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Paraquat induces extrinsic pathway of apoptosis in A549 cells by induction of DR5 and repression of anti-apoptotic proteins, DDX3 and GSK3 expression

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ABSTRACT

Paraquat (PQ) is a bipyridyl derivative herbicide known to cause lung toxicity partly through induction of apoptosis. Here we demonstrated that PQ caused apoptosis in A549 cells. PQ increased cleavage of caspase-8 and Bid, indicating caspase-8 activation and truncated Bid, the two key mediators of extrinsic apoptosis. Additionally, PQ treatment caused an increase in DR5 (death receptor-5) and caspase-8 interaction, indicating formation of DISC (death-inducing signaling complex). These results indicate that PQ induces apoptosis through extrinsic pathway in A549 cells. Moreover, PQ drastically increased DR5 expression and membrane localization. Furthermore, PQ caused prominent concentration dependent reductions of DDX3 (the DEAD box protein-3) and GSK3 (glycogen synthase kinase-3) which can associate with DR5 and prevent DISC formation. Additionally, PQ decreased DR5-DDX3 interaction, suggesting a reduction of DDX3/GSK3 anti-apoptotic complex. Inhibition of GSK3, which is known to promote extrinsic apoptosis by its pharmacological inhibitor, BIO accentuated PQ-induced apoptosis. Moreover, GSK3 inhibition caused a further decrease in PQ-reduced DR5-DDX3 interaction. Taken together, these results suggest that PQ may induce extrinsic pathway of apoptosis in A549 cells through upregulation of DR5 and repression of anti-apoptotic proteins, DDX3/GSK3 leading to reduction of anti-apoptotic complex.

1. Introduction

Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride: PQ) is an effective, fast-acting and nonselective herbicide and is one of the most widely used herbicides in the world. Due to its high toxicity in humans, it has been banned in some countries. However, it is still used in many developing countries, especially in Asia (Dinis-Oliveira et al., 2008). PQ intoxication is often fatal, primarily due to pulmonary damage as a consequence of its accumulation in the lung (Dinis-Oliveira et al., 2008). The mechanism underlying PQ pulmonary toxicity is not yet fully understood, but many studies have shown that treatment with PQ in rodents causes oxidative stress-related apoptosis in the lungs (Dinis-Oliveira et al., 2007; He et al., 2012; Kim et al., 2013; Seo et al., 2014), indicating the contribution of oxidative stress and apoptosis in its toxicity. PQ has been shown to cause mitochondrial membrane damage resulting in cytochrome-C release and subsequent caspase-3 activation (Dinis-Oliveira et al., 2007; Fujimori et al., 2012; He et al., 2012; Hong et al., 2013; Seo et al., 2014), which suggests that PQ induces intrinsic pathway of apoptosis. Extrinsic apoptotic cell death by PQ was also evidenced as it caused activation of caspase-8, which is an initiation caspase in the extrinsic pathway of apoptosis (Dinis-Oliveira et al., 2007). Additionally, PQ-induced extrinsic apoptosis was indirectly indicated by the evidence that it induced upregulations of Bid, a pro-apoptotic Bcl2 protein that plays an important role in extrinsic apoptosis (Fei et al., 2008) and Fas ligand (CD95L) (Vogt et al., 1998), and degradation of c-FLIP, which is a caspase-8 inhibitory protein (Wilkie-Grantham et al., 2013). These findings clearly indicate that PQ can cause both the intrinsic and extrinsic apoptosis.

Protein complex formation plays a central role in extrinsic pathway of apoptosis. Upon death receptor stimulation by its ligand, the death

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receptor forms tetrameric complex, which subsequently recruiting FADD (Fas associated with death domain protein) and caspase-8 to form DISC (death inducing signaling complex) and leading to caspase-8 activation and initiation of apoptosis. On the other hand, recruitment of c-FLIP to DISC through interaction with FADD hinders caspase-8 activation (Lavrik and Krammer, 2012), and association of DDX3, a member of the DEAD box protein family blocks DISC formation (Li et al., 2006; Sun et al., 2008). Recently, DDX3 was reported to bind with GSK3 (glycogen synthase kinase-3) and c-IAP1 (cellular inhibitor of apoptosis protein 1) resulting in counteraction of death receptor inducing apoptosis (Sun et al., 2008). It is noteworthy to note that PQ treatment resulted in alteration of GSK3 activity (Songin et al., 2011a; Songin et al., 2011b), suggesting the contribution of GSK3 activity in PQ toxicity. However, attribution of GSK3 in PQ-induced extrinsic apoptosis is remained to be investigated.

The limited knowledge regarding the mechanism underlying PQ-induced extrinsic apoptosis led us to examine the effects of PQ on proteins in extrinsic anti-apoptotic complex. In order to elucidate the molecular mechanism of PQ pulmonary toxicity, we used human lung adenocarcinoma A549 cells as an in vitro model. The results showed that, in A549 cells, PQ induced extrinsic apoptosis, upregulated DR5 (death receptor 5 also known as TRAIL-R2:TNF-related apoptosis-inducing ligand receptor 2) but reduced level of proteins, DDX3 and GSK3, which are members of anti-apoptotic complex, and DR5-DDX3 interactions. These findings indicate that PQ may induce extrinsic pathway of apoptosis by upregulation of DR5 and reduction of DDX3/GSK3 anti-apoptotic complex.

2. Materials and methods

2.1. Cell culture and treatment

The human lung adenocarcinoma A549 cells (ATCC, USA) were cultured in RPMI1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (JR Scientific Inc., USA), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Gibco, USA) at 37 °C in a humidified 5% CO_2 incubator. Cells were treated with PQ for 48 h. Where indicated, cells were pretreated with GSK3 inhibitor, 6-bromoindirubin-3′-oxime (BIO) for 30 min prior to 48 h treatment with PQ.

2.2. Apoptosis detection

Apoptosis was detected by phosphatidylserine externalization or DNA fragmentation using annexin V binding assay or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, respectively.

For annexin V binding assay, A549 cells were treated with indicated treatments, and harvested using trypsin. Then, apoptotic cells were detected by using annexin V-FITC apoptotic detection kit I (BD Biosciences, USA) according to the manufacturer's recommendation. After staining, cells were immediately analyzed by flow cytometer (FACSCanto™, BD Biosciences, USA).

For TUNEL assay, A549 cells (3×10^5 cells) were seeded onto a cover slip, and allowed to attach overnight. Cells were treated with 5 μM BIO for 30 min, followed by a 48 h treatment with 500 μM PQ. At the end of treatment, cells were fixed with 4% paraformaldehyde. Apoptosis was detected by using FragEL™ DNA fragmentation detection kit (Calbiochem, Germany) as recommended by the manufacturer. Briefly, the fragmented DNA was labeled with biotin-dNTP using TdT enzyme. Then, the nick end labeled DNA was detected by incubation with streptavidin-peroxidase, followed by DAB (3,3′ diaminobenzidine) staining and methyl green counter staining. The coverslip was mounted onto a microscope slide. The slides were viewed using a microscope (Nikon Eclipse TS100, Nikon, USA). 200–300 cells from random fields were counted for each condition, and the number of TUNEL positive apoptotic cells was recorded.

2.3. Immunoblot analysis

At the end of treatment, cells were lysed with ice-cold lysis buffer containing 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 20 mM sodium fluoride, 100 nM okadaic acid and protease cocktail inhibitor (Calbiochem, Germany). The cell lysates were sonicated then centrifuged at 16,000 × *g* for 15 min at 4 °C to remove insoluble material, and the resulting supernatants were stored at − 80 °C until use. The protein concentration was determined with the Bradford assay (Bio-Rad, USA). For immunoblotting, the cell lysate was mixed with 2X Laemmli sample buffer (125 mM Tris, pH 6.8, 20% glycerol, 4% SDS and 0.004% bromophenol blue and 5% β-mercaptoethanol), and incubated in boiling water bath for 5 min. Equivalent aliquots of protein lysate were loaded in SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with primary antibody overnight at 4 °C with agitation, followed by incubation with an appropriate secondary antibody conjugated with horseradish peroxidase (Bio-Rad, USA). Antibodies used were mouse anti-caspase-8, rabbit anti-cleaved caspase-3, rabbit anti-Bid, rabbit anti-DR5 and rabbit anti-c-IAP1 (Cell Signaling Technology, USA), mouse anti-PARP and mouse anti-GSK3β (BD Bioscience, USA), mouse anti-DDX3 (Santa Cruz Biotechnology, USA), mouse anti-GSK3α/β (Millipore, USA), and mouse anti-β-actin (Sigma-Aldrich, USA). For immunoprecipitation, 500 μg protein lysate was incubated with 2.5 μg DR5 antibody overnight at 4 °C with agitation. Thereafter, DR5 immunocomplex was recovered by incubation with protein G magnetic beads (Merck Millipore, USA) for 10 min, and DR5 protein was eluted by boiling in sample buffer for 10 min. The immunoprecipitated samples were subjected to immunoblot using anti-caspase-8 or anti-DDX3 antibody, after which the membrane was stripped and reprobed with anti-DR5. Protein band density was analyzed using densitometer (ImageScanner III, GE healthcare, UK), and quantified using Image Quant TL software (GE Healthcare, UK). The level of DDX3 co-immunoprecipitated with DR5 was normalized with DR5 derived from the same blot, and the level of normalized DR5-DDX3 complex was expressed as fold of control.

2.4. Measurement of DR5 mRNA

DR5 mRNA was measured by real-time RT-PCR. Total RNA was extracted using RNeasy mini kit (Qiagen, Germany) following the manufacturer's instruction. RNA samples were treated with RNase-Free DNase (Qiagen, Germany) to remove residual DNA contamination. Real-time RT-PCR was performed using RNA-direct SYBR Green Real-time PCR Master Mix (Toyobo, Japan) in Applied Biosystems StepOnesPlus Real-time PCR (Life Technologies, USA). The primers for DR5 (5′-GGGAGCCGCTCATGAGGAAGTTGG-3′ and 5′-GGCAAGTCTCTCTCCCAGCGTCTC-3′) and housekeeping gene, GAPDH (5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGAT GGGATTTC-3′) are described in Kong et al., 2015 and Watcharasit et al., 2012, respectively. The RT-PCR conditions were as follows; reverse transcription (RT activation at 90 °C, 30 s, RT at 61 °C, 20 min, RT deactivation at 95 °C, 1 min); followed by 40 cycles of real-time PCR (15 s of denaturing at 95 °C, 15 s of annealing at 60 °C, 30 s and extension at 72 °C, 30 s). Relative mRNA expression was calculated when normalized with the level of reference gene (GAPDH) according to the ΔΔCT method.

2.5. Measurement of membrane DR5

To investigate presentation of DR5 on cell membrane, A549 cells were treated with 500 μM PQ for 48 h and then harvested by trypsinization. The cells were washed with phosphate buffer saline (PBS) and incubated with PE-conjugated anti-human DR5 (CD262) antibody (BioLegend®, USA), recognizing extracellular domain of DR5