Available online at www.sciencedirect.com
SCIENCE DIRECT®
Toxicology 194 (2003) 19–33
TOXICOLOGY
www.elsevier.com/locate/toxicol

Cadmium directly induced the opening of membrane permeability pore of mitochondria which possibly involved in cadmium-triggered apoptosis

Min Li [a,1], Tian Xia [b,1], Chun-Sun Jiang [c], Lin-Jiang Li [c], Juan-Ling Fu [a], Zong-Can Zhou [a,*]

[a] *Department of Toxicology, Health Science Center, Peking University, Beijing 100083, PR China*
[b] *Department of Physiology and Biophysics, College of Life Science, Peking University, Beijing 100871, PR China*
[c] *National Key Lab of Biomembrane and Membrane Biotechnology, Institute of Zoology, Academia Sinica, Beijing 100080, PR China*

Received 20 January 2003; received in revised form 6 May 2003; accepted 25 July 2003

Abstract

The mitochondrial damage induced by cadmium has been well established, but its mechanism and its relationship with cadmium-induced apoptosis are elusive until now. Our research showed that cadmium could directly lead to the dysfunction of isolated mitochondria from mouse liver, including the inhibition of respiration, the opening of permeability transition pore (PTP), the loss of transmembrane potential, and the release of cytochrome *c*. These mitochondrial changes were completely suppressed by Bcl-x_L and Ruthenium Red (RR). Bongkrekic acid (BK), an inhibitor of the PTP opening directly via adenine nucleotide translocator (ANT), also completely inhibited the PTP opening and loss of transmembrane potential. However, cyclosporin A (CsA), another inhibitor of the PTP opening indirectly via ANT, had not any inhibitory effect. When cadmium being pre-incubated with proteins containing abundant thiol groups, its effect was partially reversed. These results revealed that mitochondria pathway may involve in cadmium-induced apoptosis, and cadmium caused the PTP opening possibly through its binding to thiol groups of ANT. Furthermore, the mechanism of the PTP opening induced by cadmium was probably distinct from that of the calcium-induced PTP opening.

Keywords: Cadmium; Mitochondria; Membrane permeability transition pore (PTP); Apoptosis

Abbreviations: PTP, membrane permeability transition pore; VDAC, voltage-dependent anion channel; ANT, the adenine nucleotide translocator; CyP-D, cyclophilin D; CCCP, carbonylcyanide *p*-chlorophenylhydrazone; CsA, cyclosporin A; Rh123, Rhodamine 123; H_2DCFDA, dichlorodihydrofluorescein diacetate; BCECF, 2′,7′-bis(caboxyethyl)-5(6)-carboxyfluorescein acetoxymethylester; BK, Bongkrekic acid; $\Delta\Psi_m$, mitochondrial transmembrane potential; pH_m, mitochondrial matrix pH; NAD(P), adenine dinucleotide phosphate; BSA, bovine serum albumin; GSH, glutathione

* Corresponding author. Tel.: +86-10-6209-1531.
E-mail address: zhouzc@bjmu.edu.cn (Z.-C. Zhou).
[1] Contributed equally to this work.

0300-483X/$ – see front matter © 2003 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/S0300-483X(03)00327-5

1. Introduction

Mitochondria, power plants in cells, play crucial roles in controlling cell life and death. In this process, membrane permeability transition pore (PTP) in mitochondria behaves like a switch to decide cell's fate (Wallace, 1999). The opening of PTP can lead to the collapse of mitochondrial transmembrane potential ($\Delta\Psi_m$), the release of proteins that activate caspases, the disruption of adenosine triphosphate (ATP) production and the induction of ROS sometimes due to incomplete oxygen reduction (Green and Reed, 1998). Therefore, in cell death, mitochondria are the possible common target and the PTP is the "bull's-eye" for many stimuli.

Cadmium, potent toxic environmental pollutant, can induce both apoptosis and mitochondrial injury. Cadmium could directly damage isolated mitochondria, prevent respiration and cause mitochondrial swelling and loss of inner membrane potential (Al Nasser, 2000; Rikans and Yamano, 2000). As well as, in our previous study, the decrease of Bcl-x_L and the collapse of mitochondrial membrane potential were observed in cells treated with cadmium (Li et al., 2000); and Bcl-2 could suppressed cadmium-triggered cell death (Kim et al., 2000). As well known, the members of anti-apoptotic Bcl-2 family exert their protection mainly through maintaining the integrity of mitochondria (Adamas and Cory, 1998). Therefore, the mitochondrial damage possibly plays a pivotal role in cadmium-induced apoptosis. However, there is lack of direct relationship between apoptosis and mitochondria in cadmium toxicity.

Cadmium ions have a high affinity to thiol groups and easily form cadmium–thiol complexes (Hultberg et al., 1997; Vairavamurthy et al., 2000). Through the interaction with cadmium ions, thiol-containing molecules, such as glutathione (GSH) and metallothionein, could protect cells and body from toxicity of cadmium (Diaz-cruz et al., 1997; Furey et al., 1986). On the other hand, since thiol groups are usually involved in the function of many enzymes, structure proteins and receptors, the cadmium–thiol complexes possibly disturbed many functions of cells (Hultberg et al., 1998; Bandyopadhyay et al., 1997). As well as, the modification of thiol groups in mitochondria has been report to induce the PTP opening (Zazueta et al., 1998).

In our research, we observed the effect of cadmium on purified mitochondria and tried to clarify the mechanism of mitochondrial damage induced by cadmium and its relationship with cadmium-induced apoptosis. The results suggested that cadmium could directly damage mitochondria and this mitochondrial damage possibly involved in apoptosis induced by cadmium.

2. Materials and methods

2.1. Material

Cadmium chloride, BSA, ADP, carbonylcyanide *p*-chlorophenylhydrazone (CCCP), cyclosporin A (CsA), Rhodamine 123 (Rh123) and dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Sigma (St. Louis, MO). Sucrose and IPTG were obtained from Life Technologies (Grand Island, NY). 2′,7′-Bis(caboxyethyl)-5(6)-carboxyfluorescein acetoxymethylester (BCECF) was obtained from Molecular Probes (Eugen, OR). Bongkrekic acid (BK) was purchased from BIOMOL Research Lab, Inc. Glutathione Sepharose 4B was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Triton X-100 and HEPES were products of Merck. Anti-cytochrome *c* antibody was purchased from BD PharMingen. pGEX-2TK expression vector with the full-length human Bcl-x_L was a gift from Dr. Y. Tsujimoto (Osaka university, Japan) and selenoprotein P was generously presented from Dr. J.G. Yang (Sydney University, Australia).

2.2. Isolation of mitochondria

Livers of Balb/c mice were homogenized with a glass-Teflon Potter homogenizer. Mitochondria were isolated in medium (250 mM sucrose, 2 mM HEPES, pH 7.4, 0.1 mM EDTA, 0.1% fatty acid-free BSA), as described previously (Graham and Rickwood, 1997a). Purified mitochondria were resuspended in same buffer and stored on ice for use. Protein content of mitochondria was determined by biuret method.

2.3. Expression and purification of Bcl-x_L

Human Bcl-x_L was expressed as Glutathione *S*-transferase (GST) protein in *Escherichia coli* Strain

BH 5α and purified on a Glutathione Sepharose 4B column (Narita et al., 1998; Smith and Johnson, 1998). Purified protein was suspended in the buffer composed of 20 mM HEPES-K^+ (pH 7.4) and 1 mM DTT. The protein content was estimated by measuring the absorbance at 280 nm.

2.4. Measurement of oxygen consumption

By using a Clark oxygen electrode (YSI Model 53 Oxygen Monitor, USA), mitochondrial respiration was monitored in media (225 mM sucrose, 20 mM KCl, 5 mM $MgCl_2$, 10 mM KH_2PO_4, 10 mM HEPES (pH 7.4) and 2 μM rotenone) at 25 °C (Graham and Rickwood, 1997b).

2.5. Determination of mitochondrial permeability transition

Mitochondrial swelling, the result of mitochondrial permeability transition, was determined by the decrease in 90° light scattering method (Hunter and Haworth, 1979). Mitochondrial suspension was monitored at 520 nm in media P (250 mM sucrose, 2 mM Tris, pH 7.4, 0.5 mM KH_2PO_4 and 4.2 mM potassium succinate to energize mitochondria) by using a spectrofluorimeter (Jobin-Yvon FluoroMax-2).

2.6. Measurement of mitochondrial transmembrane potential ($\Delta\Psi_m$)

Mitochondrial transmembrane potential was estimated by measuring the $\Delta\Psi_m$-dependent uptake of Rhodamine 123, using a spectrofluorimeter (Jobin-Yvon FluoroMax-2) operated at excitation and emission wavelengths of 500 and 527 nm, respectively (Narita et al., 1998; Emaus et al., 1986) . Isolated mitochondria were incubated at 25 °C in media P.

2.7. Determination of mitochondrial matrix pH (pH_m)

Matrix pH of mitochondria in media P was measured with pH-sensitive probes, BCECF (Kapus et al., 1989). Mitochondria were incubated with 5 μM BCECF/AM for 5 min at 2 °C. After centrifugation (10,000 × *g*; 10 min), mitochondria were resuspended for use. The fluorescence of BCECF was determined by using a spectrofluorimeter (Jobin-Yvon FluoroMax-2) with excitation at 500 nm and emission at 530 nm.

2.8. Determination of ROS production

Mitochondrial ROS production was evaluated by using H_2DCF as previously described with some modification (Bejma and Ji, 1999). Mitochondria were loaded with 5 μM H_2DCFDA for 15 min at 25 °C. After centrifugation (10,000 × *g*; 10 min), the pellet was resuspended for use. By using a spectrofluorimeter (Jobin-Yvon FluoroMax-2) with excitation and emission wavelengths of 499 and 525 nm, respectively, the rate of the conversion from H_2DCF to DCF was determined in assay buffer (225 mM sucrose, 20 mM KCl, 5 mM $MgCl_2$, 10 mM KH_2PO_4, 2 mM Tris (pH 7.4) and 4.2 mM succinate as substrate).

2.9. Cytochrome c release assay

Purified mitochondria (1 mg protein/ml; 50 μl/tube) were incubated in media P with presence of cadmium or/and other inhibitors for 5 min at 25 °C. After centrifugation, aliquots supernatant were subjected to SDS–PAGE gel electrophoresis (12%). Western blot analysis of cytochrome *c* was carried out by using a specific monoclonal antibody (Liu et al., 1996).

3. Results

3.1. Suppression of mitochondria respiration by cadmium

In reaction system containing 1 mg mitochondrial protein/ml, the effects of 1, 2.5 and 5 μM cadmium on mitochondrial respiration were observed (Fig. 1A). The results illustrated that cadmium could suppress mitochondrial oxygen consumption depending on concentration. 5 μM cadmium completely suppressed mitochondria respiration, but 1 μM cadmium had little effect. Because of different effects on mitochondrial respiration, a basic and vital function of mitochondria, 1 and 5 μM cadmium were often used in this study. Although its ion radius and valence is very similar to that of cadmium ion, calcium had different effect on

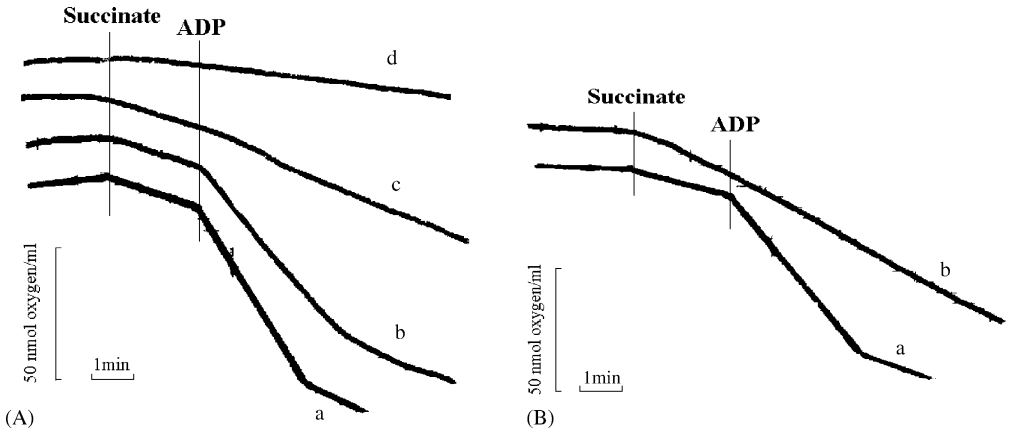

Fig. 1. The effect of cadmium and calcium on the mitochondrial respiration. (A) In presence of different concentrations of cadmium, succinate-stimulated state 4 and ADP-stimulated state 3 respiration of mitochondria was measured by a Clark oxygen electrode as described in Section 2. *Trace a* was normal control, and *traces b*, *c* and *d* were delineated in presence of 1, 2.5 and 5 μM cadmium, respectively. (B) Mitochondrial respiration was measured in presence of 100 μM calcium as described in A. *Trace a* was normal control, and *trace b* was delineated with 100 μM calcium.

mitochondrial respiration. 100 μM calcium induced the uncoupling of mitochondria (Fig. 1B).

Mitochondria are main source of ROS in cells. H_2DCFDA, which has been used to detect the overall oxidative stress both in cell (Culcasi et al., 1994) and in mitochondria (Bejma and Ji, 1999), was employed to observed the ROS production induced by cadmium. Fig. 2 showed that regardless of mitochondrial

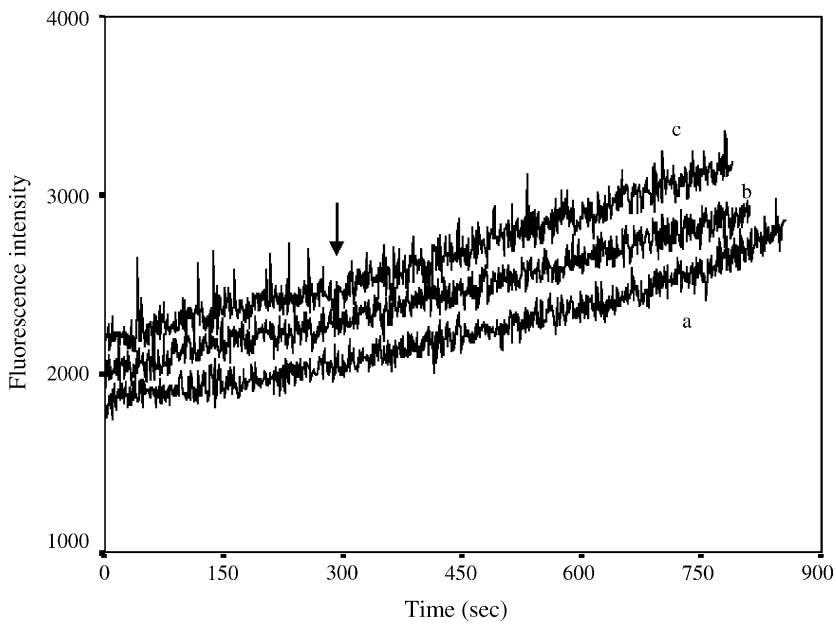

Fig. 2. ROS production in mitochondria treated with cadmium. Mitochondria were loaded with DCFH-DA and then the rate of the conversion from DCFH to DCF was determined at excitation and emission wavelengths of 488 and 525 nm, respectively. *Trace a* was normal control, and *traces b* and *c* were delineated with 1 and 5 μM cadmium, respectively. Where indicated, cadmium was added (*arrow*).

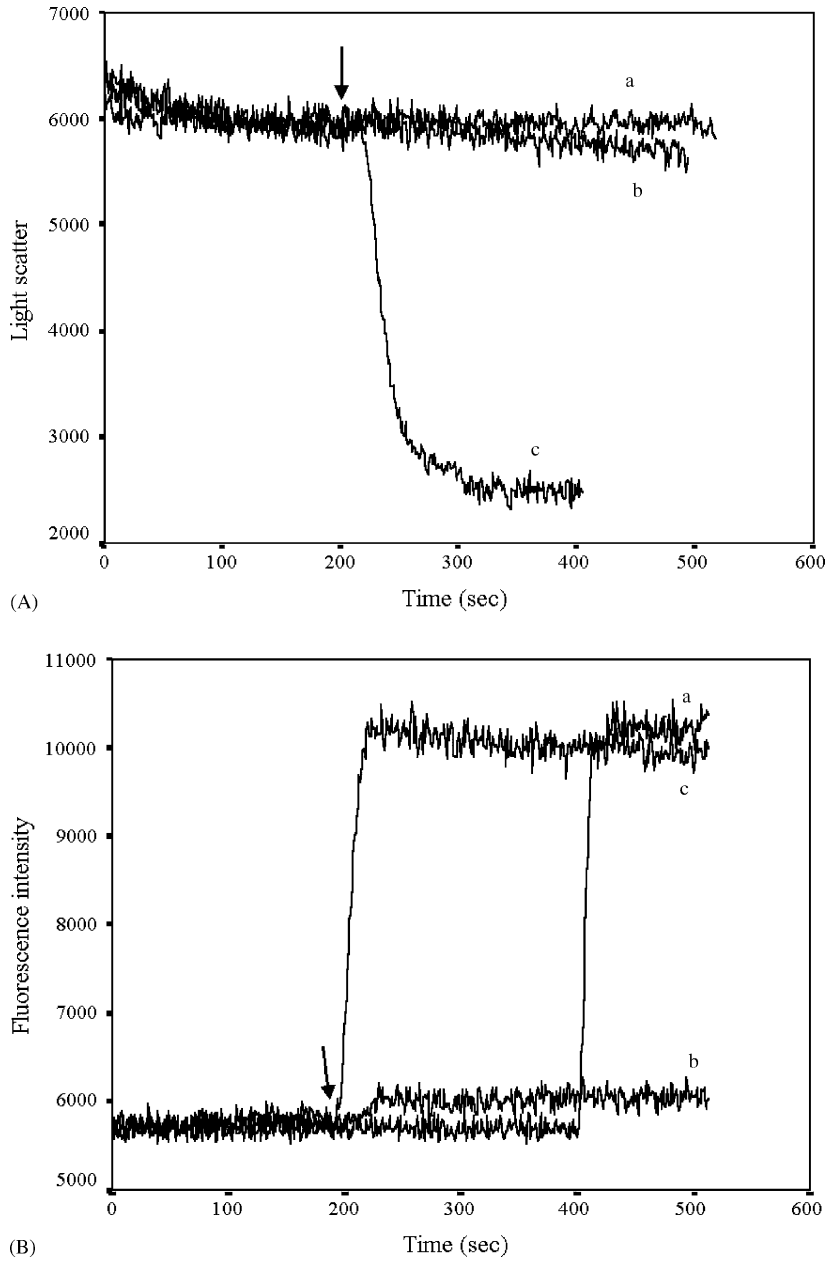

Fig. 3. Induction of opening of PTP, loss of membrane potential and decrease of pH_m induced by cadmium. (A) Mitochondrial swelling was monitored at 520 nm in absence or presence of cadmium as described in Section 2. *Trace a* was normal control, and *traces b* and *c* were delineated with 1 and 5 μM cadmium, respectively. *Arrow* indicated that cadmium was added. (B) The fluorescence of Rhodamine 123 (Rh123), showing mitochondrial transmembrane potential, was monitored at excitation and emission wavelengths of 500 and 527 nm, respectively. *Trace a* was normal control in which CCCP (uncoupling agent, 3 μM) was added as positive control at 400 s and *traces b* and *c* were delineated with 1 and 5 μM cadmium, respectively which was added at 200 s (*arrow*). (C) Matrix pH of mitochondria was measured with BCECF as described in Section 2. *Trace a* was normal control, and *traces b* and *c* were delineated with 1 and 5 μM cadmium, respectively which was added at 200 s (*arrow*).

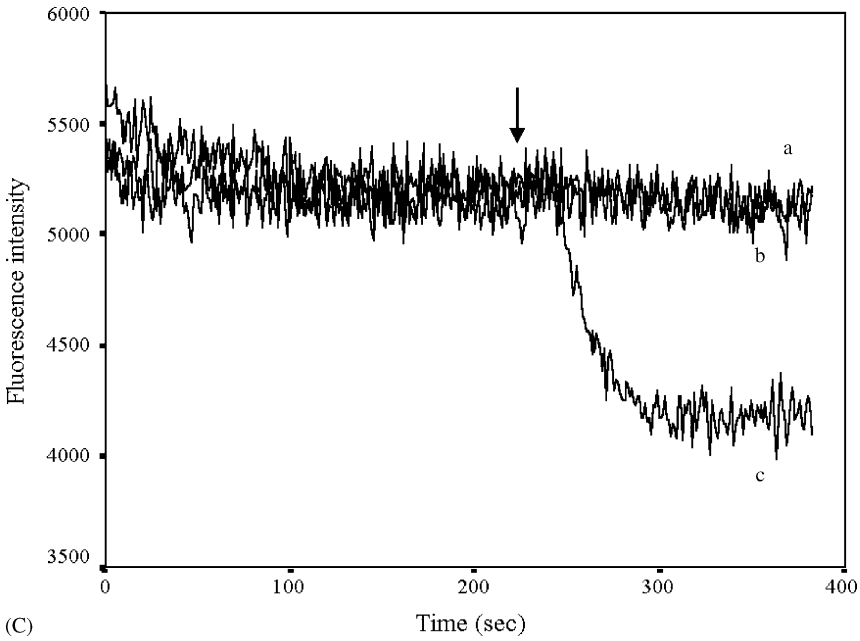

Fig. 3. (*Continued*).

respiration, 1 and 5 μM cadmium did not cause any change of the rate of the conversion from H_2DCF to DCF by auto-oxidation. Therefore, cadmium may not directly stimulate the mitochondrial ROS production.

Because of its uncoupling, 100 μM calcium did not change the rate of H_2DCF conversion to DCF too (data not shown). This suggested that the over-loading of calcium in mitochondria did not induce ROS production.

3.2. Induction of opening of PTP, loss of membrane potential and decrease of pH_m

Mitochondrial swelling is the direct result of the PTP opening (Kroemer and Reed, 2000; Martinou and Green, 2001), so it is a very good index for mitochondrial permeability transition. In our study, the rapid decrease of light scatter at 520 nm happened at once when 5 μM cadmium was added into mitochondrial suspension, but 1 μM cadmium did not have this effect (Fig. 3A). This result elucidated that cadmium can directly trigger the opening of PTP.

Usually, the collapse of transmembrane potential ($\Delta\Psi_m$) ensue from the opening of PTP (Zamzami and Kroemer, 2001). In order to observe the change of $\Delta\Psi_m$ of mitochondria exposed cadmium, Rh123, which is cationic lipophilic dye and can enter mitochondria depending on voltage, was employed. Fig. 3B illustrated that 5 μM other than 1 μM cadmium drastically destroyed $\Delta\Psi_m$ and caused the sharp increase of fluorescent intensity of Rh123.

Transmembrane potential ($\Delta\Psi_m$) is generated from electrochemical proton gradient. The PTP opening can lead to influx of proton into mitochondrial matrix and decrease matrix pH (pH_m) (Ichas and Mazat, 1998). Our results indicated that 5 μM cadmium violently decrease the fluorescent intensity of BCECF to show the acidification in mitochondria, but 1 μM cadmium did not have this effect (Fig. 3C).

As previous reports, the release of cytochrome *c*, a cofactor in the activation of caspases, is a direct result from the opening of PTP (Green and Reed, 1998; Bossy-Wetzel and Green, 1999). In our study, 5 μM but not 1 μM cadmium caused the release of cytochrome *c* (Fig. 8).

In the induction of opening of PTP, loss of $\Delta\Psi_m$ and decrease of pH_m, 100 μM calcium was very similar to cadmium (data not shown).

3.3. Inhibition of Ruthenium Red and Bcl-x_L on opening of PTP and loss of membrane potential

Because cadmium can enter cells through Ca^{2+} channel, we wonder whether cadmium enter mitochondrial via similar channel and take its effect inside other than outside mitochondria. Hence, we observed the effect of Ruthenium Red (RR), a specific inhibitor of calcium uniporter which is main calcium channel in mitochondrial membrane (Bernadi, 1999). 1 μM Ruthenium Red could completely inhibited the opening of PTP, the loss of $\Delta\Psi_m$ and the release of cytochrome *c* induced by 5 μM cadmium (Figs. 4A, B and 8).

As mentioned above, cadmium can induce apoptosis and the mitochondrial damage, however, there is lack the direct relationship between them. Anti-apoptotic members of Bcl-2 family have been reported to inhibit the PTP opening directly and indirectly (Shimizu et al., 2000; Tsujimoto and Shimizu, 2000). Therefore, we observe the effect of Bcl-2 on the mitochondrial damage induced by cadmium. Figs. 4 and 8 indicated that Bcl-x_L (16 μg/ml) completely inhibited the cadmium-induced PTP opening, loss of $\Delta\Psi_m$ and the release of cytochrome *c*. However, although it contained 1 mM DTT, the only buffer used to dissolve Bcl-x_L was not effective on the opening induced by cadmium (data not shown).

Ruthenium Red and Bcl-x_L had the same effect on the PTP opening and loss of $\Delta\Psi_m$ induced by 100 μM calcium (data not shown).

3.4. Different effects of cyclosporin A and Bongkrekic acid on the cadmium-induced PTP opening

Cyclosporin A and Bongkrekic acid are two known inhibitors of the PTP opening (Leoffler and Kroemer, 2000; Jurgensmeier et al., 1998). However, they had different effect on the cadmium-induced PTP opening. 1 μM CsA, even up to 10 μM, could not inhibit the opening of PTP induced by 5 μM cadmium (Fig. 5A). Moreover, it could not also prevent the release of cytochrome *c* (Fig. 8). In contrast to CsA, BK could suppress cadmium-triggered PTP opening and presented concentration-dependent manner (Fig. 5A).

Comparing with cadmium, the PTP opening induced by 100 μM calcium could be strongly inhibited by both CsA and BK (Fig. 5B).

Besides CsA and BK, we also observed effects of other inhibitors. ADP, endogenous inhibitor of PTP, could not inhibit the PTP opening induced by cadmium (data not shown). Inhibitors of mitochondrial respiratory chain, antimycin A, potassium cyanide and myxothiazol, could completely suppressed the PTP opening induced by either cadmium or calcium (Fig. 5C). These results illustrated that the PTP opening induced by cadmium was dependent on the integrity of mitochondrial respiratory chain.

3.5. Suppression of thiol-containing proteins on the PTP opening induced by cadmium

How to induce the mitochondrial PTP opening by cadmium? Possibly, it was related to the interaction between thiol group and cadmium. In order to validate our speculation, we choose glutathione and proteins containing abundant thiol groups, selenoprotein P and bovine serum albumin (BSA). GSH and BSA had not any protection on mitochondria, when they pre-incubate with mitochondria for 5 min and then 5 μM cadmium was added into mitochondrial suspension (data not shown). In contrary, if they pre-incubated with cadmium for 5 min and then was added into mitochondrial suspension together, mitochondrial PTP opening was delayed and partially suppressed (Fig. 6A and B). Selenoprotein P, pre-incubating with either mitochondria or cadmium, could suppress the cadmium-induced PTP opening in very similar degree. Moreover, 3 μg/ml selenoprotein P completely inhibited the cadmium-triggered PTP opening (Fig. 5C). These results revealed that GSH, BSA and selenoprotein P antagonize effects of cadmium due to their binding to cadmium. Furthermore, selenoprotein P was most effective possibly due to its thiol groups and selenium (Sies and Arteel, 2000).

3.6. The closing state of PTP is very important factor for maintaining the respiratory function of mitochondria

The closing state of PTP is very helpful to maintain the mitochondrial transmembrane potential (Ichas and Mazat, 1998). Therefore, it probably is very important factor to maintain the mitochondrial respiratory. In our study, the protective effect of some inhibitors of PTP opening on the respiratory was observed. Both Bcl-x_L

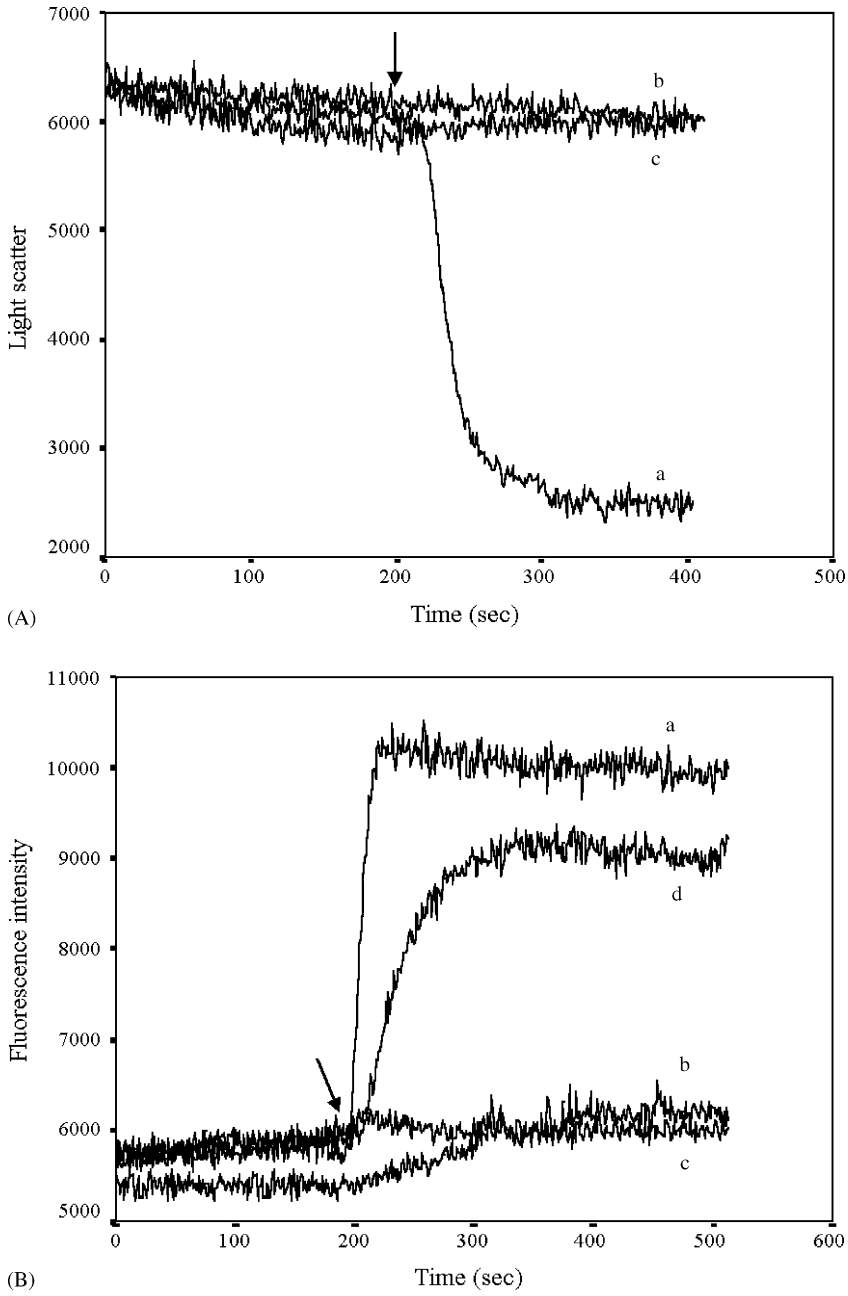

Fig. 4. Inhibition of Bcl-x_L and Ruthenium Red (RR) on the opening of PTP, loss of membrane potential. (A) As described in Fig. 3, mitochondrial swelling was monitored in presence of Bcl-x_L (16 μg/ml) or RR (1 μM). *Trace a* was delineated with only 5 μM cadmium, and *traces b* and *c* were with 5 μM cadmium in presence of Bcl-x_L (16 μg/ml) or RR (1 μM), respectively. *Arrow* indicated that cadmium was added. (B) As described in Fig. 3, the loss of membrane potential was determined in presence of Bcl-x_L (16 μg/ml) or RR (1 μM) or CsA (1 μM). *Trace a* was delineated with only 5 μM cadmium, and *traces b* and *c* were with 5 μM cadmium in presence of Bcl-x_L (16 μg/ml) or RR (1 μM), respectively. *Trace d* was with cadmium in presence of CsA (1 μM) and CCCP (3 μM) was added at 400 s. Cadmium 5 μM was added at 200 s (*arrow*).

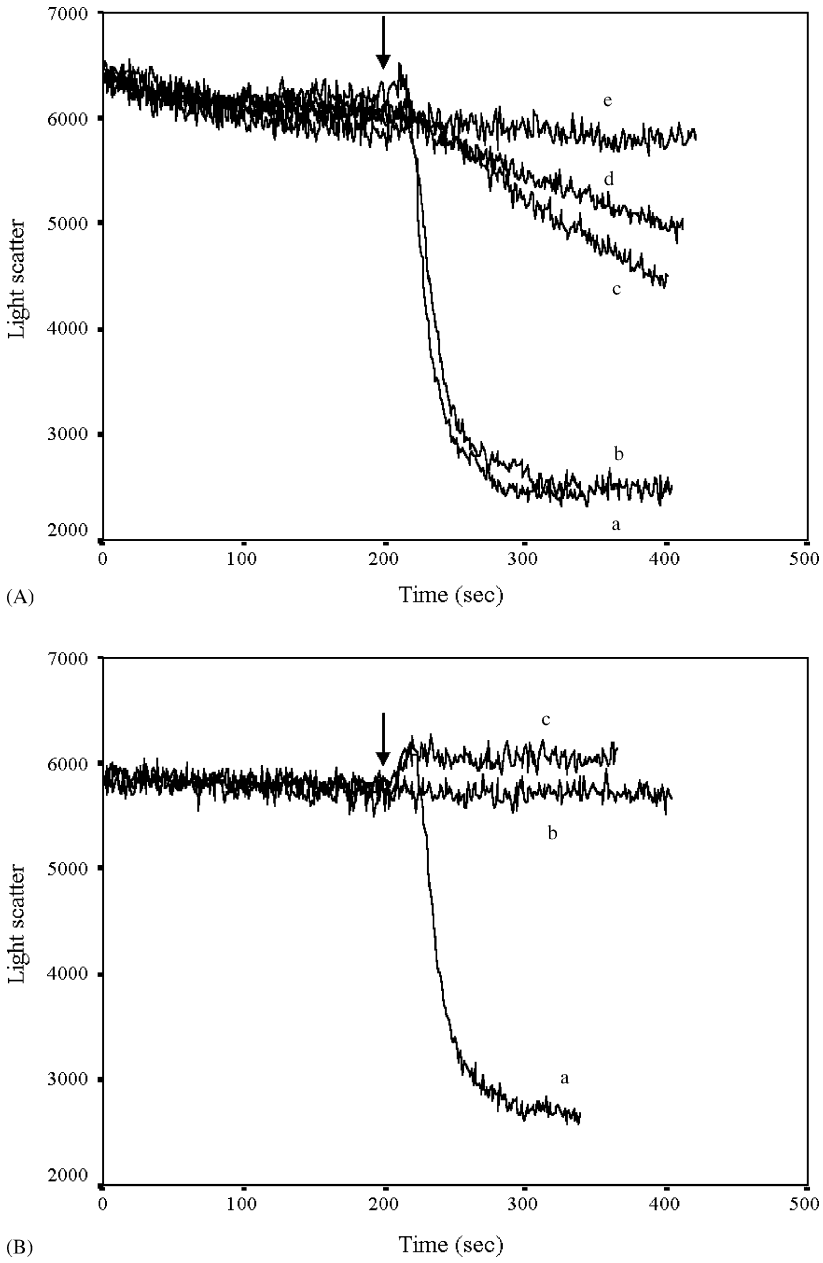

Fig. 5. The effect of cyclosporin A (CsA) and Bongkrekic acid (BK) and respiratory chain inhibitors on the opening of PTP induced by cadmium or calcium. (A) As described in Fig. 3, mitochondrial swelling was monitored in presence of CsA or BK. *Trace a* was delineated with only 5 μM cadmium, *trace b* was with 5 μM cadmium in presence of CsA (1 μM), and *traces c*, *d* and *e* were with 5 μM cadmium in presence of 12.5, 25 and 50 μM BK, respectively. *Arrow* indicated the point where cadmium was added. (B) As described in Fig. 3, mitochondrial swelling was determined in presence of CsA (1 μM) or BK (50 μM). *Trace a* was delineated with only 100 μM calcium, and *traces b* and *c* was with 100 μM calcium in presence of BK or CsA, respectively. Calcium was added at 200 s (*arrow*). (C) The effect of some inhibitor of respiratory chain on the mitochondrial swelling induced by cadmium as in Fig. 3. *Trace a* was delineated with only 5 μM cadmium, and *traces b*, *c* and *d* were with 5 μM cadmium in the presence of antimycin A (10 μM), potassium cyanide (1 mM) and myxothiazol (3 μg/ml), respectively. Cadmium was added at 200 s (*arrow*).

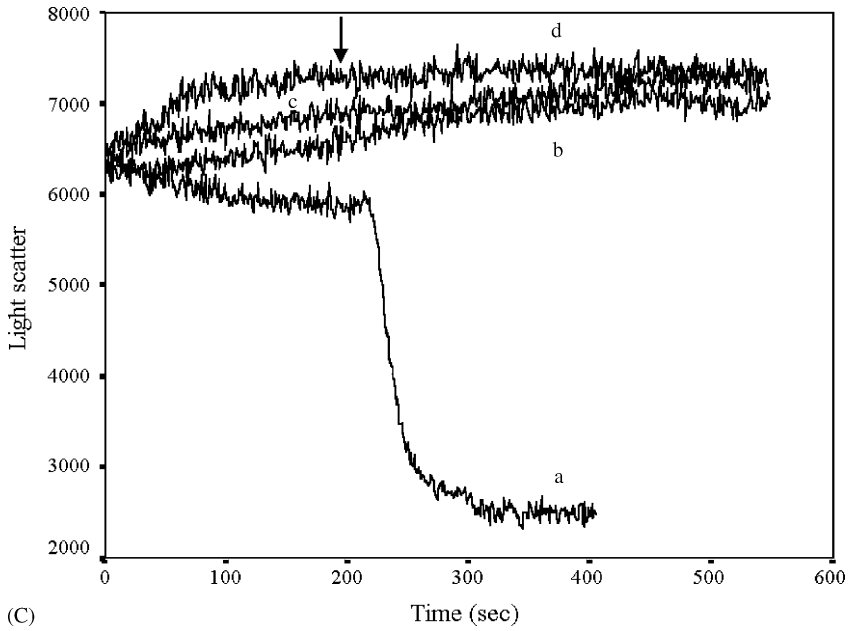

Fig. 5. (*Continued*).

and Ruthenium Red could suppress the respiratory inhibition induced by cadmium, but cyclosporin A had no any effect (Fig. 7A). As well as, Bcl-x_L, RR and CsA all inhibited the uncoupling of calcium (Fig 7B).

4. Discussion

It has been report that cadmium can lead to cell death (Li et al., 2000; Tsangaris and Tzortzatou-Stathopoulou, 1998), but the mechanism is not very clear yet. The change of mitochondria permeability transition has been known to be a common and crucial mechanism in cell death (Lemasters et al., 1998). Via the opening of PTP, cytochrome *c*, apoptosis inducing factor (AIF) and procaspase 2, 9 release from mitochondria and take part in startup of apoptosis (Shimizu et al., 1999; Susin et al., 1999). Our study showed that cadmium could directly lead to the dysfunction of mitochondria, including the inhibition of respiration, the opening of PTP, the loss of transmembrane potential, and the release of cytochrome *c*. Therefore, mitochondrial damage probably lies in a main position in cadmium-induced apoptosis (Fig. 8).

The PTP is a protein-complex, which lies in the contact site between inner and outer membrane of mitochondria. Although its exact composition is unclear, PTP is thought to contain hexokinase, voltage-dependent anion channel (VDAC), creatine kinase, the adenine nucleotide translocator (ANT) and cyclophilin D (CyP-D) (Zamzami and Kroemer, 2001). Many inducers and inhibitors of apoptosis act on the VADAC or ANT, such as Ca^{2+}, oxidant agent, Bcl-2 family members etc (Zamzami and Kroemer, 2001; Susin et al., 1998). Our research revealed that cadmium probably acted on ANT to trigger the opening of PTP. There are two evidences for this conclusion in our study. (1) Ruthenium Red, which is specific inhibitor of Ca^{2+} uniporter on mitochondria, could completely inhibit the mitochondrial dysfunction. It suggested that cadmium entered mitochondria via Ca^{2+} uniporter and only took an effect inside mitochondria. ANT lied in the inner membrane and was easily attacked by cadmium. (2) Bongkrekic acid, a specific inhibitor of ANT, also could completely suppress the cadmium-induced PTP opening. Zazueta's group has also deducted that ANT possibly involved in the cadmium-induced PTP opening (Zazueta et al., 2000).

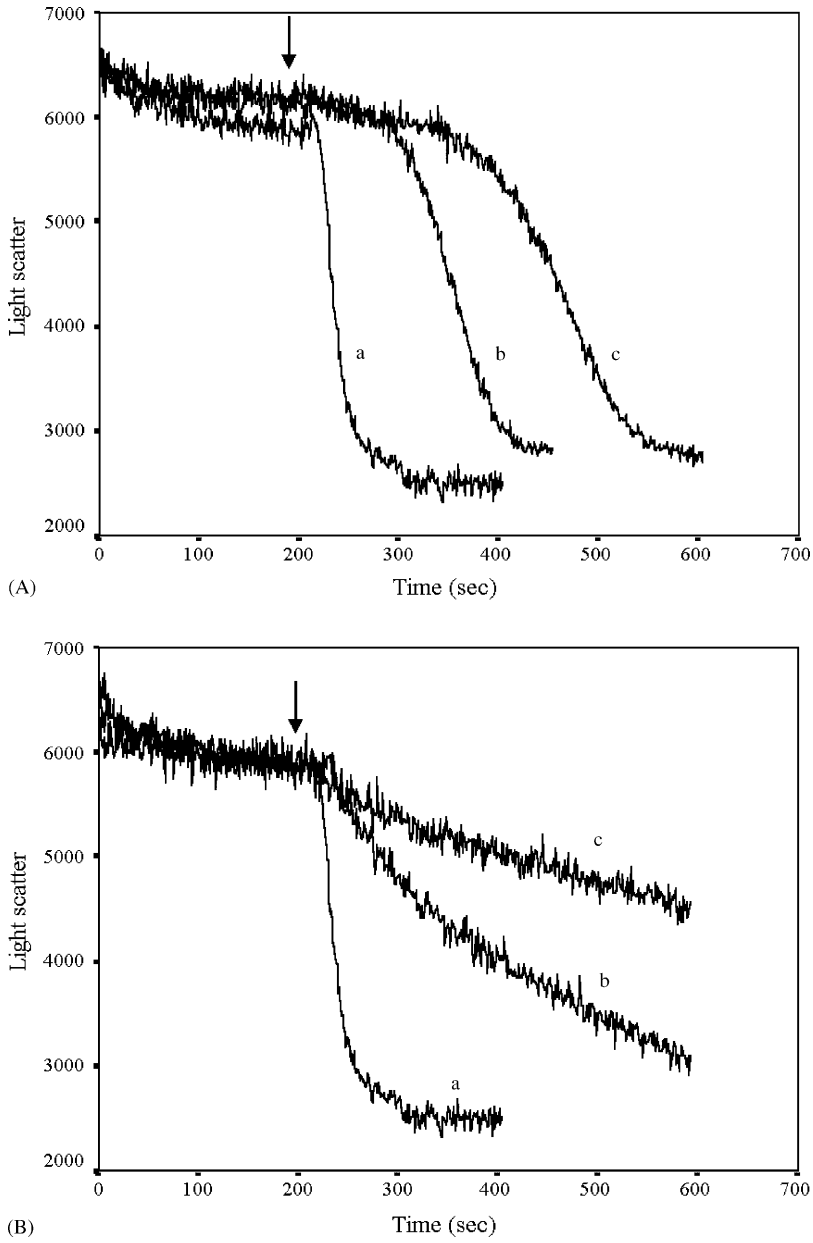

Fig. 6. Inhibition of thiol-containing proteins on the PTP opening induced by cadmium. (A) 30 and 60 mM glutathione (GSH) were pre-incubated with cadmium (5 μM) for 5 min, and then the mixture were added into mitochondrial suspension at 200 s (*arrow*). The PTP opening was measured as described in Fig. 3. *Trace a* was delineated with only 5 μM cadmium, *traces b* and *c* showed the effect of 30 or 60 mM GSH on the PTP opening. (B) 30 and 60 μg/ml BSA were pre-incubated with cadmium (5 μM) for 5 min, and then the mixture were added into mitochondrial suspension at 200 s (*arrow*). The PTP opening was measured as described in Fig. 3. *Trace a* was delineated with only 5 μM cadmium, and *traces b* and *c* showed the effect of 30 or 60 μg/ml BSA on the PTP opening. (C) 5 and 10 μg/ml selenoprotein were pre-incubated with cadmium (5 μM) for 5 min, and then the mixture were added into mitochondrial suspension at 200 s (*arrow*). The PTP opening was measured as described in Fig. 3. *Trace a* was delineated with only 5 μM cadmium, and *traces b* and *c* showed the effect of 5 or 10 μg/ml selenoprotein on the PTP opening.

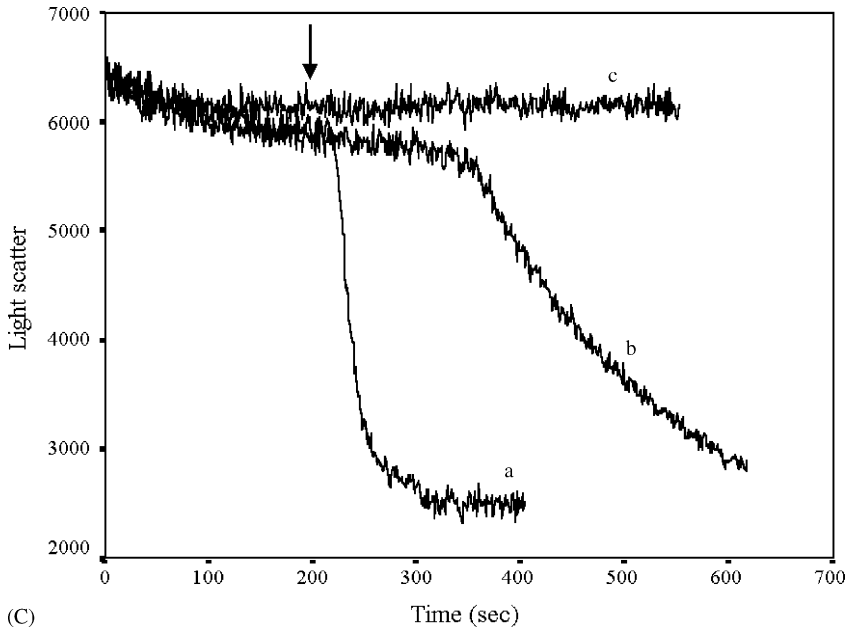

Fig. 6. (*Continued*).

ANT, ADP/ATP carrier, works as a dimmer in mitochondrial inner membrane. Each monomer of ANT possesses four cysteine residues (Majima et al., 1995, 1993). Therefore, through its high affinity to thiol groups, cadmium may directly modify thiol groups of ANT and finally make PTP open. Furthermore, when thiol-containing proteins (GSH, BSA, selenoprotein P) are pre-incubated with cadmium, they can delay

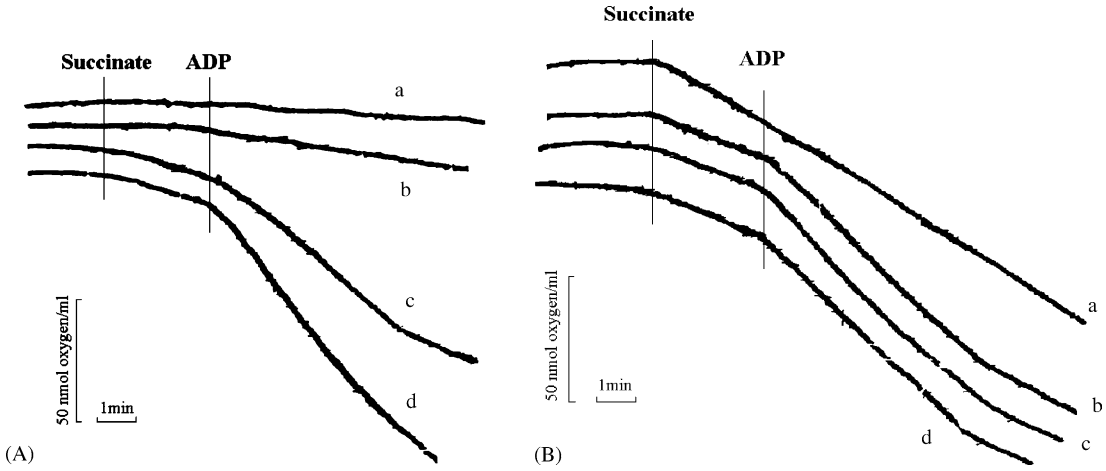

Fig. 7. Inhibition of PTP-opening inhibitors on the dysfunction of mitochondrial respiration induced by cadmium or calcium. (A) In presence of 5 μM cadmium without or with PTP-opening inhibitors, succinate-stimulated state 4 and ADP-stimulated state 3 respiration of mitochondria was measured by a Clark oxygen electrode as described in Section 2. *Trace a* is 5 μM cadmium, and *traces b*, *c* and *d* showed that 5 μM cadmium was with CsA (1 μM), Bcl-x_L (16 μg/ml) or RR (1 μM), respectively. (B) In presence of 100 μM calcium without or with PTP-opening inhibitors, the oxygen consumption of mitochondria was monitored by a Clark oxygen electrode as described in Section 2. *Trace a* is 100 μM calcium; and *traces b*, *c* and *d* showed that 100 μM calcium was with CsA (1 μM), Bcl-x_L (16 μg/ml) or RR (1 μM), respectively.

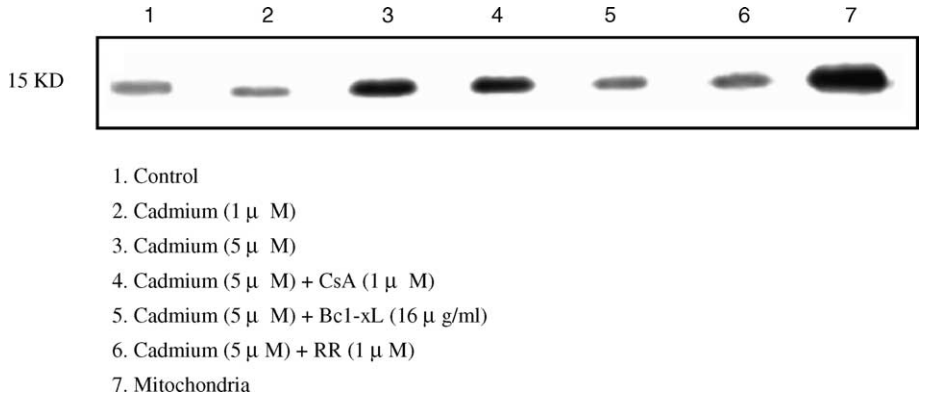

Fig. 8. Release of cytochrome *c* induced by cadmium. The mitochondria was incubated with cadmium in absence or presence of some inhibitors for 5 min. After centrifugation, aliquots of the supernatant were subjected to SDS–PAGE and immunoblotted as described in Section 2.

and suppress the PTP opening. This demonstrated that thiol-containing proteins possibly decreased the available cadmium (free cadmium) to bind to the thiol group in ANT. As previous reports, thiol reagents can form a disulfide bridge between two vicinal thiol residues of the ANT monomers and promote the PTP opening, and they can overcome the inhibitory effect of Bcl-2 (Halestrap et al., 1997; Costantini et al., 2000). These results also support our speculation, that cadmium induced the opening of PTP not through forming disulfide bridge but just binding to vicinal thiol groups (Cys–s–Cd–s–Cys). Because in our study, Bcl-x_L could completely suppress the effect of cadmium, and there is not ROS production during cadmium-treatment. In addition, cadmium suppressed the mitochondrial respiration possibly also through its binding to thiol groups.

Bongkrekic acid and cyclosporin A directly or indirectly act on ANT and inhibit the PTP opening (Zamzami and Kroemer, 2001). BK can directly lock ANT at m-state (one of two conformations of ANT) and inhibit the pore opening (Crompton, 1999). CsA probably occupy the active site of cyclophilin D (Mikol et al., 1994; Alberg and Schreiber, 1993) and forms a VDAC–ANT–CyP D–CsA complex to block the pore opening (Crompton, 1999; Woodfied et al.,

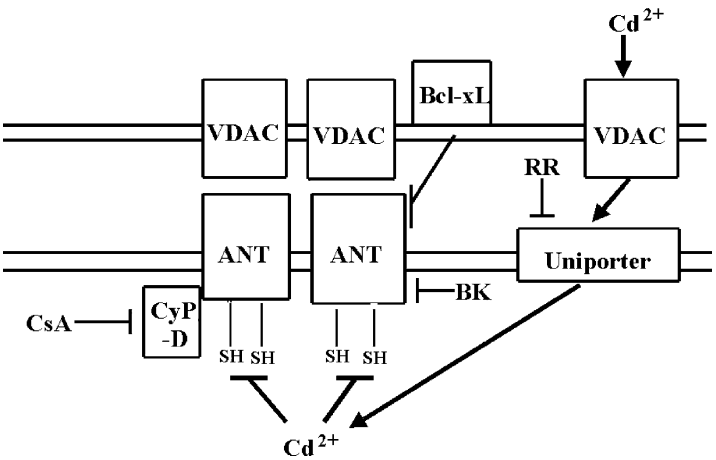

Fig. 9. Scheme of the pathway of cadmium-induced PTP opening. Cadmium enter mitochondria via Ca^{2+} uniporter, then binding to thiol groups in adenine nucleotide translocator (ANT), and finally induce the opening of PTP, loss of membrane potential and decrease of pH_m.

1998). In our studies, BK and CsA have different effects on the PTP opening induced by cadmium. Possibly since their binding positions is difference and they have direct or indirect effect on ANT conformation. Although Ca^{2+} and Cd^{2+} are very similar in some action (Beyersmann and Hechtenberg, 1997), the mechanism of the PTP opening induced by them is different. Calcium-induced PTP opening was CsA-dependent but cadmium was not.

In our study, the possible mechanism of the PTP opening caused by cadmium is expounded (Fig. 9). Cadmium may enter mitochondria via Ca^{2+} uniporter and interact with crucial thiol groups of ANT to induce the PTP opening and cytochrome *c* release. Furthermore, because of inducing the mitochondrial PTP opening and the release of cytochrome *c*, cadmium could probably induce cell death via mitochondria pathway besides Ca^{2+} and caspase-8 pathway in apoptosis of U937 cells, respectively in our previous study (Li et al., 2000).

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 30070663).

References

Adamas, J.M., Cory, S., 1998. The Bcl-2 protein family: arbiters of cell survival. Science 281, 1322–1326.

Al Nasser, I.A., 2000. Cadmium hepatotoxicity and alterations of the mitochondrial function. J. Toxicol. Clin. Toxicol. 38, 407–413.

Alberg, D.G., Schreiber, S.L., 1993. Structure-based design of a cyclophilin-calcineurin bridging ligand. Science 262, 248–250.

Bandyopadhyay, D., Chatterjee, A.K., Datta, A.G., 1997. Effect of cadmium on purified hepatic flavokinase: involvement of reactive-SH groups in the inactivation of flavokinase by cadmium. Life Sci. 60, 1891–1903.

Bejma, J., Ji, L.L., 1999. Aging and acute exercise enhance free radical generation in rat skeletal muscle. J. Appl. Physiol. 87, 465–470.

Bernadi, P., 1999. Mitochondrial transport of cations: channels, exchangers and permeability transition. Physiol. Rev. 79, 1127–1155.

Beyersmann, D., Hechtenberg, S., 1997. Cadmium, gene regulation, and cellular signalling in mammalian cells. Toxicol. Appl. Pharmacol. 144, 247–261.

Bossy-Wetzel, E., Green, D.R., 1999. Apoptosis: checkpoint at the mitochondrial frontier. Mutat. Res. 434, 243–251.

Costantini, P., Belzacq, A.S., Vieira, H.L., Larochette, N., de Pablo, M.A., Zamzami, N., Susin, S.A., Brenner, C., Kroemer, G., 2000. Oxidation of a critical thiol residue of the adenine nucleotide translocator enforces Bcl-2-independent permeability transition pore opening and apoptosis. Oncogene 19, 307–314.

Crompton, M., 1999. The mitochondrial permeability transition pore and its role in cell death. Biochem. J. 341 (Pt. 2), 233–249.

Culcasi, M., Lafon-Cazal, M., Pietri, S., Bockaert, J., 1994. Glutamate receptors induce a burst of superoxide via activation of nitric oxide synthase in arginine-depleted neuron. J. Biol. Chem. 269, 12589–12593.

Diaz-cruz, M.S., Mendieta, J., Tauler, R., Esteban, M., 1997. Cadmium-binding properties of glutathione: a chemometerical analysis of voltammertric data. J. Inorg. Biochem. 66, 29–36.

Emaus, R.K., Grunwald, R., Lemasters, J.J., 1986. Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties. Biochim. Biophys. Acta 850, 436–448.

Furey, W.F., Robbins, A.H., Clancy, L.L., Winge, D.R., Wang, B.C., Stout, C.D., 1986. Crystal structure of Cd, Zn metallothionein. Science 231, 704–710.

Graham, J.M., Rickwood, D., 1997a. Subcellular Fraction. A Practical Approach, 1st ed., pp. 112–113.

Graham, J.M., Rickwood, D., 1997b. Subcellular Fraction. A Practical Approach, 1st ed., pp. 120–128.

Green, D.R., Reed, J.C., 1998. Mitochondria and apoptosis. Science 281, 1309–1312.

Halestrap, A.P., Woodfied, K., Connern, C.P., 1997. Oxidative stress, thiol reagents and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocase. J. Biol. Chem. 272, 3346–3354.

Hultberg, B., Andersson, A., Isaksson, A., 1997. Copper ions differ from other thiol reactive metal ions in their effects on the concentration and redox status of thiols in HeLa cell cultures. Toxicology 117, 89–97.

Hultberg, B., Andersson, A., Isaksson, A., 1998. Alteration of thiol metabolism in human cell lines induced by low amounts of copper, mercury or cadmium ions. Toxicology 126, 203–212.

Hunter, D.R., Haworth, R.A., 1979. The Ca^{2+}-induced membrane transition in mitochondria. Arch. Biochem. Biophys. 195, 453–459.

Ichas, F., Mazat, J.P., 1998. From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high- conductance state. Biochim. Biophys. Acta 1366, 33–50.

Jurgensmeier, J.M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D., Reed, J.C., 1998. Bax directly induces release of cytochrome c from isolated mitochondria. Proc. Natl. Acad. Sci. U.S.A. 95, 4997–5002.

Kapus, A., Ligeti, E., Fonyo, A., 1989. Na^+/H^+ exchange in mitochondria as monitored by BCECF fluorescence. FEBS Lett. 251, 49–52.

Kim, M.S., Kim, B.J., Woo, H.N., Kim, K.W., Kim, K.B., Kim, I.K., Jung, Y.K., 2000. Cadmium induces caspase-mediated cell death: suppression by Bcl-2. Toxicology 145, 27–37.

Kroemer, G., Reed, J.C., 2000. Mitochondrial control of cell death. Nat. Med. 6, 513–519.

Lemasters, J.J., Nieminen, A.L., Qian, T., Trost, L.C., Elmore, S.P., Nishimura, Y., Crowe, R.A., Cascio, W.E., Bradham, C.A., Brenner, D.A., Herman, B., 1998. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim. Biophys. Acta 1366, 177–196.

Leoffler, M., Kroemer, G., 2000. The mitochondrion in cell death control: certainties and incognita. Exp. Cell Res. 256, 19–26.

Li, M., Kondo, T., Zhao, Q.L., Li, F.J., Tanabe, K., Arai, Y., Zhou, Z.C., Kasuya, M., 2000. Apoptosis induced by cadmium in human lymphoma U937 cells through Ca^{2+}-calpain and caspase-mitochon. J. Biol. Chem. 275, 39702–39709.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R., Wang, X., 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86, 147–157.

Majima, E., Shinohara, Y., yamaguchi, N., Hong, Y.M., Terata, H., 1993. Characterization of cysteine residues os mitochondrial ADP/ATP carrier with SH-reagent eosin 5-maleimide and *N*-ethylmaleimide. J. Biol. Chem. 268, 22181–22187.

Majima, E., Ikawa, K., Takeda, M., Hashimoto, M., Shinohara, y., Terata, H., 1995. Translocation of loops regulates transport activity of mitochondrial ADP/ATP carrier deduced from formation of a specific intermolecular disulfide bridge catalyzed by cooper-*o*-phenanthroline. J. Biol. Chem. 270, 29548–29554.

Martinou, J.C., Green, D.R., 2001. Breaking mitochondral barrier. Nat. Rev. 2, 63–67.

Mikol, V., Kallen, J., Walkinshaw, M.D., 1994. X-ray structure of a cyclophilin D/cyclosporin complex: comparison with cyclosporin A and delineation of its calcineurin-binding domain. Proc. Natl. Acad. Sci. U.S.A. 91, 5183–5186.

Narita, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R.J., Matsuda, H., Tsujimoto, Y., 1998. Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. Proc. Natl. Acad. Sci. U.S.A. 95, 14681–14686.

Rikans, L.E., Yamano, T., 2000. Mechanisms of cadmium-mediated acute hepatotoxicity. J. Biochem. Mol. Toxicol. 14, 110–117.

Shimizu, S., Narita, M., Tsujimoto, Y., 1999. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 399, 483–487.

Shimizu, S., Konishi, A., Kodama, T., Tsujimoto, Y., 2000. BH4 domain of antiapoptotic Bcl-2 family members closes voltage-dependent anion channel and inhibits apoptotic mitochondrial changes and cell death. Proc. Natl. Acad. Sci. U.S.A. 97, 3100–3105.

Sies, H., Arteel, G.E., 2000. Interaction of peroxynitrite with selenoproteins and glutathione peroxidase mimics. Free Radic. Biol. Med. 28, 1451–1455.

Smith, D.B., Johnson, K.S., 1998. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione *S*-transferase. Gene 67, 31–40.

Susin, S.A., Zamzami, N., Kroemer, G., 1998. Mitochondria as regulators of apoptosis: doubt no more. Biochim. Biophys. Acta 1366, 151–165.

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Brenner, C., Larochette, N., Prevost, M.C., Alzari, P.M., Kroemer, G., 1999. Mitochondrial release of caspase-2 and -9 during the apoptotic process. J. Exp. Med. 189, 381–394.

Tsangaris, G.T., Tzortzatou-Stathopoulou, F., 1998. Cadmium induces apoptosis differentially on immune system cell lines. Toxicology 128, 143–150.

Tsujimoto, Y., Shimizu, S., 2000. Bcl-2 family: life-or-death switch. FEBS Lett. 466, 6–10.

Vairavamurthy, M.A., Goldenberg, W.S., Ouyana, S., Khalid, S., 2000. The interaction of hydrophilic thiols with cadmium: investigation with a simple model, 3-mercaptopropionic acid. Mar. Chem. 70, 181–189.

Wallace, D.C., 1999. Mitochondrial diseases in man and mouse. Science 283, 1482–1488.

Woodfied, K., Ruck, A., Brdiczka, D., Halestrap, A.P., 1998. Direct demonstration of a specific interaction between cyclophilin-D and the adenine nucleotide translocase confirm their role in the mitochondrial permeability transition. Biochem. J. 336, 287–290.

Zamzami, N., Kroemer, G., 2001. The mitochondrion in apoptosis: how Pandora's box opens. Nat. Rev. 2, 67–71.

Zazueta, C., Reyes-Vivas, H., Zafra, G., Sanchez, C.A., Vera, G., Chavez, C.A., 1998. Mitochondrial permeability transition as induced by cross-linking of the adenine nucleotide translocase. Int. J. Biochem. Cell Biol. 30, 517–527.

Zazueta, C., Sanchez, C., Garcia, N., Correa, F., 2000. Possible involvement of the adenine nucleotide translocase in the activation of the permeability transition pore induced by cadmium. Int. J. Biochem. Cell Biol. 32, 1093–1101.