induce ER stress. 4-PBA is a chemical chaperone that helps in protein folding and trafficking within the ER, so it alleviates ER stress and acts as an ER stress inhibitor (15,16). To investigate this hypothesis, we examined many ER stress markers. To our knowledge, this is the first study focusing on RFP induced adaptive UPR.

Materials and methods

Drugs. RFP (Prod #R3501), 4-PBA (Prod #SML0309) and TM (Prod #SML1287) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RFP was dissolved in a <0.1% DMSO (Sigma-Aldrich) solution, and the final concentration was 100 mg/ml. 4-PBA was dissolved in sterile ddH₂O, and its final concentration was 1 M. The final concentration of TM was 5 mg/ml in DMSO.

Cell culture. Human normal hepatocyte cell line L02 was purchased from the Central Laboratory of the Xiangya School of Central South University (Hunan, China). Cells were cultivated in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin according to the manufacturer's instructions. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. Half of the growth medium was changed each day. L02 cells at 60-70% confluency after the fourth passage were plated for the experiments.

MTT assay. The cell survival rate was determined using the conventional MTT assay. Briefly, L02 cells were seeded at a density of 5x10³ cells/well in a 96-well plate, and the cells were incubated in serum-free and antibiotic-free medium for 24 h before stimulation. After 24 h, the culture supernatant was replaced with fresh RPMI-1640 medium and added with different drugs of different concentration for 24 or 48 h. Then the medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS) buffer. Then 90 µl of RPMI-1640 medium and 10 µl of the MTT solution was added to each well, and the cells were incubated for 4 h at 37°C. MTT (Sigma-Aldrich) was dissolved in PBS and filter sterilized at a concentration of 5 mg/ml. Then, the medium was removed, and 150 µl of DMSO was added to each well. The formazan dye crystals were solubilized on a shaker for 15 min at room temperature, and the absorbance at 570 nm of each well was measured using a microplate reader (Rayto, Shenzhen, China). The cell survival rate was determined by comparing the number of viable cells to that of the controls, which was defined as 100% cell viability.

Western blot analysis. After treatment, L02 cells were collected and lysed with RIPA lysis buffer: 50 mM Tris-HCl (pH 8.0), 1% Triton X-100, 150 mM NaCl, 10% SDS, 100 mM methylenediaminetetraacetic acid (EDTA), 10% NP-40 and 10% sodium deoxycholate. Equal amounts of protein for each

sample were loaded with a known molecular weight marker onto 8 and 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. After gel electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes for 150 min at 80 V. After blocking the membranes in 5% nonfat milk for 1 h at room temperature, the membranes were washed with PBST (0.01 M PBS and 0.1% Tween-20) three times, for 10 min each time. Then, the membranes were probed with the primary antibodies at a dilution of GRP78 (1:1,000), PERK (1:500), p-PERK (1:500), eukaryotic initiation factor 2α (eIF2α) (1:1,000), p-eIF2α (1:1,000), ATF4 (1:1,000), CHOP (1:500), IRE1 (1:500), p-IRE1 (1:500), XBP-1U (1:500), XBP-1S (1:500), ATF6 (1:1,000) and α-tubulin (1:4,000) in blocking buffer for 2 h at room temperature or incubated overnight at 4°C. The antibodies against GRP78, ATF6, IRE1, p-IRE1, XBP-1U and CHOP were obtained from Abcam (Cambridge, UK). The antibodies against PERK, ATF4, eIF2α and p-eIF2α were from Cell Signaling Technology (Beverly, MA, USA). The antibody against p-PERK was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The antibody against XBP-1S was from BioLegend (San Diego, CA, USA). The monoclonal anti-α-tubulin antibody was purchased from Sigma-Aldrich. Then, the membrane was washed three times again, and subsequently, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (rabbit or mouse, 1:4,000) for 1 h at room temperature and washed. The membranes were detected using the Gel Catcher 3400 (Shanghai, China), the ECL were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and the data were analyzed with a gel documentation system from Clinx (Shanghai, China).

Immunofluorescence staining. L02 cells were cultured on the circular slide in a 24-well plate. After treatment, the slides were removed and were washed twice with PBS, fixed with 200 µl 4% polyformaldehyde for 15 min. The slides were washed with PBS three times, for 5 min each time, then the slides were treated with 200 µl of 0.1% Triton X-100 for 15 min, blocked with 200 µl 5% BSA for 30 min. After that, the slides were incubated with the specific primary antibodies at a dilution of 1:50 in PBS buffer, followed by staining with the secondary antibodies at a dilution of 1:200 in PBS buffer in dark. DAPI with concentration of 1 µg/ml was added to each slide for 10 min. After that, the slides were washed and mounted with 80% glycerol. The slides were viewed with Olympus IX73P2F (SN: 2J44787). In each group, immunofluorescence images were captured with the same light exposure times.

Flow cytometric assay (FCA). After treatment, the L02 cells were subjected to Annexin V-FITC binding and propidium iodide (PI) staining according to the manufacturer's protocol as follows. Samples were collected after trypsinization, and the cells were washed twice with cold PBS buffer. Then, 400 µl of Annexin V binding buffer was added to each flow tube, and then 5 µl of Annexin V-FITC was added to the culture on ice for 15 min in the dark. After that, 10 µl propidium iodide (PI) was added to the culture on ice for 5 min in the dark. Then, the samples were analyzed by flow cytometer within 1 h. The model of the flow cytometer was BD FACSCanto™ II (SN:V33896301389; BD Biosciences, San Jose, CA, USA).

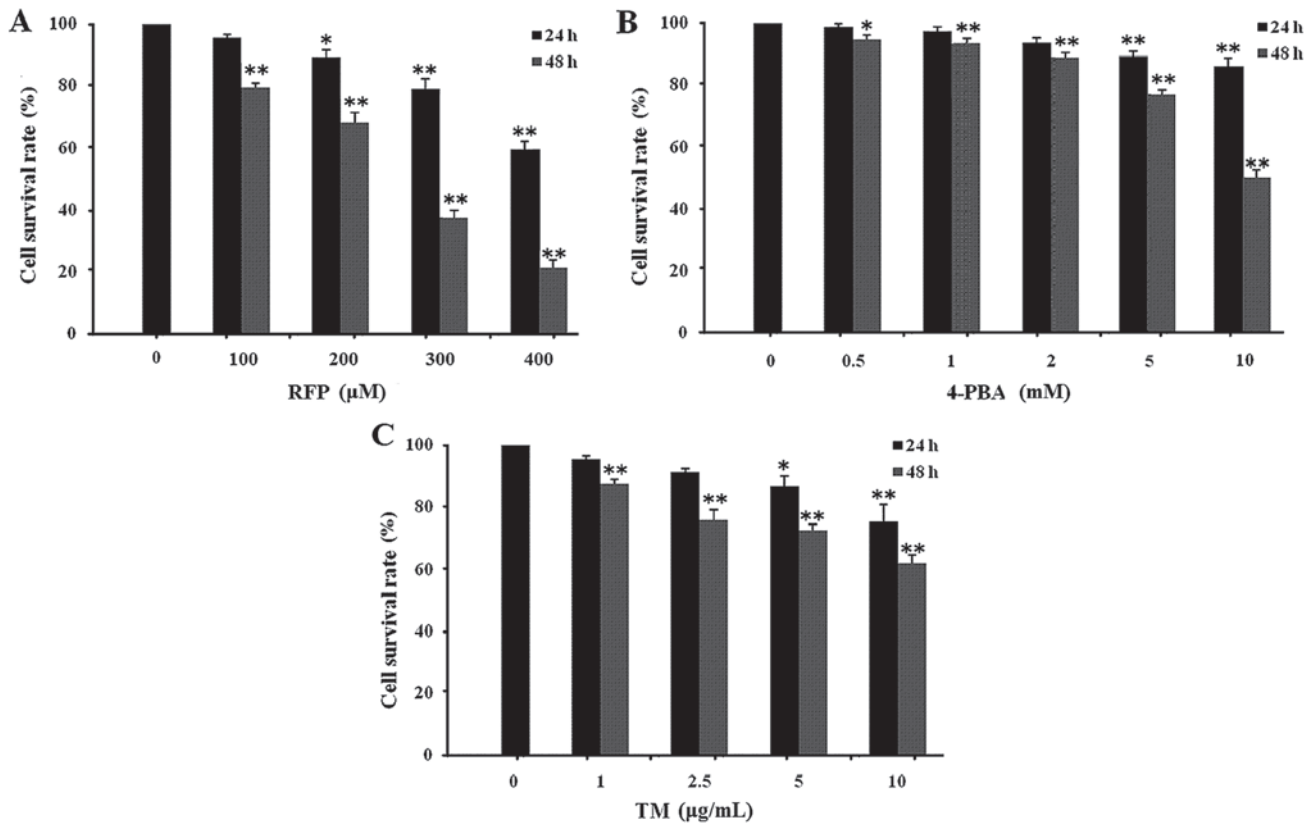


Figure 1. The impact of (A) rifampicin (RFP), (B) 4-phenylbutyrate (4-PBA), and (C) tunicamycin (TM) on the cell survival rate of L02 cells. (A) The cell survival rate of L02 cells exposed to different concentrations (100, 200, 300 and 400 μM) at different times (24 and 48 h) of RFP. The cell survival rates were measured using an MTT assay, and the results showed that with the increase in the concentration and treatment time of RFP, the L02 cell survival rate decreased gradually. A similar trend was observed in (B) 4-PBA-treated L02 cells and (C) TM-treated cells. The experiment was repeated three times, and the data represent the mean \pm SEM. * $p < 0.05$ and ** $p < 0.01$ compared with the control.

Cells negative for both Annexin V-FITC and PI staining were considered live cells. PI-positive cells were considered to be necrotic. Annexin V-FITC positive cells were considered to be in early apoptosis, and cells positive for both Annexin V-FITC and PI were considered to be in late apoptosis. The total number of apoptotic cells was defined as the sum of the early apoptotic and late apoptotic cells. All experiments were repeated at least three times.

Statistical analysis. Statistical analyses were performed using the Statistical Package for the Social Science (SPSS version 12.0; SPSS, Chicago, IL, USA) software. Experimental results are presented as the mean \pm standard error of the mean (SEM) derived from three independent experiments. Comparisons between groups were made using a one-way analysis of variance (ANOVA) followed by the least-significant difference (LSD) test. Probability values < 0.05 were considered significant.

Results

Effect of RFP, 4-PBA, and TM on the survival rate of L02 cells. In order to observe the cell toxicity of these three drugs, L02 cells were treated with RFP at different concentration (100, 200, 300 and 400 μM), 4-PBA at different concentration (0.5, 1, 2, 5 and 10 mM), TM at different concentration (1, 2.5, 5 and 10 $\mu\text{g/ml}$), both at different time-points (24 and 48 h). The

results of the MTT assay demonstrated that with the increase in the concentration of these three drugs and treatment time, the L02 cell survival rate decreased gradually, thus showing a dose- and time-dependent effect (Fig. 1). When the concentration of RFP was higher than 100 μM , the concentration of 4-PBA was higher than 2 mM, and the concentration of TM was higher than 2.5 $\mu\text{g/ml}$, the cell survival rate was significantly inhibited. The IC_{50} value for RFP of 24 h was $\sim 548.91 \mu\text{M}$ and at 48 h was $\sim 234.96 \mu\text{M}$. Referring to previous studies (17,18) we ascertain RFP of 100 μM , 4-PBA of 2 mM, and TM of 2.5 $\mu\text{g/ml}$ as the test concentrations in the following experiment.

RFP effect on GRP78 protein expression in L02 cells. Because GRP78 plays an important role in initiating the adaptive ER stress and inhibiting apoptotic ER stress (19), we first observed the change in the protein expression of GRP78 in RFP-treated L02 cells. Western blot analysis showed that RFP treatment upregulated the expression of GRP78 in a dose-dependent and time-dependent manner. As shown in Fig. 2A, when incubated with increasing concentrations of RFP ranging from 25 to 100 μM for 6 h, L02 cells showed a dose-dependent induction of GRP78, with 100 μM RFP GRP78 protein expression was significantly elevated ($p < 0.05$) and TM was obviously higher than 100 μM RFP ($p < 0.01$). As shown in Fig. 2B, 100 μM RFP induced a significant increase in the protein expression of GRP78 beginning at 6 h ($p < 0.05$) and continued until 24 h ($p < 0.01$).

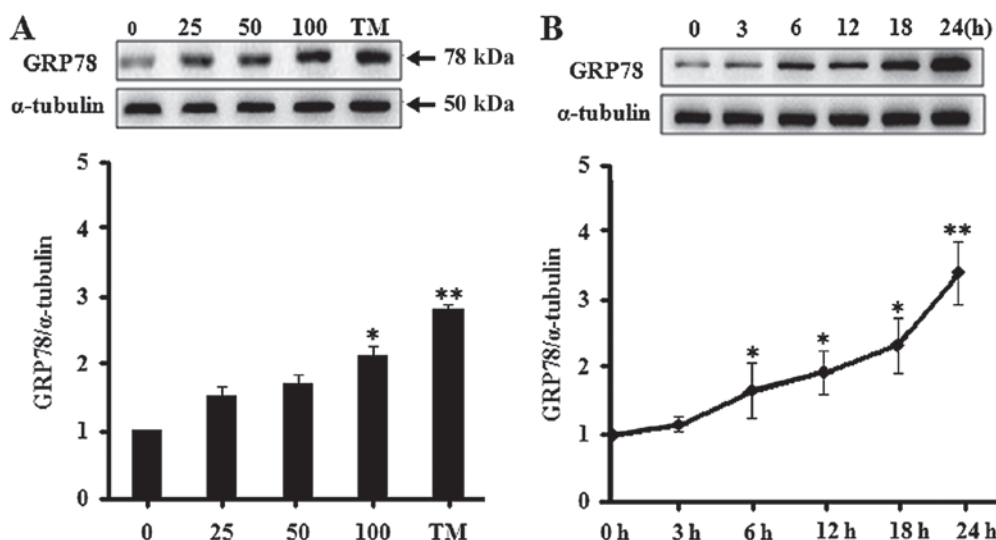


Figure 2. The impact of rifampicin (RFP) on the protein expression of glucose-regulated protein 78 (GRP78) in L02 cells. (A) Cells were treated with 25-100 μ M RFP or tunicamycin (TM) (2.5 μ g/ml) for 6 h. Western blot analysis showed that RFP upregulated GRP78 protein expression at 100 μ M. (B) Cells were treated with 100 μ M rifampicin for the indicated periods of time, and RFP significantly upregulated GRP78 protein expression at 6, 12, 18 and 24 h. The experiment was repeated three times, and the protein expression was normalized relative to the control cells, in which protein expression was deemed to be 1. The data are presented as the mean \pm SEM. * p <0.05 and ** p <0.01 compared with the control.

RFP effect on the IRE1 α -XBP1, PERK and ATF6 pathways in L02 cells. As known, the UPR includes three pathways, and we observed the effect of RFP on IRE1 α pathway, PERK pathway, and ATF6 pathway protein expression. As shown in Fig. 3A, cells were treated with RFP and TM for 6 h, there was a similar trend of p-eIf2 α protein expression with GRP78. RFP of 50 μ M induced p-eIf2 α protein expression (p <0.05) and 100 μ M RFP was stronger (p <0.01), while TM still had a more powerful activation. The other ER stress protein expression did not change significantly. Next we observed RFP and TM treatment for 24 h (Fig. 3B) 100 μ M RFP still increased GRP78, p-eIf2 α protein expression, while TM increased GRP78, p-eIf2 α , ATF4, XBP-1U and XBP-1S protein expression.

4-PBA inhibits ER stress protein expression in RFP-treated L02 cells. To determine whether 4-PBA could inhibit RFP and TM induced ER stress protein expression, L02 cells were treated with 2 mM 4-PBA for 2 h, followed by 100 μ M RFP and TM for 24 h. 4-PBA could cut down the expression level of GRP78 protein (all p <0.05) (Fig. 4). RFP had no activated effect on IRE1 α , p-IRE1 α , XBP-1U, XBP-1S protein expression, TM activated XBP-1U, XBP-1S protein expression, but the value of XBP-1S/XBP-1U had no difference between the other groups. 4-PBA could inhibit expression of XBP-1S protein obviously. After given RFP, p-eIf2 α /eIf2 α level was increased (p <0.05), 4-PBA could cut down the level of p-eIf2 α /eIf2 α (p <0.05). TM increased p-eIf2 α /eIf2 α level and ATF4 protein expression level (all p <0.05), 4-PBA could inhibit expression of these two proteins (all p <0.05). RFP and TM had no effect on ATF6 protein expression.

4-PBA effect on protein localization of GRP78 and p-eIf2 α in RFP-treated L02 cells. The expression of GRP78 and p-eIf2 α protein was minor in the control group, and the localization was mainly in the cytoplasm (Fig. 5). RFP treatment showed

increased staining in the cytoplasm, and after 4-PBA treatment showed decreased staining in the cytoplasm.

4-PBA effect on cell apoptosis survival and cell survival in RFP-treated L02 cells. We determined 4-PBA effect on RFP-treated cells by using FCA and MTT assay. We found that incubation of L02 cells with 100 μ M RFP and TM for 24 h had no obvious influence on the number of apoptotic cells compared to the control (Fig. 6A). After treatment with 4-PBA, the number of apoptotic cells significantly increased (p <0.01). Our MTT assay showed that 4-PBA decreased cell survival rate both in RFP treatment group and TM treatment group (all p <0.01) (Fig. 6B).

Discussion

DILI is often responsible for boxed warnings, drug non-approval or drug withdrawal, even in acute liver failure (ALF) (20). Many drugs cause hepatotoxicity, including over-the-counter (OTC) drugs (21), herbs and dietary supplements (22-24). The symptoms of DILI range from mildly elevated liver enzymes to severe hepatic damage like ALF. Mortality from DILI in individuals with pre-existing liver disease or concomitant severe skin reactions is significantly higher than patients without (25). Risk factors in DILI including drug factors such as threshold dose and lipophilicity, reactive metabolites and oxidative stress, mitochondrial hazards, hepatobiliary transporter inhibition, and host factors such as age, gender, race, genetic variants, nutrition, alcohol, smoking, underlying liver disease, comorbidities, drug-drug interactions and gut flora (26,27).

Antimicrobials, including antibacterial agents and anti-tuberculosis (anti-TB) agents, have been found to be the most common drugs leading to DILI (28). Anti-TB drugs, including RFP, isoniazid (INH) and pyrazinamide (PZA) probably carry some of the highest DILI risk of any medication, but are

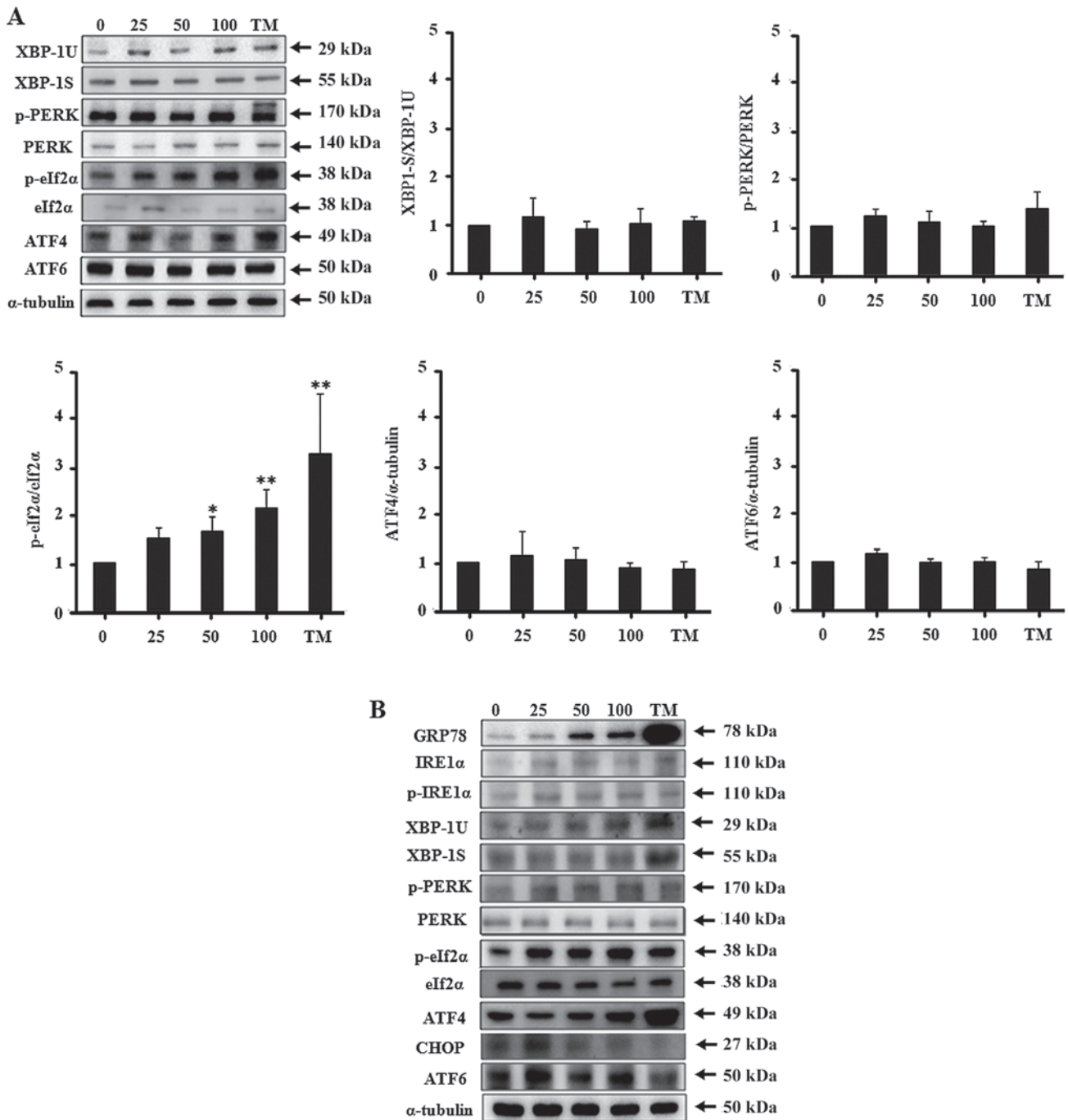


Figure 3. The impact of rifampicin (RFP) treatment for (A) 6 h and (B) 24 h on IRE1 α pathway, PKR-like ER kinase (PERK) pathway, and ATF6 pathway in L02 cells. (A) Cells were treated with 25-100 μ M RFP or tunicamycin (TM) (2.5 μ g/ml) for 6 h, western blot analysis showed that from 50 μ M RFP started to show an upregulated trend of p-eukaryotic initiation factor 2 α (eIf2 α) protein expression. The experiment was repeated three times, protein expression was relative to control cells, in which protein expression was deemed to be 1. Data are presented as mean \pm SEM. * p <0.05 and ** p <0.01 compared with the control. (B) Western blot analysis showed that glucose-regulated protein 78 (GRP78) and p-eIf2 α protein expression were elevated in RFP treatment group, while GRP78, p-eIf2 α , ATF4, X-box binding protein-1U (XBP-1U) and XBP-1S protein expression were elevated in TM treatment group.

less commonly prescribed compared to amoxicillin-clavulanate (29). Anti-TB drug-induced liver injury is a major cause of DILI in India and much of the developing world (30). RFP may occasionally disturb the bilirubin uptake in a dose-dependent manner, resulting in jaundice or unconjugated hyperbilirubinemia without hepatocellular damage (31). RFP occasionally cause hepatocellular injury has also been reported. The latency injury of RFP is 0-3 month. A unique feature of anti-TB drugs is the adaptation or tolerance to the drugs, which means the

patients present elevation of transaminases and/or bilirubin, without any clinical symptoms, which resolves with continuation of the drugs (30). Exposure to specific drugs may trigger physiologic adaptive responses. The activation of survival genes, including those that regulate antiinflammatory, antioxidant, and antiapoptotic pathways, may alleviate toxin-related harmful responses. Such injury may also stimulate protective adaptation and hepatocyte proliferation (31). Some evidence in human suggests that this adaptation response last for months

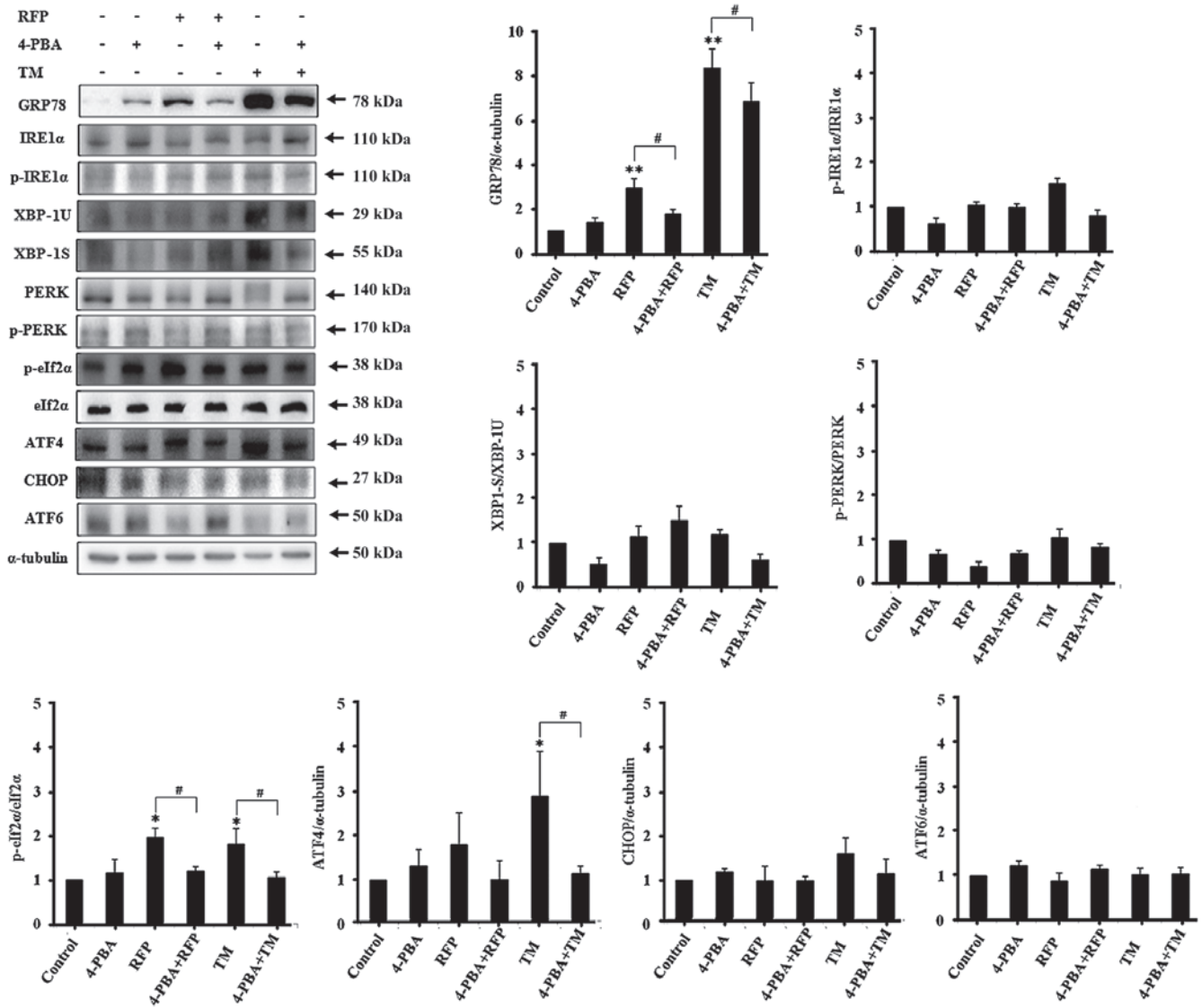


Figure 4. 4-Phenylbutyrate (4-PBA) inhibits glucose-regulated protein 78 (GRP78) and p-eukaryotic initiation factor 2α (eIf2α) protein expression in rifampicin (RFP) treated L02 cells. Cells were treated with 2 mM 4-PBA for 2 h, followed by 100 μM RFP or tunicamycin (TM) (2.5 μg/ml) for 24 h. Western blot analysis showed that RFP upregulated GRP78 and p-eIf2α protein expression. 4-PBA could cut down the expression level of GRP78 and p-eIf2α protein induced by RFP. The experiment was repeated three times, protein expression was relative to control cells, in which protein expression was deemed to be 1. Data are presented as mean ± SEM. *p<0.05 and **p<0.01 compared with the control; #P<0.05 compared between the groups.

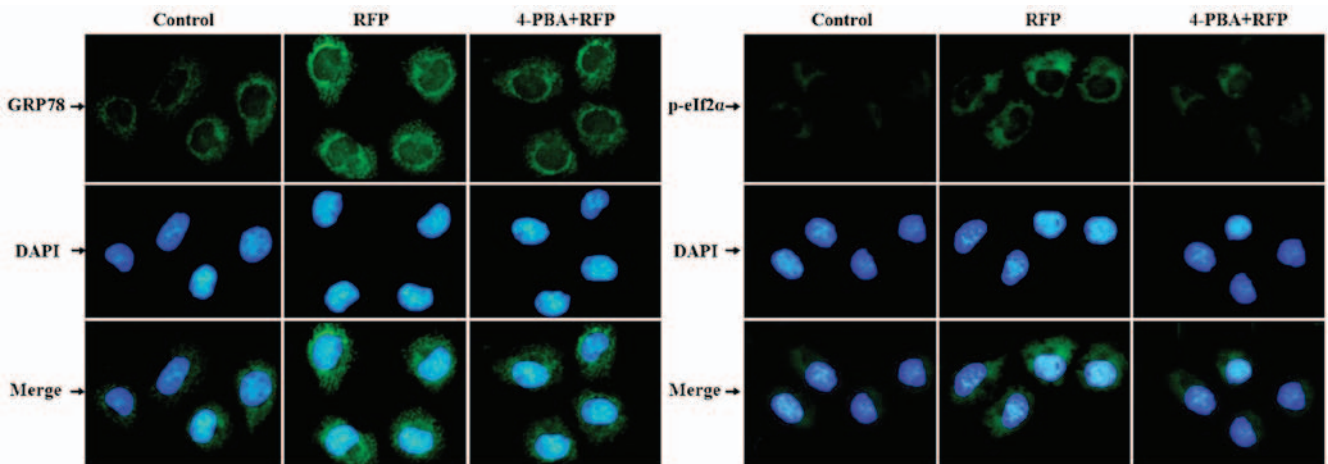


Figure 5. Immunostaining of glucose-regulated protein 78 (GRP78) and p-eukaryotic initiation factor 2α (eIf2α) (green) in L02 cells (x200). Cells were treated with 2 mM 4-phenylbutyrate (4-PBA) for 2 h, followed by 100 μM rifampicin (RFP) for 24 h. The nuclei were counterstained with DAPI (blue), the final merged image is a representative of three independent experiments.

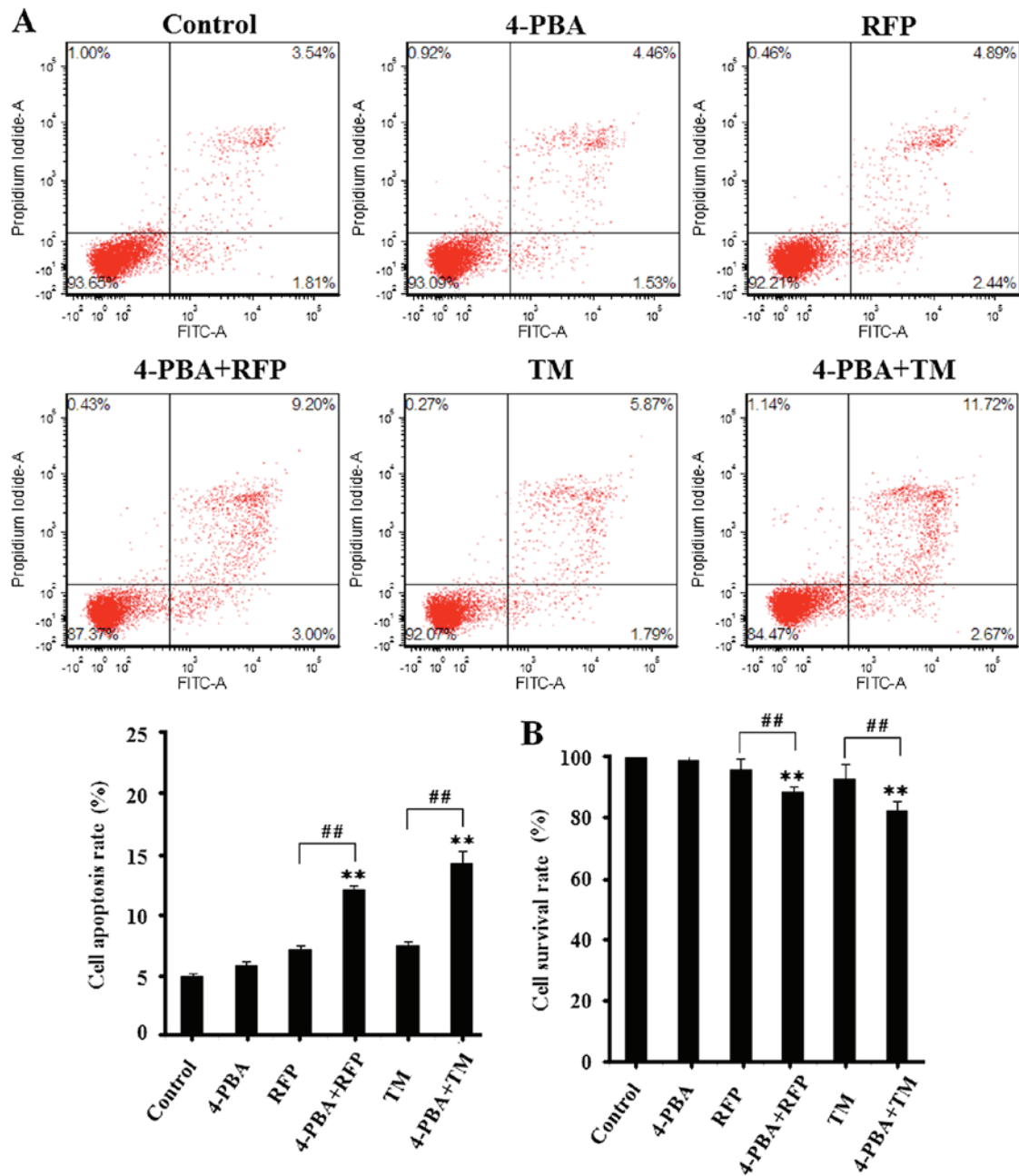


Figure 6. 4-Phenylbutyrate (4-PBA) increases rifampicin (RFP) induced L02 cells apoptosis and decreases cell viability. Cells were treated with 2 mM 4-PBA for 2 h, followed by 100 μ M RFP or tunicamycin (TM) (2.5 μ g/ml) for 24 h. (A) FCA showed that RFP cotreatment with 4-PBA increased apoptosis obviously. (B) MTT assay showed that RFP cotreatment with 4-PBA decreased cell viability obviously. The same trend was found in TM treatment group. Data are present as mean \pm SEM of three independent experiments. ** $p < 0.01$ compared with the control; ## $p < 0.01$ compared between the groups.

after a mild DILI occurred (32). Thus, awareness of the phenomenon of adaptation is critical in tuberculosis treatment, helps to avoid unnecessary drug withdrawal so as to prevent the deterioration of the condition.

Recent studies suggested that ER stress plays an important role in DILI, in addition to oxidative stress, mitochondrial dysfunction, and many other mechanisms (33). ER stress activates an adaptive response termed the UPR to restore the ER homeostasis. If restored the ER homeostasis is unsuccessful, the UPR initiates a proapoptotic response to clear dysfunctional cells (34). The UPR has three main function: reduction in protein translation to decrease ER protein load; increase the ability of protein folding and export capacity of the ER,

and activation of ER-associated degradation (ERAD) (9). An adaptive UPR helps to maintain ER homeostasis, it also regulates dynamic interactions between ER and mitochondria and autophagy to support ER function (35). Activation of the UPR promotes axonal regeneration after peripheral nerve injury (36). Inhibition of the UPR by hypoxia reduces ER-to-Golgi protein trafficking and increases cell death in mouse β cells (37). The ability to maintain the adaptive UPR in islets may protect obese mice against diabetes development (38).

In this study, we found that RFP upregulated GRP78 expression in a dose- and time-dependent manner, indicating that RFP was a GRP78 inducer. GRP78 belongs to the heat shock 70

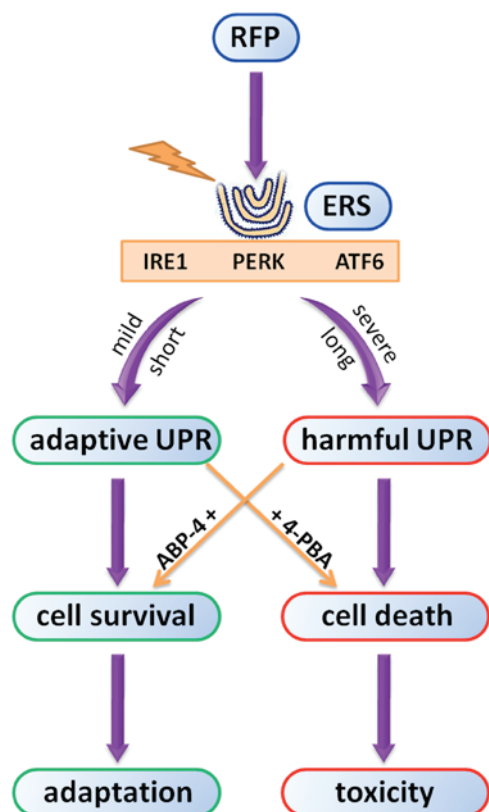


Figure 7. Possible schematic model of how unfolded protein response (UPR) affects rifampicin (RFP)-induced hepatotoxic effects. When the degree of endoplasmic reticulum (ER) stress induced by RFP is relatively mild and the duration is short, adaptive UPR was activated to promote cell survival. At this time 4-phenylbutyrate (4-PBA) inhibits the UPR increased cell death instead. If the degree of ER stress induced by RFP is relatively severe and the duration is long, harmful or injurious UPR was activated leading to cell death. At this point 4-PBA inhibition of UPR is good for cell survival. Cell survival leads to adaption of the drug, and cell death contributed to the drug toxicity.

protein (HSP70) family, during ER stress GRP78 binds the unfolded proteins releasing the three UPR sensors (39). Due to its multiple functional roles in protein folding, controlling the activation of transmembrane ER stress sensors, and ER calcium binding, GRP78 is a central regulator of ER homeostasis. Study shows that GRP78 is required for cell proliferation (40). Overexpression of GRP78 enhanced cell proliferation in chondrocyte development, and inhibited ER stress-mediated apoptosis (41). GRP78 heterozygosity attenuates diet-induced obesity and insulin resistance by promoting adaptive UPR (42). Liver GRP78 was required for neonatal survival, and a loss of GRP78 in the adult liver greater than 50% caused dilation of the ER compartment and an ER stress response, with the increase of apoptosis at the same time. 4-PBA could alleviate these disorders. Therefore, it is convincing that GRP78 is an important factor in adaptive UPR and as a protection.

The UPR is initiated by activation of specialized sensors including IRE1 α , PERK and ATF6. The contribution of each pathway to the disease process is complex and highly dependent on the disease-triggering mechanisms and the cell type affected (36). IRE1 α catalyzes the splicing of the mRNA encoding for XBP1, resulting in the production of an active transcription factor termed XBP1s, which is essential for the expression of genes involved in protein secretion, folding and ERAD. PERK

phosphorylation of eIF2 α represses global protein synthesis, lowering influx of nascent polypeptides into the stressed ER, coincident with preferential translation of ATF4. In cultured cells, ATF4 induces transcriptional expression of genes directed by the PERK arm of the UPR, including genes involved in resistance to oxidative stress, amino acid metabolism, and the proapoptotic transcription factor CHOP (GADD153/DDIT3) (43). ATF6 is also important for cell growth and ER stress-mediated apoptosis. We investigated the effects of RFP on the protein expression of the three pathways. We found that RFP had no significant effect on the protein expression except for p-eIF2 α . p-eIF2 α is primarily a stress signal, it evokes adaptive or apoptotic responses depending on its cellular location, coincident signaling activities, changes in gene expression, and inter-protein interactions (44). p-eIF2 α reduces general translation initiation, but for select transcripts such as ATF4, it will facilitate the preferential translation (45). p-eIF2 α is more relevant to acute toxicity, the activation time start early (46). p-eIF2 α triggers an adaptation process in glucose-deficient cells, contributing to cell survival (47). Other studies also shown that p-eIF2 α plays a cytoprotective role and contribute to cell survival (48-50).

TM is generally used to induce ER stress experimentally as it is an N-linked glycosylation inhibitor. TM significantly increased the expression of XBP-1S, p-eIF2 α and GRP78 in the cerebral cortex and cerebellum of mouse (51). TM increased the expression of p-eIF2 α and ATF4 in neuroblastoma cells (52). GRP78 was elevated in TM-treated HepG2 and SMMC-7721 cells (53). GRP78, p-PERK, XBP-1S, p-IRE1 were activated by TM in skeletal muscle cells (54). In this study, we found that TM upregulated GRP78, XBP-1U, XBP-1S, p-eIF2 α and ATF4, suggested that the ER stress is also widely activated.

4-PBA, an aromatic fatty acid analog, is used to treat urea cycle disorders. Moreover, 4-PBA is also a chemical chaperone and histone deacetylase inhibitor. 4-PBA significantly reduced GRP78, ATF4 in a rat unilateral ureteral obstruction (UUO) model (55). 4-PBA reduced levels of both p-eIF2 α and CHOP, reduced apoptosis in mice of liver ischemia reperfusion injury (56). 4-PBA reduced p-eIF2 α expression in mouse livers exposed to PFOA (57). In this study, we found that 4-PBA reduced levels of both GRP78 and p-eIF2 α induced by RFP, reduced levels of both GRP78, p-eIF2 α and ATF4 induced by TM, along with the increased cell apoptosis rate and the decreased cell survival rate, this shows that after the inhibition of UPR, cell damage is more serious. This proves that when the degree of UPR induced by RFP is relatively mild, adaptive UPR is helpful for cell survival which is associated with upregulated expression of GRP78 and p-eIF2 α . We speculate that microscopic changes of cells may appear early after given RFP, the cells reduce damage by some adaptive responses, including adaptive UPR, which contribute to cell survival and the adaption of the drug (Fig. 7). Future animal studies may further verify this mechanism.

Acknowledgements

Not applicable.

Funding

We are grateful for funding support by the National Natural Science Foundation of China, grant no. 81370529.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JX designed the research, WZ performed the research, and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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