Toxicology in Vitro 23 (2009) 288–294

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

The protection of selenium on ROS mediated-apoptosis by mitochondria dysfunction in cadmium-induced LLC-PK$_1$ cells

Yi-jing Zhou, Shi-ping Zhang, Chang-wei Liu, Yun-qing Cai *

Department of Nutrition and Food Hygiene, Nanjing Medical University, 140 Han-zhong Road, Nanjing 210029, The People's Republic of China

ARTICLE INFO

Article history:
Received 14 July 2008
Accepted 11 December 2008
Available online 24 December 2008

Keywords:
Selenium
Cadmium
Apoptosis
Reactive oxygen species (ROS)

ABSTRACT

Selenium, an essential trace element, showed the significant protective effects against liver and kidney damage induced by some heavy metals. However, the mechanism how selenium suppresses cadmium (Cd)-induced cytotoxicity remains unclear. In this study, we investigated the protective mechanism of selenium on Cd-induced apoptosis in LLC-PK$_1$ cells via reactive oxygen species (ROS) and mitochondria linked signal pathway. Studies of PI and Annexin V dual staining analysis demonstrated that 20 μM Cd-induced apoptosis as early as 18 h. A concomitant by the generation of ROS, the loss of mitochondrial membrane potential, cytochrome *c* (cyt *c*) release, activation of caspase-9, -3 and regulation of Bcl-2 and Bax were observed. *N*-acetylcysteine (NAC, 500 μM), a free radical scavenger, was used to determine the involvement of ROS in Cd-induced apoptosis. During the process, selenium played the same role as NAC. The anti-apoptosis exerted by selenium involved the blocking of Cd-induced ROS generation, the inhibition of Cd-induced mitochondrial membrane potential collapse, the prevention of cyt *c* release, subsequent inhibition of caspase activation and the changed level of Bcl-2 and Bax. Taken together, we concluded that Cd-induced apoptosis was mediated by oxidative stress and selenium produced a significant protection against Cd–induced apoptosis in LLC-PK$_1$ via ameliorating the mitochondrial dysfunction.

1. Introduction

Cadmium (Cd) is a long lifetime occupational and environmental pollutant, which has been classified by the International Agency for Research on Cancer (IARC, 1993) as type I carcinogen in humans. It has been well established that chronic exposure to cadmium causes irreversible kidney damage and renal tubular dysfunction (Nishijo et al., 2006; Horiguchi et al., 2006).

Although, Cd is a redox-inactive metal, it was reported to induce the formation of reactive oxygen species (ROS) in a wide variety of cells (Hart et al., 1999; Stachura et al., 2000; Thevenod et al., 2000). ROS are known to affect mitochondrial membrane potential and trigger a series of mitochondria-associated events including apoptosis (Chatterjee et al., 2008; Pathak and Khandelwal, 2007). High level of ROS in the mitochondria can result in free radical attack of membrane phospholipids, preceding mitochondrial membrane depolarization. Mitochondria depolarization, considered as an irreversible step in the apoptosis process, can trigger a cascade of caspases (Pelicano et al., 2003; Stohs and Baghi, 1995). The mitochondrion is a major subcellular compartment where the Bcl-2 family members interact with each other or exert function independently (Huppertz et al., 2006). The Bcl-2 family of proteins include proteins that can either inhibit (Bcl-2, Bcl-xL, etc.) or favor (Bax, Bak, Bad, etc.) apoptosis. Bcl-2 is an anti-apoptotic protein, which is predominantly present in mitochondria, prevents apoptosis by suppressing oxyradical-mediated membrane damage and stabilizing mitochondrial membrane potential (Bruce-Keller et al., 1998; Desagher and Martinou, 2000). The proapoptotic protein Bax regulates the mitochondrial pathway by triggering the release of apoptotic factors such as cytochrome *c* from the mitochondrial subsequent to the death signal (Hsu et al., 1997).

Selenium (Se), an essential trace element, is involved in functions of several enzymes and proteins such as glutathione peroxidases (GPx), selenoprotein P and thioredoxin reductases (Stadtman, 1991). GPx is an antioxidant enzyme which protects membrane lipids and macromolecules against the oxidative damage generated by peroxides (Hsu et al., 1997). Most of the selenoproteins have ROS scavenging activities, so that the action of

Abbreviations: $CdCl_2$, cadmium chloride; cyt *c*, cytochrome *c*; DCF, 2,7-dichlorofluorescein; DCFH-DA, 2,7-dichlorofluorescein diacetate; FITC, Annexin V-fluorescein isothiocyanate; GPx, glutathione peroxidases; GSH, glutathione; JNK, c-Jun *N*-terminal kinase; MAPK, mitogen-activated protein kinases; NAC, N-acetyl-L-cysteine; PBS, phospate buffered saline; PI, propidium iodide; ROS, reactive oxygen species; SAPK, stress-activated protein kinase; Se, selenium; $\Delta\psi m$, mitochondrial membrane potential.

* Corresponding author. Tel.: +86 2586662941; fax: +86 2586662930.
E-mail address: Cai2941@163.com (Y.-q. Cai).

0887-2333/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2008.12.009

selenium has been known as an antioxidant system in cell survival (Stadtman, 1991). There are some reports showed that renal proximal tubular damage caused by prolonged Cd exposure was suppressed by treatment with antioxidants, such as Vitamin E, glutathione, and *N*-acetylcysteine (NAC), indicating that oxidative stress plays a critical role in Cd-induced nephrotoxicity (Acharya et al., 2008; Shaikh and Tang, 1999). However, the underlying mechanisms involved in the cytoprotective effects of selenium against Cd-induced apoptosis remains to be elucidated.

In our previous report, we clarified the protective effects of selenium on Cd-induced apoptosis in LLC-PK_1 cells through the c-jun *N*-terminal kinase (JNK) phosphorylation activation (Liu et al., 2007). The JNK, also known as stress-activated protein kinase (SAPK), belongs to the mitogen-activated protein kinases (MAPK) superfamily. Some reports showed that MAPK signal pathways seemed to be responsible for mitochondria mediated cell apoptosis and ROS production was lined to JNK phosphorylation activation (Liu and Huang, 2006; Zhou et al., 2008). Therefore, we hypothesized such protective effects of selenium against Cd-induced apoptosis might involve ROS and mitochondrial medicated-pathway. This study was conducted to explore the protection mechanism of selenium on Cd-induced apoptosis in LLC-PK_1 cells via ROS and mitochondria function.

2. Materials and methods

2.1. Materials

Cadmium chloride, Sodium selenite and *N*-acetyl-L-cysteine (NAC) (Sigma Chemical Co., St. Louis, MO, USA) were dissolved in water, sterilized with 0.22 μm filters, and added to cultures at the indicated time and concentrations. Cell culture reagents were obtained from GIBCO Life Technology (Grand Island, NY). Antibodies specific for the bcl-2, bax and anti-rabbit IgG, were purchased from Cell Signaling Technology (Beverly, MA). β-Actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Annexin V-FITC Apoptosis Detection kit from Pharmingen (Becton Dickinson Company). The 6-carboxy-2′,7′ dichlorodihydrofluorescein diacetate (DCFH-DA) and caspase-3, 9 activity kit were obtained from Beyotime Institute of Biotechnology (Haimen, China), Rhodamine123 (Rh 123) was purchased from Molecular Probes (Eugene, OR, USA). All other reagents unless indicated were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture and chemical treatments

LLC-PK_1 cells, a porcine renal epithelial cell line, generously provided by Dr Zhou XM (Uniformed Services University, USA). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2, 95% air at 37 °C. The cells were seeded at a density of 1.5×10^6 cells on 10 cm petridishes and used for the drug exposures after overnight culture. Cd (100 mM), selenite (100 mM) and NAC (100 mM) were used respectively as stock solutions in distilled water, and these were further diluted with a medium to the desired concentrations. The cells were pretreated with selenite and NAC for 0.5 h prior to the addition of Cd.

2.3. Assessment of apoptotic and necrotic cells

Flow cytometry was used to assess the membrane and nuclear events during apoptosis. The assay was performed with a two color analysis of FITC-labeled Annexin V binding and PI uptake using the Annexin V-FITC Apoptosis Detection kit. Briefly, after the treatment period the harvested cells were suspended in a binding buffer (1×). An aliquot of 100 μl was incubated with 5 ul of Annexin V-FITC and 5 ul of PI for 15 min in dark, 400 μl binding buffer (1×) was added to each sample. The stained cells were analyzed directly by flow cytometry using the Cell Quest program (Becton Dickinson, Franklin, NJ).

2.4. Reactive oxygen species (ROS) measurement

The production of peroxides was measured by staining cells with the fluorescent probe 2,7-dichlorofluorescein diacetate (DCFH-DA). This dye is a stable nonpolar compound that readily diffuses into cells and yields DCFH. Intracellular H_2O_2 or OH in the presence of peroxidase change DCFH to the highly fluorescent compound 2,7-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of peroxides produced by the cells. After the treatment, cells were harvested, washed with phosphate-buffered saline (PBS pH 7.2), and incubated with 10 μmol/L DCFH-DA for 0.5 h at 37 °C. And then ROS generation was measured by the fluorescence intensity (FL-1,530 nm) of 10,000 cells.

2.5. Measurement of mitochondrial membrane potential ($\Delta\psi m$)

For the detection of mitochondrial membrane potential, Cd, Se or NAC treated cells were incubated with Rh 123 (5 mg/ml) for 60 min in dark at 37°C, harvested and suspended in PBS. The mitochondrial membrane potential was measured by the fluorescence intensity (FL-1, 530 nm) of 10,000 cells.

2.6. Cytochrome c quantification by ELISA

Cytochrome *c* was determined using a quantitative immunoassay (DCDCO, R&D Systems, Wiesbaden, Germany). Briefly, untreated, Cd, Se or NAC treated cells (1.5×10^6 cells/ml) were washed in PBS and treated with lysis buffer for 1 h at room temperature. Following centrifugation at 1000*g* for 15 min, supernatants were removed and 200 μl of a 1:100 dilution was used for a sandwich enzyme-linked immunosorbent assay. Following incubation with substrate solution in the dark for 30 min the reaction was stopped. The optical density of each well was determined on an enzyme-linked immunosorbent assay reader at 450 nm. The amount of cytochrome *c* was determined according to a calibration curve.

2.7. Caspase-3,-9 activity assay

Caspase-3 and -9 activities were measured through cleavage of a colorless substrate specific for caspase-3 (Ac-DEVD-*p*NA) or caspase-9 (Ac-LEHD-*p*NA) releasing the chromophore, *p*-nitroaniline (*p*NA). Assays were carried out according to manufacturer's instructions. To evaluate the activity of caspase-3,-9, cell lysates were prepared after their respective treatment with various designated treatments. Assays were performed on 96-well microtitre plates by incubating 10 μl protein of cell lysate per sample in 80 μl reaction buffer [1% NP-40, 20 mM Tris–HCl (pH 7.5), 137 mM Nad and 10% glycerol] containing 10 μl caspase-3 substrate (Ac-DEVD-*p*NA, 2 mM) or caspase-9 substrate (Ac-LEHD-*p*NA, 2 mM). Lysates were incubated at 37 °C for 4 h. Samples were measured with an ELISA reader at an absorbance of 405 nm.

2.8. Western blotting

Cells were grown at 5×10^4 cells/well in 6-well microplates and incubated with Cd, Se or NAC for indicated time and concentrations. Following treatment, cells were washed with PBS, and total cell lysates were prepared by scraping in 200 ul of lysis buffer

[20 mM Tris–HCl (pH 8.0), 1 mM sodium orthovanadate, 10% glycerol, 1mM phenylmethylsulfonyl fluoride, 2 mM ethylenediaminetetraacetate (EDTA), 1% Triton X-100, 50 mM β-glycerolphosphate, 10 mg/ml each of aprotinin, leupeptin, and pepstatin]. Fifty micrograms of proteins determined by Bradford assay was electrophoretically separated using a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) gel and transferred to PVDF membrane and then immunoblotted with the corresponding antibodies. Immunodetection was performed using enhanced chemiluminescence (ECL) detection kit (Cell Signaling Technology, Beverly, MA).

2.9. Statistical analysis

Data were expressed as means ± SD and analyzed using one-way analysis of variance (ANOVA) procedures among treatment groups. Statistically significant differences were reported as $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{\#\#}p < 0.01$. Data with values of $p < 0.05$ were generally accepted as statistically significant.

3. Results

3.1. The effects of selenium on Cd-induced apoptosis

To observe whether selenium is capable of inhibiting Cd-induced apoptosis, cells were pretreated with different concentrations of selenium (5, 10 and 20 μM) or NAC (500 μM), then incubated them with 20 μM of Cd. After the treatment, cells were stained with PI and FITC-labeled Annexin V (AV-FITC) to analyze the percentage of apoptotic cells with flow cytometry. At 18 h, the total (early + late) apoptotic cells drastically enhanced from 2.05% (control) to 82.33% at 20 μM Cd (Fig. 1A and B). However, selenium or NAC pretreatment before Cd exposure to the cells resulted in protection against Cd-induced apoptosis, as compared to that observed in the cells treated with Cd alone (Fig. 1C–F). The apoptotic population decreased with the advancing dose of selenium, a 5.7-fold lowering in the apoptotic population by 20 μM selenium and 4-fold by NAC at 18 h were observed. The results showed the protective effect of selenium for Cd-induced apoptosis.

3.2. The effects of selenium on Cd-induced ROS generation

The cells were stained with DCFH-DA to examine the production of ROS in the LLC-PK_1 cells and the DCF fluorescence was measured by flow cytometer. We determined that ROS was peaked within 12 h in the LLC-PK_1 cells after Cd treatment, and then at 18 h, when DNA damage was intense, the ROS decreased (Fig. 2A). To further investigate the protective role of selenium on Cd-induced ROS production, the 12 h time period for the evaluation of ROS was selected based on our experiments, because maximum DCF fluorescence was observed at this time point. Selenium pretreatment before Cd exposure to the cells resulted in the recovery of ROS generation levels to basal levels, as reflected by the decrease of DCF fluorescence staining from 194 to 31 at 20 μM selenium (Fig. 2B). NAC as a thiol antioxidant can raise intracellular glutathione levels and thereby protect cells from the effects of ROS (Aruoma et al., 1989). As shown in Fig. 2, NAC also effectively inhibited the Cd-induced ROS generation, the fluorescence intensity of DCF decreased from 194 to 24. Therefore, the results suggested that selenium may abolish Cd-induced ROS generation.

3.3. The effects of selenium on Cd-induced mitochondrial membrane potential ($\Delta\psi m$) reduction and the Cd-induced cyt c release

ROS production may be accompanied by the reduction of $\Delta\psi m$, so we monitored the change of $\Delta\psi m$ in cells following treatment using Rh 123 and flow cytometry. A decrease in $\Delta\psi m$ occurred as early as 3 h, and the loss in $\Delta\psi m$ became significant at 6 h and 12 h (Fig. 3A). Pretreatment with selenium reversed the reduction of $\Delta\psi m$ resulted from Cd as indicated by a decrease in Rh 123 fluorescence. Concurrently, NAC was found to be capable of restoring the drop of $\Delta\psi m$ caused by Cd to its normal state (Fig. 3B). Concomitantly, Cd-induced cyt *c* release was also observed in LLC-PK_1 cells, represent as significant increase of cytosolic cyt *c* concentration (Fig. 3C). And cyt *c* in cytosolic markedly increased by Cd was restored by selenium or NAC treatment. These results indicated that selenium exerted cytoprotective effects against Cd-induced apoptosis by inhibiting the reduction of $\Delta\psi m$ and cyt *c* release.

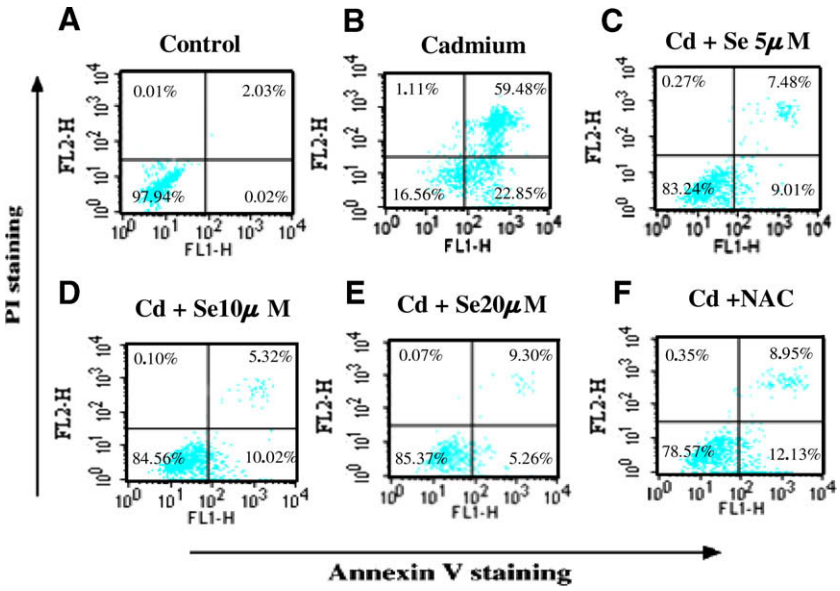

Fig. 1. The effects of selenium and NAC on cadmium-induced apoptosis. LLC-PK_1 cells were treated with Cd (20 μM) for 18 h or pretreated with Se (5, 10, 20 μM) or NAC (500 μM) for 0.5 h, then exposed to 20 μM Cd for 18 h, cell distribution analyzed using Annexin V binding and PI uptake. The FITC and PI fluorescence were measured using flow cytometer with FL-1 and FL-2 filters, respectively. LL, living cells (Annexin V^-/PI^-); LR, early apoptotic/primary apoptotic cells (Annexin V^+/PI^-); UR, late apoptotic/secondary apoptotic cells (Annexin V^+/PI^+); UL, necrotic cells (Annexin V^-/PI^+).

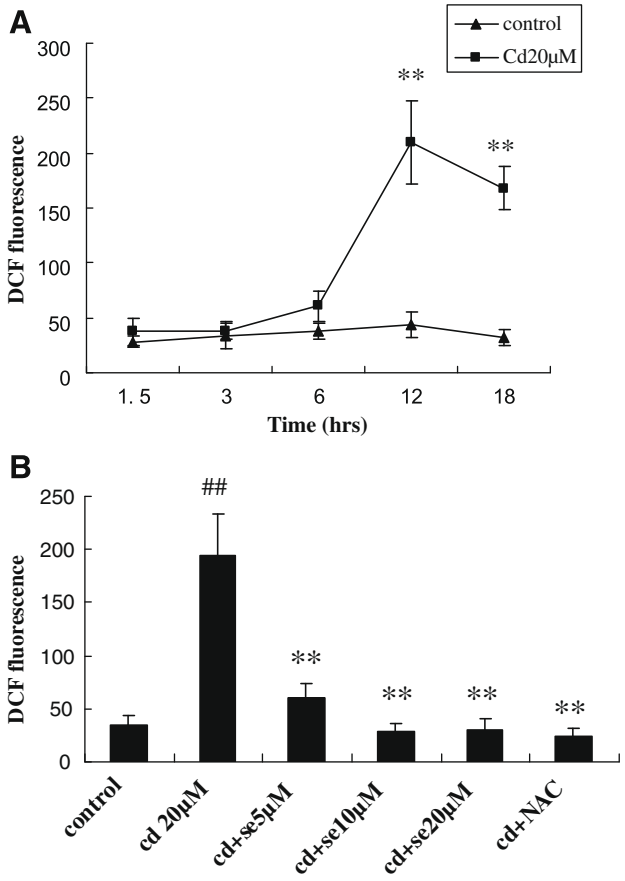

Fig. 2. The effects of selenium and NAC on cadmium-induced ROS generation. (A) Time-course analysis of ROS production in the LLC-PK$_1$ cells. After cells were treated with 20 μM of Cd for the indicated times, the cells were stained with DCFH-DA. Intracellular ROS was monitored by flow cytometry. (B) Cells were pre-incubated with 5, 10, 20 μM selenium or 500 μM NAC selenium for 0.5 h, and then treated with 20 μM Cd for 12 h. Mean fluorescence obtained from the histogram statistics. Each bar represents mean ± S.D. (n = 3). $^{\#\#}p < 0.01$ compared to control; $^{**}p < 0.01$, $^{*}p < 0.05$ compared to Cd treated group, using one-way ANOVA.

3.4. The effects of selenium on Cd-induced caspase-9, -3 activation

Since caspase-9, -3 activation is predominantly triggered by the changes of mitochondrial membrane potential (Δψm). We further investigated the effects of selenium on Cd-induced caspase activation. Cells were pre-incubated with 5, 10, 20 μM selenium or 500 μM NAC for 0.5 h before treatment with 20 μM Cd, respectively. After 12 h of treatment of Cd, the caspase activity was quantified. As shown in Fig. 4A, all the doses of selenium showed a significant ($p < 0.01$) decrease in caspase-9 activity as compared to Cd alone treated cells in a dose-dependent manner. A significantly 2.0-fold increase for levels of caspase-3 activity was shown in the LLC-PK$_1$cells with Cd exposure. When the cells were pretreatment with selenium, this enzyme activity significantly ($p < 0.01$) decreased in a dose-dependent manner, 20 μM selenium exhibiting maximum affect (Fig. 4B). Similarly, a significant decrease for the casapase-9, -3 activity was found in the cells with NAC pretreatment. As described above, the results may suggest that the elevation of Cd-induced casapase-9, -3 activation was mediated by oxidative stress and selenium has a suppressive effect on the Cd-related activation of caspases.

3.5. The effects of selenium on Bcl-2 and Bax protein expression

Bcl-2 family has been associated with mitochondrial function during apoptosis, therefore the level of Bcl-2 and Bax proteins were

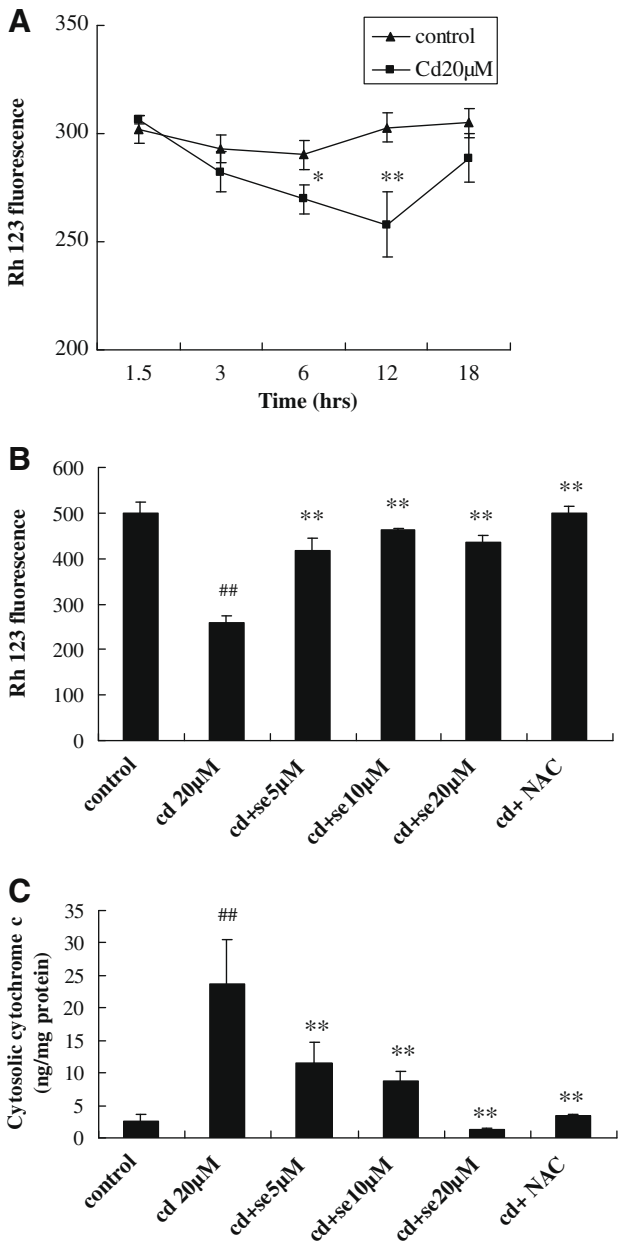

Fig. 3. The effects of selenium and NAC on cadmium-induced mitochondrial membrane potential reduction and the Cd-induced cyt c release. (A) Time-course analysis of mitochondrial membrane potential in the LLC-PK$_1$ cells. After cells were treated with 20 μM of Cd for the indicated times, Rh 123 was added and incubated for 1 h. The fluorescence was measured using a flow cytometer with FL-1 filter. (B) Cells were pre-incubated with 5, 10, 20 μM selenium or 500 μM NAC for 0.5 h, and then treated with 20 μM Cd for 12 h. Mean fluorescence obtained from the histogram statistics. (C) Cells were treated with 5, 10, 20 μM selenium or 500 μM NAC 0.5 h prior to 12 h of 20 μM Cd treatment. Cytoplasmic cyt c was determined by ELISA. Each bar represents mean ± S.D. (n = 3). $^{\#\#}p < 0.01$ compared to control; $^{**}p < 0.01$, $^{*}p < 0.05$ compared to Cd treated group, using one-way ANOVA.

examined by western blot analysis in the presence or absence of selenium. As shown in Fig. 5A, Bax protein in the cell lysate from LLC-PK$_1$ cells treated with 20 μM Cd for 12 h caused almost 8-fold increase as compared to control. However, pretreatment with 10, 20 μM of selenium or 500 μM NAC induced a marked reduction in amount of Bax. In Fig. 5B, with 20 μM Cd for 12 h, the amount of Bcl-2 in the cell lysate caused a 71% decrease compared to control. On the contrary, LLC-PK$_1$ cells which were pretreated with selenium (5, 10, 20 μM) showed up-regulation for the level of

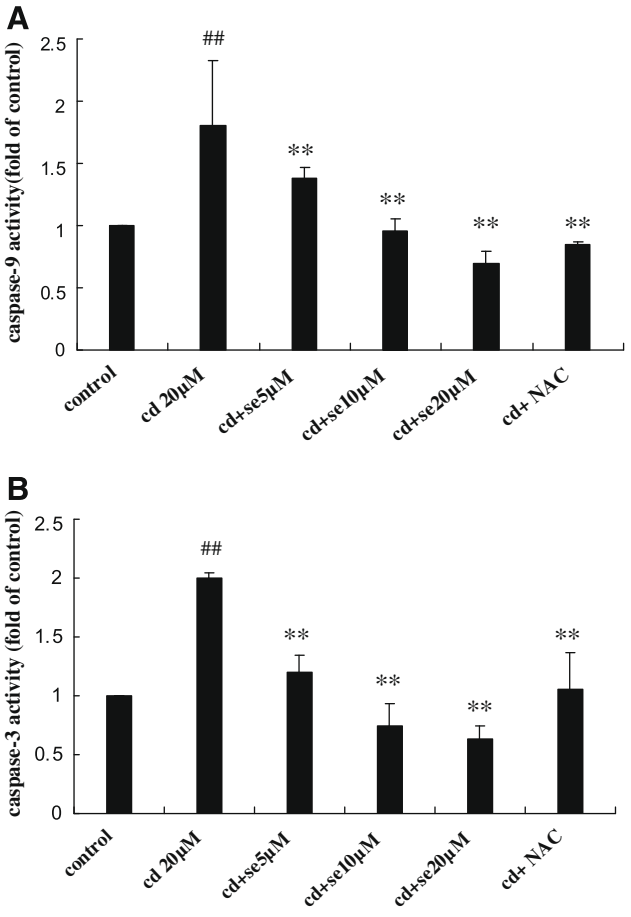

Fig. 4. Effects of selenium and NAC on cadmium-induced caspase-9 (A), -3 (B) activation. The LLC-PK_1 cells were treated with Cd (20 μM) for 12 h or pretreated with Se (5, 10, 20 μM) or NAC (500 μM) for 0.5 h, then exposed to 20 μM Cd for 12 h, respectively. The relative activities of caspase-9, -3 shown were calculated from the average of three experiments. Each value was expressed as the ratio of caspase-9, -3 activation levels to control level, and the value of control was set to 1. Each bar represents mean ± S.D. ($n = 3$). $^{\#\#}p < 0.01$ compared to control; $^{**}p < 0.01$, $^{*}p < 0.05$ compared to Cd treated group, using one-way ANOVA.

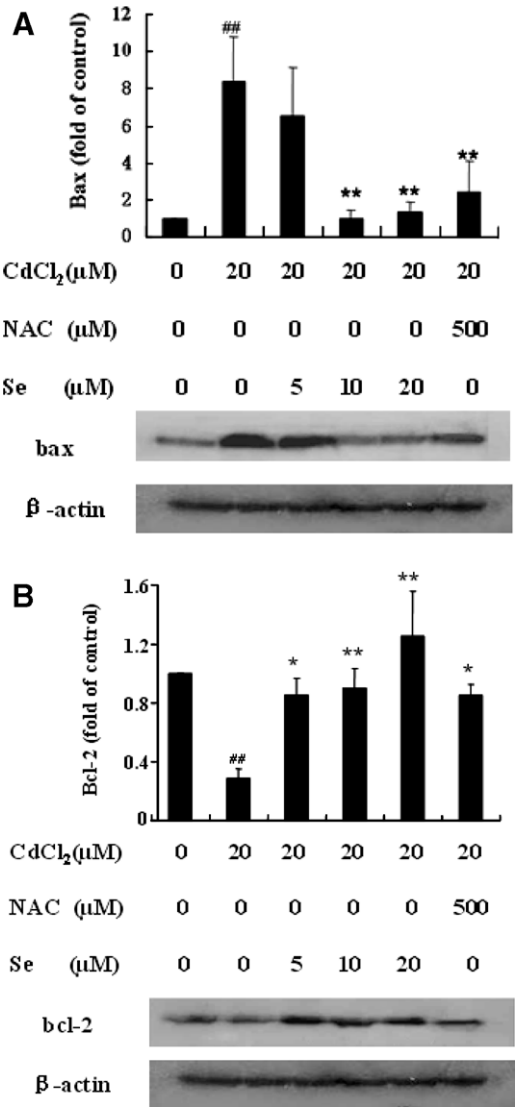

Fig. 5. The effects of selenium and NAC on cadmium-induced Bax (A) and Bcl-2 (B) protein expression. LLC-PK_1 cells were treated with Cd (20 μM) for 12 h or pretreated with Se (5, 10, 20 μM) or NAC (500 μM) for 0.5 h, then exposed to 20 μM Cd for 12 h, respectively. Bax and Bcl-2 were analyzed by the Western blot. Intensity of each band was quantified by densitometry. The relative activities of kinases shown were calculated from the average of three experiments. The protein expression from control group was designated as 1, and the others were expressed as folds compared with the control. Each bar represents mean ± S.D. ($n = 3$). $^{\#\#}p < 0.01$ compared to control; $^{**}p < 0.01$, $^{*}p < 0.05$ compared to Cd treated group, using one-way ANOVA.

Bcl-2 protein in a dose-dependent manner. The 20 μM selenium exhibiting 4.5-fold increase of Bcl-2 protein compared to Cd treated group. The level of Bcl-2 protein was also significantly increased in the groups of pretreatment with NAC at 500 μM.

4. Discussion

Numerous studies have reported that the Cd-induced apoptosis related to oxidative stress in various cells. Our previous studies also showed that Cd-induced apoptosis was through JNK signal pathways, which associated with ROS in the LLC-PK_1 cells (Liu et al., 2007). In this study, further protective effects of selenium as an anti-oxidant against Cd-induced apoptosis have been observed to clarify the protection mechanism of selenium on Cd-induced apoptosis mediated by ROS via mitochondria function in the LLC-PK_1 cells.

The generation of ROS, changes in mitochondrial membrane potential and caspase-9 activation are known be major elements of the mitochondrial pathway of apoptosis (Gupta et al., 2003; Fan et al., 2005). ROS was predominantly produced in the mitochondria, then leading to the free radical attack of membrane phospholipids and loss of mitochondrial membrane potential, which leads to increase of cytoplasmic cyt *c* released from damaged mitochondria, activation of caspases and then resulting in apoptosis (Nicholls and Budd, 2000; Kim et al., 2003; Belyaeva et al., 2006). Our results indicated that 20 μM Cd-induced apoptosis through the generation of ROS, the collapse of mitochondrial membrane potential, the accumulation of the cytosolic cyt *c* and the activation of caspase-9, -3. However, in our study ROS scavengers NAC had a suppressive effect on the Cd-related apoptosis, suggesting ROS may play a crucial role in Cd-induced apoptosis in the LLC-PK_1 cells. The presence of NAC effectively blocked the Cd-induced intracellular ROS generation, mitochondrial membrane potential collapse, cyt *c* release and the activation of caspase-9, -3, indicating that Cd-induced mitochondrial dysfunction may be regulated indirectly by ROS mediated signaling pathway.

Selenium is known to provide protection from ROS-induced cell damage, and the proposed mechanism mainly invokes the functions of glutathione peroxidases (GPxs) and selenoprotein P (SelP).

Selenium has been linked to regulatory functions in cell growth, survival and cytotoxicity, as well as transformations possibly involving redox regulation, chemical toxicity. Some reports showed that selenium can ameliorat the kidney damage induced by $HgCl_2$ injection (El-Shenawy and Hassan, 2008), and selenium also had the hepatoprotective effects against cadmium toxicity in rats (Newairy et al., 2007). Those reports showed the protection of selenium treatment might be associated with recovering inhibition of glutathione peroxidase and thioredoxin reductase activities, decreasing free radical-mediated lipid peroxidation and regenerate the glutathione (Gan et al., 2002). In our previous report, we observed that 10 μM selenium as an antioxidant agent played a cytoprotective role, which was mediated by the phosphorylation of JNK in Cd-induced apoptosis (Liu et al., 2007). In this study, we used the lower concentration of selenium and noted that even 5 μM selenium obviously protected against Cd-induced apoptosis. Furthermore, pretreatment of LLC-PK_1 cells with selenium significantly inhibited the apoptosis due to ROS and mitochondrial dysfunction. An important observation in this study showed that pretreatment of selenium in the LLC-PK_1 cells was determined to inhibit Cd-induced ROS generation. We found that selenium can induce the total inhibition of Cd-induced mitochondrial membrane potential collapse, the prevention of cyt *c* release and significantly attenuated the casapase-9, -3 activities. These findings, taken together, indicated that protective effects of selenium against Cd-induced apoptosis involve ROS and mitochondria linked signal pathway.

Bcl-2 is known as an upstream effecter molecule in the apoptotic pathway and is identified as a potent suppressor of apoptosis (Hockenbery et al., 1993; Tsujimoto and Shimizu, 2000). In addition, it was observed that Bcl-2 promotes cell survival by preserving the integrity of the external mitochondrial membrane, which prevents the release of cyt *c* from mitochondria and hence induce cell death (Desagher and Martinou, 2000; Kawakami et al., 2008). Bax is a 21-kDa protein promoting mitochondrial membrane permeability, which has been demonstrated to accelerate apoptotic cell death (Wolter et al., 1997; Neuzil et al., 2006). Our studies revealed Bcl-2 protein expression was decreased significantly and Bax protein expression was increased as early as 12 h following exposure to Cd. On the contrary, in our study NAC was shown to completely inhibit the Cd-induced apoptosis pathway via the Bcl-2 up-regulation and Bax downregulation. These results suggested that Cd-induced ROS generation was capable of modulating Cd-induced apoptosis via regulation of Bcl-2 and Bax. Furthermore, selenium markedly reduced Cd-induced apoptosis in LLC-PK_1 cells by the regulations of Bcl-2 and Bax. These results also provide further evidences for the anti-apoptotic properties of selenium.

In conclusion, our results emphasized that selenium had an ability to inhibit Cd-induced apoptosis in LLC-PK_1 cells and provided mechanistic evidence that cytoprotective effects was mediated through blocking ROS generation, restoring the mitochondrial membrane potential collapse, the prevention of cyt *c* release, subsequent inhibition of caspase activation and the changed expression of Bcl-2, Bax. Although other pathways may be involved, this requires further investigation. Further works in vivo studies are required to determine whether selenium could be an effective therapeutic agent for the toxicity of Cd.

Acknowledgements

The authors gratefully thank Ai-Ping Li and Dr. Shou-Yu, Wang (Nanjing Medical University, China) for their expert advices and critical reading of the manuscript, Dr. Yu Wu, Yin Zhou, Hui Xu and Juan Zhang (Nanjing Medical University, China) for technical assistance.

References

Acharya, U.R., Mishra, M., Patro, J., Panda, M.K., 2008. Effect of vitamins C and E on spermatogenesis in mice exposed to cadmium. Reproductive Toxicology 25, 84–88.

Aruoma, O.I., Halliwell, B., Hoey, B.M., Butler, J., 1989. The antioxidant action of *N*-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Radical Biology and Medicine 6, 593–597.

Belyaeva, E.A., Dymkowska, D., Wieckowski, M.R., Wojtczak, L., 2006. Reactive oxygen species produced by the mitochondrial respiratory chain are involved in Cd^{2+}-induced injury of rat ascites hepatoma AS-30D cells. Biochimica et Biophysica Acta 1757, 1568–1574.

Bruce-Keller, A.J., Begley, J.G., Fu, W., Butterfield, D.A., Bredesen, D.E., Hutchins, J.B., Hensley, K., Mattson, M.P., 1998. Bcl-2 protects isolated plasma and mitochondrial membranes against lipid peroxidation induced by hydrogen peroxide and amyloid beta-peptide. Journal of neurochemistry 70 (1), 31–39.

Chatterjee, S., Kundu, S., Bhattacharyya, A., 2008. Mechanism of cadmium induced apoptosis in the immunocyte. Toxicology Letters 177, 83–89.

Desagher, S., Martinou, J.C., 2000. Mitochondria as the central control point of apoptosis. Trends in Cell Biology 10, 369–377.

El-Shenawy, S.M., Hassan, N.S., 2008. Comparative evaluation of the protective effect of selenium and garlic against liver and kidney damage induced by mercury chloride in the rats. Pharmacological Reports 60 (2), 199–208.

Fan, T.J., Han, L.H., Cong, R.S., Liang, J., 2005. Caspase family proteases and apoptosis. Acta Biochimica et Biophysica Sinica 37, 719–727.

Gan, L., Liu, Q., Xu, H.B., Zhu, Y.S., Yang, X.L., 2002. Effects of selenium overexposure on glutathione peroxidase and thioredoxin reductase gene expressions and activities. Biological Trace Element Research 89, 165–175.

Gupta, S., Yel, L., Kim, D., Kim, C., Chiplunkar, S., Gollapudi, S., 2003. Arsenic trioxide induces apoptosis in peripheral blood T lymphocyte subsets by inducing oxidative stress: a role of Bcl-2. Molecular Cancer Therapeutics 2 (8), 711–719.

Hart, B.A., Lee, C.H., Shukla, G.S., Shukla, A., Osier, M., Eneman, J.D., Chiu, J.F., 1999. Characterization of cadmium-induced apoptosis in rat lung epithelial cells; evidence for the participation of oxidant stress. Toxicology 133, 43–58.

Hockenbery, D.M., Oltvai, Z.N., Yin, X.M., Milliman, C.L., Korsmeyer, S., 1993. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 75, 241–251.

Horiguchi, H., Oguma, E., Kayama, F., 2006. Cadmium and cisplatin damage erythropoietin-producing proximal renal tubular cells. Archives of Toxicology 80, 680–686.

Hsu, Y.T., Wolter, K.G., Youle, R.J., 1997. Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proceedings of the National Academy of Science of the United States of America 94, 3668–3672.

Huppertz, B., Kadyrov, M., Kingdom, J.C., 2006. Apoptosis and its role in the trophoblast. American Journal of Obstetrics and Gynecology 195, 29–39.

IARC (International Agency for Research on Cancer), 1993. Cadmium and cadmium compounds. CAS No. 7440-43-9. International Agency for Research on Cancer. Monographs. IARC Press, Lyon.

Kawakami, M., Inagawa, R., Hosokawa, T., Saito, T., Kurasaki, M., 2008. Mechanism of apoptosis induced by copper in PC12 cells. Food and Chemical Toxicology 46, 2157–2164.

Kim, S.C., Cho, M.K., Kim, S.G., 2003. Cadmium-induced non-apoptotic cell death mediated by oxidative stress under the condition of sulfhydryl deficiency. Toxicology Letters 144, 325–336.

Liu, Y., Zhang, S.P., Cai, Y.Q., 2007. Cytoprotective effects of selenium on cadmium-induced LLC-PK_1 cells apoptosis by activating JNK pathway. Toxicology in Vitro 21, 677–684.

Liu, Z.M., Huang, H.S., 2006. As_2O_3-induced c-Src/EGFR/ERK signaling is via Sp1 binding sites to stimulate p21WAF1/CIP1 expression in human epidermoid carcinoma A431 cells. Cell Signal 18, 244–255.

Neuzil, J., Wang, X.F., Dong, L.F., 2006. Molecular mechanism of 'mitocan'-induced apoptosis in cancer cells epitomizes the multiple roles of reactive oxygen species and Bcl-2 family proteins. FEBS Letters 580 (22), 5125–5129.

Newairy, A.A., El-Sharaky, A.S., Badreldeen, M.M., Eweda, S.M., Sheweita, S.A., 2007. The hepatoprotective effects of selenium against cadmium toxicity in rats. Toxicology 242, 23–30.

Nicholls, D.G., Budd, S.L., 2000. Mitochondria and neuronal survival. Physiological Reviews 80 (1), 315–360.

Nishijo, M., Morikawa, Y., Nakagawa, H., Tawara, K., Miura, K., Kido, T., Ikawa, A., Kobayashi, E., Nogawa, K., 2006. Causes of death and renal tubular dysfunction in residents exposed to cadmium in the environment. Occupational and Environmental Medicine 63, 545–550.

Pathak, N., Khandelwal, S., 2007. Role of oxidative stress and apoptosis in cadmium induced thymic atrophy and splenomegaly in mice. Toxicology Letters 169, 95–108.

Pelicano, H., Feng, L., Zhou, Y., Carew, J.S., Hileman, E.O., Plunkett, W., 2003. Inhibition of mitochondrial respiration: a novel strategy to enhance drug-induced apoptosis in human leukemia cells by a reactive oxygen species-mediated mechanism. Journal of Biological Chemistry 278 (39), 37832–37839.

Shaikh, Z.A., Tang, W., 1999. Protection against chronic cadmium toxicity by glycine. Toxicology 132, 139–146.

Stachura, A., Slotwinska, M., Kaminska, T., Sniezko, R., Paduch, R., Abramezyk, D., Filar, J., Kandefer-Szerszen, M., 2000. The inhibitory effect of zinc on cadmium

induced cell apoptosis and reactive oxygen species (ROS) production in cell cultures. Toxicology 145, 159–171.
Stadtman, T.C., 1991. Biosynthesis and function of selenocysteine-containing enzymes. Journal of Biological Chemistry 266 (25), 16257–16260.
Stohs, S.J., Baghi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radical Biology and Medicine 18, 321–336.
Thevenod, F., Friedmann, J.M., Katsen, A.D., Hauser, I.A., 2000. Up-regulation of multidrug resistance P-glycoprotein via nuclear factor-kappa B activation protects kidney proximal tubule cells from cadmium and reactive oxygen species-induced apoptosis. Journal of Biological Chemistry 275, 1887–1896.
Tsujimoto, Y., Shimizu, S., 2000. Bcl-2 family: life-or-death switch. FEBS Letters 466, 6–10.
Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xi, X.G., Youle, R.J., 1997. Movement of Bax from the cytosol to mitochondria during apoptosis. Journal of Cell Biology 139 (5), 1281–1292.
Zhou, J., Ye, J., Zhao, X., Li, A., Zhou, J., 2008. JWA is required for arsenic trioxide induced apoptosis in HeLa and MCF-7 cells via reactive oxygen species and mitochondria linked signal pathway. Toxicology and Applied Pharmacology 230, 33–40.