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Pyrrolidine dithiocarbamate (PDTC)/Cu complex induces lung epithelial cell apoptosis through mitochondria and ER-stress pathways

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ABSTRACT

Pyrrolidine dithiocarbamate (PDTC) is widely used in pesticides, fungicides, insecticides, and herbicides. Copper (Cu) is a toxic heavy metal in the environment, and an essential trace metal element in the body, which is involved in many biological processes as a catalytic cofactor. The present study is designed to investigate the cellular toxicity of PDTC, $CuCl_2$, and PDTC/Cu complex exposure in lung alveolar epithelial cells that serve primary structural and functional roles in the lungs. The results showed that PDTC or $CuCl_2$ alone did not affect cell viability, but PDTC/Cu complex significantly decreased lung alveolar epithelial cell viability. PDTC/Cu complex also significantly increased intracellular copper concentration, but PDTC or $CuCl_2$ alone had low levels of copper. PDTC/Cu complex dramatically enhanced the JNK protein phosphorylation and ERK protein phosphorylation proteins. PDTC/Cu complex did not affect the p38 protein phosphorylation. PDTC/Cu complex was capable of activating the apoptosis-related caspases including caspase-9, caspase-7, and caspase-3, which could be reversed by the addition of JNK inhibitor SP600125 or transfection of MAPK8 short hairpin RNA. PDTC/Cu complex also increased cytosolic cytochrome *c* and decreased mitochondrial transmembrane potential. The Bcl-2 mRNA and protein expressions were decreased in lung epithelial cells treated with PDTC/Cu complex, which could be reversed by SP600125. Furthermore, PDTC/Cu complex could trigger the expressions of ER stress-associated signaling molecules including Grp78, Grp94, caspase-12, ATF4, and CHOP, which could be reversed by SP600125. Taken together, these results indicate that exposure to PDTC/Cu complex induces cytotoxicity and apoptosis in alveolar epithelial cells via the mitochondria- and ER-stress-related signaling pathways.

1. Introduction

Recently, many studies have discussed the biological effects of dithiocarbamate–metal complexes because of their wide application in pesticides, fungicides, insecticides, and herbicides (Yamamoto et al., 2007; Boers et al., 2008). Pyrrolidine dithiocarbamate (PDTC) is a low-molecular weight thiol compound that functions as a metal chelator and antioxidant. For example, PDTC demonstrated protective effects against paraquat-induced pulmonary damage (Chang et al., 2009). In addition, PDTC has been found to inhibit NF-κB and inflammatory cytokines, protecting against many diseases including obstructive uropathy and neuropathic disorders (Chuang et al., 2009; Yang et al., 2009). Furthermore, PDTC has also been used in chelating therapy for metal intoxication (Atanasov et al., 2003). However, recent studies have shown that PDTC can induce cell death in several cell types (Chen et al., 2000, 2008), and toxic effects have been observed when PDTC is added to cultured HL-60 cells (Chen et al., 2008).

Copper (Cu) is an essential trace element that functions as a catalytic cofactor and is involved in many biological processes

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(Krewski et al., 2010) and plays an important role in the oxidant defense system (Stern, 2010). However, Cu is also present in our environment, and an excess of Cu may have adverse effects on our health (Chen et al., 2008). Cu overload may lead to the generation of unbound ionic Cu^{2+}, inducing hydroxyl radicals, oxidative stress, and displacement of other essential metal cofactors from metalloenzymes. The recommended amount of dietary intake of Cu is 20 μg/kg body weight per day for adults and 50 μg/kg body weight per day for infants (Stern, 2010). Blood is an important target of drug exposure and environmental chemicals (Chen et al., 2008), and previous studies have shown that chronic exposure to high doses of Cu (e.g., 200 μg/g in pigs) can lead to gastrointestinal damage, liver cirrhosis, hemolysis, and damage to renal tubules and the central nervous system (Linder and Hazegh-Azam, 1996). Another report has also indicated that excessive Cu accumulation (1 g/L in drinking water) decreases superoxide dismutase (SOD) activity and glutathione (GSH) levels and increases malondialdehyde (MDA) concentrations in brain tissues (Ozcelik and Uzun, 2009). Because PDTC is widely used in agriculture and Cu is present throughout the environment, chronic exposure to both PDTC and Cu is likely.

The lungs receive most of the venous bloodstream and implement blood–air exchange. Previous studies have not explored PDTC, Cu, or PDTC/Cu complex toxicity in lung epithelial cells. Thus, we used lung cells to investigate the consequences of exposure to PDTC, Cu, and PDTC/Cu complex. Lung epithelial apoptosis has been demonstrated in several lung functional disorders and diseases, such as lung fibrosis (Yalcin et al., 2009), emphysema (Diab et al., 2010), and acute lung injury (Meng et al., 2010).

Mitogen-activated protein kinase (MAPK) pathways are involved in lung inflammation and injury, including idiopathic pulmonary fibrosis (Yoshida et al., 2002). A previous study has reported that MAPKs are conserved enzymes that connect cell surface receptors to critical regulatory targets within the cell and respond to chemical and physical stresses (Chang and Karin, 2001). There are three major groups of MAPKs, including extracellular signal-regulated kinase (ERK)-1/2, c-jun N-terminal kinase (JNK)-1/2/3, and p38 MAPK. JNK and p38 are involved in apoptotic signaling. Yoshida et al. (2002) and Penna et al. (2009) demonstrated that endoplasmic reticulum (ER) stress can enhance phosphorylation of eIF2alpha and JNK during apoptotic signaling.

The ER is responsible for the synthesis, folding, assembly, and modification of cytoplasmic and membrane proteins (Zhang et al., 2010). Activation of the ER stress response upregulates ER resident chaperones and other regulatory components of the secretary pathway. Previous studies have reported that under stress conditions, unfolded proteins accumulate in the ER and sequester Grp78, preventing the secretion of incompletely assembled immunoglobulins. This causes the release of activating transcription factor 6 (ATF6), protein kinase-like endoplasmic reticulum kinase (PERK), and IRE-1, promoting their activity. Enhanced ER stress also leads to a decrease in the inner mitochondrial transmembrane potential (MMP) and release of cytochrome *c* associated with the intrinsic apoptotic pathway (Breckenridge et al., 2003). Cytochrome *c* binds to apoptotic protease activating factor 1 (Apaf-1) to form a cytochrome *c*–Apaf-a complex. In the presence of ATP or dATP, this complex recruits and activates procaspase-9, which then activates other caspases (Desagher and Martinou, 2000).

The lungs provide blood–air exchange for the body, and lung epithelial cells compose the alveolar wall that facilitates this exchange. In this study, we evaluated the toxicity of the environmental pollutants PDTC, $CuCl_2$, and PDTC/Cu complex in lung epithelial cells and found that PDTC/Cu complex likely promotes cytotoxicity by induction of apoptosis.

2. Materials and methods

2.1. Cell line

Rat lung epithelial-derived L2 cells (CCL-149) were purchased from ATCC. The cells were cultured in a humidified chamber with a 5% CO_2/95% air mixture at 37 °C. The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) containing 1% penicillin–streptomycin (Gibco/Invitrogen, Carlsbad, CA, USA).

2.2. Cell viability assay

Cells were washed with PBS and detached from dishes using trypsin. They were then cultured in 24-well plates (2×10^5 cells/well) and treated with PDTC, $CuCl_2$, or PDTC/Cu complex for 24 h. After incubation, the medium was removed and replaced with a fresh medium containing 30 μL of 2 mg/mL 3-(4,5-dimethyl thiazol-2-yl-)-2,5-diphenyl tetrazolium bromide (MTT). After incubation for 4 h, the medium was removed, and 1 mL of dimethyl sulfoxide (Sigma, St. Louis, MO, USA) was added to dissolve the blue formazan crystals. Following mixing, 150 μL was transferred to a 96-well plate. An enzyme-linked immunosorbent assay reader (Thermo Fisher Scientific, Waltham, MA, USA) was used for fluorescence detection at a wavelength of 570 nm.

2.3. Intracellular copper concentration

To determine the copper levels in cells, cells were cultured in 10 cm^2 dishes and treated with indicated compounds for 24 h. The cells were harvested and placed in a 15 mL polyethylene tube, and 0.5 mL of a 3:1 mixture of hydrochloric acid (35%) and nitric acid (70%) was added. The cell mixtures were heated at 50 °C. After cooling, the copper contents were determined by inductively coupled plasma mass spectrometry (ICP-MS).

2.4. Plasmid and transfection

RNAi reagents were obtained from the National RNAi Core, Institute of Molecular Biology, and Genomic Research Center, Taiwan. A short hairpin RNA (shRNA) was designed to target the specific sequence of human MAPK8 (Clone ID: TRCN0000001056; target sequence: 5′-GCCCAGTAATATAGTAGTAAA-3′). The shRNA was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the recommendations of the manufacturer. Before transfection, the cells were seeded without antibiotics, and the efficiency of transfection (approximately 80%) was determined using an equal amount of a plasmid encoding the green fluorescent protein driven by the cytomegalovirus promoter.

2.5. Cytosol cytochrome c detection

Cells were homogenized with a pestle and mortar in 0.4 M mannitol, 25 mM MOPS (pH 7.8), 1 mM EGTA, 8 mM cysteine, and 0.1% (w/v) bovine serum albumin (BSA). Cell debris was then removed via centrifugation at $6000 \times g$ for 2 min. The supernatant was then removed and recentrifuged at $12{,}000 \times g$ for 15 min to pellet mitochondria, and the supernatant (cytosol) was stored for Western blot analysis.

2.6. Western blot analysis

Cells were treated with the indicated compounds for various time periods. Afterwards, 50 μg of protein from each cell lysate was subjected to electrophoresis on 10% (w/v) SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were then blocked in PBST (PBS and 0.05% Tween 20) containing 5% nonfat dry milk for 1 h. After blocking, the membranes were incubated with antibodies against phospho-JNK, phospho-ERK1/2, phospho-p38, JNK1, ERK1/2, p38, cytochrome *c*, caspase 9, caspase 7, caspase 3, caspase 12, Grp78, Grp94, and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then washed with 0.1% PBST and incubated with secondary antibodies conjugated to horseradish peroxidase for 45 min. The antibody-reactive bands were revealed using enhanced chemiluminescence reagents (Amersham Biosciences, Sweden) and exposed to radiographic film (Kodak, Rochester, NY, USA).

2.7. Quantitative real-time PCR

This method was performed as previously described (Lu et al., 2010). L2 cells were treated with $CuCl_2$, PDTC, or PDTC/Cu complex for the indicated times, and total RNA was extracted. Total RNA (5 μg) was heated to 90 °C for 5 min to remove any secondary structures and then rapidly placed on ice. Samples were then reverse transcribed into cDNA using AMV RTase (Promega, Madison, WI, USA) at 42 °C in a reaction buffer containing 2.5 mM dNTPs, 40 U/μL RNasin (Promega, Madison, WI, USA), 100 nmol random-hexamer primers, 1× RTase buffer; 30 U AMV RTase in nuclease-free water at a final volume of 20 μL. The mixture was incubated at 42 °C for 60 min. Samples were then denatured at 95 °C for 10 min and placed on ice. Primers for rat Bcl-2, Grp78, Grp94, ATF4, and C/EBP homologous protein (CHOP) are as follows: Bcl-2 forward 5′-CTTTGTGGAACTGTACGGCCCCAGCATGCG-3′ and reverse

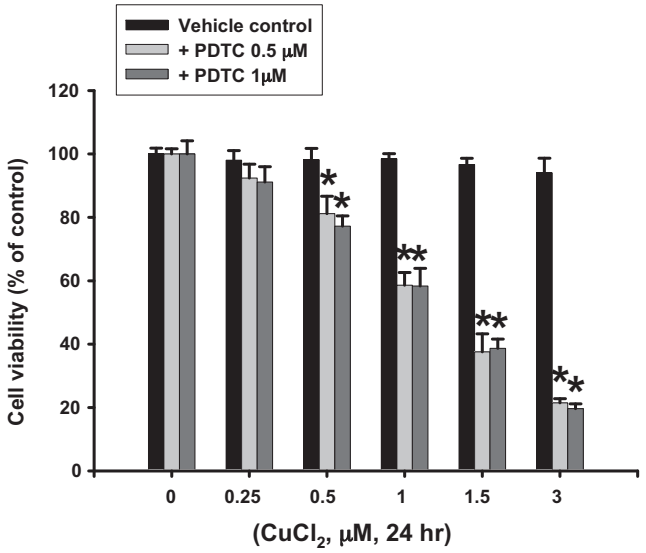

Fig. 1. Effects of PDTC, $CuCl_2$, and PDTC/Cu complex on cell viability in lung epithelial cell-derived L2 cells. Cells were treated with $CuCl_2$ (0–3 μM) with or without PDTC (0 μM, 0.5 μM, or 1 μM) for 24 h. The cell viability was determined via MTT assay. All data are presented as means ± SEM from four independent experiments performed in triplicate. $^*P < 0.05$ as compared to vehicle control.

5′-ACAGCCTGCAGCTTTGTTTCATG-GTACATC-3′ (Bozec et al., 2004), Grp78 forward 5′-TGATAATCAGCCCACCGTAACA-3′ and reverse 5′-GGAGGGATTCCAGTCAGATCAA-3′ (Urban et al., 2009), Grp94 forward 5′-AAGGTCATTGTCACGTCGAAA-3′ and reverse 5′-GTGTTTCCTCTTGGGTCAGC-3′ (Pirot et al., 2006), ATF4 forward 5′-GTTGGTCAGTGCCTCAGACA-3′ and reverse 5′-CATTCGAAACAGAGCATCGA-3′ (Cardozo et al., 2005), and CHOP forward 5′-CCAGCAGAGGTCACAAGCAC-3′ and reverse 5′-CGCACTGACCACTCTGTTTC-3′ (Cardozo et al., 2005). Each sample was detected using a real-time Sybr Green PCR reagent (Invitrogen, Carlsbad, CA, USA) with transgene-specific primers in a 25 μL reaction volume, and amplification was performed using an ABI Prism 7900HT real-time thermal cycler (Applied Biosystems, Carlsbad, CA, USA). Cycling conditions were 2 min at 50 °C, 10 min at 95 °C, 40 cycles at 92 °C for 30 s, and 60 °C for 1 min. Real-time fluorescence detection was performed during the 60 °C annealing/extension step of each cycle. Melt-curve analysis was performed on each primer set to ensure that no primer dimers or non-specific amplification was present under the optimized cycling conditions. The fold difference in mRNA expression between treatment groups was determined using the relative quantification method utilizing real-time PCR efficiencies and normalized to the β-actin gene, thus comparing relative C_T changes between control and experimental samples. Prior to conducting statistical analyses, the fold change from the mean of the control group was calculated for each individual sample, including individual control samples to assess variability within the group.

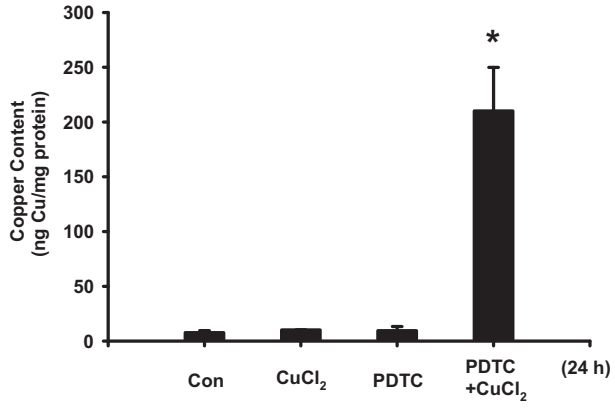

Fig. 2. Intracellular copper contents in PDTC, $CuCl_2$, and PDTC/Cu complex exposed lung epithelial cell-derived L2 cells. Cells were treated with PDTC (0.5 μM), or $CuCl_2$ (1 μM) with or without PDTC for 24 h. Intracellular copper contents were determined by inductively coupled plasma mass spectrometry (ICP-MS). All data are presented as means ± SEM from eight independent experiments performed in triplicate. $^*P < 0.05$ as compared to vehicle control.

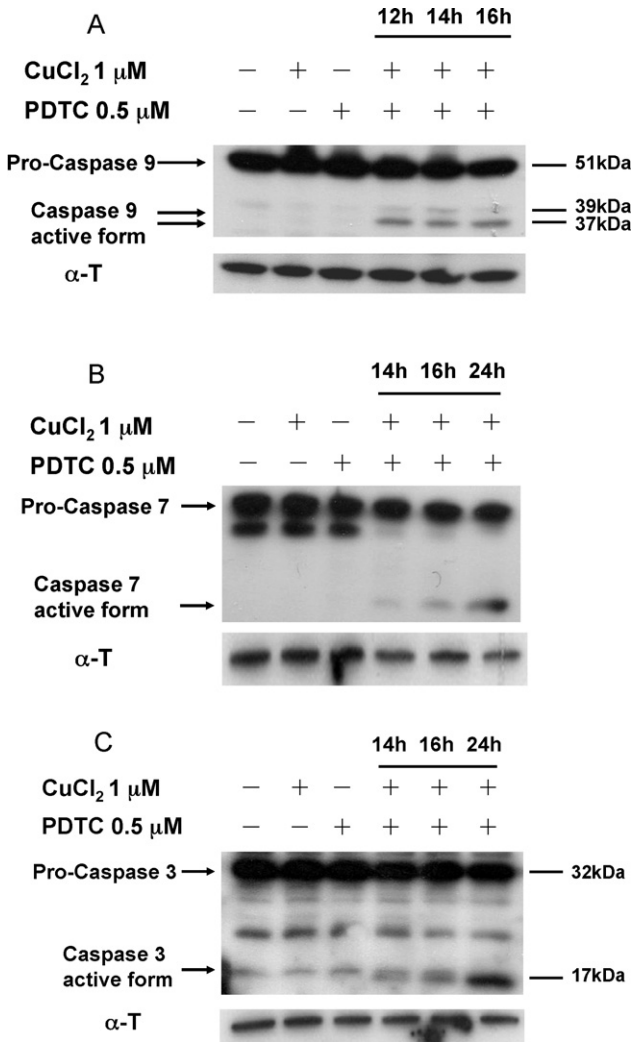

Fig. 3. Effects of PDTC, $CuCl_2$, and PDTC/Cu complex on caspase-9, caspase-7 and caspase-3 proteins expression in lung epithelial cell-derived L2 cells. Cells were treated with PDTC (0.5 μM), or $CuCl_2$ (1 μM) with or without PDTC for various time points. The Western blot analysis was used to detect (A) caspase-9, (B) caspase-7 and (C) caspase-3 protein expressions in lung epithelial cell-derived L2 cells. All data are representative of three independent experiments.

2.8. MMP analysis

The detection of MMP was performed as previously described (Chen et al., 2006). After the cells were treated with $CuCl_2$, PDTC, or PDTC/Cu complex, they were harvested at the indicated time points and washed twice with PBS. The cells were then treated with 40 nM $DiOC_6$ for 30 min and analyzed in a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

2.9. Statistical analysis

These data are expressed as the means ± SEM. Statistical significance was assessed using the paired Student's *t* test (Sigma Plot 10.0; Systat Software, San Jose, CA, USA). One-way ANOVA was used for multiple groups analysis, and Duncan's post hoc test was applied to identify group differences. A *P* value of less than 0.05 was considered significant. The statistical package SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

3. Results

3.1. Effects of PDTC, $CuCl_2$, and PDTC/Cu complex on cell viability, intracellular copper contents, MAPKs and caspase-9, -7 and -3 proteins expression in L2 alveolar epithelial cells

To examine the effects of PDTC, $CuCl_2$, and PDTC/Cu complex on lung alveolar epithelial cell viability, MTT assays were used to

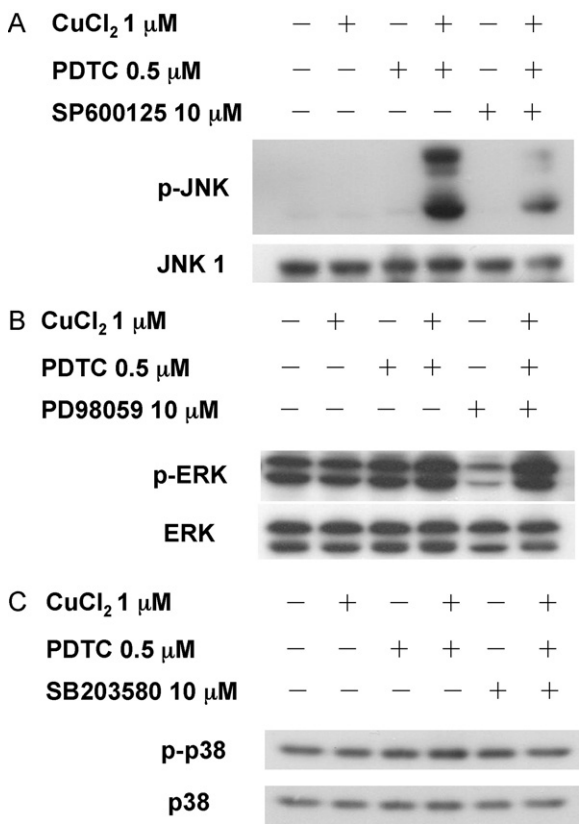

Fig. 4. Effects of PDTC, $CuCl_2$, and PDTC/Cu complex on MAPK protein expression in lung epithelial cell-derived L2 cells. Cells were pretreated with JNK inhibitor (SP600125), ERK inhibitor (SB203580), and p38 inhibitor (PD98059) for 30 min, respectively. After cells were treated with PDTC (0.5 μM), or $CuCl_2$ (1 μM) with or without PDTC for 1 h, the MAPK proteins, including JNK, ERK, and p38 phosphorylation, were analyzed by Western blot. All data are representative of three independent experiments.

detect cytotoxicity in L2 cells. The cells were treated with various doses of $CuCl_2$ with or without PDTC (0 μM, 0.5 μM, or 1 μM) for 24 h. The results showed that the viability of cells was not altered in the PDTC or $CuCl_2$ treatment groups, but viability was decreased in cells treated with the PDTC/Cu complex (Fig. 1). These experiments demonstrated that the PDTC/Cu complex might be cytotoxic to L2 cells.

We next investigated whether PDTC/Cu complex induced copper accumulation in L2 cells. Results showed that exposed to PDTC/Cu complex resulted amount of copper accumulation in cells. However, there were low levels of copper in control, PDTC, and $CuCl_2$ groups. The levels of copper concentration were 7.65 ± 1.95 (ng/mg protein), 10.14 ± 0.18 (ng/mg protein), and 9.51 ± 3.73 (ng/mg protein) in control, PDTC, and $CuCl_2$ groups, respectively. However, there was 210.08 ± 39.81 (ng/mg protein) in PDTC/Cu complex administered cells (Fig. 2).

To investigate the possible induction of apoptotic signaling, we determined the expression of caspase-9, caspase-7, and caspase-3 proteins in L2 cells following treatment with PDTC, $CuCl_2$, or PDTC/Cu complex. After the cells were treated with PDTC (0.5 μM) and $CuCl_2$ (1 μM) for 12 h, 14 h, or 16 h, the cleaved form of caspase-9 was significantly increased (Fig. 3A). Furthermore, the cleaved forms of caspase-7 and caspase-3 were also increased after the cells were treated with PDTC/Cu complex for 14 h, 16 h, and 24 h (Fig. 3B and C).

In the next experiments, MAPK signaling induced by the PDTC/Cu complex was evaluated by Western blot analysis. We further demonstrated that PDTC/Cu complex dramatically enhanced

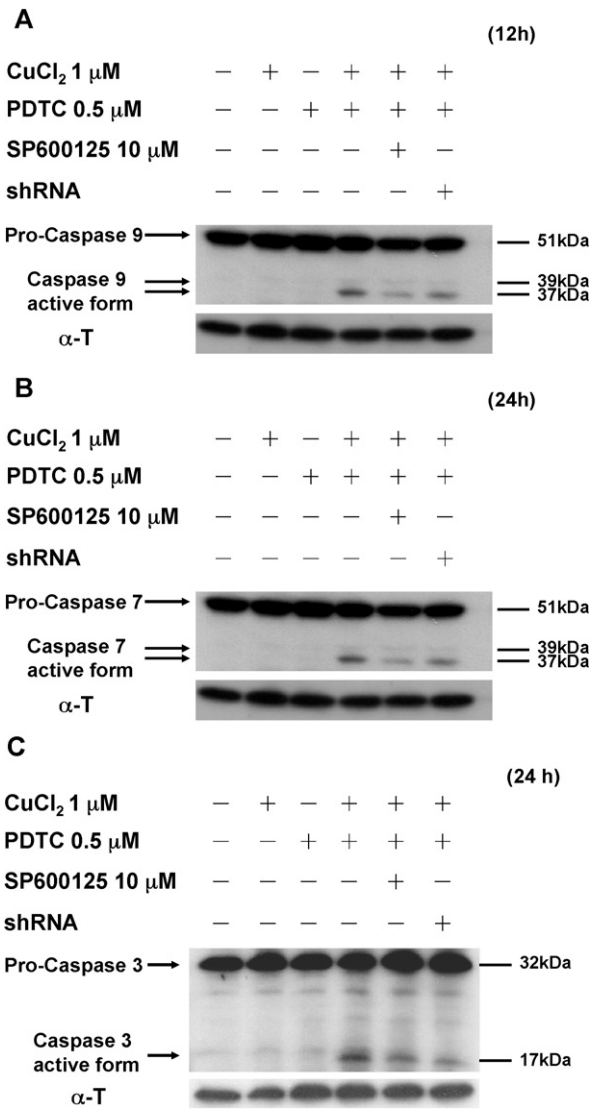

Fig. 5. Effects of PDTC, $CuCl_2$, and PDTC/Cu complex on caspase protein expressions in lung epithelial cell-derived L2 cells. Cells were pretreated with JNK inhibitors, SP600125 (10 μM), or transfected with human MAPK8 short hairpin RNA (shRNA). After cells were treated with PDTC (0.5 μM), or $CuCl_2$ (1 μM) combined with or without PDTC for 1 h, Western blot analysis was used to detect (A) caspase-9, (B) caspase-7 and (C) caspase-3 protein expression in lung epithelial cell-derived L2 cells. All data are representative of three independent experiments.

JNK and ERK phosphorylation proteins expression. These effects induced by PDTC/Cu complex could be inhibited by JNK inhibitor, SP600125, but not by ERK inhibitor, SB203580 (Fig. 4A and B). Besides, there are no effects on p38 phosphorylation protein expression (Fig. 4C). A previous study reported that JNK phosphorylation plays an important role during the induction of the intrinsic mitochondrial apoptotic pathway (Dhanasekaran and Reddy, 2008). These results indicated that the cytotoxicity of PDTC/Cu complex might involve JNK protein regulation.

Next we determined if PDTC/Cu complex-induced cell death signaling required the JNK phosphorylation pathway. After the cells were treated with PDTC/Cu complex, the cleaved form of caspase-9 was detected at 12 h and the cleaved forms of caspase-7 and caspase-3 were detected at 24 h. These PDTC/Cu complex-induced response could be reversed by addition of the JNK inhibitor, SP600125, or by transfection with a MAPK8 shRNA (Fig. 5A–C). These results indicated that PDTC–Cu complex induced cell death via the JNK phosphorylation pathway.

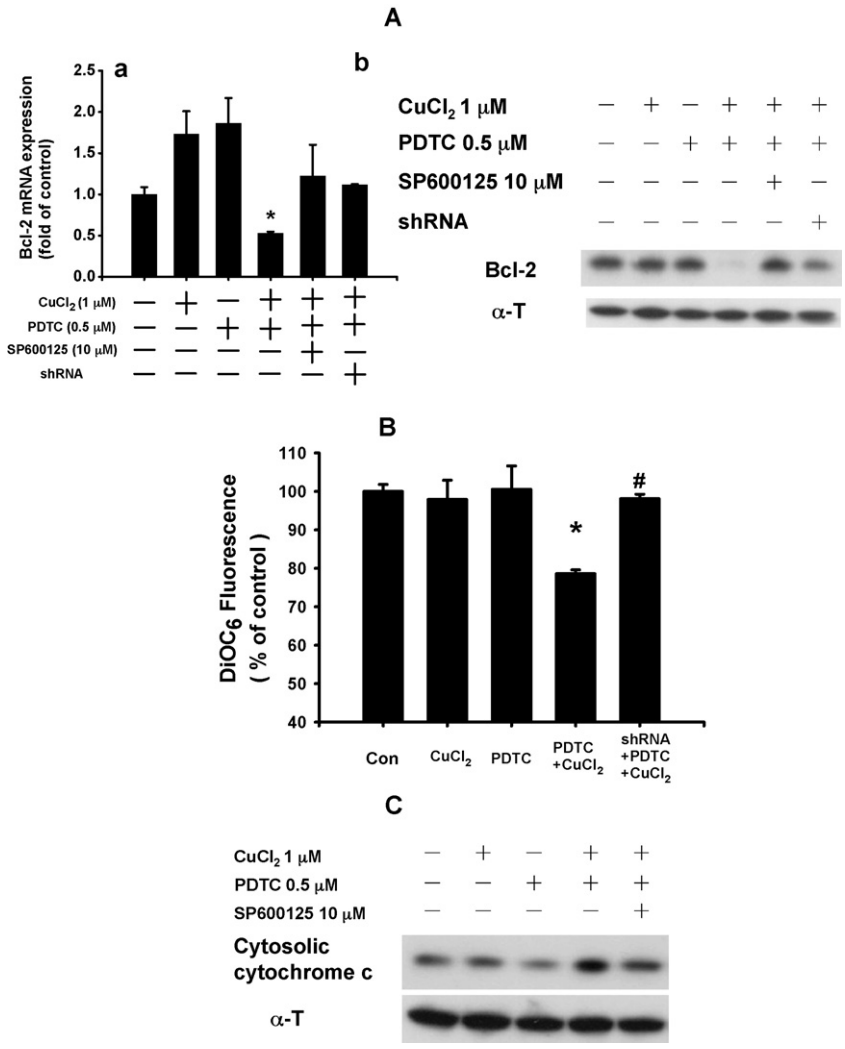

Fig. 6. Bcl-2 mRNA and protein expression, mitochondria transmembrane potential (MMP), and cytochrome *c* release in PDTC, $CuCl_2$, and PDTC/Cu complex treated L2 cells. (A) Cells were treated with PDTC (0.5 μM), $CuCl_2$ (1 μM), or PDTC/Cu complex with or without addition of the JNK inhibitor, SP600125 (10 μM), for 6 h, or with or without transfection of human MAPK8 short hairpin RNA (shRNA). (a) Bcl-2 mRNA and (b) protein expression were determined by quantitative real-time PCR and Western blot analysis, respectively. (B) Cells were treated as described above, and flow cytometry was used to detect alterations in MMP. (C) After cells were treated as described above, their cytosolic fractions were separated and cytochrome *c* release was determined via Western blot analysis. Data in (A-a) and (B) are expressed as mean ± SEM ($n = 4$ for each group). *$P < 0.05$ as compared with vehicle control group. Data in (A-b) and (C) are representative of three independent experiments.

3.2. *Effects of PDTC, $CuCl_2$, and PDTC/Cu complex on Bcl-2 expression, MMP, and cytosolic cytochrome c release in L2 alveolar epithelial cells*

To further investigate cell signaling in PDTC, $CuCl_2$, and PDTC/Cu complex treated L2 cells, we determined the effects of PDTC (0.5 μM), $CuCl_2$ (1 μM), and PDTC/Cu complex on Bcl-2 mRNA and protein expression. The results showed that PDTC and $CuCl_2$ did not alter Bcl-2 mRNA or protein expression. However, cells treated with PDTC/Cu complex demonstrated significantly decreased Bcl-2 mRNA and protein levels, and addition of the JNK inhibitor, SP600125, and transfection with a MAPK8 shRNA, reversed these effects (Fig. 6A-a and A-b). A previous study has shown that changes in the expression of Bcl-2 family proteins correlated with a decrease in MMP and release of cytochrome *c* in arsenic-trioxide-induced cell apoptosis (Zhong et al., 2009). Thus, we next investigated possible alterations in MMP and cytochrome *c* release in cells treated with PDTC, $CuCl_2$, or PDTC/Cu complex. The MMP did not change in PDTC or $CuCl_2$ treated cells. However, the MMP was significantly decreased in cells treated with PDTC/Cu complex (Fig. 6B). Our results also showed that neither PDTC nor $CuCl_2$ induced cytochrome *c* release in L2 cells. However, cells treated with PDTC/Cu complex demonstrated a dramatic increase in cytochrome *c* release (Fig. 6C). The effects of PDTC/Cu complex on both MMP and cytochrome *c* release were reversed by addition of the JNK inhibitor, SP600125 (Fig. 6B and C).

3.3. *Effects of PDTC, $CuCl_2$, and PDTC/Cu complex on the ER stress pathway in L2 alveolar epithelial cells*

We next investigated the role of the ER stress pathway in the cytotoxicity of PDTC/Cu complex in L2 cells. We first examined the expression of Grp78, Grp94, and procaspase-12 proteins by Western blot. Results showed that the Grp78 and Grp94 protein levels were significantly increased following 8–24 h of PDTC (0.5 μM) and Cu (1 μM) treatment. In contrast, procaspase-12 levels were decreased after treatment with PDTC/Cu complex for 8–24 h (Fig. 7). Furthermore, mRNAs encoding proteins related to the ER stress pathway, including Grp78, Grp94, ATF4, and CHOP, were dramatically increased after the cells were treated with PDTC/Cu complex, and these effects could be inhibited by addition of the JNK inhibitor, SP600125 (Fig. 8A–D). These results indicated that PDTC/Cu com-

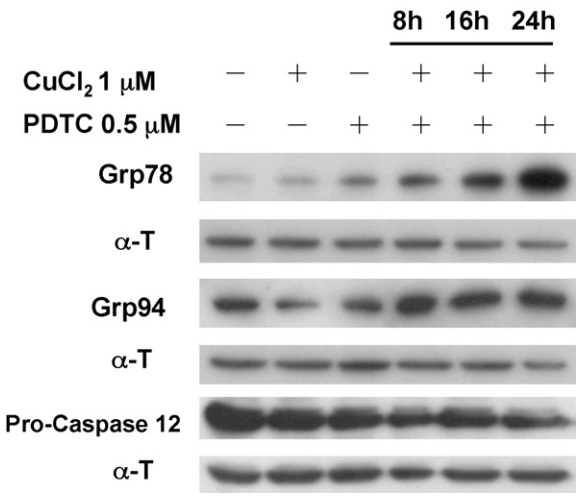

Fig. 7. Effects of PDTC, $CuCl_2$, and PDTC/Cu complex on Grp78, Grp94, and procaspase-12 protein expression in L2 cells. Cells were treated with PDTC (0.5 μM), $CuCl_2$ (1 μM), or PDTC/Cu complex for 8–24 h and then assayed via Western blot analysis to determine procaspase-12, Grp78, and Grp94 protein levels. All data are representative of three independent experiments.

plex induced ER stress signaling and that this effect required the JNK phosphorylation pathway in L2 cells.

4. Discussion

Many studies have reported that PDTC and $CuCl_2$ accumulation disrupted antioxidant systems and induced cell damage in many cell types. The effects of PDTC- and $CuCl_2$-induced cell damage have been related to the ROS-triggered apoptosis pathway (Chen et al., 2008). Despite many studies having reported on toxicity of PDTC and $CuCl_2$ in several kinds of cells, there have been no studies that have discussed PDTC, $CuCl_2$, or PDTC/Cu complex toxicity in type-2 alveolar epithelial cells. Specifically, the precise action and mechanism of PDTC/Cu complex induced cell damage in alveolar epithelial cells have not been clarified. In the present study, we investigate the cytotoxicity of PDTC/Cu complex and its possible mechanisms of cell death in alveolar epithelial cells.

Apoptotic signaling is divided into two major cell-death pathways, including intrinsic and extrinsic pathways. A previous study showed that activation of intrinsic apoptotic signaling includes a release of mitochondria cytochrome *c*, an increase in Apaf-1 expression, a decrease in pro-apoptotic Bcl-2 family proteins, and an activation of caspases and cleavage of their cellular substrates. The extrinsic signaling pathway is mediated by death receptors and subsequent activation of the caspase cascade (Putcha et al., 2002). Recently, it has been shown that alveolar epithelial cell death promotes the progression of pulmonary fibrosis (Waisberg et al., 2010).

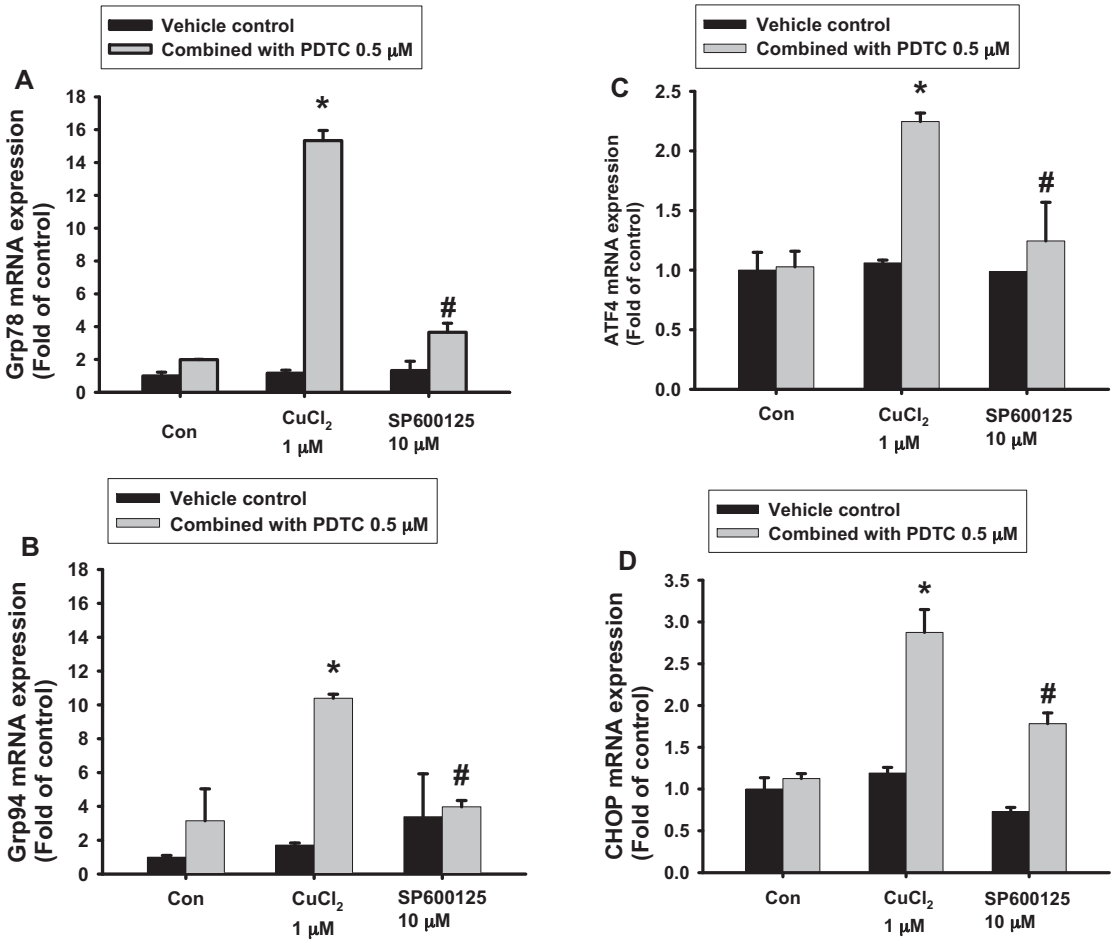

Fig. 8. The mRNA expression of Grp78, Grp94, ATF4, and CHOP in PDTC, $CuCl_2$, and PDTC/Cu complex treated L2 cells. Cells were pretreated with the JNK inhibitor, SP600125, for 30 min and then treated with PDTC (0.5 μM), $CuCl_2$ (1 μM), or PDTC/Cu complex for 24 h. mRNA levels were determined by quantitative real-time PCR. All data are expressed as mean ± SEM ($n = 4$ for each group). *$P < 0.05$ as compared to the vehicle control group.

Type II alveolar epithelial cells are responsible for surfactant synthesis and secretion (Yang et al., 2003). In our study, we found that treatment of cells with PDTC or $CuCl_2$ did not have any effect on cell viability. However, when cells were exposed to PDTC and $CuCl_2$ simultaneously, this PDTC/Cu complex significantly reduced the viability and activation of caspase-9, -7 and -3 in L2 cells. Furthermore, the PDTC/Cu complex significantly increased intracellular copper concentration, but PDTC or $CuCl_2$ alone had low levels of copper. PDTC increased copper entering to cells. Therefore, these results indicated that the formation of PDTC/Cu complex was cytotoxic to L2 cells and increased intracellular copper levels. Furthermore, PDTC/Cu complex induced apoptotic effects in L2 cells.

The MAPK/JNK signaling cascade promotes cell death in several cell types (Dinh and Van De Water, 2009; Mizote et al., 2010). The MAPK pathway is known to be involved in many biology and physiology functions, such as proliferation, survival, differentiation, and locomotion. A total of nine MAPKs have been described in mammalian cells, including ERK1/2, ERK3, ERK4, ERK5, ERK6/p38MAPKg, ERK7, ERK8, JNK1/2/3, and p38MAPKa/b/d. MAPKs are activated by a cascade of protein phosphorylation and can subsequently phosphorylate a large number of substrate proteins to regulate downstream of signaling (Engstrom et al., 2010). The JNKs belong to the superfamily of MAP kinases that are involved in many biological processes, including cell proliferation, differentiation, and apoptosis. Recently, studies have reported that JNKs can be activated by growth factors, cytokines, and stress factors. Thus, JNKs seem to play an important role in regulating apoptotic signaling (Dhanasekaran and Reddy, 2008). In our study, we found that PDTC/Cu complex significantly enhanced JNK and ERK phosphorylation protein expression, and these effects could be reversed by addition of the JNK inhibitor, SP600125, but not ERK inhibitor. Furthermore, PDTC/Cu complex could not promote the p38 phosphorylation protein expression. Thus, we concluded that PDTC/Cu complex induced JNK phosphorylation-related apoptotic signaling.

A previous study has reported that JNK phosphorylation plays an important role in induction of the mitochondrial intrinsic apoptotic pathway (Dhanasekaran and Reddy, 2008). Current evidence suggests that mitochondria play a pivotal role in caspase activation through the release of cytochrome *c* (Desagher and Martinou, 2000). Another study also showed that mitochondrial apoptotic signaling is related to MAPKKK function within the JNK/stress-activated protein kinase (SAPK) and p38 MAPK signaling pathways (Chang et al., 1998). For example, it has been reported that cardiocyte apoptosis is attenuated by inhibition of JNK phosphorylation and by TNF-α, caspase-8, Bax, and cytochrome *c* through mitochondria pathway (Zhang et al., 2009a,b). However, there have been no studies that have explored the relationship of JNK and mitochondria in lung alveolar cells. Here, we report that treatment with PDTC/Cu complex significantly increased Bcl-2 mRNA and protein expression in L2 cells. Furthermore, activation of caspase-9, caspase-7, and caspase-3 was detected after these cells were treated with PDTC/Cu complex, and addition of a JNK phosphorylation inhibitor reversed these effects. We suggested that PDTC/Cu complex induced cell death signaling through a JNK and mitochondria dependent pathway in L2 cells.

Apoptosis is known to involve the proteolytic activation of caspase cysteine proteases, which is regulated by Bcl-2 family proteins. Bcl-2 family proteins are localized in the ER membrane and have been shown to influence ER homeostasis and ER membrane permeability. ER proteins interact with Bcl-2 family proteins to activate death effectors or influence the sensitivity of mitochondria to apoptotic transitions (Breckenridge et al., 2003). Among the ER-resident molecular chaperones, Grp78 is a highly conserved 78-kDa protein that has 60% amino-acid homology with the 70-kDa heat-shock protein (HSP70). Grp94 is the most abundant glycoprotein in ER and has 50% amino-acid homology with HSP90. Both Grp78 and Grp94 have been identified as ER indicators (Zhang et al., 2009a,b). ATF4 is a basic leucine zipper transcription factor, and upregulation of ATF4 occurs during the recovery of cellular stress, growth arrest, and DNA damage (Whitney et al., 2009). The transcription factor CHOP is also known as growth-arrest and DNA-damage inducible gene 153 (GADD153). This protein is a member of the C/EBP transcription factor family that heterodimerizes with other C/EBPs and can be induced by ATF4. CHOP is known to promote apoptotic cell death (Szegezdi et al., 2006). In idiopathic pulmonary fibrosis, ER stress induces type II alveolar epithelial cell apoptosis through activation of ATF6, ATF4, CHOP, and X-box binding protein 1 (XBP1) (Korfei et al., 2008). It has also been shown that anticancer drugs increase CHOP and Grp78 expressions to induce ER stress-related apoptosis in A549 cells (Lin et al., 2008). In our experiments, we found that PDTC/Cu complex decreased procaspase-12 and increased Grp78 and Grp94 protein expressions in L2 cells. The mRNAs of Grp78, Grp94, ATF4, and CHOP were also increased by PDTC/Cu complex treatment. All of these effects could be reversed by addition of the JNK inhibitor. Thus, these results indicated that the ER stress pathway was activated by PDTC/Cu complex and that this was influenced by JNK phosphorylation.

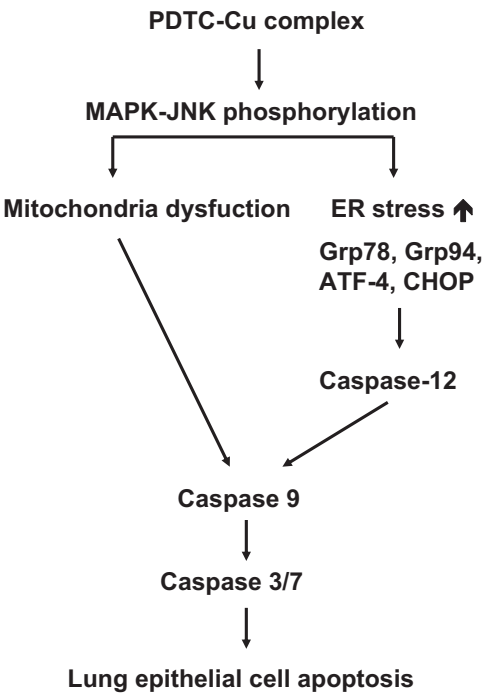

Fig. 9. Schematic representation of the proposed intracellular signaling leading to PDTC–Cu complex-induced cell death in alveolar lung epithelial cells.

5. Conclusion

Collectively, we presented evidence showing that PDTC/Cu complex decreased cell viability and Bcl-2 expression and increased JNK phosphorylation expression in L2 cells. Furthermore, a decrease in MMP, increased release of cytochrome *c*, and activation of caspase-9, caspase-7, and caspase-3 were involved in PDTC/Cu complex-induced cell death. Furthermore, increased levels of Grp78, Grp94, ATF4, and CHOP and decreased procaspase-12 were reported in the JNK phosphorylation induced apoptotic pathway (Fig. 9). Our results indicated that PDTC/Cu complex exposure promoted alveolar epithelial cell apoptosis through JNK phosphorylation-dependent mitochondria and ER stress pathways. These observations provide evidence that PDTC/Cu complex is an

environmental risk factor for lung functional disorders and diseases, such as lung fibrosis, emphysema, and acute lung injury.

Conflict of interest statement

All authors declare that there are no conflicts of interest in this study.

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