Toxicology in Vitro 46 (2018) 58–65

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Eukaryotic translation initiation factor 2 subunit α (eIF2α) inhibitor salubrinal attenuates paraquat-induced human lung epithelial-like A549 cell apoptosis by regulating the PERK-eIF2α signaling pathway

Rui Wang, Da-Zhuang Sun, Chun-Qing Song, Yong-Min Xu, Wei Liu, Zhi Liu, Xue-Song Dong*

Department of Emergency, The First Affiliated Hospital, China Medical University, Shenyang 110001, China

ARTICLE INFO

Keywords:
Paraquat
eIF2α inhibitor
Salubrinal
Endoplasmic reticulum stress
Apoptosis
PERK

ABSTRACT

Paraquat (PQ), as one of the most widely used herbicides in the world, can cause severe lung damage in humans and animals. This study investigated the underlying molecular mechanism of PQ-induced lung cell damage and the protective role of salubrinal. Human lung epithelial-like A549 cells were treated with PQ for 24 h and were pre-incubated with salubrinal for 2 h, followed by 500 μM of PQ treatment. Silencing eIF2α gene of the A549 cells with siRNA interference method was conducted. Cell morphology, cell viability, apoptosis and caspase-3 activity were assessed by different assays accordingly thereafter. The expression of PERK, p-PERK, ATF6, c-ATF6, IRE1α, p-IRE1α, CHOP, GRP78, p-eIF2α and β-actin was assayed by western blot. The data showed that PQ significantly reduced A549 cell viability, changed cell morphology, induced cell apoptosis and significantly upregulated the levels of GRP78, CHOP, p-PERK, c-ATF6 and p-IRE1α. However, 30 μM salubrinal could attenuate the effects of PQ on damages to A549 cells through upregulating p-eIF2α. In contrast, knocking down eIF2α gene inhabited the effects of salubrinal. These results suggest that PQ-induced A549 cell apoptosis involved endoplasmic reticulum (ER) stress, specially the PERK-eIF2α pathway. Salubrinal attenuated A549 cells from PQ-induced damages through regulation of the PERK-eIF2α signaling.

1. Introduction

Paraquat (1, 1′-dimethyl-4, 4′-bipyridinium dichloride; PQ) is a bipyridyl compound, and is one of the most widely used herbicides worldwide. However, PQ is highly toxic to mammals including animals and human beings, even if ingested in very small amounts (Gunnell et al., 2007). Furthermore, the intentional and accidental ingestion of PQ is fatal. In many countries, PQ has become a popular suicide pesticide or PQ pot due to its ease of access, extremely low cost, and high mortality rate (Hwang et al., 2002). Ingestion of PQ would rapidly uptake into the blood stream and lead to multiple organ damage (Onyon and Volans, 1987). For example, PQ causes severe damage to lung epithelial cells and induces acute respiratory distress syndrome (ARDS) in experimental animals and human beings, as well as alveolar hemorrhage, interstitial edema and inflammation, eventually resulting in lung fibrosis (Fukuda et al., 1985; Takahashi et al., 1994; Tomita et al., 2007). ARDS and pulmonary interstitial fibrosis are the most important causes of death in patients. Chronic PQ exposure is also linked to the development of Parkinson's disease (Kamel, 2013; Wang et al., 2011). Therefore, determining the underlying molecular mechanism responsible for PQ-induced organ damage would provide useful information to control the unwanted side effects of this herbicide. However, this study mainly focused on lung damage induced by PQ.

Alveolar epithelial cells are cells that line the alveoli of the lungs and play an important role in the oxygen-carbon dioxide exchange function of the lungs. The alveolar epithelium contains a continuous layer of cells that can be classified into two types: flattened type-I pneumocytes that cover 95% of the alveolar surface, and type-II pneumocytes that cover < 5% of the alveolar surface. Type-II pneumocytes produce a pulmonary surfactant (phospholipoprotein) for proper lung inflation, and these can also proliferate and generate both type-I and type-II cells when damage occurs in lung cells (Hirota and Martin, 2013). Alveolar epithelial cells are also reported to produce and secrete a number of different biological molecules such as cytokines and growth factors, enzymes, or matrix proteins (Mason, 2006). Alveolar cell apoptosis and regeneration are considered to be the key events in pulmonary fibrosis (Liu et al., 2013; Noble et al., 2012). A549 is a human lung adenocarcinomic epithelial cell line frequently used for different *in vitro* studies of lung functions. For example, a previous study

* Corresponding author.
E-mail address: sxying8655@sina.com (X.-S. Dong).

http://dx.doi.org/10.1016/j.tiv.2017.10.006
Received 18 March 2017; Received in revised form 16 August 2017; Accepted 2 October 2017
Available online 03 October 2017

utilized this cell line to investigate PQ-induced apoptosis (Cappelletti et al., 1998).

The endoplasmic reticulum (ER) is an intracellular organelle that regulates synthesis, folding, post-translational modification, and the delivery of functional proteins for secretion (Ron and Walter, 2007). However, the accumulation of unfolded proteins in the lumen of ER could cause ER stress, and induce unfolded protein response (UPR) to (i) halt protein translation and restore normal cell function, (ii) degrade misfolded proteins, and (iii) activate the molecular signaling pathway to increase protein folding. However, if such event is not achieved, UPR would induce cells to undergo apoptosis (Gong et al., 2017). Previous studies have demonstrated that the ER stress-related pathway regulate PQ-induced alveolar cell damage (Chen et al., 2012), while ambiphilic bile acid and sodium tauroursodeoxycholate with chemical chaperone activity is able to prevent PQ-induced A549 cell death by suppressing ER stress response (Omura et al., 2013). Furthermore, salubrinal, a small molecular compound, inhibits the activity of the α-subunit of eukaryotic initiation factor 2 (eIF2α) through the selective inhibition of eIF2α dephosphorylation, in order to maintain or restore homeostasis in ER (Boyce et al., 2005; Wiseman and Balch, 2005). Previous studies have reported that salubrinal is able to attenuate kainic acid-induced hippocampal cell apoptosis (Kim et al., 2014). In a rat myocardial infarction model, salubrinal protects cardiomyocytes from apoptosis by suppressing eIF2α dephosphorylation (Li et al., 2015). Furthermore, salubrinal also protects cardiomyocyte apoptosis induced by tunicamycin and hypoxia (Liu et al., 2012). In lung models, salubrinal protects human bronchial epithelial cell apoptosis induced by cigarette smoke extracts through the regulation of protein kinase R (PKR)-like ER kinase (PERK)-eIF2α signaling (Yuan et al., 2012).

Thus, in this study, we investigated the molecular mechanism of PQ-induced lung cell damage and the protective role of eIF2α inhibitor salubrinal in lung epithelial cells using an *in vitro* model of human lung epithelial-like A549 cells. We expect to provide insightful information regarding the molecular mechanism responsible for PQ-induced damage to lung cells and molecular targets for the future management of PQ toxicity in lungs.

2. Materials and methods

2.1. Cell line, culture and treatment

Human lung adenocarcinomic epithelial-like A549 cells were obtained from the Experimental Center of China Medical University (Shenyang, China). Cells were seeded in a 6-well cell culture plate with Dulbecco's modified Eagle's medium (DMEM) and high glucose supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution containing 10,000 U/ml of penicillin and 10,000 μg/ml of streptomycin. Then, cells were cultured in a humidified incubator with 5% CO_2 at 37 °C.

For PQ treatment, cells were seeded at a density of 1.0×10^5 overnight in a six-well dish, and treated with various concentrations of PQ (Sigma, St. Louis, MO, USA) or vehicle for 24 h. For the salubrinal protection assay, cells were pre-treated with various concentrations of salubrinal (Sigma) or vehicle for 2 h, and treated with and without 500 μM of PQ for 24 h. Control cells were treated with 0.1% (v/v) solvent dimethyl sulfoxide (DMSO) as vehicle control.

2.2. Cell viability MTT assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. After A549 cells were grown in 24-well plates and treated with salubrinal and/or PQ at different time points, 20 μl of MTT (5 mg/ml) was added to each well, and cells were further incubated for an additional 4 h at 37 °C. Next, the medium was carefully removed and replaced with 150 μl of DMSO, and the plate was gently shaken to dissolve the formazan crystals formed by MTT. Absorbance rate was measured at 490 nm using a microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Cell morphology and transmission electron microscopy

A549 cells were grown on 48-well plates and treated with PQ or vehicle, as described above. Then, cells were for assessment of cell morphology under an inverted microscope (Nikon TS100, Tokyo, Japan).

For transmission electron microscopy, A549 cells were washed with ice-cold phosphate buffered saline (PBS) twice, fixed in 4% glutaraldehyde, post-fixed with 1% OsO_4, stepwise dehydrated in increasing concentrations of ethanol, and embedded in Epon 812 epoxy resin (TAAB, Reading, UK). Then, ultrathin sections were prepared and stained with uranyl acetate and lead citrate, and viewed under a transmission electron microscope (JEOL, Tokyo, Japan).

2.4. Flow cytometric Annexin V-FITC/PI assay

An Annexin V-FITC/PI kit (Dojindo, Tokyo, Japan) was used to assess PQ-induced apoptosis and salubrinal protection. In brief, after treatment, cells were detached by trypsin without ethylene diaminetetra acetic acid (EDTA), washed and centrifuged with PBS three times, fixed in 70% ethanol, stained with Annexin V-FITC/PI, and measured using a flow cytometer (BD FACSCalibur, Becton Dickinson Co., Franklin Lakes, NJ, USA).

2.5. Caspase-3 activity assay

Caspase-3 activity in treated or untreated cells was measured using a Caspase-3 Colorimetric Assay Kit (Nanjing KeyGEN Biotech Institute, Nanjing, China), according to manufacturer's instructions. Briefly, after treatment, cells were assayed for caspase-3 activity using the colorimetric assay kit. Cleavage of the Ac-DEVD-pNA substrate by caspase-3 pNA was quantified spectrophotometrically at 405 nm using an ELISA reader. The change in optical density was directly proportional to caspase-3 activity.

2.6. Western blot analysis

For PQ on the induction of ER stress response in A549 cells, cells were treated with 500 μM of PQ for 3, 6, 12 and 24 h, or with vehicle. For the effect of salubrinal, A549 cells were pretreated with 1, 5, or 30 μM of salubrinal or vehicle for 2 h and treated with or without 500 μM of PQ for 24 h. Subsequently, cells were collected, washed with ice-cold BPS three times, and centrifuged at 2000 rpm for 10 min. Then, the cell pellets were lysed in 100 μL of lysis buffer containing 1% phenylmethane sulfonyl fluoride (PMSF) for 30 min on ice, and centrifuged at 12,000 rpm for 10 min at 4 °C. The protein concentration was measured using a bicinchoninic acid (BCA) kit (Beyotime Institute of Biotechnology, Shanghai, China). Next, 40 μg of protein samples were separated in 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis gel at 100 V for 100 min, and transferred onto polyvinylidene fluoride membrane at 200 mA for 120 min. The membrane was blocked with 5% skim milk solution in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) for 1 h at room temperature, and incubated with primary antibodies against PERK (Abcam, Cambridge, UK), p-PERK (Abcam), ATF6 (Abcam), c-ATF6 (Abcam), IRE1α (Abcam), p-IRE1α (Abcam), CHOP (Abcam), GRP78 (Abcam), p-eIF2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (Proteintech Group Inc., Chicago, IL, USA) in TBST overnight at 4 °C, according to manufacturer recommendations.

On the next day, the membranes were washed with TBST three times for 10 min each, further incubated with horseradish peroxidase-conjugated goat anti rabbit IgG or goat anti-mouse IgG at room temperature for 2 h, briefly incubated with enhanced chemiluminescence