Effect of endoplasmic reticulum calcium on paraquat-induced apoptosis of human lung type II alveolar epithelial A549 cells

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Abstract. The present study aimed to explore the role of endoplasmic reticulum calcium (ER Ca^{2+}) in the apoptosis of human lung type II alveolar epithelial A549 cells induced by paraquat (PQ) \textit{in vitro}. PQ significantly elevated the intracellular Ca^{2+} concentration. Treatment with the Ca^{2+}-ATPase inhibitor thapsigargin significantly increased PQ-induced cytotoxicity, elevated the intracellular level of Ca^{2+}, and increased the apoptosis rate, the protein expression of glucose-regulated protein 78 (GrP78) and c/EBP homologous protein (cHoP), and the activities of caspase-7 and caspase-12 in PQ-treated cells. By contrast, treatment with heparin, an inositol 1,4,5-triphosphate receptor inhibitor, remarkably attenuated cytotoxicity and decreased the intracellular level of Ca^{2+}, the apoptosis rate and the expression levels of GrP78, cHoP and caspases. In conclusion, PQ impaired the regulating function of ER Ca^{2+} and resulted in an excessive increase of intracellular Ca^{2+}. Therefore, influencing the Ca^{2+} signaling in the ER influenced the apoptosis of A549 cells via the ER stress pathway.

Introduction

Paraquat (PQ), also known as 1,1'-dimethyl-4,4'-bipyridinium dichloride, is a highly effective quaternary ammonium herbicide that has been widely used in agriculture worldwide (1). Its misuse by humans can result in multiple organ dysfunction and lung injury, and this compound has an extremely high fatality rate with no specific antidote available (2). To date, the mechanism involved in the toxic effect of PQ remains unknown. Certain studies have indicated that the apoptosis of alveolar epithelial cells is a key in the early manifestation of pulmonary fibrosis caused by PQ (3,4). Previous studies have also revealed that PQ induced the apoptosis of human lung type II alveolar epithelial A549 cells mediated by the endoplasmic reticulum stress (ERS) pathway (5).

Calcium (Ca^{2+}), an important signal transduction molecule in the endoplasmic reticulum (ER), drives the cell apoptosis by ERS and mitochondrial apoptosis pathway (6). The highest concentration of Ca^{2+} in cells is found in the ER, and thus the ER serves an important role in maintaining the Ca^{2+} stability. Ca^{2+} homeostasis in cells is achieved through the Ca^{2+}-ATP enzyme pump in the ER, which absorbs Ca^{2+} from the cytoplasm, and inositol 1,4,5-triphosphate receptor (IP3R), which regulates the release of Ca^{2+} from the ER (7). When the Ca^{2+} homeostasis in the ER is damaged, physiopathological changes in cells occur, such as ERS, changes in mitochondrial membrane permeability, the release of cytochrome c, and dysregulation of B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein, which results in cell apoptosis (8,9). However, it is unclear whether ER Ca^{2+} plays an important role during the apoptosis of human lung type II alveolar epithelial A549 cells induced by PQ.

Therefore, in the present study, the Ca^{2+}-ATP enzyme inhibitor thapsigargin and the IP3R inhibitor heparin were used to preprocess A549 cells, followed by exposure to PQ to induce cell apoptosis. The cell activity, nuclear form, Ca^{2+} concentration, apoptosis rate, expression levels of the ER stress marker proteins glucose-regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP), and changes in caspase-7/12 activity were detected to determine whether ER Ca^{2+} was involved in the PQ-induced apoptosis of human lung type II alveolar epithelial A549 cells induced by PQ.

Materials and methods

Cells and reagents. The A549 cell line was obtained from The Chinese Academy of Sciences Cell Bank. RPMI-1640 medium was purchased from GE Healthcare Life Sciences (Hyclone; Logan, UT, USA) and fetal bovine serum (FBS) was from Gemini Bio-Products, Inc. PQ and trypsin were purchased from Merck KGaA (Sigma-Aldrich), heparin and thapsigargin were obtained from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China), while Hoechst 33258 stain was from Beyotime Institute of Biotechnology (Shanghai, China). The Annexin V-FITC/PI kit was purchased from...
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**Results**

**Changes in cell activity.** Compared with the control group, there were no evident changes in the heparin and thapsigargin.
groups, which indicated that the concentrations of heparin and thapsigargin used in the present study exerted no cytotoxicity (Fig. 1). However, the cell activity in the PQ group was significantly decreased, which indicated that the concentration of PQ used in the present study had a strong toxic effect on A549 cells. Compared with the PQ group, the cell activity was significantly increased in the heparin + PQ group, while cell activity was significantly decreased in the thapsigargin + PQ group. These results suggested that the inhibition of ER Ca\(^{2+}\) release was able to alleviate the cytotoxicity exerted by PQ, while the inhibition of ER Ca\(^{2+}\) absorption resulted in aggravation of cytotoxicity (Fig. 1). Taken together, these findings indicated that the cytotoxicity of PQ is correlated with the Ca\(^{2+}\) signal.

Changes in nuclear form. Compared with the control group, there were no evident changes in the nuclear form in the heparin and thapsigargin groups (Fig. 2). By contrast, characteristic changes, such as karyopyknosis, karyorrhexis and nuclear apoptosis, appeared at different degrees in the PQ, heparin + PQ and thapsigargin + PQ groups. Compared with the PQ group, fewer changes were observed in the nuclear form in the heparin + PQ group, while the nuclear form changes in the thapsigargin + PQ group were more prominent (Fig. 2). These findings indicated that Ca\(^{2+}\) signal had an effect on the A549 cell apoptosis induced by PQ.

Changes in cell apoptosis rate. Compared with the control group, there were no evident changes in the heparin and thapsigargin groups, which indicated that the concentrations of heparin and thapsigargin used in the experiments of the present study had no significant cytotoxic effect. However, in the PQ group, the cell apoptosis rate was significantly enhanced, which suggested that the concentration of PQ used in the present study was able to strongly induce A549 cell apoptosis (Fig. 3). In order to determine whether the Ca\(^{2+}\) signals of ER affect the rate of cell apoptosis induced by PQ, the apoptosis rate was measured in PQ-treated cells that were preprocessed with thapsigargin or heparin. As presented in Fig. 3, the cells apoptosis rates in the PQ, heparin + PQ and thapsigargin + PQ groups were increased by different levels compared with the control group. When compared with the PQ group, the cell apoptosis rate was significantly decreased in the heparin + PQ group, but was significantly increased in the thapsigargin + PQ group. These results further revealed that the Ca\(^{2+}\) signal of ER participated in the A549 cell apoptosis induced by PQ.

Changes in Ca\(^{2+}\) concentration in cells. Ca\(^{2+}\) is known to be an important signal transduction factor in eukaryotic cells, and maintaining its homeostasis serves an important role in the normal physiological activity of cells (10). Ca\(^{2+}\) overload in cells is usually an early manifestation of cell apoptosis and death. The Ca\(^{2+}\) influx from outside the cell, the activities of Ca\(^{2+}\) stores in the cells, and changes in Ca\(^{2+}\) spatial distribution may cause Ca\(^{2+}\) overload in cells (11). In order to investigate the influence of PQ on Ca\(^{2+}\) concentration in cells, a Fluo-3 AM fluorescent probe was used to detect the changes in Ca\(^{2+}\) concentration. The results revealed that, compared with the control group, the Ca\(^{2+}\) concentration in cells treated with PQ was significantly increased, indicating that PQ induced Ca\(^{2+}\) overload in cells (Fig. 4). Heparin, an inhibitor of IP3R in the ER, and thapsigargin, an inhibitor of sarco-ER Ca\(^{2+}\)-ATPases in the ER, were used to determine the association between Ca\(^{2+}\) overload in the cells and Ca\(^{2+}\) in the ER. The results indicated that, compared with the PQ group, Ca\(^{2+}\) fluorescence intensity was significantly decreased in the heparin + PQ group, indicating that heparin prevented the release of Ca\(^{2+}\) in the ER and significantly reversed the increase in Ca\(^{2+}\) concentration induced by PQ. By contrast, a marked increase in Ca\(^{2+}\) fluorescence intensity was observed in the thapsigargin + PQ group as compared with the PQ group (Fig. 4). These aforementioned results suggested that Ca\(^{2+}\) overload in the cells existed during the A549 cell apoptosis induced by PQ, and that the ER served an important role in the imbalance of Ca\(^{2+}\) and outflow of Ca\(^{2+}\).

Changes in GRP78 and CHOP protein levels. In order to determine whether PQ induced ERS in A549 cells, the expression levels of two relevant proteins, GRP78 and CHOP, were determined. The results demonstrated that, compared with the control group, there were no evident changes in the heparin and thapsigargin groups, whereas the levels of the two proteins were significantly increased in the PQ group (Fig. 5). This suggested that PQ significantly induced ERS during the induction of A549 cell apoptosis. Compared with the PQ group, the expression levels of GRP78 and CHOP were significantly decreased in the heparin + PQ group and significantly increased in the thapsigargin + PQ group (Fig. 5). These results indicated that the intervention of Ca\(^{2+}\) release and absorption in the ER influenced the ERS.

Changes in caspase-7/12 activity. Caspase-7 and caspase-12 are members of the Caspase protein family, and are defined as the executive proteins in cell apoptosis. The activation of caspase-7 and caspase-12 is necessary for the ERS-induced cell apoptosis. In the present study, the expression levels of caspase-7 and caspase-12 in each group was detected to determine whether PQ was able to promote Ca\(^{2+}\) release and reduce Ca\(^{2+}\) absorption, and influence and trigger A549 cell apoptosis mediated by ERS. As shown in the Fig. 6, the activities of caspase-7
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Figure 2. A549 cells were processed accordingly in the different groups for 48 h, and then changes in the nuclear form were observed by Hoechst 33258 staining. Magnification, x200. PQ, paraquat.

Figure 3. A549 cells were processed accordingly in the different groups for 48 h, and then changes in apoptosis rate were detected using a flow cytometer. (A) Flow cytometric analysis, and (B) statistical analysis of the apoptosis rate in each group. *P<0.01 vs. control group; **P<0.01 vs. PQ group. PQ, paraquat.

Figure 4. A549 cells were processed accordingly in the different groups for 48 h, and then changes in Ca²⁺ concentration in the cells were detected using a flow cytometer. (A) Flow cytometric analysis and (B) statistical analysis of Ca²⁺ fluorescence intensity in each group. *P<0.01 vs. control group; **P<0.01 vs. PQ group. PQ, paraquat; Ca²⁺, calcium.
and caspase-12 were increased by different degrees in the PQ, heparin + PQ and thapsigargin + PQ groups as compared with the control group. However, compared with the PQ group, the activities of caspase-7 and caspase-12 were significantly decreased in the heparin + PQ group, but significantly increased in the thapsigargin + PQ group (Fig. 6). These results revealed that intervention of Ca^{2+} signals in the ER has an effect on A549 cell apoptosis mediated by the ER stress induced by PQ.

Discussion

The lung is the main target organ of PQ poisoning. Lung damage caused by PQ mainly manifests as pulmonary congestion, bleeding, edema, the formation of a hyaline membrane, degeneration, hyperplasia and fibrosis, resulting in acute lung injury and acute respiratory distress syndrome. Even when patients have survived the acute phase, they may succumb due to respiratory failure caused by irreversible pulmonary fibrosis (12,13). Certain studies have suggested that the apoptosis of lung epithelial cells serves an important role in lung injury caused by PQ (3,4). Therefore, it would be of great importance to illustrate the mechanism of lung epithelial cell apoptosis induced by PQ.

Cell apoptosis is an ordered, programmed and active type of cell death, and is the normal physiological response of the cell nucleus stimulated by specific signals. However, abnormal cell apoptosis is associated with the occurrence of numerous diseases (14). The cell apoptosis regulation mechanism is complex, and the cell apoptosis pathway differs according to the different environments, types of cells or stimulations (15).

Ca^{2+} signals are responsible for various basic cell functions, and once the concentration of Ca^{2+} or its regulation function becomes abnormal, Ca^{2+} homeostasis is lost. Hence, a series of cascade reactions are activated, and cell apoptosis or death finally occurs (16,17). The ER is a membranous duct system distributed in the cytoplasm and is involved in material transportation in cells. Ca^{2+} concentration in cells is mediated by the ER, since Ca^{2+} is mainly stored in the ER. At present, studies have indicated that among all organelles, the ER is mainly in charge of the dynamic equilibrium of Ca^{2+} (18).

The present study suggested that PQ exposure was able to increase the Ca^{2+} concentration in cells and cause Ca^{2+} overload. When cells were preprocessed with thapsigargin to inhibit the ER from absorbing Ca^{2+}, the Ca^{2+} concentration in the cytoplasm was further increased, indicating that the increase of Ca^{2+} concentration in the cytoplasm is correlated with the decrease in Ca^{2+} in the ER. By contrast, when cells were preprocessed with heparin to inhibit the release of Ca^{2+} in the ER, the Ca^{2+} concentration in the cytoplasm was decreased, indicating that the decrease in Ca^{2+} concentration in the cytoplasm is correlated with the decreased release of Ca^{2+} in the ER. These results suggested that PQ damaged the balance of ER Ca^{2+} release and absorption, and led to Ca^{2+} overload in the cytoplasm. Ca^{2+} overload in the cytoplasm can trigger the mitochondrial pathway apoptosis (19). In the current study, MTT assay, Annexin V-FITC/Pi double staining and Hoechst 33258 staining were conducted to further reveal that thapsigargin evidently enhanced the cytotoxicity and apoptosis levels induced by PQ treatment. By contrast,
heparin was observed to inhibit the release of Ca\(^{2+}\) in the ER and evidently reverse the cell apoptosis induced by PQ. These findings further indicated that PQ induced cell apoptosis by damaging the Ca\(^{2+}\) homeostasis in the ER.

GRP78 is the molecular chaperone of ERS, and serves a critical role in maintaining ER protein synthesis, proper protein folding and Ca\(^{2+}\) homeostasis in cells; thus, it is an important marker of ERS (20). CHOP, as the enhancer binding protein and homologous protein of CCAAT, is a pro-apoptotic protein that is highly expressed during ERS, while it exhibits low expression in the absence of ERS; therefore, it can serve as a marker protein of ERS (21). An increasing number of studies have suggested that cell apoptosis induced by ERS is implemented by the activation of caspase-7 and caspase-12 (22,23). In the present study, PQ was found to markedly increase the expression levels of GRP78 and CHOP, which are relevant markers of ERS, as well as enhance the activities of caspase-7 and caspase-12 (as summarized in Fig. 7). Preprocessing with thapsigargin significantly increased the expression levels of GRP78 and CHOP, and the activities of caspase-7 and caspase-12 in PQ-treated cells. By contrast, preprocessing with heparin markedly reversed the effects of PQ in cells. These findings indicated that the intervention of Ca\(^{2+}\) had an effect on A549 cell apoptosis mediated by PQ through the ER pathway.

All apoptotic pathways eventually activate caspase-3, and thus caspase-3 is a non-specific marker for certain apoptotic pathways. Other Caspases, such as caspase-8 and caspase-9, that are activated by special apoptotic pathways have been reported (24,25). Caspase-12, which is located in the adventitia of the ER, is a key molecule that mediates ERS-induced apoptosis and is only correlated with the mechanism of ERS-mediated apoptosis. There are several methods of caspase-12 activation induced by ERS, including Ca\(^{2+}\)-dependent calpain activation and the caspase-7 pathway. Therefore, caspase-12 and the associated caspase-7 were investigated in the present study.

In conclusion, the results of the present study revealed that PQ had an effect on A549 cells by damaging the regulatory function of Ca\(^{2+}\) in the ER, which consequently resulted in Ca\(^{2+}\) overload and ERS, and triggered cell apoptosis. Furthermore, the study results suggested that, at the early stages of PQ poisoning, a considerable therapeutic strategy may be to prevent the ER Ca\(^{2+}\) release, maintain the Ca\(^{2+}\) homeostasis in the ER and cytoplasm, and prevent the apoptosis of lung epithelial cells.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors’ contributions**

XSD designed the study. QC analyzed the data. CY made substantial contributions to conception and design. CQS, DZS and YMX conducted the experiments. All authors reviewed, edited and approved the final version of the manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.
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