Matrix cadmium accumulation depolarizes mitochondria isolated from mouse brain

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Cadmium (Cd^{2+}) is an environmental contaminant commonly found in industrial settings with a biological half-life of 30 years. Although the accumulation and subsequent cytotoxicity of Cd^{2+} in nervous tissue is well documented, it is unclear exactly how Cd^{2+} kills cells. One potential mechanism involves inhibition of cellular energy production. In this study, we used fluorescence microscopy to monitor the effects of Cd^{2+} on mitochondrial membrane potential ($\Delta\Psi_m$) in individual mitochondria isolated from mouse brain. Mitochondria were attached to microscopy glass and loaded with rhodamine 123, a fluorescent indicator that collects in energized and respiring mitochondria, i.e., those with a robust $\Delta\Psi_m$. We found that Cd^{2+} at relatively low concentrations quickly and completely abolished $\Delta\Psi_m$. Cd^{2+} actions were concentration-dependent, and were relatively potent and efficacious when compared to calcium (Ca^{2+}) and zinc (Zn^{2+}). Moreover, the Ca^{2+} uniporter blocker ruthenium red protected against Cd^{2+}-induced depolarization, suggesting that matrix entry of Cd^{2+} through this traditional Ca^{2+} pathway is necessary for its effect. These results demonstrate that Cd^{2+} substantially inhibits mitochondrial function and provide important insight regarding the mechanism of Cd^{2+}-mediated neurotoxicity.

Abbreviations: $\Delta\Psi_m$ – mitochondrial membrane potential; Rh123 – rhodamine 123; FCCP – carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone; RR – ruthenium red; ROS – reactive oxygen species

Keywords: fluorescence microscopy; mitochondrial membrane potential; oxidative stress; calcium uniporter

Introduction

Industrial exposure to cadmium (Cd^{2+}) comes primarily from rechargeable nickel-cadmium batteries, although it is also incorporated into pigments and plastics stabilizers. Because Cd^{2+} is retained in soil and water, human populations are exposed to it primarily through ingesting vegetables, meats and seafood (Järup, 2003). Moreover, plasma Cd^{2+} levels are 4-5 times more elevated in smokers compared to non-smokers, mainly from accumulation in tobacco (Järup et al., 1998). Although Cd^{2+} has no known biological purpose, it can accumulate in tissues and cause toxicity at even low levels. For example, acute Cd^{2+} exposure through inhalation can cause inflammation of lung tissue (Seidal et al., 1993), whereas chronic exposure can damage kidney tubules (Hellström et al., 2001). Its effects on the skeletal system were first reported in Japan in the 1950s where exposure to Cd^{2+}-contaminated water in rice fields produced brittle bones and consequent joint and spine pains. This was the so-called itai-itai or "ouch-ouch" disease, to describe the discomfort caused by the metal-induced osteoporosis and associated skeletal pains (Alfvén et al., 2000).

Although Cd^{2+} effects are more immediately destructive in the kidneys and liver, chronic Cd^{2+} exposure caused factory workers to develop poor olfactory sensation (Rose et al., 1992), headaches, dizziness (Shukla and Singhal, 1984), hyperactivity, reduced attention

and memory loss (Hart et al., 1989). Furthermore, Ong et al. (2006) demonstrated that animals undergoing neurodegeneration accumulate more Cd^{2+} in the brain compared to controls, suggesting that exposure to contaminant metals may be more detrimental in individuals who have suffered brain injuries. Accumulation of Cd^{2+} in the brain appears to involve injury to the blood-brain barrier (Shukla et al., 1996). While the cellular mechanisms by which it elicits neurotoxicity are still unclear, Cd^{2+} may use existing ion pathways to enter mammalian cells and gain access to intracellular targets (Ong et al., 2006). Given that Cd^{2+} complexes with many Zn^{2+}-binding proteins and enzymes, one possible explanation is that Cd^{2+} displaces Zn^{2+} and therefore interferes with many normal biological processes (Sabolíc et al., 2010). Moreover, Cd^{2+} is known to compete with Ca^{2+} and disrupt Ca^{2+} channels (Marchetti et al., 1991) and Ca^{2+}-binding proteins (Usai et al., 1999), and inhibits the release of excitatory neurotransmitters while enhancing the release of inhibitory ones (Minami et al., 2001).

Given that many target organs of Cd^{2+} are energy-intensive tissues, understanding its mechanism on mitochondria is important. Mitochondria are not only generators of ATP, but also play an important role in Ca^{2+} buffering and production of free radicals. However, excess levels of Ca^{2+} and other cations adversely affect mitochondrial function. It is well established that high levels of free Ca^{2+} contribute to excitoxicity through mitochondrial damage (Nicholls and Budd, 2000). Evidence shows that Zn^{2+} might also contribute to neurodegeneration (Choi and Koh, 1998), possibly by inhibiting mitochondria (Dineley et al., 2003). Although Cd^{2+} effects on mitochondria are even more uncertain, several studies have noted that Cd^{2+} inhibits mitochondrial respiration (Dorta et al., 2003), dissipates $\Delta\Psi_m$ (Bolduc et al., 2004; López et al., 2006), elevates production of reactive oxygen species (ROS; Wang et al., 2004; López et al., 2006), damages mitochondrial DNA (Cannino et al., 2008), and initiates cell death via apoptosis (López et al., 2006). However, most of these studies were conducted in mitochondria isolated from kidney, liver, and intestinal tissue. Furthermore, experiments in brain tissue were conducted in primary neurons and thus show the effects of Cd^{2+} on the whole cell, as opposed to mitochondria exclusively.

In this study, we investigated the effects of Cd^{2+} on isolated brain mitochondria by comparison to biologically relevant ions, Ca^{2+} and Zn^{2+}. Based on the similar intracellular behaviors of Cd^{2+} and Zn^{2+}, we hypothesized that Cd^{2+} depolarized mitochondria through a mechanism similar to Zn^{2+}. We used a novel paradigm of isolated mouse brain mitochondria that were attached to microscopy glass and incubated with the potentiometric probe, rhodamine 123 (Rh123) and exposed to various concentrations of metals and drugs. Using fluorescence microscopy to monitor real-time changes in $\Delta\Psi_m$, we show that Cd^{2+} depolarizes mitochondria immediately, rapidly and completely, whereas both Ca^{2+} and Zn^{2+} depolarize mitochondria in a delayed, slower and incomplete manner. The Cd^{2+} effect elicits a concentration-dependent inhibition of $\Delta\Psi_m$ and is dependent on its matrix accumulation through the Ca^{2+} uniporter. Our results suggest that Cd^{2+} behaves much like Ca^{2+}, and not Zn^{2+}, in its ability to inhibit mitochondrial function in neural tissue.

Material and Methods

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified. BALB/c mice were bred and housed according to the guidelines provided by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures using mouse tissue were approved by the Institutional Animal Care and Use Committee at Francis Marion University.

Isolation of Brain Mitochondria

Adult mice were euthanized by CO_2 and brains extracted after decapitation. Brain mitochondria were isolated according to the protocol described by Vergun et al. (2003) with minor changes as indicated. Brain tissues were homogenized using a glass/glass Dounce homogenizer in an isolation buffer containing (in mM): 225 mannitol, 75 sucrose, 0.5 ethylenediaminetetraacetic acid (EDTA), 5 4-(2-

hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 1 mg/ml fatty acid free bovine serum albumin (BSA), with pH adjusted to 7.4 using KOH. The homogenate was centrifuged at 1300g for 10 minutes at 2°C. The supernatant was transferred to a new tube and centrifuged at 10,000g for 10 minutes. The subsequent supernatant was discarded, the pellet re-suspended in additional buffer, and spun down at 10,000g for 10 minutes. As a deviation from the original protocol, the subsequent supernatant was disposed and the pellet again re-suspended in isolation buffer containing all the ingredients as above minus the EDTA and BSA and centrifuged a final time at 10,000g for 10 minutes. The final pellet was added to a fresh tube and kept on ice for the duration of the experiments. Experiments were performed up to 6 hours after isolation. For each preparation, two adult brains were used to provide mitochondria to carry out 10-12 experiments. Approximately 40-50 animals in total were used for these studies.

Fluorescence Microscopy

Microscopy experiments were performed according to the protocol by Vergun et al. (2003) at room temperature in a mitochondrial incubation buffer containing (in mM): 125 KCl, 2 K_2HPO_4, 5 HEPES, 5 $MgCl_2$, 5 glutamate, and 5 malate, with pH adjusted to 7.0 with KOH. Fifteen microliters of mitochondrial suspension at a concentration of 20 mg/ml protein were placed on a 25-mm glass coverslip for exactly 3 minutes to allow the mitochondria to attach. The coverslips were placed in a 308 µl/mm RC-40HP high profile open bath perfusion chamber (Warner Instruments, LLC, Hamden, CT) and immediately mounted onto the microscope stage. Mitochondria were perfused with buffer at a rate of 5 ml per minute. Unattached mitochondria were washed out of the chamber leaving behind a single layer of attached, isolated brain mitochondria. Mitochondria were perfused with the potentiometric dye rhodamine 123 (Rh123; Invitrogen, Carlsbad, CA) at a concentration of 100 nM in order to visualize $\Delta\Psi_m$. Rh123 accumulates in mitochondria with a strong $\Delta\Psi_m$ and is lost as $\Delta\Psi_m$ is diminished, therefore healthy mitochondria appear bright, whereas depolarized mitochondria lose the dye and become dim. To monitor the effects of ions on $\Delta\Psi_m$, concentrations of $CaCl_2$, $ZnSO_4$ or $CdCl_2$ were diluted in incubation buffer from 1000× stock solutions and perfused for 5 minutes. For control experiments, mitochondria were perfused with incubation buffer alone for 5 minutes. At the end of each experiment, mitochondria were perfused for 2-3 minutes with 250 nM FCCP to induce complete depolarization. For experiments involving ruthenium red (RR), mitochondria were perfused with RR for 2 minutes prior to treatment with ion in the presence of RR.

A BX50WI Olympus Optical (Tokyo, Japan) microscope fitted with an Olympus Optical LUM PlanFI 100× water immersion quartz objective was used to detect fluorescence changes. Excitation light was provided by a 75 W xenon lamp-based monochromator (T.I.L.L. Photonics GmbH, Martinsried, Germany). Emitted light was detected with a CCD camera (Orca; Hamamatsu, Shizuoka, Japan). Rh123 was illuminated at 490 nm and light passed through a 500-nm long pass dichromatic mirror and a 535/25 nm band pass filter (Omega Optical, Brattleboro, VT). Simple PCI 6.2 software (Compix, Inc., Cranberry, PA) was used to record and analyze mean fluorescence intensity in each mitochondrion. Each coverslip possessed 80-100 individual mitochondria; objects that were smaller than 0.3 µm were not analyzed. Background fluorescence from mitochondria- and debris-free areas was subtracted from all the signals. To calculate percent depolarization, the change in Rh123 fluorescence upon metal exposure was divided by the fluorescence change upon FCCP treatment. All experiments were repeated three to six times using mitochondria from at least three different animals.

Statistical Analysis

Data was analyzed using PRISM 4.03 (Graph Pad Software, San Diego, CA). All data are presented as mean ± S.E. Comparisons were made using Student's t-test, with p values of less than 0.05 regarded as significant.

Results

Fluorescence visualization of Cd^{2+}-induced depolarization in isolated brain mitochondria.

Using Rh123, $\Delta\Psi_m$ was detected in isolated brain mitochondria perfused with Cd^{2+}. Representative fluorescence micrographs were taken of mitochondria exposed to 10 μM $CdCl_2$ (Figure 1). Panel A represents a field of healthy mitochondria, as evinced by the many bright fluorescent bodies, while panel B is the same field 5 minutes after Cd^{2+} exposure. Most mitochondria in the field have diminished fluorescence. This confirms that Cd^{2+} depolarizes mitochondria uniformly. It should be noted that this effect is comparable to that seen with the mitochondrial uncoupler carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP), which is used as a positive control for complete mitochondrial depolarization. Panels C and D show the same field of mitochondria before and after 250 nM FCCP exposure, respectively. This treatment effectively and consistently depolarized all mitochondria within a given population.

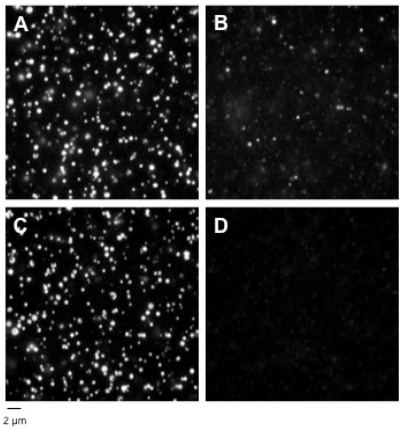

Figure 1. Cd^{2+} causes loss of membrane potential in isolated mitochondria. $\Delta\Psi_m$ was monitored by perfusing mitochondria with buffer containing Rhodamine 123 (100 nM). Fluorescence micrographs were taken of the same field before (A) and after (B) a 5-minute treatment with 10 μM $CdCl_2$. Images (C) and (D) represent mitochondria before and after, respectively, a 3-minute treatment of 250 nM FCCP, which served as a positive control for complete depolarization.

Cd^{2+} exposure causes immediate, rapid and complete mitochondrial depolarization.

Next we demonstrated the dynamics of ion-induced mitochondrial depolarization over time (Figure 2). Upon a 5-minute exposure to $CdCl_2$, mitochondria depolarize very quickly (Figure 2B). This is similar to depolarization observed upon FCCP treatment at the end of the positive control condition (Figure 2A). In contrast, mitochondrial depolarization after 5-minute exposures to equimolar concentrations of $CaCl_2$ or $ZnSO_4$ (Figure 2C and 2D, respectively) show that these ions produce a delayed, slower and incomplete depolarization. We note that the Cd^{2+} effect is consistent throughout the entire mitochondrial population, whereas the effect of Ca^{2+} or Zn^{2+} appears more variable.

Compared to Ca^{2+} and Zn^{2+}, Cd^{2+} is a more potent depolarizer of mitochondria.

To investigate the concentration response of Cd^{2+}, isolated mitochondria were exposed to varying concentrations of $CdCl_2$ (Figure 3A). Given that the effects of Ca^{2+} and Zn^{2+} on mitochondrial function are better documented, we compared the extent of mitochondrial depolarization to an equimolar concentration range of $CaCl_2$ (Figure 3B) and $ZnSO_4$ (Figure 3C). Cd^{2+}, like Ca^{2+} but not Zn^{2+}, appears to have a significant effect compared to the negative control buffer at concentrations as low as 1 μM. However, Cd^{2+} causes more profound depolarization with increasing concentrations. Both Ca^{2+} and Zn^{2+} produce concentration-dependent depolarization, but they do not achieve 100% depolarization even at 100 μM concentrations. Cd^{2+} causes complete depolarization at a 10-fold lower concentration. We calculated IC_{50} values from the concentration response curves: IC_{50} for $CdCl_2$ = 1.059 μM, IC_{50} for $CaCl_2$ = 10.07 μM and IC_{50} for $ZnSO_4$ = 10.18 μM. These data suggests that Cd^{2+} is a much more potent mitochondrial toxin compared to Ca^{2+} or Zn^{2+}.

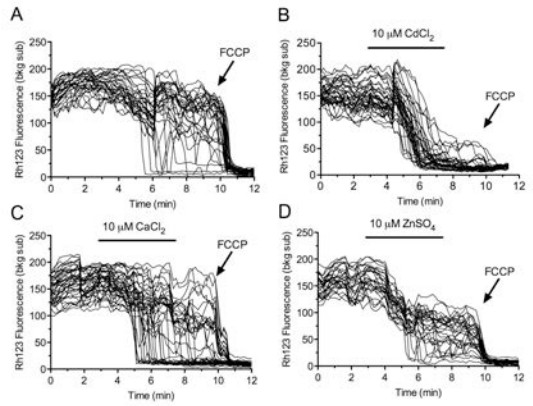

Figure 2. A comparison of the effect of equimolar concentrations of ion on $\Delta\Psi_m$. Graphs represent time-dependent changes in Rh123 fluorescence after 5-minute treatment with 10 μM $CdCl_2$ (B), $CaCl_2$ (C), or $ZnSO_4$ (D). Note: (A) represents the negative control where mitochondria were perfused with buffer alone. All experiments were concluded with perfusion of FCCP (250 nM) to demonstrate complete depolarization. Each line represents fluorescence changes measured in a single mitochondrion. All conditions were done at least three to six times from at least three different mitochondrial preparations.

Cd^{2+}-mediated mitochondrial depolarization requires its matrix entry through the Ca^{2+} uniporter.

It is well known that Ca^{2+} causes mitochondrial dysfunction by entering the matrix through the Ca^{2+} uniporter in the inner membrane. This has been pharmacologically confirmed as mitochondria pretreated with the uniporter blocker RR are protected against Ca^{2+}-induced depolarization (Votyakova and Reynolds, 2005). To determine if the mechanism of Cd^{2+}-mediated depolarization also involves matrix entry through the uniporter, we pretreated mitochondria with RR prior to Cd^{2+} (Figure 4). As shown, RR prevents Cd^{2+}-induced depolarization. As expected, RR prevents mitochondrial depolarization with 30 μM $CaCl_2$. However, RR only modestly reduces depolarization with 30 μM $ZnSO_4$ and does not significantly protect mitochondria. The inhibitor's limited protection against Zn^{2+} is consistent with our previous work (Malaiyandi et al., 2005; Devinney et al., 2009), and suggests that Zn^{2+} depolarizes mitochondria by acting at a site external to the inner membrane. From the results of the present study, we conclude that Cd^{2+} mediates mitochondrial depolarization through a mechanism more similar to Ca^{2+}.

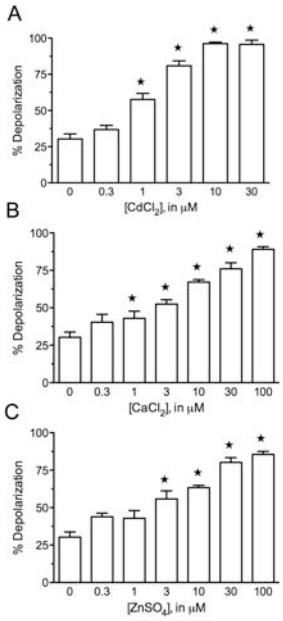

Figure 3. Cd^{2+} is a more potent mitochondrial toxin compared to Ca^{2+} or Zn^{2+}. Mitochondria were exposed for 5-minutes to varying concentrations of $CdCl_2$ (0-30 μM), $CaCl_2$ (0-100 μM) or $ZnSO_4$ (0-100 μM). Data is represented as the mean % depolarization ± SE of experiments performed at least three to six times from at least three different mitochondrial preparations. Comparisons were made between metal-treated conditions and controls using Student's t-tests with $p < 0.05$ as considered significant (★). IC_{50} for $CdCl_2$ = 1.059 μM, IC_{50} for $CaCl_2$ = 10.07 μM and IC_{50} for $ZnSO_4$ = 10.18 μM.

Discussion

In this study, we demonstrate a concentration-dependent loss of $\Delta\Psi_m$ upon acute Cd^{2+} exposure in mitochondria isolated from mouse brain. Individual mitochondria attached to microscopy glass loaded with the potentiometric dye Rh123 depolarized in response to a 5-minute exposure to Cd^{2+}. This paradigm has certain advantages over previous studies on isolated mitochondria in suspension, because it allows the addition and removal of solutions when desired and investigates direct metal interaction with mitochondria, without the confounds of other cellular components (Vergun et al., 2003). Using this model, we compared the effects of Cd^{2+} to those of Ca^{2+} and Zn^{2+}. It had been shown previously that nanomolar levels of Ca^{2+} are effective at depolarizing isolated rat brain mitochondria (Vergun and Reynolds, 2005), whereas we have shown that micromolar levels of Zn^{2+} are required to produce depolarization to the same extent (Malaiyandi et

al., 2005). Here, we demonstrate that Cd^{2+} is more potent compared to Ca^{2+} or Zn^{2+}, as indicated by calculated IC_{50} values (Figure 3). This is in agreement with previous work, showing that, compared to Ca^{2+}, Cd^{2+} more strongly inhibited respiration in mitochondria isolated from rainbow trout liver (Adiele et al., 2010). With respect to the kinetics of depolarization, Ca^{2+} and Zn^{2+} effects were delayed in onset and, once underway, slower. However, with Cd^{2+} the depolarization was rapid and complete (Figure 2). In our previous work, Ca^{2+} was far more effective at depolarizing rat brain mitochondria compared to what we see in mouse brain, which could be attributed to a species difference in sensitivity to Ca^{2+} (Vergun and Reynolds, 2005; Malaiyandi et al., 2005). This finding is consistent with the work of Panov et al. (2007) who demonstrated that 70% more Ca^{2+} was necessary to depolarize brain mitochondria isolated from mouse compared to rat, because of the ability of mouse brain mitochondria to efficiently sequester Ca^{2+}. However, the effects of Zn^{2+} in both animal models were comparable.

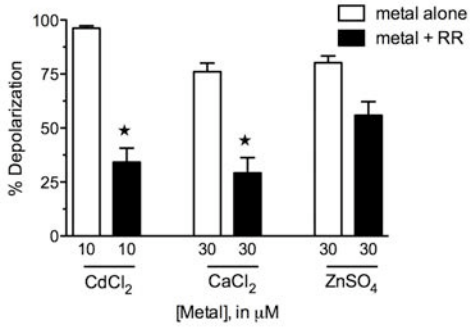

Figure 4. Cd^{2+} depolarizes mitochondria by entry through the Ca^{2+} uniporter. Preservation of $\Delta\Psi_m$ with the uniporter blocker ruthenium red (RR) was tested in mitochondria exposed to 10 μM $CdCl_2$ or 30 μM $CaCl_2$ or $ZnSO_4$. Mitochondria were pretreated with 2 μM RR for 2 minutes before a combined 5-minute treatment of both RR and metal (solid bars). In controls, mitochondria were exposed for 5-minutes to metal alone (open bars). Data represent the mean % depolarization ± SE of experiments performed at least 3 to 6 times from at least 3 different mitochondrial preparations. Comparisons were made between RR treatment and controls using Student's t-tests with $p < 0.05$ as considered significant (★).

Given the strong association between Cd^{2+} and Zn^{2+}-binding proteins (Sabolíc et al., 2010), we expected interactions between the uniporter and Cd^{2+} to be similar to that of Zn^{2+}. Our results, however, suggest similar mechanisms for Ca^{2+} and Cd^{2+} effects on mitochondria. Upon treatment with the Ca^{2+} uniporter blocker, RR, mitochondria are protected from the effects of Cd^{2+}, suggesting that like Ca^{2+}, Cd^{2+} import into the matrix is necessary for depolarization (Figure 4). Consistent with our previous findings, mitochondria are not much protected from Zn^{2+} in the presence of RR, suggesting that Zn^{2+} entry is not essential, and that Zn^{2+} acts at an external site (Malaiyandi et al., 2005). Similar to our present findings, Lee et al. (2005) show that mitochondria isolated from rat kidney cortex do not swell in the presence of Cd^{2+} and uniporter inhibitors.

The mechanism for Cd^{2+}-induced mitochondrial injury may go beyond simply sharing a common pathway with Ca^{2+}. Some groups hypothesize that Cd^{2+}-mediated toxicity may involve a disruption of cellular Ca^{2+} homeostasis. For instance, 3T3 cells treated with Cd^{2+} showed impaired Ca^{2+} homeostasis and structural abnormalities in mitochondria and ER (Biagioli et al., 2008). Furthermore, another study hypothesized that Ca^{2+} and Cd^{2+} may cooperate to impair mitochondrial respiration (Adiele et al., 2010). However, it is unclear whether Cd^{2+} operates or inhibits normal Ca^{2+} pathways. In the case of the Ca^{2+} uniporter, it appears that Cd^{2+} entry through this mechanism is necessary to injure mitochondria.

Another hypothesized mechanism for the effect of Cd^{2+} is the promotion of reactive oxygen species production (ROS). There is evidence that Cd^{2+} could act on electron transport chain sites to increase ROS. Using isolated guinea pig mitochondria, Wang et al. (2004) showed that complexes II and III were most sensitive to inhibition by Cd^{2+}, with complex III producing more ROS in response to Cd^{2+}. One interesting finding comes from ROS effects on mitochondrial membrane potential. In cortical neurons, lower Cd^{2+} exposure induced ROS production, but disrupted $\Delta\Psi_m$ at much higher concentrations (López et al., 2006). In

another study, Cd^{2+} exposure in a human intestinal cell line disrupted $\Delta\Psi_m$ that was not associated with cellular ROS production (Bolduc et al., 2004). This suggests that (1) there may be concentration-dependent effects of Cd^{2+} in disrupting the two parameters, and (2) ROS effects may be compensated by cellular antioxidants, whereas mitochondria may not be protected from higher Cd^{2+} concentrations.

In conclusion, the findings of this study demonstrate that in individual mitochondria isolated from mouse brain, (i) Cd^{2+} is a more potent mitochondrial toxin compared to Ca^{2+} and Zn^{2+}, and that (ii) it accumulates in mitochondria by an established Ca^{2+} pathway. To further elucidate the mechanism of Cd^{2+}, it would be necessary to visualize its import and determine whether prevention of Cd^{2+} entry preserves mitochondrial function. Because Cd^{2+} toxicity affects other highly active organs, this mitochondrial model provides a unique system to study the bioenergetic consequences of cadmium poisoning.

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