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Sodium tauroursodeoxycholate prevents paraquat-induced cell death by suppressing endoplasmic reticulum stress responses in human lung epithelial A549 cells

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ABSTRACT

Paraquat is a commonly used herbicide; however, it is highly toxic to humans and animals. Exposure to paraquat causes severe lung damage, leading to pulmonary fibrosis. However, it has not been well clarified as how paraquat causes cellular damage, and there is no established standard therapy for paraquat poisoning. Meanwhile, endoplasmic reticulum stress (ERS) is reported to be one of the causative factors in many diseases, although mammalian cells have a defense mechanism against ERS-induced apoptosis (unfolded protein response). Here, we demonstrated that paraquat changed the expression levels of unfolded protein response-related molecules, resulting in ERS-related cell death in human lung epithelial A549 cells. Moreover, treatment with sodium tauroursodeoxycholate (TUDCA), a chemical chaperone, crucially rescued cells from death caused by exposure to paraquat. These results indicate that paraquat toxicity may be associated with ERS-related molecules/events. Through chemical chaperone activity, treatment with TUDCA reduced paraquat-induced ERS and mildly suppressed cell death. Our findings also suggest that TUDCA treatment represses the onset of pulmonary fibrosis caused by paraquat, and therefore chemical chaperones may have novel therapeutic potential for the treatment of paraquat poisoning.

1. Introduction

Although highly toxic to humans and animals, paraquat (1,1′-dimethyl-4,4′-bipyridinium chloride) is one of the most commonly used herbicides in the world. Paraquat poisoning can result in fatal damage to multiple organs, especially the lungs, where it causes pulmonary fibrosis via development of excessive fibrotic responses [1,2]. Paraquat toxicity is characterized by the development of lung edema and epithelial cell damage, which progresses to fibroblast proliferation and accumulation [3,4]. The pathogenesis of paraquat toxicity is probably due to a result of lung epithelial cell death [5]. In the present study, we suspected that epithelial cell death from paraquat poisoning was probably due to endoplasmic reticulum (ER) stress (ERS)-induced apoptosis.

ER is a cellular organelle with several important functions, including the synthesis, folding and glycosylation of proteins. Various stresses, such as oxidative stress and hypoxia, lead to the accumulation of unfolded proteins in ERS, which can induce apoptosis [6]. ERS is reportedly responsible for the onset of diseases such as Parkinson's disease [7], diabetes mellitus [8,9], and rheumatoid arthritis [10,11], suggesting that unfolded proteins may play an etiological role in certain diseases. Sodium tauroursodeoxycholate (TUDCA), a low-molecular-weight compound and a human bile salt, has chemical chaperone activity, which is a prerequisite in resolving ERS by reducing the load of misfolded proteins [12]. Thus, TUDCA improves protein-folding capacity in ER and facilitates trafficking of mutant proteins, resulting in the suppression of ERS-induced cell death [12,13]. We have previously reported that 4-phenyl butyrate, a chemical chaperone, rescues cells from ERS-induced cell death, and suppresses the upregulation of ERS-related molecules, such as immunoglobulin H (IgH) chain-binding protein (Bip) and glucose-regulated protein (GRP) 94, as

Abbreviations: ER, endoplasmic reticulum; ERS, endoplasmic reticulum stress; UPR, unfolded protein response; HRD1, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase degradation 1; SEL1L, suppressor/enhancer lin12 1-like; ERAD, endoplasmic reticulum associated degradation; TUDCA, sodium tauroursodeoxycholate; GRP, glucose-regulated protein; Bip, immunoglobulin H (IgH) chain binding protein; PERK, protein kinase RNA-like endoplasmic reticulum kinase; eIF2α, eukaryotic initiation factor 2α; IRE1, inositol-requiring protein-1; ATF6, activating transcription factor 6; XBP1, X-box binding protein 1.

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well as relevant events such inhibiting phosphorylation of the eukaryotic initiation factor 2α (eIF2α) through its chemical chaperone activity and so on [14].

Mammalian cells react to the presence of unfolded proteins by inducing an unfolded protein response (UPR) against ERS, which represses protein synthesis by triggering phosphorylation of the eIF2α via activation of the protein kinase RNA-like ER kinase (PERK) [15], and which induces ER chaperones to promote appropriate folding of proteins via the activation of activating transcription factor 6 (ATF6) [16]. In addition, degradation of unfolded proteins is induced by ER-associated degradation (ERAD) via activation of the inositol-requiring protein-1 (IRE1)-X-box binding protein 1 (XBP1) pathway or ATF6 pathway in order to avoid accumulation of unfolded proteins in cells. During ERS, *XBP1* mRNA is alternatively spliced and activated by IRE1α [17], and ATF6 is activated, resulting in a cleaved form [18]. In the ERAD system, ubiquitin ligase (E3) mediates the ubiquitination of unfolded proteins for their degradations [19,20]. ERAD-associated E3, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase degradation (HRD) 1, is reported to prevent ERS-induced apoptotic cell death [21]. We have previously shown that suppressor/enhancer lin12 1-like (SEL1L) interacts with and stabilizes HRD1, indicating that the HRD1–SEL1L complex promotes the degradation of unfolded proteins [22–24]. It has also been reported that HRD1 is induced via the IRE1α-XBP1 and ATF6 pathways, while SEL1L is induced via the ATF6 pathway [25].

Based on these previous findings, we hypothesized that paraquat causes ERS and induces ERS-related molecules/events in a human pulmonary epithelial cell line (A549) to inflict cell death. Moreover, TUDCA rescues cells from paraquat-induced cytotoxicity via its chemical chaperone activity. In this study, we investigated if ERS-related molecules/events (especially ERAD-related molecules) were involved in paraquat-induced cell death, and further examined whether TUDCA could prevent paraquat-induced cell death via ERS suppression.

2. Materials and methods

2.1. Reagents and antibodies

Paraquat, TUDCA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay reagents and anti-HRD1 polyclonal antibody (pAb; C-terminal) were commercially obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). The anti-parkin pAb, anti-caspase-3 (Asp175) pAb, anti-eIF2α pAb, anti-phospho-eIF2α (Ser51) pAb, and anti-β-actin pAb were commercially procured from Cell Signaling Technology, Inc. (Danvers, MA, USA). The anti-SEL1L monoclonal antibody (mAb), anti-ATF6 mAb, anti-KDEL mAb (SPA-827) and anti-C/EBP homologous protein (CHOP) mAb were products of Enzo Life Sciences International, Inc. (Plymouth Meeting, PA, USA), IMGENEX Corporation (San Diego, CA, USA), StressGen Biotechnologies (Ann Arbor, MI, USA) and Thermo Fisher Scientific, Inc. (Waltham, MA, USA), respectively. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG from sheep and anti-rabbit IgG from donkey were purchased from GE Healthcare (Piscataway, NJ, USA) for use as secondary antibodies.

2.2. Cell culture

Human pulmonary type-II-like epithelial A549 cells in Dulbecco's modified Eagle's medium [supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Biowest S.A.S., Nuaillé, France) containing penicillin (100 units/ml) and streptomycin (100 μg/ml) (GIBCO, Invitrogen Corporation, Grand Island, NY, USA)] were seeded in a 6-well plate at a density of 1.1×10^5 cells/cm^2, and cultured in a humidified incubator (5% CO_2/95% air atmosphere) at 37 °C.

2.3. RNA preparation and reverse transcription (RT)

RNA was isolated from cells using the QIAshredder Kit and RNeasy Plus Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the corresponding manufacturer's instructions. Random-primed cDNAs were prepared from 2 μg of total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Prepared cDNAs were used as template for quantitative real-time polymerase chain reactions (PCRs) and RT-PCR.

2.4. Quantitative real-time PCR

Quantitative real-time PCR was performed as previously described [17]. Briefly, a cDNA sample (5 ng) was placed in 96-well microtiter plates containing the reaction mixture and specific primers for the mRNA sequences of target genes. The protocol consisted of an initial denaturation step at 95 °C for 5 min, followed by 45–55 cycles at 95 °C for 10 s, 60 °C for 25 s, and 72 °C for 10 s before cooling the reaction mixture to 40 °C. The mRNA levels of each gene were quantified relative to the second-derivative comparative Ct method using Lightcycler 480 SW 1.5 software (Roche Diagnostics GmbH), and the differences in gene expression levels were calculated by normalizing to the expression levels in untreated cells (controls).

2.5. Analysis of XBP1 mRNA splicing

XBP1 mRNA-splicing analysis was performed as described previously [17]. Briefly, PCR was performed with forward and reverse primers to amplify the cDNA of *XBP1* using a Takara PCR Thermal Cycler Personal (Takara Bio Inc., Otsu, Japan) after RT performance of total cellular mRNA. Fragments of unspliced (*XBP1*-u) and spliced (*XBP1*-s) *XBP1* were detected as 442-bp and 416-bp nucleotides, respectively. *β-Actin* was used as the loading control.

2.6. Immunoblotting

Immunoblotting analyses were performed as previously described [17]. Briefly, cells (after rinsing with ice-cold PBS) were lysed in 100 μl of ice-cold lysis buffer containing 20 mM HEPES (pH 7.4), 120 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 10 mM sodium fluoride, 2 mM sodium vanadate, and a protease inhibitor cocktail (EDTA-free complete type; Roche Diagnostics GmbH) before removal of cellular debris from the lysate by centrifugation at 15,000 ×*g* for 20 min at 4 °C. After boiling with Laemmli buffer for 5 min, proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred onto a polyvinylidene fluoride membrane. The membrane was incubated with the corresponding antibodies in 10 mM Tris-buffered saline with 0.1% Tween 20 (TBST; pH 7.5), washed in TBST, and then probed with HRP-conjugated anti-mouse or anti-rabbit IgG antibodies (dilution of 1:10,000). The membrane was incubated with a chemiluminescent reagent (ECLplus, GE Healthcare), and the proteins were visualized with LumiViewer EX140 (AISIN, Aichi, Japan).

2.7. Cell viability

A549 cells were treated with paraquat, TUDCA, and paraquat/TUDCA for 24 h. Cell viability was measured using the MTT assay according to a previously described method [17].

2.8. Statistical analyses

The data were represented as the mean ± standard error of the mean (SEM) and were analyzed by the one-way analysis of variance (ANOVA) method, followed by the Dunnett's correction for multiple comparisons.

3. Results

3.1. Paraquat-induced cell death and activated caspase-3 in human lung epithelial A549 cells

In order to assess the cytotoxicity of paraquat, we treated A549 cells with various concentrations (0–0.5 mM) of paraquat for 24 h before measuring the cell viability using the MTT assay method (Fig. 1A). Paraquat exposure inflicted A549 cell death in a concentration-dependent manner, and drastically decreased cell viability at 0.2–0.3 mM. In addition, treatment with paraquat significantly increased the levels of cleaved caspase-3, the activated form of proapoptotic protein caspase-3 (Fig. 1B).

3.2. Paraquat changed the expression of UPR-related molecules in A549 cells

To clarify the involvement of ERS responses in paraquat-induced A549 cell death, the mRNA expression of UPR-related genes was investigated. Paraquat in concentrations up to 0.15 mM increased *CHOP* and *BIP* mRNA levels, where the former is an ERS-associated apoptosis factor, while the latter is an ERS-inducible molecular chaperone (Fig. 2A and B). In addition, ubiquitin ligase *HRD1* mRNA levels were significantly upregulated ($p < 0.01$) following exposure to 0.15 mM paraquat, whereas its mRNA levels fell below the baseline level when the cells were treated with paraquat at doses above 0.15 mM (Fig. 2C). Similar results were obtained for *SEL1L* mRNA levels (Fig. 2D), which have been previously reported to stabilize HRD1 proteins [23]. In contrast,

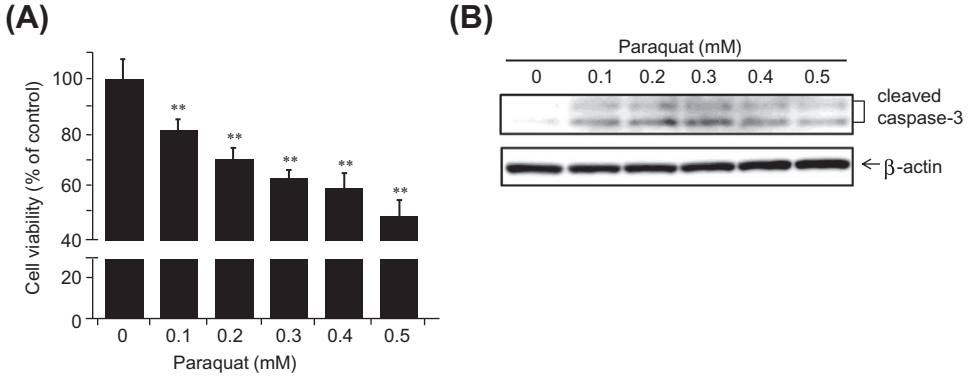

Fig. 1. Paraquat-induced cell death and caspase-3 activation. Human lung epithelial A549 cells were treated with paraquat (0–0.5 mM) for 24 h. (A) Cell viability was determined by the colorimetric MTT assay. Data are expressed as the mean ± standard error of the mean (SEM) of at least three independent experiments. (B) Representative immunoblot images of cleaved caspase-3 and β-actin are shown. Differences where *p*-values less than 0.01 (**) were considered significant when compared with controls using the Dunnett's test for one-way ANOVA.

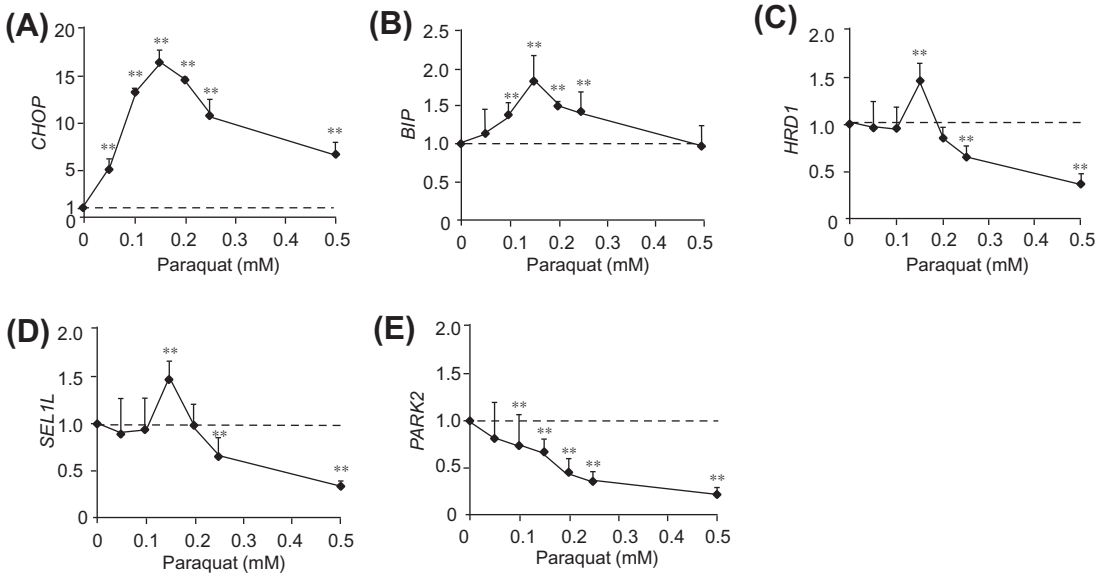

Fig. 2. Effects of paraquat on mRNA expression levels of unfolded protein response (UPR) genes ((A): *CHOP*, (B): *BIP*, (C): *HRD1*, (D): *SEL1L*, and (E): *PARK2*). A549 cells were treated with paraquat (0–0.5 mM) for 24 h. The mRNA expression levels were measured with the TaqMan-based quantitative real-time PCR assay. Each mRNA expression level was normalized to 18S rRNA and plotted relative to the control value (designated as 1.0). Data are expressed as the mean ± SEM of at least three independent experiments. When compared with controls, differences where *p*-values less than 0.05 (*) or 0.01 (**) were considered significant using the one-way ANOVA and Dunnett's tests, respectively.

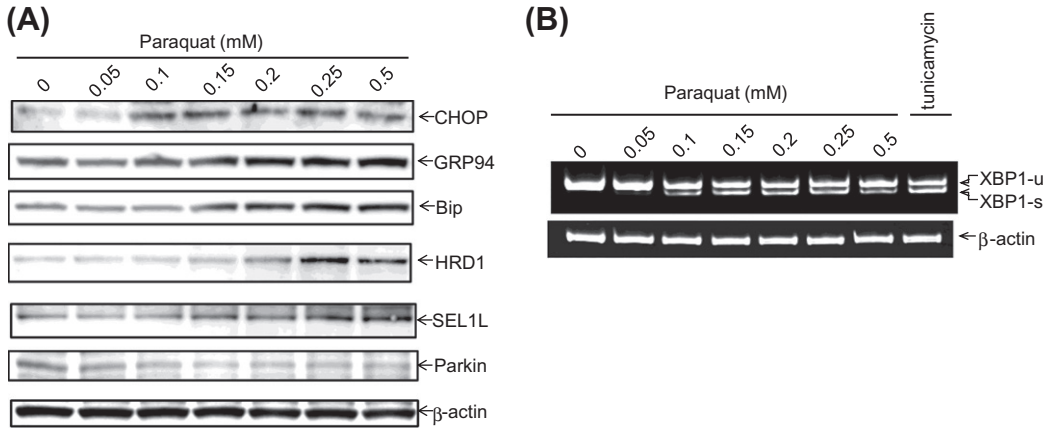

Fig. 3. Paraquat changed UPR-related protein levels and induced splicing of *XBP1* mRNA. (A) A549 cells were treated with paraquat (0–0.5 mM) for 24 h before being subjected to immunoblotting analysis with each antibody. Representative immunoblot images from at least three independent experiments using CHOP, GRP94, Bip, HRD1, SEL1L, parkin, and β-actin are shown. (B) A549 cells were treated with paraquat (0–0.5 mM) for 24 h. Total cellular RNA was prepared and subjected to RT-PCR using specific primers (*XBP1* and β-*actin*). The PCR products from spliced and unspliced mRNAs are indicated as *XBP1*-s and *XBP1*-u, respectively. Representative images from at least three independent experiments are shown. Tunicamycin (5 μg/ml), an ERS-inducer, was used as a positive control for *XBP1* splicing.

the mRNA levels of *PARK2*, also known as ERAD-related ubiquitin ligase [26], were reduced in a concentration-dependent manner in the presence of 0–0.5 mM paraquat (Fig. 2E).

When we next examined if these alterations in mRNA levels were also observed at the protein level, we confirmed that Bip and GRP94 proteins, ERS-sensitive molecular chaperones, and CHOP protein were increased following paraquat treatment (Figs. 3A and S1). An elevated HRD1 protein expression was also observed in a concentration-dependent manner. A result similar to that for HRD1 protein was obtained by immunoblotting analysis of SEL1L. As observed for the mRNA levels, parkin protein levels declined in a concentration-dependent manner as well.

3.3. Paraquat activated XBP1 mRNA, eIF2α and ATF6 proteins

To confirm if the upregulation of UPR components, especially ERAD components, was due to the ERS response, we examined the induction effect of paraquat on splicing of *XBP1* mRNA. The results indicated that paraquat induced *XBP1* mRNA activation in the concentration range of 0.1–0.5 mM (Fig. 3B). Tunicamycin, an ERS-inducer, was used as a positive control for XBP1 activation. We also found that eIF2α and ATF6 were also activated by exposure to paraquat (Fig. 4A).

3.4. TUDCA prevented cell death caused by exposure to paraquat by the alleviation of ERS responses

We assessed TUDCA cytotoxicity using the MTT assay in A549 cells, and found that TUDCA alone did not lead to cell death at concentrations between 0 and 0.5 mM (data not shown). Next, we tested via the MTT assay whether TUDCA prevented A549 cell death under paraquat-induced ERS. Paraquat exposure (0.25 mM for 24 h) resulted in cell death; the cell viability was 69% compared with controls. However, concomitant treatment of paraquat with TUDCA significantly prevented paraquat-induced cell death in a concentration-dependent manner (Fig. 4B). Furthermore, when we tested if TUDCA rescued cells from paraquat-induced ERS responses and apoptosis by immunoblotting analysis of ERS-marker proteins and cleaved caspase-3, the Bip, GRP94, HRD1 and SEL1L levels were upregulated, and caspase-3 was cleaved by paraquat (Figs. 4A and S2). However, the paraquat-induced increment of protein levels and cleavage of caspase-3 were suppressed by TUDCA. We also confirmed that TUDCA suppressed paraquat-induced decreases in parkin protein. Furthermore, TUDCA treatment suppressed the activation of ATF6 and phosphorylation of eIF2α induced by paraquat.

4. Discussion

Paraquat is highly toxic to humans; however, the molecular mechanisms of paraquat poisoning that causes cell death and pulmonary fibrosis have not been established. Although many approaches to treat paraquat poisoning have been reported, an effective treatment for paraquat injury has not been identified to date. In this study, we demonstrated that exposure to paraquat increased the expression of UPR-related molecules/events and induced ERS via the IRE1-XBP1, ATF6, and eIF2α pathways. In addition, treatment of a human pulmonary cell line (A549) with TUDCA, a chemical chaperone, moderately rescued paraquat-induced cell death.

Human lung epithelial A549 cells, an *in vitro* model cell line reported to be useful for the investigatation of injury due to pulmonary fibrosis [27–29], were treated with paraquat at concentrations of up to 0.5 mM. The present study confirmed that exposure to paraquat at doses of up to 0.3 mM caused caspase-3-dependent cell death in a concentration-dependent manner. However, exposure to paraquat at doses above 0.3 mM appeared to cause cell death in a caspase-3-independent manner, since cleaved caspase-3 levels did not increase in cells treated with these paraquat doses, although cell viability continued to decrease. Thus, lower doses of paraquat induced cell death by caspase-3 activation (i.e., apoptosis). From this result, we assumed that paraquat caused apoptosis resulting from ERS response, which has recently been reported to be one of the causative factors in caspase-3 activation [6,30].

Next, we observed that paraquat induced ERS by upregulating mRNAs of *CHOP* and *BIP*, proteins of CHOP, as well as Bip and GRP94. The different expression levels observed between mRNA and protein levels might be due to disruption of the cellular protein quality control system when exposed to high concentrations of paraquat. Thus, paraquat at lower concentrations would activate the ERS response, inducing the UPR genes (including ERAD components) to promote appropriate folding of proteins or degradation of unfolded proteins. As a result, cell death was inhibited in eukaryotic cells. However, various cellular organelles (including ER,

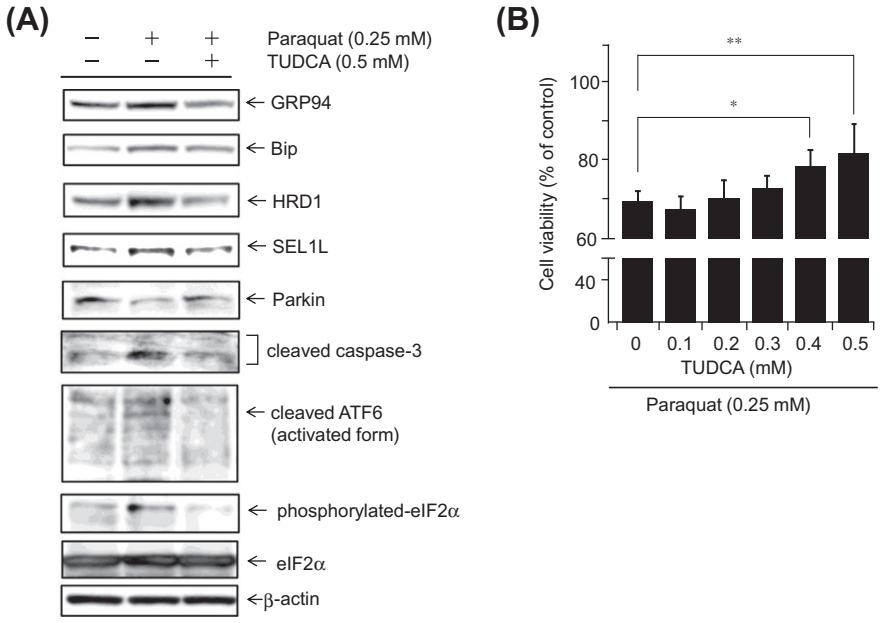

Fig. 4. TUDCA suppressed paraquat-induced ERS and cell death. (A) A549 cells were treated with 0.25 mM paraquat and/or 0.5 mM TUDCA for 24 h. Representative immunoblot images of GRP94, Bip, HRD1, SEL1L, parkin, cleaved caspase-3, phosphorylated-eIF2α, eIF2α, ATF6 (cleaved form), and β-actin are portrayed. (B) A549 cells were treated with 0.25 mM paraquat and TUDCA (0–0.5 mM) for 24 h. Cell viability was determined by the colorimetric MTT assay. Data are expressed as the mean ± SEM of at least three independent experiments. Differences where *p*-values less than 0.05 (∗) or 0.01 (∗∗) were considered significant when compared with the 0.25 mM paraquat-treated cells by the Dunnett's test for one-way ANOVA.

mitochondria, and cytosol) could be severely damaged when exposed to high doses of paraquat, leading to a reduced turnover of UPR proteins. Hence, the overall protein levels are considered to increase superficially at high concentrations of paraquat in spite of a decrease in mRNA levels.

Parkin (*PARK2*) was downregulated at both the mRNA and protein levels, although E3, HRD1 and SEL1L (a HRD1 stabilizer [23]) were temporally upregulated. These differences may be accounted for by the following two hypotheses: (1) the HRD1–SEL1L complex prevented cell death caused by paraquat; and (2) parkin was not involved in paraquat toxicity. HRD1 should be a more important E3 than parkin for influencing unfolded substrates, since it has been reported that unfolded proteins do not accumulate on a massive scale in *Parkin* knockout mice [31]. As such, the HRD1–SEL1L complex would have an important role in preventing paraquat toxicity. Furthermore, *PARK2* mRNA and parkin protein levels were downregulated in a concentration-dependent manner in this study, suggesting that parkin could serve as an index for paraquat poisoning.

The most important finding in this study was that TUDCA, a chemical chaperone that prevents ERS-induced apoptosis [12,13], prevented cell death and caspase-3 activation resulting from exposure to low levels of paraquat in a concentration-dependent manner, although the protective effect was limited in its potency. Furthermore, TUDCA suppressed paraquat-induced ERS response through the inactivation of ATF6 and eIF2α. Our present finding is supported by an *in vivo* study which shows that TUDCA inhibits the paraquat-induced apoptosis of cardiac myocytes [32]. Thus, we inferred that TUDCA would suppress cell death by inhibiting pulmonary fibrosis caused by exposure to paraquat *in vivo*, although it may be necessary to develop an appropriate administration route or chemical manipulation/design to effectively utilize TUDCA (or its derivatives) at useful doses/potency *in vivo*. However, TUDCA could not suppress cell death induced by higher concentrations of paraquat (data not shown), indicating that TUDCA might be effective in treating low-dose paraquat poisoning. Another chemical chaperone, 4-Phenyl butyrate, protects cell death when administered after the application of an ERS-inducer *in vivo* [33]. In short, it is possible that TUDCA treatment may be effective even if given after low-dose paraquat exposure.

In summary, this study suggests that ERS response may be involved in the pathogenesis of paraquat poisoning, and that parkin may serve as a useful index/parameter for paraquat poisoning. In addition, TUDCA inhibited the ERS response, and led to modest suppression of caspase-3 activation and cell death caused by exposure to paraquat. Presently, there are no efficient therapeutic agents for paraquat poisoning. If the protective effects of TUDCA against pulmonary fibrosis can be definitively demonstrated *in vivo*, TUDCA can be used as a therapeutic agent for paraquat poisoning and pulmonary fibrosis related to ERS in animals and humans.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.131.

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