Cadmium is Acutely Toxic for Murine Hepatocytes: Effects on Intracellular Free Ca$^{2+}$ Homeostasis

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Summary
We studied cadmium toxicity in murine hepatocytes in vitro. Cadmium effects on intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) were assayed, using a laser scanning confocal microscope with a fluorescent probe, Fluo-3/AM. The results showed that administration of cadmium chloride (CdCl$_2$, 5, 10, 25 μM) resulted in a dose-dependent decrease of hepatocyte viability and an elevated aspartate aminotransferase (AST) activity in the culture medium (p<0.05 for 25 μM CdCl$_2$ vs. control). Significant increases of lactate dehydrogenase (LDH) activities in 10 and 25 μM CdCl$_2$-exposed groups were observed (p<0.05 and p<0.01, respectively). A greatly decreased albumin content and a more malondialdehyde (MDA) formation also occurred after CdCl$_2$ treatment. The Ca$^{2+}$ concentrations in the culture medium of CdCl$_2$-exposed hepatocytes were significantly decreased, while [Ca$^{2+}$]$_i$ appeared to be significantly elevated (p<0.05 or p<0.01 vs. control). We found that in Ca$^{2+}$-containing hydroxyethyl piperazine ethanesulfonic acid-buffered salt solution (HBSS) only, CdCl$_2$ elicited [Ca$^{2+}$]$_i$ increases, which comprised an initially slow ascent and a strong elevated phase. However, in Ca$^{2+}$-containing HBSS with addition of 2-aminoethoxydiphenyl borane (2-APB), CdCl$_2$ caused a mild [Ca$^{2+}$]$_i$ elevation in the absence of an initial rise phase. Removal of extracellular Ca$^{2+}$ showed that CdCl$_2$ induced an initially slow [Ca$^{2+}$]$_i$, rise alone without being followed by a markedly elevated phase, but in a Ca$^{2+}$-free HBSS with addition of 2-APB, CdCl$_2$ failed to elicit the [Ca$^{2+}$]$_i$ elevation. These results suggest that abnormal Ca$^{2+}$ homeostasis due to cadmium may be an important mechanism of the development of the toxic effect in murine hepatocytes. [Ca$^{2+}$]$_i$ elevation in acutely cadmium-exposed hepatocytes is closely related to the extracellular Ca$^{2+}$ entry and an excessive release of Ca$^{2+}$ from intracellular stores.

Key words
Cadmium • Toxicity • Hepatocyte • Calcium ion • Homeostasis

Introduction
Cadmium (Cd), is an extremely important industrial and environment pollutant, which results in the pollution of water, air and soil. It can accumulate in the bodies of plants and animals, and can enter the human body by food chain, followed by slow accumulation in the body. Such accumulation in many tissues and systems produces chronic and long-term pathogenic effects with multiple toxicity in various organs and cytotoxicity, such
as hepatic injury, testicular damage, pulmonary edema, renal dysfunction, osteomalacia, development of cancer, etc. (Goering et al. 1995, Satarug et al. 2000, Lecoeur et al. 2002, Chen et al. 2003). It has been demonstrated that the major site of the initial cadmium accumulation and toxicity in the body is the liver (Elez et al. 2001). Our previous observations revealed that acute administration of cadmium to rats results in significant elevation of cadmium accumulation in the liver, testis and heart. Ten-fold to 20-fold higher values were reported in the liver than in testes or heart, increasing proportionally to dosage used and time exposure to cadmium (Chen et al. 2003). Reports have been accumulated that cadmium can destroy the hepatic cell membrane and induce lipid peroxidation, affect the configuration and function of mitochondria and impair energy metabolism, damage DNA synthesis, transformation and expression (Koizumi et al. 1996, Risso-de Faverney et al. 2004, Hsiao and Stapleton 2004). Furthermore, there is evidence suggesting that changes of intracellular cation homeostasis are closely related to the mechanism of hepatic cell injury (Gasbarrini et al. 1992, Carini et al. 1995). Especially, the elevation of intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) is associated with the development of cell damage (Ueda et al. 2000, Orrenius et al. 1992). Cadmium can interfere for uptake with essential metal ions including calcium (Ca), zinc (Zn) and copper (Cu), and especially affect Ca\(^{2+}\) signaling in hepatic cells (Blazka and Shaikh 1992, Dundjerski et al. 2000, Baker et al. 2003). Thus the effect of cadmium on intracellular Ca\(^{2+}\) homeostasis may be an important factor in the development of hepatocellular damage.

In the present study, to further clarify the hepatocellular toxicity and injury in Cd-exposed hepatocytes, hepatocyte viability and its malondialdehyde (MDA) content as well as lactate dehydrogenase (LDH) activity in cultured medium, were assayed. We also investigated the effect of cadmium on hepatocyte [Ca\(^{2+}\)]\(_i\), under a laser scanning confocal microscope (LSCM) using Fluo-3/AM as an intracellular free Ca\(^{2+}\) fluorescent probe. In particular, we explored the effects of cadmium on Ca\(^{2+}\) signaling in hepatocytes to reveal its possible mechanisms.

**Material and Methods**

**Reagents**

HepatoZYME-SFM medium was from GIBCO (Invitrogen Co., USA) and type IV collagenase was purchased from Worthington Co., USA. 2-aminoethoxydiphenyl borane (2-APB) was obtained from Calbiochem Co., USA. Fluo-3/AM and Con A were from Fluka Co., USA. The ethylene glycol-bis (β-amino ethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), hydroxyethyl piperazine ethanesulfonic acid (HEPES) and L-glutamine were from Amresco Co., USA. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were from Sigma Chemical Co., USA. Cadmium chloride (CdCl\(_2\).2.5H\(_2\)O, analytical pure) was from Shanghai Tingxin Chemical Engineering Factory (Shanghai, China). Other chemicals were purchased from local commercial sources and were of analytical grade quality.

HEPES-buffered salt solution (HBSS) contained (mM): NaCl 135; KCl 5; CaCl\(_2\) 1.8; MgCl\(_2\) 1; HEPES 20; glucose 5 (pH 7.4). Ca\(^{2+}\)-free HBSS contained no Ca\(^{2+}\) plus 1 mM EGTA.

**Isolation of hepatocytes**

ICR neonatal mice, 1-day-old, were obtained from the Experiment Animal Center of Nanjing Medical University, Nanjing, China. The mouse pups were sacrificed to collect livers under sterile conditions. The livers were quickly removed into an ice-cold D-Hank’s solution. Thereafter, the freshly isolated cells, whose erythrocytes had been dissolved with 0.85 % amchlor solution, were hepatocytes which were diluted to 1×10\(^6\) cells/ml in HepatoZYME-SFM medium containing L-glutamine (2 mM) and 100 U/ml penicillin/streptomycin for experiment of exposure to CdCl\(_2\) after preculture for 24 h. 

**Assay for viability of administered CdCl\(_2\) in hepatocytes**

Hepatocyte suspensions were seeded at 0.1 ml/well in 96-well flat-bottomed plates and randomly divided into a normal control group and three groups treated with CdCl\(_2\) (5, 10, 25 μM, respectively) with six replicates of each group. Cells in the control group received D-Hank’s solution at the same dose. The cultures were maintained for 12 h at 37 °C with 5 % CO\(_2\)
humidified air. Then, 0.01 ml (5 mg/ml) of MTT reagent was added into each well and incubated for 4 h. After the incubation, the incubation precipitates were dissolved with 0.1 ml of SDS. The optical density (OD) values were measured by spectrophotometry at 570 nm using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc. Winooski, Vermont, USA).

**Biochemical analysis of hepatocytes and culture medium after exposure to CdCl\(_2\)**

Hepatocyte suspensions were seeded at 2 ml/well in 6-well flat-bottomed plates and randomly divided into a normal control group and three treated groups with CdCl\(_2\) as described above. The specimens from hepatocytes and culture medium of each group were collected at 12 h after exposure to CdCl\(_2\). MDA and protein contents in the cells were assayed, and LDH and aspartate aminotransferase (AST) activities as well as albumin and Ca\(^{2+}\) contents in the culture medium, respectively, were detected by spectrophotometry using commercial kits (Nanjing Jiancheng Biotechnology Institute, China). The procedures indicated by the kits were performed strictly according to the manufacturer’s protocol.

**Laser scanning confocal microscope analysis of [Ca\(^{2+}\)]\(_i\) in CdCl\(_2\)-exposed hepatocytes**

The effect of CdCl\(_2\) exposure on [Ca\(^{2+}\)]\(_i\) in hepatocytes were first evaluated. Fluo-3/AM was chosen to use as an intracellular free Ca\(^{2+}\) fluorescent probe for analysis of [Ca\(^{2+}\)]\(_i\) in CdCl\(_2\)-exposed living hepatocytes under LSCM. In short, 20-µl samples of hepatocyte suspension were collected from each group 12 h after the experiment and loaded with 15 µl of 40 µM Fluo-3/AM for 30 min at 37 °C, and then washed 3 times with D-Hank’s solution to remove the extracellular Fluo-3/AM as described by Chen et al. (2005). To determine the [Ca\(^{2+}\)]\(_i\) distribution in hepatocytes, LSCM analysis was performed with a Bio-Rad MRC 1024 laser scanning confocal imaging system (Bio-Rad, Cambridge, MA).

The parameters of LSCM were set up to a magnification 40x, the excited light to 488 nm, the emission light 522/35 nm, the pinhole 10-40 nm and the power at 30 %. Thereafter, scan-time series menu was used to scan the hepatocytes repeatedly for monitoring the dynamic changes in [Ca\(^{2+}\)]\(_i\) with green fluorescence. [Ca\(^{2+}\)]\(_i\) levels were represented with fluorescent intensity (FI). Time series of optical sections through a cell was obtained with a XY-step. Total images were scanned in each experiment and the data were stored on disks for analysis.

To understand the possible mechanism of [Ca\(^{2+}\)]\(_i\) alteration in CdCl\(_2\)-treated hepatocytes, hepatocyte suspensions precultured for 24 h were loaded with Fluo-3/AM, then washed and resuspended in Ca\(^{2+}\)-containing HBSS or in Ca\(^{2+}\)-free HBSS with/without 100 µM of 2-APB. This was followed by monitoring cellular [Ca\(^{2+}\)]\(_i\) under LSCM as described above. After attaining stable values of [Ca\(^{2+}\)]\(_i\) fluorescence, administration of CdCl\(_2\) (10 µM) was performed with continuous recording at 2-s intervals for 500 s.

**Statistical analysis**

Data were represented as mean values ± standard error. Statistical analysis was performed by Student’s t-test (STATISTICA, Statsoft Inc., Tulsa, USA) on a conventional personal computer.

**Results**

**Changes of AST and LDH activities as well as albumin and Ca\(^{2+}\) contents in culture medium of CdCl\(_2\)-exposed hepatocytes**

As shown in Figure 1a, AST activity increased in culture medium in hepatocytes administered CdCl\(_2\) and significantly higher activity (p<0.05) occurred in 25 µM CdCl\(_2\)-treated group compared to that in control group. LDH activity in culture medium was higher in CdCl\(_2\)-treated groups than in the control group and significantly higher values were found in 10 and 25 µM CdCl\(_2\)-treated groups (p<0.05 and p<0.01, respectively) (Fig. 1b). A greatly decreased albumin content was observed after CdCl\(_2\) treatment (Fig. 1c). As demonstrated in Figure 1d, the Ca\(^{2+}\) concentrations in culture medium of CdCl\(_2\)-exposed hepatocytes showed significant decreases compared to control cells (p<0.05 or p<0.01).

**Changes of viability and MDA content in CdCl\(_2\)-exposed hepatocytes**

Administration of CdCl\(_2\) resulted in a dose-dependent decrease of hepatocyte viability and a significantly lower value in 25 µM CdCl\(_2\)-treated hepatocytes (p<0.05) compared to that in control cells (Fig. 2a). The MDA content in CdCl\(_2\)-treated hepatocytes greatly increased compared to that in control cells (Fig. 2b).
Fig. 1. Changes of AST and LDH activities as well as albumin and Ca\(^{2+}\) contents in culture medium of CdCl\(_2\)-exposed murine hepatocytes. Primary murine hepatocytes were exposed to 5, 10, 25 µM CdCl\(_2\), respectively, and control cells received D-Hank’s solution at the same dose. Spectrophotometry was used for biochemical analysis in culture medium. (a) changes of AST activity. (b) changes of LDH activity. (c) changes of albumin content. (d) changes of Ca\(^{2+}\) concentration. Results are presented as mean ± S.E. (n=6). * p<0.05, ** p<0.01 (Student’s t-test). CdCl\(_2\) treatment groups vs. control group.

Fig. 2. Changes of viability and MDA content in CdCl\(_2\)-exposed murine hepatocytes. (a) hepatocyte viability was evaluated using an MTT assay. (b) changes of MDA formation in hepatocytes was detected by spectrophotometry. Results are presented as mean ± S.E. (n=6). * p<0.05 (Student’s t-test). CdCl\(_2\) treatment groups vs. control group.

**Manifestation and changes of hepatocyte [Ca\(^{2+}\)]\(_{i}\), after exposure to CdCl\(_2\)**

Treatment of hepatocytes with 5-25 µM CdCl\(_2\) altered hepatocyte [Ca\(^{2+}\)]\(_{i}\) handling, showing significantly stronger [Ca\(^{2+}\)]\(_{i}\), fluorescent intensities at 12 h (panels C, D, E vs. A, respectively) and 24 h (panels F, G, H vs. B, respectively) after the experiment under LSCM (Fig. 3a). Significantly higher [Ca\(^{2+}\)]\(_{i}\), in CdCl\(_2\)-treated hepatocytes
than in control cells at 12 h and 24 h (p<0.05 or p<0.01) and a dose-dependent increase at 12 h after the experiment are shown in Figure 3b.

As shown in Figure 4, administration of 10 μM CdCl₂ to freshly precultured hepatocytes loaded with Fluo-3/AM was performed in Ca²⁺-containing HBSS with/without 2-APB and in Ca²⁺-free HBSS with/without 2-APB to further understand the effects of CdCl₂ on Ca²⁺ signaling in hepatocytes. We observed that in Ca²⁺-containing HBSS without 2-APB, CdCl₂ elicited [Ca²⁺] increases which comprised an initially slow rise (0-100 s) and a strongly elevated phase (100-378 s), followed by a slow decay at a high level (Fig. 4a). However, in Ca²⁺-containing HBSS with 2-APB, a membrane-permeable inhibitor of inositol triphosphate receptor (IP₃R) (Braun et al. 2003), CdCl₂ caused a mild [Ca²⁺] elevation of the absence of a slow rise phase (Fig. 4b). Removal of extracellular Ca²⁺ reduced CdCl₂-induced [Ca²⁺], signals, showing a slow rise alone without a subsequent elevated phase (Fig. 4c). Furthermore, in Ca²⁺-free HBSS containing 2-APB, CdCl₂ failed to elicit [Ca²⁺] elevation, which was similar to the consequence of administered D-Hank’s solution instead of CdCl₂ in hepatocytes (data not shown).

**Discussion**

In this study, our group has shown that the administration of CdCl₂ resulted in a dose-dependent decrease of murine hepatocyte viability and an elevated AST activity in the culture medium at 5-25 μM (p<0.05 for 25 μM CdCl₂ vs. control). LDH activity in the culture medium for hepatocytes exposed to CdCl₂ was greatly enhanced and significantly higher values were observed in 10 and 25 μM CdCl₂-exposed groups (p<0.05 and p<0.01, respectively), which is consistent with previous findings showing that cadmium induces the increase in LDH leakage as a sensitive indicator of cytotoxicity (Martel et al. 1990, Sarkar et al. 1995). Furthermore, a greatly decreased albumin content also occurred after CdCl₂ treatment, indicating a decline of hepatic cell function (Doumas and Peters et al. 1997, Peters 1985). Reports have been accumulated that cadmium can destroy hepatic cell membranes and induce lipid peroxidation, thereby affecting the configuration and function of mitochondria and disturbing energy metabolism, impairing DNA synthesis, transformation and expression (Koizumi et al. 1996, Risso-de Faverney et al. 2004, Hsiao and Stapleton 2004). Previous studies of Stacey et al. (1980) have demonstrated that cadmium can lead to lipid peroxidation in isolated rat hepatocytes. However, Manzl et al. (2003) reported that cadmium does not cause acute short-term toxic effects in hepatocytes from the rainbow trout, Oncorhynchus mykiss. Our present study has shown greater MDA formation in CdCl₂-exposed murine hepatocytes than in control cells, suggesting that...
an enhanced lipid peroxidation reaction of hepatocytes exposed to cadmium does exist. Taken together, such findings reveal that acute administration of CdCl$_2$ to in vitro murine hepatocytes results in a strongly toxic effect by inducing cellular lipid peroxidation and injury, thereby eliciting its decreased viability and malfunction.

Earlier studies have indicated that changes of intracellular cation homeostasis are closely related to the mechanism of hepatic cell injury (Gasbarrini et al. 1992, Carini et al. 1995). Especially, the [Ca$^{2+}$]$_i$ elevation is associated with the development of cell damage (Orrenius et al. 1992, Ueda et al. 2000). Cadmium can interfere for uptake with essential metal ions, such as Ca$^{2+}$, Zn$^{2+}$ and Cu$^{2+}$, and especially affect Ca$^{2+}$ signaling in hepatocytes (Blazka and Shaikh 1992, Dundjerski et al. 2000, Baker et al. 2003). In this study, Fluo-3/AM was chosen to use as an intracellular free Ca$^{2+}$ fluorescent probe to evaluate the effect of cadmium on [Ca$^{2+}$]$_i$ in murine hepatocytes. Digital imaging of living mussel hemocytes loaded with Fura-2/AM or Fluo-3/AM demonstrated that Cd$^{2+}$ induced rise in probe fluorescence (Marchi et al. 2000). In particular, Cd$^{2+}$ produced the strongest probe signal rise in free solution, but the lowest fluorescence increase in cells, indicating that Fura-2/AM or Fluo-3/AM can be a suitable tool to record the effect of Cd$^{2+}$ on [Ca$^{2+}$]$_i$ in living cells. However, in the presence of cadmium Fura-2/AM it is impossible to calibrate the [Ca$^{2+}$]$_i$ measurements conducted with cell suspension (Manzl et al. 2003). Recently, further reports indicated that Fura-2/AM can be used in determination of intracellular Cd$^{2+}$ level due to its very high affinity for Cd$^{2+}$ (Le et al. 2005). Thus Fluo-3/AM, but not Fura-2/AM, was chosen for LSCM analysis of [Ca$^{2+}$]$_i$ in Cd-exposed hepatocytes. We observed that treatment of hepatocytes with 5-25 μM CdCl$_2$ resulted in abnormal hepatocyte [Ca$^{2+}$]$_i$ handling, showing that significantly higher [Ca$^{2+}$]$_i$ was present in CdCl$_2$-treated hepatocytes than in control cells at 12 h and 24 h after the experiment (p<0.05 or p<0.01). This fact suggests that abnormal Ca$^{2+}$ homeostasis due to cadmium may be an important mechanism of the development of hepatocellular damage.

Martinez-Sánchez et al. (2004) have reported that cells regulate their Ca$^{2+}$ homeostasis by a complex interplay between three cellular mechanisms: Ca$^{2+}$ influx and efflux through the plasma membrane, Ca$^{2+}$ uptake and release from intracellular stores and intracellular Ca$^{2+}$ buffering. [Ca$^{2+}$]$_i$ increase is usually caused by Ca$^{2+}$ mobilization from intracellular stores and/or Ca$^{2+}$ entry from the extracellular space (Jan et al. 2001). Our current study revealed that the Ca$^{2+}$ concentrations in the culture medium of CdCl$_2$-exposed hepatocytes were significantly lower, whereas there appeared significant [Ca$^{2+}$]$_i$ elevations in CdCl$_2$-exposed hepatocytes compared to those in control cells (p<0.05 or p<0.01). This suggests that a part of [Ca$^{2+}$]$_i$ elevation may be closely related to extracellular Ca$^{2+}$ entry after CdCl$_2$ treatment.

![Dynamic [Ca$^{2+}$]$_i$ changes of acutely administered CdCl$_2$ in in vitro murine hepatocytes.](image-url)
To better understand the effects of cadmium on \([\text{Ca}^{2+}]_i\) signaling in isolated living murine hepatocytes and to provide diverse information for clarifying the mechanism of Cd-induced hepatotoxicity, the present study further investigated the dynamic \([\text{Ca}^{2+}]_i\) changes after in vitro administration of CdCl\(_2\) to murine hepatocytes which occurred in Ca\(^{2+}\)-containing HBSS or in Ca\(^{2+}\)-free HBSS with/without 100 \(\mu\text{M}\) of 2-APB. We found that in Ca\(^{2+}\)-containing HBSS only, administration of CdCl\(_2\) elicited \([\text{Ca}^{2+}]_i\) increases which comprised an initially slow ascent and a subsequent strongly elevated phase, which were followed by a slow decay at a high level. However, in Ca\(^{2+}\)-containing HBSS with the addition of 2-APB, CdCl\(_2\) caused a mild \([\text{Ca}^{2+}]_i\) elevation without an initial \([\text{Ca}^{2+}]_i\) rise, suggesting that the initial rise may be due to the release of Ca\(^{2+}\) from intracellular stores. It is evident that 2-APB, a membrane-permeable inhibitor of IP\(_3\)R which plays a key role in Ca\(^{2+}\) release from endoplasmic reticulum (ER) (Hajnóczky and Thomas 1997), has extensively been used to investigate the effects of IP\(_3\)-induced Ca\(^{2+}\) release and Ca\(^{2+}\) influx in a number of cell systems (Bilmen et al. 2002, Braun et al. 2003). and also appears to be an inhibitor of capacitative calcium entry channels (Ma et al. 2000, Broad et al. 2001, Gregory et al. 2001). Thus blocking the IP\(_3\)R by 2-APB may abolish the initial Ca\(^{2+}\) release from ER and affect the following Ca\(^{2+}\) influx from extracellular space, leading thus to a mild \([\text{Ca}^{2+}]_i\) elevation. The latter effect may also be partly due to the inhibition of corresponding Ca\(^{2+}\)-influx channels by 2-APB (Ma et al. 2000).

Our further observations showed that the removal of extracellular Ca\(^{2+}\) reduced CdCl\(_2\)-induced \([\text{Ca}^{2+}]_i\) signals, showing an initially slow \([\text{Ca}^{2+}]_i\) rise alone without a subsequent strongly elevated phase. On the other hand, in Ca\(^{2+}\)-free HBSS with addition of 2-APB, CdCl\(_2\) failed to elicit any \([\text{Ca}^{2+}]_i\) elevation (including an initially slow rise) during the entire experiment period, which was similar to a consequence of administered D-Hank’s instead of CdCl\(_2\) in hepatocytes (data not displayed). These findings strongly support our assumption that a major part of \([\text{Ca}^{2+}]_i\), elevation in CdCl\(_2\)-exposed hepatocytes deals with the extracellular Ca\(^{2+}\) entry, whereas the initial segment of \([\text{Ca}^{2+}]_i\), elevation induced by CdCl\(_2\) is associated with an excessive release of Ca\(^{2+}\) from intracellular stores.

In conclusion, the main finding of this study is that abnormal Ca\(^{2+}\) homeostasis due to cadmium may be an important mechanism in the development of toxic effect in murine hepatocytes. \([\text{Ca}^{2+}]_i\), elevation in acutely cadmium-exposed hepatocytes is closely related to the extracellular Ca\(^{2+}\) entry and the excessive release of Ca\(^{2+}\) from intracellular stores.

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