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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].