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Spectroscopic and Microscopic Studies on the Mechanisms of Mitochondrial Toxicity Induced by Different Concentrations of Cadmium

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Abstract The deleterious action of Cd^{2+} on rat liver mitochondria was investigated in this work using spectroscopic and microscopic methods. The concentration dependence of Cd^{2+} on mitochondrial swelling, membrane potential and membrane fluidity was studied. Our aim was to detect the active sites of Cd^{2+} in the mitochondrial membrane treatments with cyclosporin A (CsA) and EGTA on the mitochondrial permeability transition (MPT) induced by low and high concentrations of Cd^{2+}. The protective effects of dithiothreitol, human serum albumin and monobromobimane^{+} on Cd^{2+}-induced MPT were also monitored. All of these investigations indicated that Cd^{2+} can directly affect MPT at two separate localization sites at different concentrations: the classic Ca^{2+} triggering site and the thiol (–SH) groups of membrane proteins matched by MPT pore opening (defined as "S" site). At the high concentration of Cd^{2+}, other free –SH groups in the mitochondrial matrix may be involved in this process. These findings were supported by transmission electron microscopy and shed light on the toxic mechanism of Cd^{2+} on mitochondria.

Keywords Mitochondria · Cadmium · Permeability transition · Fluorescence anisotropy · Binding site

Yue Zhang and Jia-Han Li contributed equally to this work.

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Abbreviations

DPH	1,6-Diphenyl-1,3,5-hexatriene
HP	Hematoporphrin
HSA	Human serum albumin
DTT	Dithiothreitol
MBM^{+}	Monobromobimane^{+}
CsA	Cyclosporin A
Rh123	Rhodamine 123
BKA	Bongkrekic acid

Introduction

Cadmium is one of the most harmful environmental pollutants and has been investigated for over 50 years. With the dramatic development in nanotechnology over the past few years (Whitesides 2005; Cuenca et al. 2006), researchers have provided extraordinary insight into the heavy metals, including cadmium, which is utilized in the construction of nanoparticles known as quantum dots (Jiang et al. 2004; Rosenthal et al. 2007). The toxic effects of cadmium on mammalian cells and tissues have attracted much attention (Deckert 2005; Rzigalinski and Strobl 2009; Lu et al. 2008). It is generally acknowledged that cadmium is not essential for the human body but highly toxic to many organs of both humans and other animals (Cannino et al. 2009). Damage to mitochondria possibly plays a pivotal role in cadmium-induced apoptosis as cadmium has been found to induce mitochondrial dysfunction and inhibit oxidative phosphorylation (Sato et al. 1978; Dorta et al. 2003). Further studies have shown that cadmium may induce matrix swelling and opening of the mitochondrial permeability transition (MPT) pore, which has been recently associated with cell damage and death (Whitesides 2005). Cadmium also alters the

activities and conformations of mitochondrial proteins (Lu et al. 2008; Al-Nasser and Al-Nasser 2000; Rikans and Yamano 2000; Lemarié et al. 2004). In addition, some scientists have suggested that the influences of cadmium (Cd^{2+}) on mitochondria at high and low concentrations are different (Diamond and Kench 1974; Cameron et al. 1986; Belyaeva and Korotkov 2003). However, the binding sites of cadmium on mitochondria are still unidentified, and the mechanism by which it operates is still poorly understood. To date, few studies have contributed to our understanding of the mechanism of action of Cd^{2+} at different concentrations on mitochondria. It is of great interest to study the dose dependence of Cd^{2+} on the function and structure of mitochondria.

Herein, the molecular mechanism of the effects of Cd^{2+} on mitochondria was further investigated. The different influences of Cd^{2+} on osmotic swelling, membrane potential and membrane fluidity of mitochondria at low and high concentrations were studied using isolated rat liver mitochondria as the model. Importantly, the binding sites of Cd^{2+} on mitochondrial membranes are suggested, as confirmed by detailed transmission electron microscopic (TEM) investigations. The present work is expected to provide a comprehensive understanding of the toxic action of Cd^{2+} on mitochondria and to shed new light on the mechanisms of Cd^{2+}-induced mitochondrial toxicity.

Materials and Methods

Chemicals

Cyclosporin A (CsA), EGTA, oligomycin, rotenone, rhodamine 123 (Rh123), 1,6-diphenyl-1,3,5-hexatriene (DPH), hematoporphrin (HP), human serum albumin (HSA), carbonylcyanide-*p*-trifluorometh-oxyphenyl hydrazone (FCCP), monobromobimane$^+$ (MBM^+), dithiothreitol (DTT) and $CdCl_2$ were purchased from Sigma (St. Louis, MO). All other reagents were of analytical reagent grade, and all solutions were prepared with asepsis double-distilled water.

Isolation of Mitochondria

Liver mitochondria from Wistar rats (200–250 g) were isolated according to standard differential centrifugation procedures. The liver tissue was briefly homogenized in medium A, containing 250 mM sucrose, 0.5 mM EGTA, 3 mM Tris (pH 7.2) (Belyaeva and Korotkov 2003; Belyaeva et al. 2001). The protein concentration was determined by the biuret method. The respiratory control ratio (RCR) was measured by a Clark electrode. Only mitochondrial suspensions that demonstrated an RCR above 3 were used.

Determination of Mitochondrial Swelling

Mitochondrial swelling was measured spectrophotometrically by monitoring the absorbance at 540 nm over 10 min at 25°C. Mitochondria (0.25 mg/ml) was suspended in 2 ml respiration buffer B (200 mM sucrose, 10 mM Tris–Mops, 20 μM EGTA–Tris, 5 mM succinate, 2 μM rotenone and 3 μg/ml oligomycin, pH 7.4) and incubated with different concentrations of Cd^{2+} (Ricchelli et al. 1999b). Spectra were recorded at room temperature on a UNICO (Dayton, NJ) 4802 double beam spectrophotometer equipped with 1.0-cm quartz cells.

Measurement of Membrane Potential

Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were indicated by the accumulation of Rh123 (250 μM) as monitored by the changes in fluorescence emission intensity (Zamzami et al. 1995). The $\Delta\Psi_m$ was assessed by an LS-55 fluorophotometer (Perkin-Elmer, Norwalk, CT) at 25°C equipped with a quartz cell of 1.0-cm path length ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 525$ nm). Mitochondria (0.5 mg/ml) were suspended in buffer B (2 ml). For analysis of single-bolus Cd^{2+} data, mitochondria were completely depolarized with FCCP at the end of the experiment. The change in fluorescence induced by FCCP was used as a standard of maximum depolarization, and other data were expressed as a percentage.

Assessment of Membrane Fluidity

Dynamic changes of the mitochondrial membranes were measured by the fluorescence anisotropic changes of HP or DPH-labeled mitochondria, respectively.

Fluorescence anisotropic (r) values were collected by measurement of I and $I_\perp$, i.e., the fluorescence intensities polarized parallel and perpendicular, respectively, to the vertical plane of polarization of the excitation beam. The anisotropy, r, is defined by the following equation:

$$r = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}} \quad (1)$$

where $G = I_\perp/I_\parallel$ is the correction factor for instrumental artifacts (Ricchelli et al. 1999b; Lakowicz 1999). Free probes in the bulk medium do not contribute to the fluorescence anisotropy since they are almost fluorimetrically silent in aqueous media.

Stock solutions of the two probes were prepared in absolute ethanol. HP (final concentration 5 μM) was added to the mitochondrial suspensions (0.5 mg/ml, buffer B) and incubated for 3 min before measuring. DPH (2 μM) required much longer incubation times (1 h). Anisotropic changes were recorded by the LS-55 fluorophotometer

at $\lambda_{ex} = 520$ nm, $\lambda_{em} = 626$ nm for HP and at $\lambda_{ex} = 340$ nm, $\lambda_{em} = 460$ nm for DPH.

TEM of Mitochondria

Mitochondria in the various experimental conditions were fixed for 30 min at 4°C using glutaraldehyde at a final concentration of 2.5% in 0.1 M cacodylate buffer, then postfixed with 1% osmium tetroxide and dehydrated. Observations were performed on a JEM-100CX TEM (JEOL, Peabody, MA).

Results

Concentration Dependence of Cd^{2+} on Mitochondrial Dysfunction

The effects of different concentrations of Cd^{2+} on mitochondrial swelling were evaluated according to the decrease in absorbance at 540 nm (A540) over 10 min. As shown in Fig. 1a, Cd^{2+} induced mitochondrial swelling at the testing concentrations with a proportional dependence of the swelling tendency on the concentration of Cd^{2+}.

The effects of Cd^{2+} on swelling of isolated mitochondria were accompanied by the changes of membrane potential measured using fluorescent probe Rh123. Rh123 can accumulate in the mitochondrial matrix, and its fluorescence will be quenched. Upon collapse of $\Delta\Psi_m$, Rh123 is released into the medium, causing an increase in the fluorescence intensity. Also, $\Delta\Psi_m$ was abolished by adding FCCP (3 μM), a potent uncoupler of oxidative phosphorylation. As shown in Fig. 1b, the values of $\Delta\Psi_m$ decreased with increasing concentrations of Cd^{2+} (Zhu et al. 2002).

Recent studies have reported that induction of MPT in rat liver mitochondria was accompanied by fluidity changes of mitochondrial membranes (Ricchelli et al. 1999b, 2005). The changes of fluorescence anisotropy (r) of mitochondria-bound dyes can evaluate the membrane fluidity changes. In order to investigate the changes of membrane fluidity during Cd^{2+}-induced mitochondrial dysfunction, the two probes HP and DPH were used to monitor the fluidity of different regions of lipid membranes.

As shown in Fig. 2a, the HP or DPH anisotropy was continuously monitored. The addition of Cd^{2+} (final concentration 50 μM) caused an obvious increase of the anisotropy of HP from 0.22 to 0.30 (Fig. 2a, curve a). The increase of anisotropy can be attributed to the decrease of Brownian motion or energy transfer between identical chromophores. Since the anisotropic changes of HP reflect the conformational variation of HP-binding regions on the mitochondrial membrane, the result indicates that the mitochondrial membrane structure was strongly disturbed

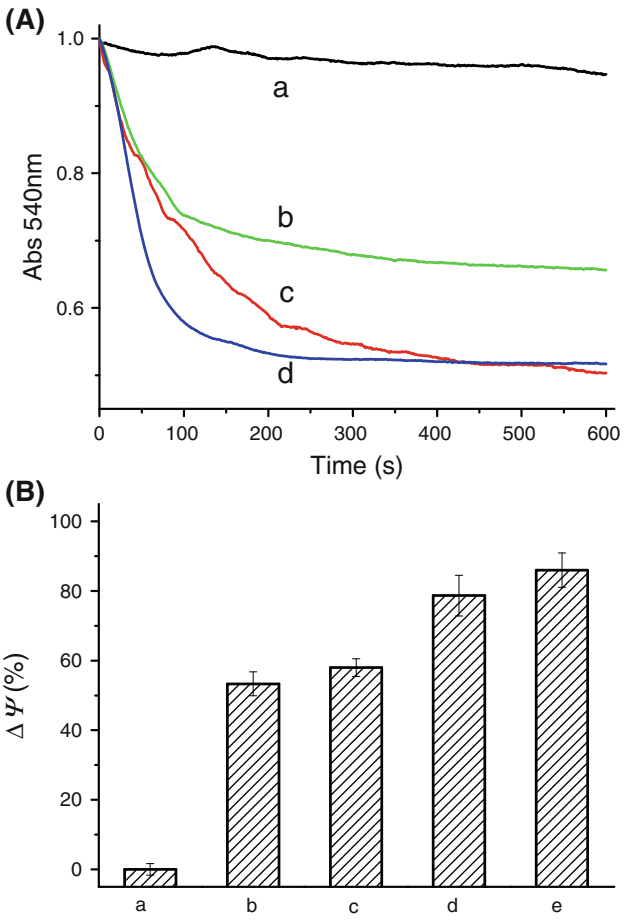

Fig. 1 Cd^{2+} induced isolated mitochondrial swelling and decreased the $\Delta\Psi_m$. **a** Mitochondrial swelling was monitored at 540 nm in the absence or presence of Cd^{2+} as described in "Determination of Mitochondrial Swelling" section. Mitochondria (0.25 mg/ml) followed by addition of Cd^{2+} at concentrations of 0 μM (*a*), 5 μM (*b*), 10 μM (*c*) and 25 μM (*d*). **b** Membrane potential was measured by the fluorescence of Rh123 as described under Measurement of Membrane Potential. Complete depolarization was caused by FCCP (3 μM). Traces *a*–*e* Mitochondrial suspensions (0.5 mg/ml) were added. *c* (Cd^{2+})/μM: 0, 10, 50, 100, 150

after uptake of Cd^{2+}. In contrast to the results obtained with HP, there was no obvious change in the anisotropy of DPH (Fig. 2a, curve b). HP accumulates mainly in polar, solvent-accessible regions of the lipid bilayer and protein regions of the inner membrane (Ricchelli et al. 1999a), while DPH is typically used to reflect fluidity changes of the hydrophobic regions of the membrane. The DPH probe lies preferentially close to the direction of acyl chains (Ricchelli et al. 1999b). The results show that Cd^{2+}-evoked mitochondrial dysfunction is accompanied by a remarkable change in membrane fluidity in the HP-related domain (protein regions), whereas the apolar lipid regions probed by DPH are not influenced by Cd^{2+}.

Based on the above results, the concentration dependence of Cd^{2+} on the anisotropic changes of HP-labeled mitochondria were studied (Fig. 2b). Interestingly we

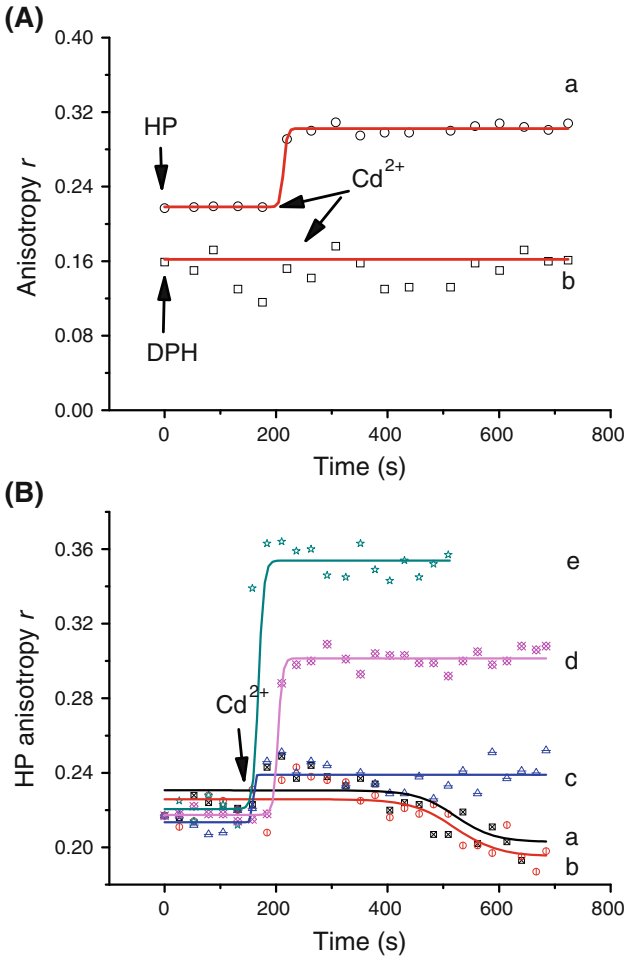

Fig. 2 Changes of mitochondria membrane fluidity induced by Cd^{2+}. **a** Time courses of the anisotropic changes of HP-labeled (curve *a*) and DPH-labeled (curve *b*) mitochondria during the MPT induced by addition of 50 μM $CdCl_2$ (*arrow*). **b** Effects of different concentrations of Cd^{2+} on the anisotropic changes of HP-labeled mitochondria. *c* (Cd^{2+})/μM; *a–e* 10, 20, 30, 50, 100

observed that the influences of Cd^{2+} on mitochondrial membrane fluidity at high and low concentrations were different. The decrease of HP anisotropy, which corresponds to the increase of membrane fluidity, was detected at low concentrations of Cd^{2+} (10, 20 μM). When the concentration of Cd^{2+} was higher than 30 μM, the membrane fluidity decreased with the increase of Cd^{2+} concentration (30, 50 and 100 μM). The phenomenon was quite different from Ca^{2+}-induced membrane fluidity changes, which decreased at increasing Ca^{2+} concentrations (see supplemental information and Ricchelli et al. 1999b).

Effects of CsA on MPT Induced by Different Concentrations of Cd^{2+}

Mitochondrial swelling and collapse of the transmembrane potential are the direct results of PT pore opening (Kowaltowski and Castilho 1997; Sanni et al. 2008). Although the structure of the MPT pore, a protein channel, has not yet been identified, CsA is considered to be a well-established inhibitor of MPT (Leoffler and Kroemer 2000; Jurgensmeier et al. 1998). The effects of CsA were evaluated at Cd^{2+}-induced mitochondrial swelling, loss of membrane potential and changes of membrane fluidity (Fig. 3). As shown in Fig. 3a, even at very low concentrations of 1 μM, CsA can notably inhibit the mitochondrial swelling caused by Cd^{2+} with low concentrations of 5 μM but cannot completely suppress the swelling induced by high concentrations of Cd^{2+} (even up to 10 μM).

Compared with swelling, CsA had the same effect on the loss of $\Delta\Psi_m$ induced by Cd^{2+} (Fig. 3b). When the concentration of Cd^{2+} was up to 110 μM, the high dose of Cd^{2+} still destroyed $\Delta\Psi_m$ in the presence of CsA. Moreover, the detection of HP anisotropic changes suggested a similar conclusion as shown in Fig. 3c. CsA prevented only the membrane fluidity changes induced by 10 μM Cd^{2+} but failed to prevent the changes at 50 μM Cd^{2+}. The CsA-inhibition results further indicate the significant difference between the high and low concentrations of Cd^{2+}-induced membrane swelling, loss of membrane potential and changes of membrane fluidity.

Effects of EGTA on MPT Induced by Different Concentrations of Cd^{2+}

It is known that mitochondria are able to reseal and fully recover energy-linked functions after the removal of calcium by chelator EGTA (Petronilli et al. 1994). Since Cd^{2+} can induce the opening of mitochondrial MPT pore, it was necessary to investigate whether the membrane can regain its original conformation and function after the removal of Cd^{2+}. The effects of Cd^{2+} on fluorescence intensity of Rh123 and the anisotropic changes of HP in the absence and presence of EGTA were measured. Comparison of two different concentrations of Cd^{2+} is shown in Fig. 4.

After the addition of EGTA into the Ca^{2+}-induced MPT medium, the mitochondrial membrane was impermeable again and the $\Delta\Psi_m$ could be readily restored (Hunter et al. 1976). In contrast, the decrease of $\Delta\Psi_m$ induced by Cd^{2+} could not be restored by EGTA and did not appear to be Cd^{2+} dose–dependent.

However, a significant difference in membrane fluidity was observed between high and low doses of Cd^{2+}/EGTA (Fig. 4b). Measurements of the addition of Cd^{2+}/EGTA indicated that the high dose Cd^{2+}-induced decrease of mitochondrial membrane fluidity was reversible (trace a), but the increase of mitochondrial membrane fluidity induced by low doses of Cd^{2+} (decrease of HP anisotropy) could not be recovered by EGTA (trace b).

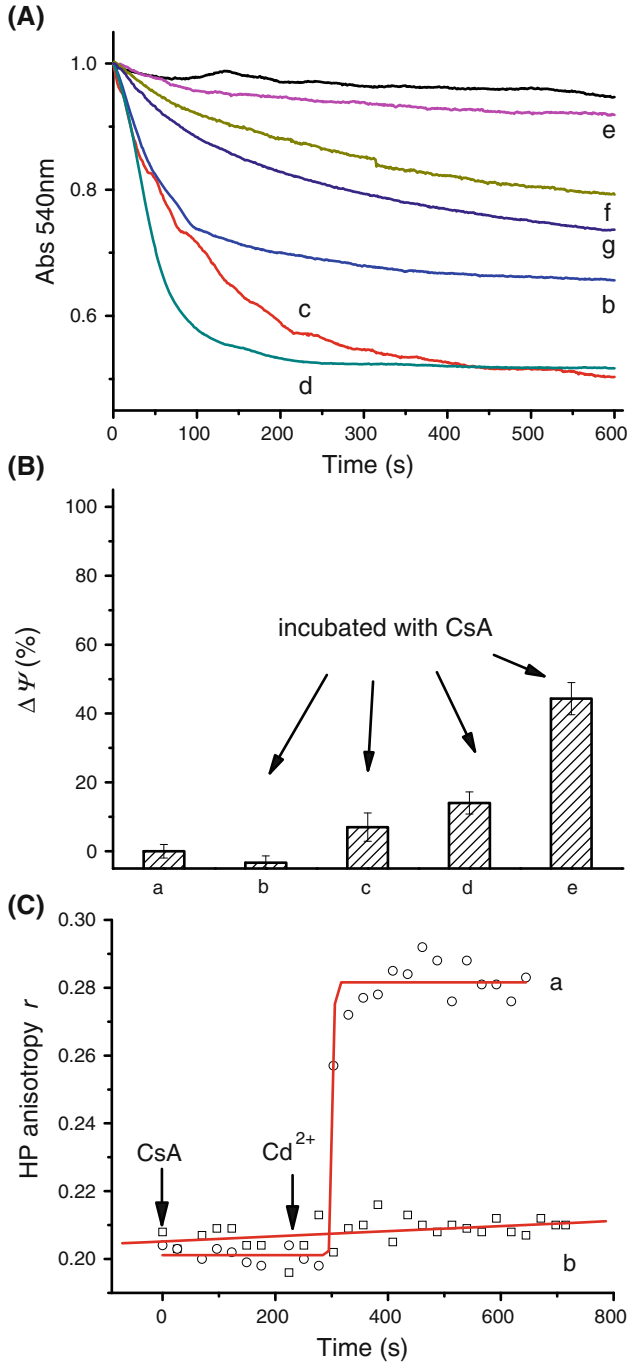

Fig. 3 Effects of CsA on MPT induced by different concentrations of Cd^{2+}. In some experiments, mitochondrial suspensions were treated with 1 μM CsA before measuring. **a** As described in Fig. 1a, mitochondrial swelling was monitored in the presence of CsA. Trace *a–d* were delineated with only Cd^{2+} at concentrations of 0 μM (*a*), 5 μM (*b*), 10 μM (*c*) and 25 μM (*d*); and traces *e–g* were with 1 μM CsA in the presence of 5 μM, 10 μM and 25 μM Cd^{2+}, respectively. **b** Membrane potential was measured in the presence of CsA. Trace *a* is the control; for traces *b–e* 1 μM CsA was added before measuring. *c* (Cd^{2+})/μM, *b–e* 0, 10, 50, 100. **c** Changes of membrane fluidity were measured in the presence of CsA. The initial medium was supplemented with 1 μM CsA, where indicated (*arrow*), and Cd^{2+} (curve *a*, 50 μM; curve *b*, 10 μM) was added

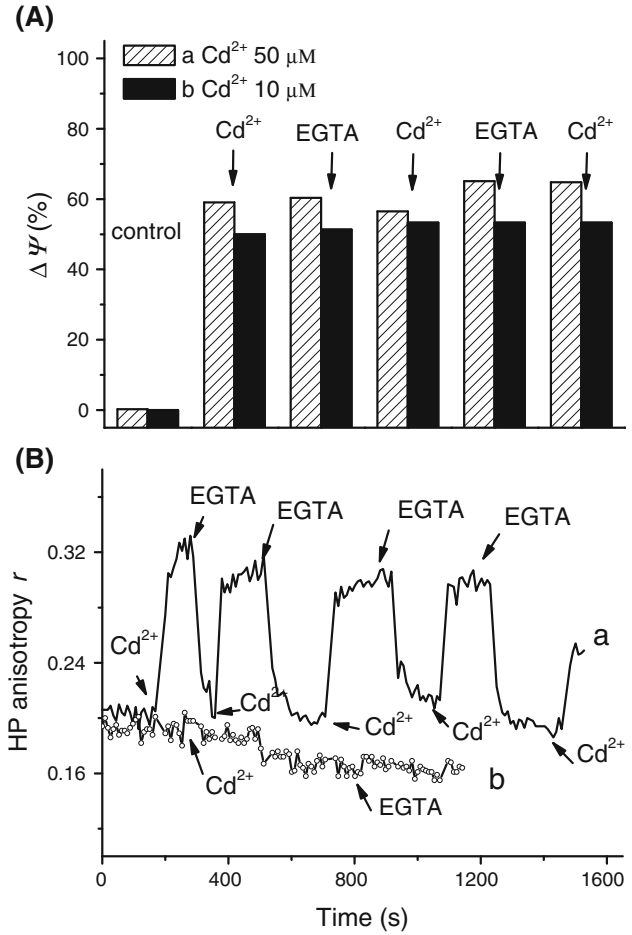

Fig. 4 **a** Membrane potential. **b** Fluidity changes of HP-labeled mitochondria during a cycle of repeated Cd^{2+}/EGTA additions. Mitochondria were suspended in NH_4Cl buffer (100 mM NH_4Cl, 10 mM Tris–Mops, 20 μM EGTA–Tris, 5 mM succinate, 2 μM rotenone, 1 mM P_i and 3 μg/ml oligomycin, pH 7.4). Where indicated (*arrows*), Cd^{2+} and EGTA (0.3 mM) were added. **a** Concentrations of Cd^{2+} were 50 μM (trace *a*) and 10 μM (trace *b*). **b** Concentrations of Cd^{2+} were 100 μM (trace *a*) and 10 μM (trace *b*)

The results suggest that there should be some differences in the mechanism between the Cd^{2+}-induced and the Ca^{2+}-induced MPT.

Effects of DTT on MPT Induced by Different Concentrations of Cd^{2+}

A disulfide reductant, DTT, was used to study Cd^{2+}-treated mitochondria at low and high concentrations. Although the swelling induced by the two concentrations of Cd^{2+} could be suppressed by DTT (Fig. 5a), Fig. 5b and c bring another interesting insight: DTT produced a complete reversal of membrane fluidity at both high and low concentrations of Cd^{2+}. But it failed to reverse the $\Delta\Psi_m$ at low concentration, while high dose Cd^{2+}-induced $\Delta\Psi_m$ collapse could be prevented. It is likely that Cd^{2+} induced

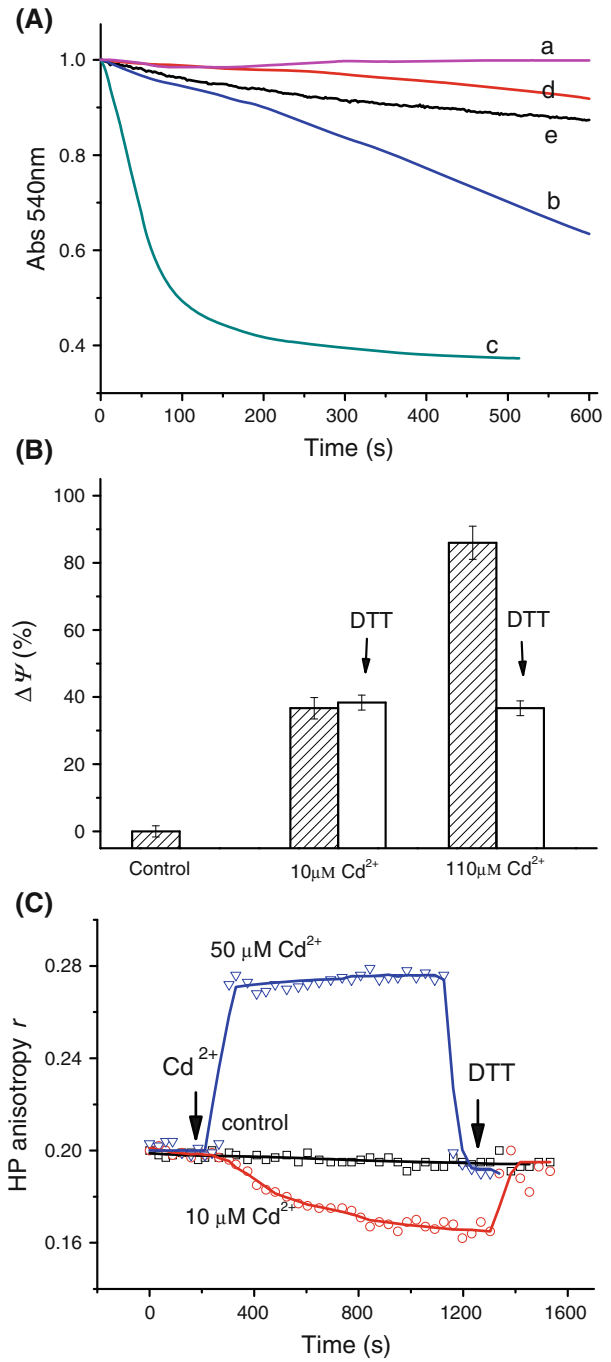

Fig. 5 Inhibition of DTT on 0.5 mg/ml mitochondrial swelling, loss of membrane potential and changes of membrane fluidity induced by different concentrations of Cd^{2+}. **a** Compared to control (trace *a*), the following additions were made: 10 μM Cd^{2+} (*b*), 50 μM Cd^{2+} (*c*), 10 μM Cd^{2+} and 0.5 mM DTT (*d*), 50 μM Cd^{2+} and 0.5 mM DTT (*e*). The mitochondrial membrane potential (**b**) and fluidity changes (**c**) were monitored as described in the legend of Fig. 1. Where indicated (*arrows*), $CdCl_2$ and DTT (0.5 mM) were added

mitochondria injury not only through affecting the Ca^{2+} channel but also through binding to thiol groups, which can be reduced by DTT (especially for high doses of Cd^{2+}).

By combining the results obtained from the protective effects of Cd^{2+}-induced MPT by CsA, EGTA and DTT, two different binding sites of Cd^{2+} on mitochondrial membranes could be confirmed.

Protective Effects

The influence of HSA (thiol-containing protein) and MBM^{+} (thiol reagent) on the deleterious action of Cd^{2+} on mitochondria were investigated. HSA, one of the most prominent proteins in plasma, has the capability to bind a wide range of endogenous and exogenous compounds. It contains abundant thiol groups and does not have any protective effect on mitochondria. In a previous study, Lu et al. (2008) measured the protective effect of bovine serum albumin (BSA) on Cd^{2+}-induced MPT using a Clark oxygen electrode. They suggested that BSA can bind to Cd^{2+} and thus possibly decrease the available Cd^{2+} to the thiol groups of membrane proteins. As shown in Fig. 6a, when HSA is added into the HP-labeled mitochondrial suspension, the observable decrease of *r* indicates that HSA can suppress the effect of Cd^{2+} on mitochondria due to its binding to Cd^{2+}. Our results agree well with Lu et al.'s experimental observation. MBM and its cationic derivative MBM^{+} are thiol reagents that selectively react with thiol groups (Kosower et al. 1979), which prevent the MPT pore opening caused by some dithiol oxidants or crosslinkers. They neither inhibit the phosphate carrier nor interfere with energy coupling, Ca^{2+} transport or ATP production and transport (Costantini et al. 1995). The process of MPT activation by low concentration of Cd^{2+} (Fig. 6b, curve a) is prevented by treatment with 0.2 mM MBM^{+}. When the concentration of Cd^{2+} is up to 50 μM (Fig. 6b, curve b), MBM^{+} is ineffective at suppressing the MPT induced by Cd^{2+}. The above fluorescence anisotropic data further proved our suggestion that interaction between Cd^{2+} and thiol groups can be involved in the Cd^{2+}-induced MPT process, and the specific thiol sites of high concentrations of Cd^{2+} on mitochondria are different from those of low concentrations.

Effects of Cd^{2+} on Mitochondrial Ultrastructure

The effects of Cd^{2+} on mitochondrial ultrastructure were investigated by TEM. Mitochondria extracted from rat liver maintained their integrity, with classical ultrastructure containing a few cristae and dense matrix (Fig. 7a) (Scalettar et al. 1991). Incubation with HP did not modify the shape of mitochondria (Fig. 7b). Following PT pore opening by 10 μM Cd^{2+}, mitochondria appeared to maintain a globular configuration with decreased matrix electron density and aggregation of membrane proteins (Fig. 7c). Furthermore, some mitochondria with other

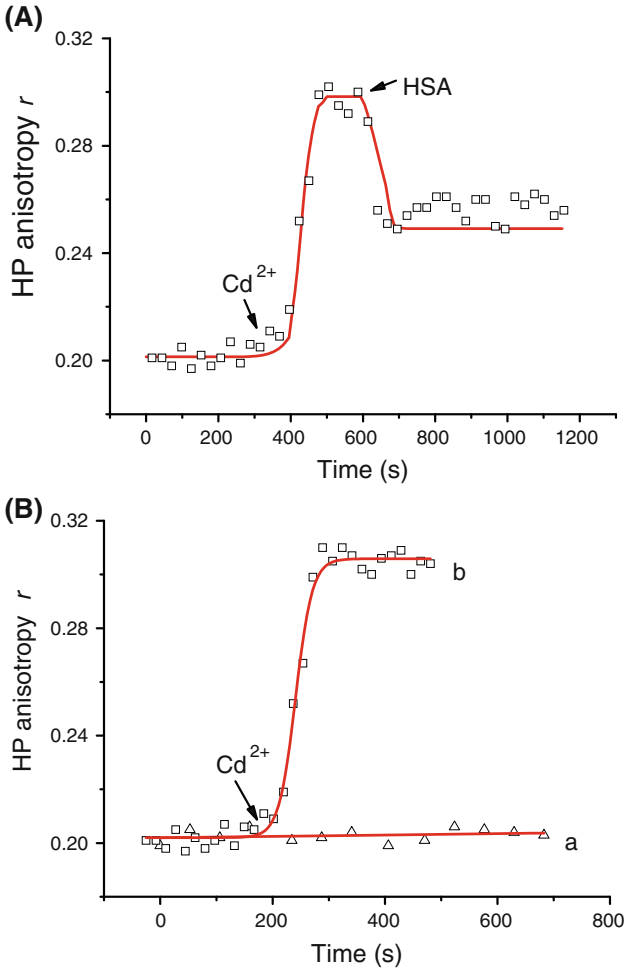

Fig. 6 Inhibition of HSA (**a**) or MBM^+ (**b**) on the PTP opening induced by Cd^{2+}. **a** Where indicated (*arrows*), $CdCl_2$ (50 μM) and HSA (6 mg/ml) were added. **b** Mitochondrial suspension was preincubated with MBM^+ (0.2 mM) for 2 min before measuring. Where indicated (*arrows*), $CdCl_2$ (curve *a*, 10 μM; curve *b*, 50 μM) was added

shapes were observed, and the outer membrane could not be clearly detected. Treatment with 80 μM Cd^{2+} caused a serious matrix swelling, with the outer membrane damaged and the typical pattern of inner membrane folding lost (Fig. 7d). The effects induced by 10 μM Cd^{2+} could be reduced by pretreatment with 1 μM CsA (Fig. 7e), but CsA could not prevent the damage induced by high concentrations of Cd^{2+} (Fig. 7f). In contrast with CsA treatment, an obvious reversion could be detected by adding DTT to the mitochondrial suspension treated with 80 μM Cd^{2+} (Fig. 7h). The aggregation of membrane proteins induced by 10 μM Cd^{2+} could also be prevented by DTT (Fig. 7g).

Discussion

This work focuses on elucidating the concentration effects of Cd^{2+} on mitochondria. Early reports (Sanni et al. 2008; Koizumi et al. 1994) showed that mitochondrion is one of the key intracellular targets for Cd^{2+}. In rat liver, the metal ion causes mitochondrial swelling, collapses membrane potential and modifies mitochondrial function. Consistently, the experiments shown in Fig. 1 appear to agree well with those reports. The effects of Cd^{2+} on swelling-activated and membrane potential remained dose-dependent, with a significant difference between the high and low concentrations of Cd^{2+}-induced membrane fluidity changes (Fig. 2b).

Figure 2a shows that the mitochondrial membrane fluidity changes induced by Cd^{2+} were specifically caused by its effect on protein sites (probed by HP), while lipid domains (probed by DPH) were unaffected. The Ca^{2+}-induced MPT pore opening was accompanied by a remarkable increase in membrane fluidity labeled by HP. It has been reported that the binding sites of HP belong to the protein regions of the inner membrane involved in MPT pore formation (Malekova et al. 2007). The increase in membrane fluidity induced by low concentrations of Cd^{2+}, as shown in Fig. 2b, indicates that low concentrations of Cd^{2+} may lead to assembly of the MPT pore. The increase in membrane fluidity also potentiates the intrinsic proton permeability of the lipid bilayer, the so-called proton leak (García et al. 1998).

CsA inhibits MPT pore opening, which appears to interact directly with cyclophilin-D (Brandham et al. 1998). It is well established that CsA can effectively inhibit the mitochondrial swelling, collapse of the membrane potential and increase of membrane fluidity induced by Ca^{2+} (Halestrap et al. 1997; Ricchelli et al. 2003). Similarly, the results (Fig. 3) show that the induced MPT with swelling, collapse of the membrane potential and increase of membrane fluidity by low concentrations of Cd^{2+} could be inhibited by CsA. Since the crystal ionic radius of Cd^{2+} is close to that of Ca^{2+} (0.097 nm for Cd^{2+}, 0.099 nm for Ca^{2+}) (Nightingale 1959), low concentrations of Cd^{2+} may be able to induce MPT via a Ca^{2+}-dependent domain.

In order to compare the mechanisms of mitochondrial dysfunction induced by these two cations, the anisotropy of HP and fluorescence of Rh123 changes during a cycle of repeated EGTA/Cd^{2+} addition were measured. It is known that mitochondria are able to reseal and fully recover energy-linked functions after removal of calcium by EGTA. Although Cd^{2+} is able to act as a Ca^{2+} agonist, the addition of the chelator EGTA into the Cd^{2+}-induced MPT medium fails to restore mitochondrial membrane potential (Fig. 4a) and the pore protein complex cannot be reconstituted to the native conformation (Fig. 4b, trace b) (Ricchelli et al. 2003). The results suggest that the mitochondrial function disturbed by low concentrations of Cd^{2+} cannot recover and the mitochondria are still injured. The difference between Cd^{2+} and Ca^{2+} indicates that Cd^{2+} induced MPT not only

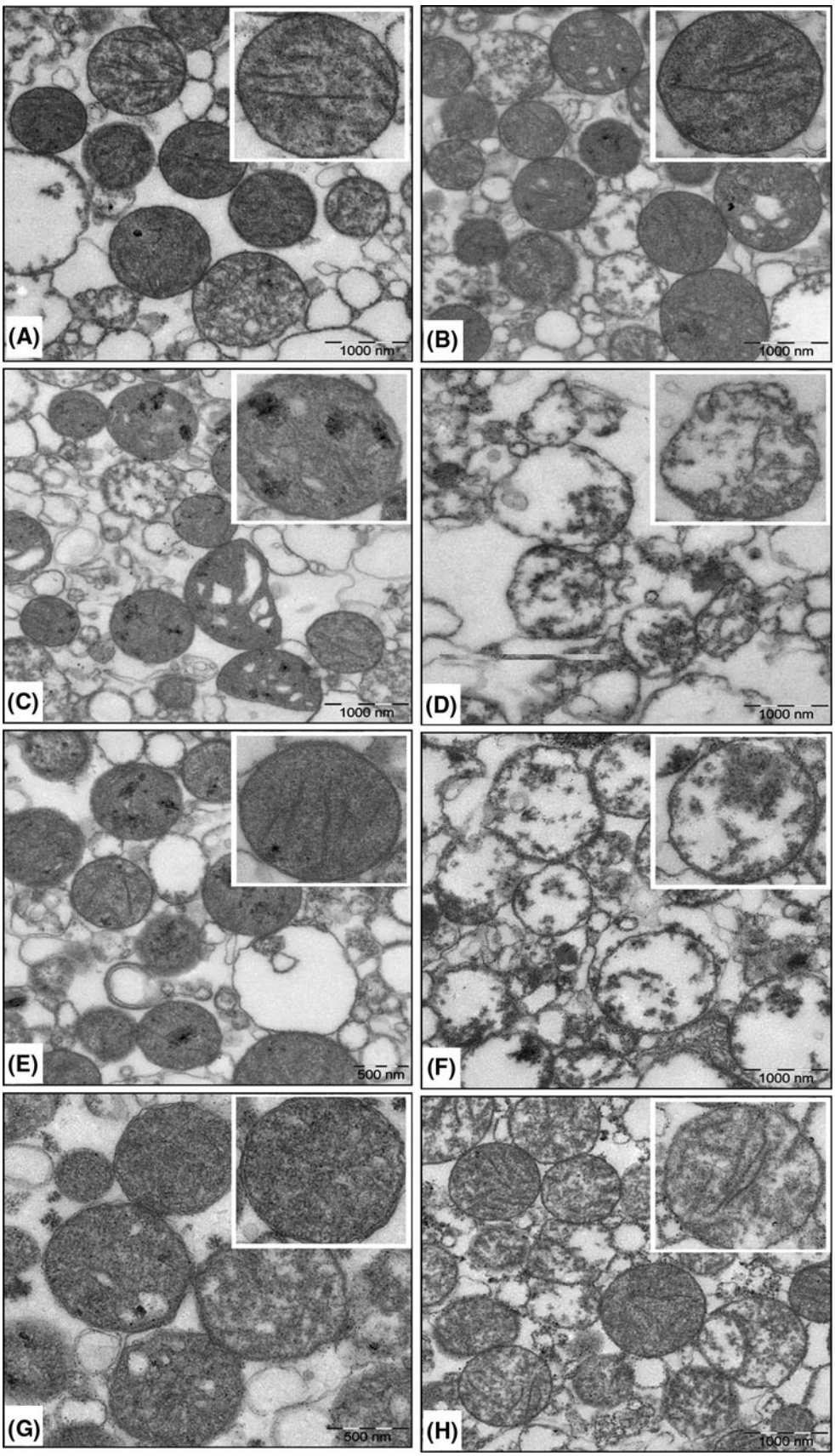

Fig. 7 Effects of Cd^{2+} on mitochondrial ultrastructure. Mitochondria (0.5 mg/ml) were incubated for 2 min at 25°C in the standard medium without (**a**) or with some additions (**b–h**). Mitochondria were treated as follows: supplemented with 3 μM HP (**b**), 10 μM Cd^{2+} (**c**) and 80 μM Cd^{2+} (**d**); pretreated with 1 μM CsA before addition of 10 μM Cd^{2+} (**e**) and 80 μM Cd^{2+} (**f**); pretreated with mM DTT before addition of 10 μM Cd^{2+} (**g**) and 80 μM Cd^{2+} (**h**)

through a Ca^{2+}-triggering site but also via some other approaches.

The "S" site, a critical dithiol, has been proposed to contribute to MPT modulation (Bernardi 1996). It has two states: (1) the MPT pore opening state, which is associated with the reduced form and can be stabilized by reaction with MBM^{+}; and (2) the PT pore closure state, which is associated with the disulfide/cross-linked species that can be reversed by a disulfide reductant such as DTT. To identify the "S" site involved in the interactions between Cd^{2+} and mitochondrial membrane, we performed a set of experiments using DTT (Fig. 5) and MBM^{+} (Fig. 6b) as "S" site protective agents.

Notably, DTT could partly recover the swelling and membrane fluidity induced by low concentrations of Cd^{2+} due to its protection on the "S" site (Fig. 5a, trace e), while it is ineffective at blocking the loss of membrane potential at low doses of Cd^{2+}. MBM^{+} also prevented the changes of membrane fluidity induced by low doses of Cd^{2+} (Fig. 6b, trace a). These results confirm that both the "S" and Ca^{2+}-triggering sites are involved in low-dose Cd^{2+}-induced MPT.

When the Cd^{2+} concentration increased to 30 μM, a new reverse observation of membrane fluidity, as shown in Fig. 2b, indicated that there are some differences between the interactions of mitochondria and Cd^{2+} at high and low doses. When high concentrations of Cd^{2+} are taken up, incubation with CsA does not consistently protect against Cd^{2+}-induced swelling (Fig. 3a, trace g), collapse of $\Delta\Psi_m$ (Fig. 3b) and changes of membrane fluidity (Fig. 3c, trace a). The results suggested that the Ca^{2+}-triggering site does not play a substantial role in the deleterious effects of high-dose Cd^{2+} on mitochondrial function. Also, with the increase in concentration of Cd^{2+} another binding model takes a leading role.

Although the restoration of membrane fluidity by addition of chelating agent EGTA (Fig. 4b, trace a) indicates that the perturbation of mitochondrial membrane structure induced by high doses of Cd^{2+} can be recovered, Fig. 4a (trace a) shows that the membrane dysfunction is irreversible.

To identify the class of thiols involved in the interactions between high doses of Cd^{2+} and mitochondrial membrane, the protective effects of MBM^{+}, DTT and HSA were also examined. Figure 5 shows that the swelling, collapse of $\Delta\Psi_m$ and decrease of membrane fluidity induced by high doses of Cd^{2+} were largely inhibited by DTT. The decrease of membrane fluidity was also partly recovered by HSA (Fig. 6a). But MBM^{+} failed to prevent the decrease of membrane fluidity induced by high doses of Cd^{2+} (Fig. 6b, trace b).

DTT is a strong reducing agent that is primarily used to protect free –SH groups from oxidation. The protective effect of DTT suggests that high doses of Cd^{2+}-evoked mitochondrial dysfunction are related to –SH groups in mitochondria. Since MBM^{+} only selectively prevents the MPT pore opening caused by dithiol oxidants or cross-linkers, the anisotropy of HP indicates that high doses of Cd^{2+} induce mitochondrial dysfunction not only through binding to "S" site but also via the interaction with other free –SH groups in mitochondria. The reversion by HSA also suggests that the inhibition is related to the interaction between Cd^{2+} and thiol groups and agrees well with our conclusion above.

Moreover, TEM provides further insight into the effects of Cd^{2+} on the mitochondrial ultrastructure (Fig. 7). Figure 7g and h shows that both injuries induced by low and high doses of Cd^{2+} could be prevented by DTT, which is consistent with the above experimental result for swelling (Fig. 5a). It also demonstrates that the different binding modes of Cd^{2+} on mitochondrial membrane can coexist. Furthermore, TEM observation provides more detailed structural changes of membrane induced by Cd^{2+} (e.g., a low dose of Cd^{2+} induces partly aggregation of membrane proteins, while a high dose induces serious aggregation reflected by a few electron-dense and a large number of electron-tenuous proteins, but both injuries can be prevented by DTT). In other words, much more information can be obtained from the mitochondrial ultrastructure; therefore, future efforts should be devoted to further understanding of the mechanism.

Conclusion

To sum up, this work presents different appearances of mitochondrial toxicity induced by low and high concentrations of Cd^{2+} due to their different binding mechanisms. We showed that low concentrations of Cd^{2+} mainly induced MPT via two distinct modes: (1) Ca^{2+}-triggering site, (2) "S" site. However, the interaction between Cd^{2+} and thiol groups plays a leading role at high concentrations of Cd^{2+}, and not only "S" site but also free –SH groups in the mitochondrial matrix can be involved in the interaction. Moreover, TEM images suggested that the high concentration of Cd^{2+} also led to aggregation of membrane proteins through nonspecific binding between Cd^{2+} and –SH groups. The repeated addition of Cd^{2+}/EGTA shows that Cd^{2+} can induce mitochondrial dysfunction at both low and high concentrations, and the injury cannot be recovered, suggesting irreversible Cd^{2+} toxicity. However, the toxicological mechanism still needs to be clarified. In conclusion, our study provides an important basis for understanding the mechanisms of Cd^{2+} toxicity to mitochondria. The above features of Cd^{2+}-induced mitochondrial dysfunction will be useful for studies on the toxicity of heavy metals.

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