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

CHUN-QING SONG, DA-ZHUANG SUN, YONG-MIN XU, CHEN YANG, QUAN CAI and XUE-SONG DONG

Department of Emergency, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning 110001, P.R. China

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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) *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 ERS 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. This was explored to provide a theoretical basis for the clinical treatment of PQ poisoning.

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

Correspondence to: Dr Xue-Song Dong, Department of Emergency, The First Affiliated Hospital of China Medical University, 155 Nanjing North Street, Heping, Shenyang, Liaoning 110001, P.R. China
E-mail: xuesongdongdr@163.com

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Dojindo Molecular Technologies, Inc. (Kumamoto, Japan), Calcium detection kit and the Caspase activity kit was from Nanjing Keygen Biotech Co., Ltd. (Nanjing, China). Antibodies against GRP78 (cat. no. 66574-1-Ig), CHOP (cat. no. 15204-1-AP) and β-actin (cat. no. HRP-60008) were purchased from ProteinTech Group, Inc. Horseradish peroxidase-conjugated goat anti-rabbit (cat. no. ab6721) goat anti-mouse immunoglobulin G secondary antibodies (cat. no. ab97040) and ECL Substrate kit (cat. no. ab133406) were purchased from Abcam.

Cell culture and grouping. A549 cells were cultured in RPMI-1640 culture solution with 10% FBS in an incubator with 5% CO_2 at 37˚C. The culture solution was changed every other day. At 70-80% confluence, cells were divided into six experimental groups, as follows: i) Control group, exposed to equal volume of PBS; ii) heparin group, preprocessed with heparin (200 μg/ml) for 2 h; iii) thapsigargin group, preprocessed with thapsigargin (4 μM) for 2 h; iv) PQ group, exposed to PQ (200 M); v) heparin + PQ group, preprocessed with heparin (200 μg/ml) for 2 h, followed by PQ (200 μM) treatment; vi) thapsigargin + PQ group, preprocessed with thapsigargin (4 μM) for 2 h, followed by PQ (200 μM) treatment. Cells in each group were cultured for 48 h, and a number of relevant indexes were then tested.

Cell activity detection by MTT assay. A total of $1x10^5$ cells/ml were seeded onto a 96-well plate, with each well containing 100 μl of solution, and cultured for 24 h. Next, the cells were treated accordingly in the different groups and continuously cultured for 48 h after treatment. A total of 20 μl MTT solution (5 mg/ml) was added into each well and mixed, and the cells were continuously cultured for 4 h in an incubator with 5% CO_2 at 37˚C. Subsequently, the supernatant was discarded, and 150 μl DMSO was added to each well. The cells were shaken in the dark for 10 min to dissolve the formazan crystals. A microplate reader at 490 nm was used to determine the optical density (OD) of each well.

Cell apoptosis detection by Hoechst 33258 staining. The culture solution was discarded after 48 h of incubation, and the cells were collected. Paraformaldehyde (4%) was added to fix for 10 min at room temperature. The fixation solution was then discarded, and the cells were washed three times with PBS. Next, Hoechst 33258 stain was added for 5 min and then discarded after washing three times with PBS. After mounting with an anti-fluorescence quenching agent the cell nuclei were observed under a microscope.

Cell apoptosis rate detection using the Annexin V-FITC/propidium iodide (PI) double-staining method. A549 cells were seeded onto a 6-well plate at a density of $1x10^6$ cells/well. When cells reached 70% confluence, they were processed accordingly in the different groups, and continuously cultured for 48 h. After the 48 h culture, cells in each group were collected, washed three times with PBS, and then incubated with a mixture containing 5 μl Annexin V-FITC and 5 μl PI for 15 min at room temperature in the dark. A flow cytometer was subsequently used for the detection of apoptosis rate.

Measurement of Ca^{2+} concentration. Cells collected from each group were placed into culture solutions with 1.25 μM Fluo-3 AM probe of the Calcium Detection Kit (Nanjing Keygen Biotech Co., Ltd.) and incubated for 30 min at 37˚C in the dark. Subsequent to incubation, the cells were washed three times with PBS and resuspended in 300 μl PBS in a flow tube. A flow cytometer was then used for the detection of Ca^{2+} concentration.

Detection of the protein expression levels of GRP78 and CHOP by western blot analysis. Cells collected from each group were washed three times with PBS and lysed with a lysis buffer consisting of dichloroacetic acid (0.1%), PMSF (1 mM), protease inhibitor cocktail (10 μM), Na_3PO_4 (1 mM), Triton X-100 (1%), NaCl (150 mM), Tris-HCl (10 mM), EDTA (1 mM) and EGTA (1 mM). Next, centrifugation was performed for 30 min at 14,000 x g at 4˚C, and the supernatant was collected and transferred into a new EP tube. The BCA method was then used to measure the protein concentration. Subsequently, 30 μg of the sample was loaded and separated by 12% SDS-PAGE at 4˚C at 70 V for 60 min. The protein was then transferred to a PVDF membrane and incubated for 2 h at room temperature with blocking solution that consisted of 5% nonfat milk powder dissolved in Tris-buffered saline/Tween-20 (TBST). Antibodies targeting GRP78 (1:500), CHOP (1:1,000) and β-actin (1:1,000) were added, and the membrane was fixed overnight at 4˚C and maintained at room temperature for 2 h. Subsequent to washing three times with TBST, secondary antibodies (1:5,000) were added to the PVDF membrane, maintained at room temperature for 2 h and washed three times with TBST. An ECL kit was used to visualize the protein bands. Finally, the image was developed and fixed, while ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to perform gray analysis.

Caspase activity detection. Cells collected from each group were washed three times with PBS. Lysis solution was added in moderation for 2 h on ice, and centrifugation was performed for 1 min at 11,000 x g at 4˚C. The supernatant was collected and transferred into a new EP tube, and the BCA method was used to measure the protein concentration. Next, 50 μl of 2X buffer solution and 5 μl caspase substrate was added to 50 μl cell lysis product (containing 150 μg of protein), and incubated for 4 h at room temperature in the dark. The OD at 405 nm was measured using a microplate reader.

Statistical analysis. SPSS software (version 20.0; IBM Corporation, Armonk, NY, USA) was used to perform the statistical analysis. The data in each group are expressed as the mean ± standard deviation. Intergroup differences were assessed by one-way analysis of variance. If equal variance was assumed, Dunnett's test was used for comparisons between the two groups, whereas if unequal variance was assumed, Dunnett's T3 test was used. $P<0.05$ was considered to indicate a statistically significant difference.

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 inhibitionof 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.

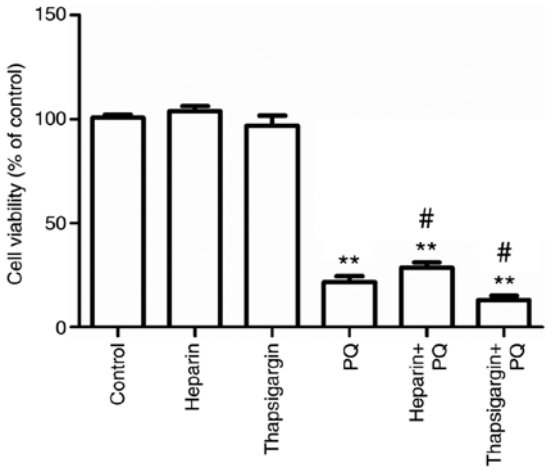

Figure 1. A549 cells were processed accordingly in the different groups for 48 h, and then MTT assay was performed to detect the cell activity. $^{**}P<0.01$ vs. control group; $^{\#}P<0.05$ vs. PQ group. PQ, paraquat.

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

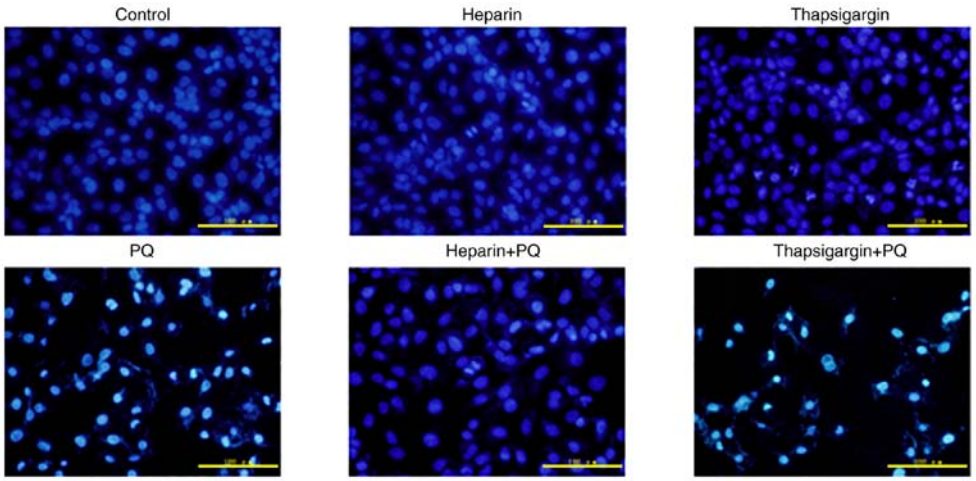

Figure 2. A549 cells were processed accordingly in the different groups for 48 h, and then changes in the nuclear form were observed by Hoechst 33258 staining. Magnification, x200. PQ, paraquat.

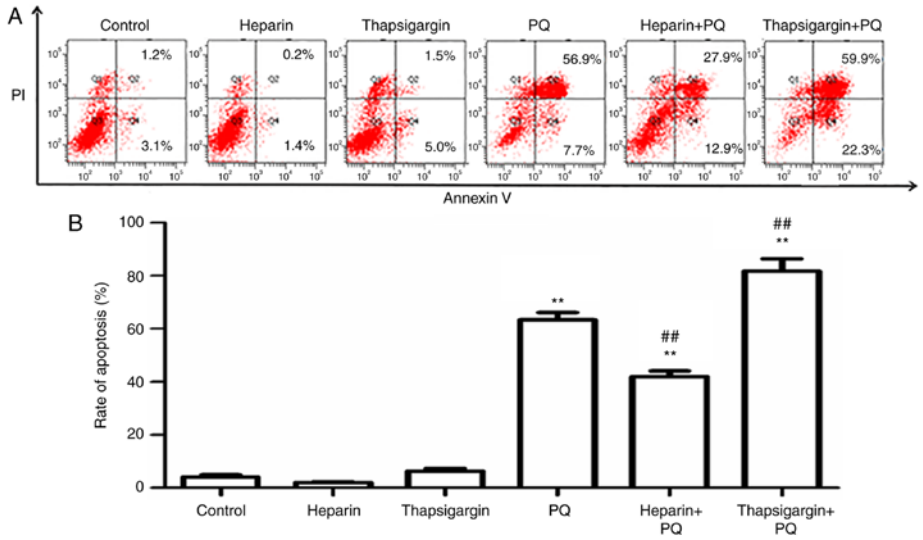

Figure 3. A549 cells were processed accordingly in the different groups for 48 h, and then changes in apoptosis rate were detected using a flow cytometer. (A) Flow cytometric analysis, and (B) statistical analysis of the apoptosis rate in each group. **P<0.01 vs. control group; ##P<0.01 vs. PQ group.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

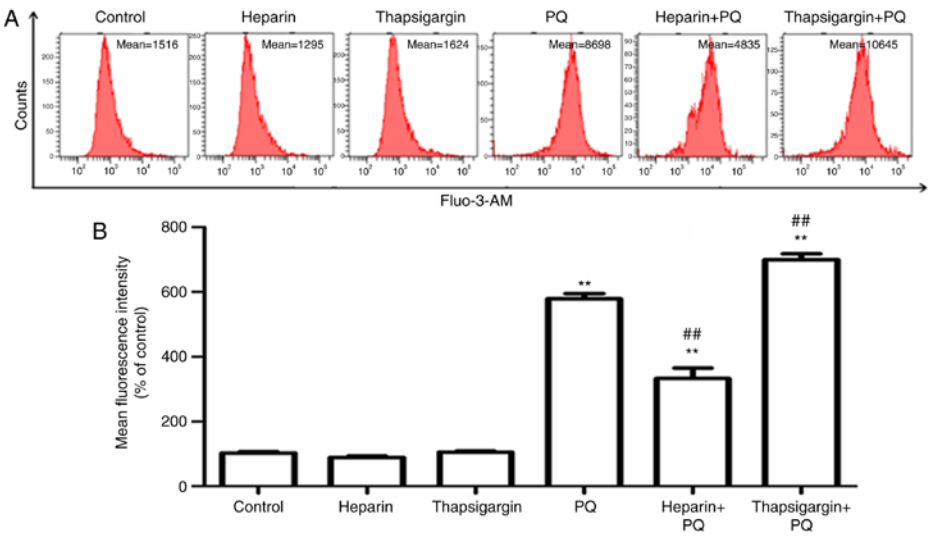

Figure 4. A549 cells were processed accordingly in the different groups for 48 h, and then changes in Ca^{2+} concentration in the cells were detected using a flow cytometer. (A) Flow cytometric analysis and (B) statistical analysis of Ca^{2+} fluorescence intensity in each group. **P<0.01 vs. control group; ##P<0.01 vs. PQ group. PQ, paraquat; Ca^{2+}, calcium.

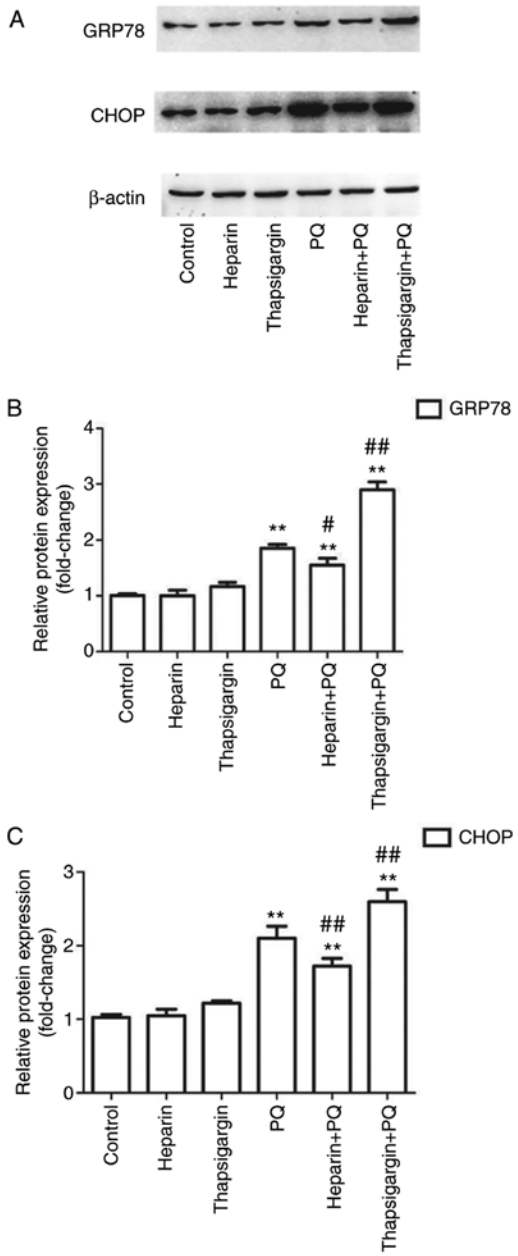

Figure 5. A549 cells were processed accordingly in the different groups for 48 h, and then the protein expression levels of GRP78 and CHOP were detected by western blotting. (A) Western blotting results are shown. Statistical analysis of the protein expression levels of (B) GRP78 and (C) CHOP. **P<0.01 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01, vs. PQ group. PQ, paraquat; GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein.

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 ERS induced by PQ.

Discussion

The lung is the main target organ of PQ poisoning. Lung damage caused by PQ mainly manifests as pulmonary congestion,

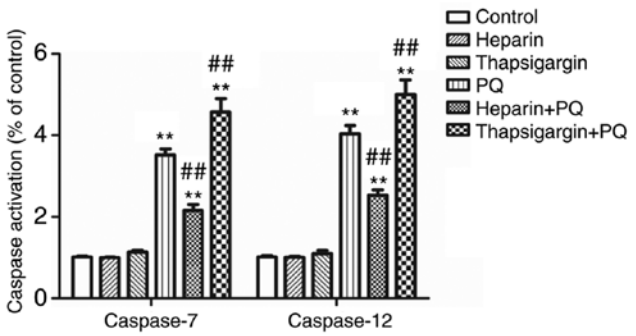

Figure 6. A549 cells were processed accordingly in the different groups for 48 h, and then changes in caspase-7 and caspase-12 activities were detected. **P<0.01 vs. control group; $^{\#\#}$P<0.01 vs. PQ group. PQ, paraquat.

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 through 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,

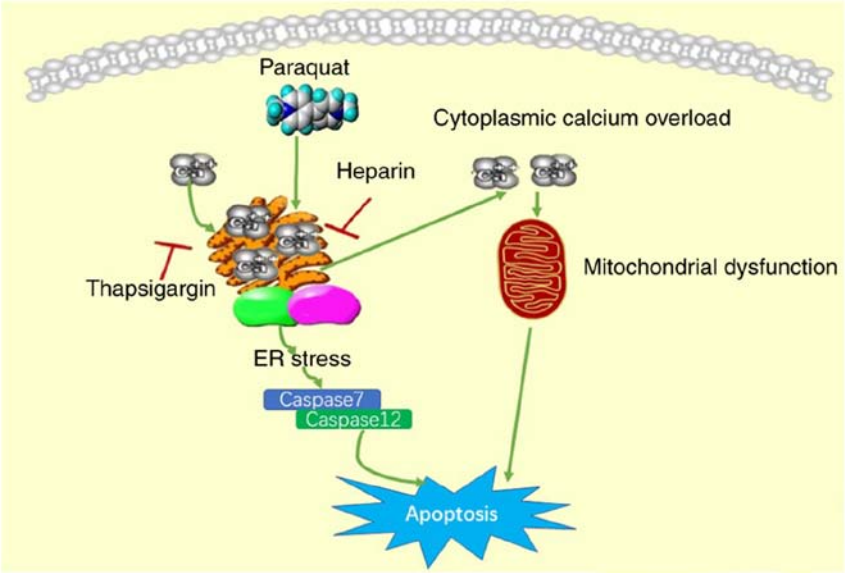

Figure 7. Mechanisms involved in ER calcium in PQ-induced A549 cell apoptosis. PQ, paraquat; ER, endoplasmic reticulum.

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, properprotein 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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