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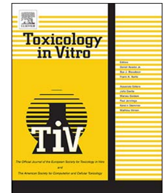

Silymarin attenuated paraquat-induced cytotoxicity in macrophage by regulating Trx/TXNIP complex, inhibiting NLRP3 inflammasome activation and apoptosis

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ABSTRACT

Oxidative stress and inflammation are involved in paraquat-induced cytotoxicity. Silymarin can exert a potent antioxidative and anti-inflammatory effect in various pathophysiological processes. The aim of this current study is to explore the protective effect and potential mechanism of silymarin in paraquat-induced macrophage injury. Cells were pretreated with different doses of silymarin for 3 h before exposure to paraquat. At 24 h after exposure to paraquat, the paraquat-induced cytotoxicity to macrophage was measured via the MTT assay and LDH release. The levels of intracellular reactive oxygen species, GSH-Px, SOD, and lipid peroxidation product malondialdehyde were measured to evaluate the oxidative effect of paraquat. NLRP3 inflammasome and cytokines secretion in macrophage exposed to paraquat at 24 h were measured via immunofluorescence microscopy, western blot or Elisa. Our results revealed that paraquat could dramatically cause cytotoxicity and reactive oxygen species generation, enhance TXNIP expression, and induce NLRP3 inflammasome activation and cytokines secretion. The pretreatment with silymarin could remarkably reduce the cytotoxicity, promote the expression of Trx and antioxidant enzymes, and suppress the TXNIP and NLRP3 inflammasome activation. In conclusion, silymarin attenuated paraquat-induced cytotoxicity in macrophage by inhibiting oxidative stress, NLRP3 inflammasome activation, cytokines secretion and apoptosis.

1. Introduction

Paraquat (1, 1′-dimethyl-4, 4′-bipyridinium dichloride, PQ) is one of the most commonly used herbicides worldwide, especially in agricultural countries. However, it can result in high mortality due to accidental or intentional oral ingestion. Redox responses have been recognized as one of most important biological processes in PQ poisoning. As a potent oxidative stress inducer, PQ can generate a great amount of reactive oxygen species (ROS) via lipid peroxidation, which damages the cellular structure and result in multiple organ dysfunction within hours to days (Gawarammana and Buckley, 2011) (Dinis-Oliveira et al., 2008). In addition, ROS may lead to progressive inflammation including immune cells infiltration and cytokines secretion (Liu et al., 2014). Based on our previous study, the cytokines including IL-1β and IL-18 secretion was significantly increased in PQ-treated macrophage (Liu et al., 2015).

Thioredoxin (Trx) mainly located in the cytoplasm and mitochondria can control cellular ROS by reducing the disulfides into thiol groups and the thioredoxin interacting protein (TXNIP) can directly bind to Trx and inhibit the ROS scavenging of thioredoxin (Devi et al., 2012; Nishiyama et al., 1999), Under conditions of oxidative stress, ROS can cause a conformational change in TXNIP and result in the dissociation of TXNIP from Trx. Therefore, the Trx/TXNIP complex plays an important role in regulating cellular redox status (Yoshihara et al., 2014).

The nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome has recently been recognized as an innate immune signaling receptor (Davis et al., 2011). NLRP3 inflammasome is extremely important in mediating cell responses to various signals including much pathogen, crystal, cellular damage, extracellular ATP release and ROS (Liu et al., 2017; Lupfer and Kanneganti, 2013; Mariathasan et al., 2006; Martinon et al., 2006; Zhou et al., 2010a, 2010b). Once NLRP3 inflammasome is activated, pro-caspase-1 would be transformed to the active form of caspase-1. Caspase-1 subsequently cleaves pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18, and the active cytokines may be released into the extracellular fluid (Zhou et al., 2010a, 2010b). Interestingly, ROS-induced TXNIP overexpression can mediate NLRP3 inflammasome activation,

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and lead to pro-inflammatory gene induction involved in inflammation (Devi et al., 2012; Zhang et al., 2015).

Silymarin is a polyphenolic flavanoid isolated from the fruits and seeds of the milk thistle (Valenzuela and Garrido, 1994), and it can exert an antioxidant and anti-inflammatory effect in different pathophysiological process without considerable side effects (Ahmad et al., 2013; Chtourou et al., 2012; Polyak et al., 2010). It was reported that silymarin can protect PQ-treated human lung adenocarcinoma A549 cell line via scavenge of ROS and induction of antioxidant Nrf2 gene expression (Podder et al., 2012). Coincidentally, we also found that silymarin attenuated PQ-induced lung injury via antioxidant activity in vivo and in vitro in our previous research (Zhao et al., 2015). Although it was reported that silymarin suppressed cellular inflammation by inducing reparative stress signaling in human liver and T cell lines (Lovelace et al., 2015), little is known about the biological effect and underlying mechanism that silymarin attenuated oxidation and inflammation in PQ-treated macrophage. In this study, we would investigate the role of Trx/TXNIP complex and NLRP3 inflammasome in the protective effect of silymarin on PQ-induced cytotoxicity in macrophage.

2. Materials and methods

2.1. Cell culture

The mouse macrophages (J774A.1 cells) were obtained from cell bank, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai, China. Cells were maintained in standard Dulbecco's Modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA)/Ham's F-12 nutrient mixture containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) in a humidified 37 °C incubator containing 5% CO_2. Nonadherent cells were carefully removed, and fresh medium was added every 2 days. For pretreatment, cells were pretreated with different concentrations of silymarin (Sigma Aldrich, St. Louis, MO, USA) for 3 h before exposure to PQ (Sigma Aldrich, St. Louis, MO, USA).

2.2. MTT assay for cell viability

The cytotoxic effect of PQ to macrophage was assessed using the 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma Aldrich, USA) assay based on cellular mitochondrial dehydrogenase activity which converts MTT to formazan crystals. PQ (Sigma Aldrich, USA) was dissolved in distilled water as stock solutions. The stock solutions were then diluted in cell culture media to the different concentrations (0, 10, 50, 100, 250, and 500 μM) prior to MTT assay. The cells were seeded into 96-well plates (1×10^4 cells per well in 100 μL of medium). The cells were exposed to the different concentrations of PQ with/without silymarin and incubated for 24 h and 48 h after the cells were adhered. At the end of the incubation period, 10 μL of 0.5 mg/mL MTT was added to each well. After incubation for an additional 4 h in a 37 °C humidified incubator, the supernatant was discarded, and 150 μL of DMSO was added. After the formazan product was dissolved, the absorbance was measured at 570 nm, using a Bio-Tek MQX 680 (Bio-Tek Instruments Inc., Winooski, VT, USA). Cellular viabilities were assessed relative to the control cells treated with medium only.

2.3. LDH activity assay

To detect PQ cytotoxicity, lactate dehydrogenase (LDH) activity was measured using a cytotoxicity detection kit (Promega, Madison, WI, USA). Macrophages were seeded on 96-well plates at a density of 1×10^4 cells/100 μL and grown to 80–90% confluence overnight. Cells were treated with different concentrations of PQ with/without silymarin for 24 h. According to the manufacturer's protocol, the LDH release assay was then performed. Briefly, 100 μL of the supernatant was transferred from each well to a 96 well plate and 100 μL of freshly prepared reaction mixture was added to each well. After 30 min of incubation at room temperature, the absorbance was measured at 490 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). The amount of LDH was expressed as a percent compared to the total amount of LDH present in cells treated with 2% Triton-X100.

2.4. Intracellular ROS measurement

To verify the oxidative stress of PQ further, the intracellular ROS level in macrophage was measured by flow cytometry using the probe 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, USA). Cells were seeded onto 6-well plates at a density of 2×10^5 cells/mL for 24 h, and then the different doses of silymarin were pretreated as mentioned above. At 24 h after PQ treatment, cells were treated with 1 mL of 10 μM DCFH-DA and incubated for 30 min at 37 °C. After washing, the fluorescence was examined by flow cytometry according to the manufacturer's instructions. The concentration of intracellular ROS in macrophage was measured in terms of intracellular fluorescent intensity (FI). The fluorescence intensity of control cells was normalized to one.

2.5. Oxidative stress markers determination

To test the oxidative stress of PQ in macrophage, the malondialdehyde (MDA) as markers of oxidative stress and activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) as antioxidant enzymes in supernatant were measured using assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The cells were seeded on 6-well plates at a density of 2×10^5 cells/mL. At 24 h after PQ treatment, the cells were kept in lysate on ice for 10 min and centrifuged at 4 °C 14,000 g for 15 min. According to the manufacturer's instructions, the absorbance of MDA, GSH-Px and SOD was measured at 532 nm, 412 nm and 560 nm at the end of reaction on a microplate reader (Bio-Rad, Hercules, CA, USA), respectively.

2.6. Elisa assays

The cells were seeded on 6-well plates at a density of 2×10^5 cells/mL and grown to 80–90% confluence overnight. Cells were treated with PQ for 24 h. Culture supernatants were prepared in order to quantify the levels of secreted cytokines including IL-1β and IL-18. Supernatants were centrifuged at 14,000 g, 4 °C, 10 min, and frozen at −80 °C. Cytokine concentrations were measured using ELISA kits (R & D Systems, USA) according to the manufacturer's recommended instructions. The levels of IL-1β and IL-18 (Abcam, Cambridge, MA, USA) in the supernatant were calculated based on a standard curve. Samples that had a concentration that exceeded the limit of the standard curve were measured after dilution.

2.7. Western blot analysis

The cells in culture dishes were washed with ice-cold phosphate buffered-saline (PBS) for 3 times and were lysed in a buffer containing 20 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 1 mM DTT, 1% Protease Inhibitor Cocktail. Cells lysates were centrifuged at 18,000 rpm for 10 min at 4 °C. Supernatants were collected, and protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Cells lysates (100 μg protein per lane) were resolved by SDS-PAGE and transferred to PVDF (Polyvinylidene Difluoride) membranes according to standard procedures. Membranes were washed in Tris buffered saline (TBS), and blocked for 1 h with TBSTween (TBST) containing 10% nonfat milk, then incubated with primary antibodies against Trx (Abcam, Cambridge, MA, USA), TXNIP (Bioss, Massachusetts, USA), NLRP3 (Abcam, Cambridge, MA, USA), Bax, Bcl-

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