Cadmium Toxicity toward Caspase-Independent Apoptosis through the Mitochondria–Calcium Pathway in mtDNA-Depleted Cells

YUNG-LUEN SHIH,[a,e] CHIEN-JU LIN,[b,c,e] SHENG-WEI HSU,[b,c] SHENG-HAO WANG,[b,c] WEI-LI CHEN,[b,c] MEI-TSU LEE,[b] YAU-HUEI WEI,[d] AND CHWEN-MING SHIH[b]

[a]*Department of Pathology and Laboratory Medicine, Shin Kong Wu Ho-Su Memorial Hospital, Taipei 111, Taiwan, ROC*

[b]*Department of Biochemistry, Taipei Medical University, Taipei 110, Taiwan, ROC*

[c]*Graduate Institute of Medical Science, Taipei Medical University, Taipei 110, Taiwan, ROC*

[d]*Department of Biochemistry and Center for Cellular and Molecular Biology, National Yang-Ming University, Taipei 112, Taiwan, ROC*

ABSTRACT: Mitochondria are believed to be integrators and coordinators of programmed cell death in addition to their respiratory function. Using mitochondrial DNA (mtDNA)-depleted osteosarcoma cells (ρ^0 cells) as a cell model, we investigated the apoptogenic signaling pathway of cadmium (Cd) under a condition of mitochondrial dysfunction. The apoptotic percentage was determined to be around 58.0% after a 24-h exposure to 25 μM Cd using flow cytometry staining with propidium iodine (PI). Pretreatment with Z-VAD-fmk, a broad-spectrum caspase inhibitor, failed to prevent apoptosis following Cd exposure. Moreover, Cd was unable to activate caspase 3 using DEVD-AFC as a substrate, indicating that Cd induced a caspase-independent apoptotic pathway in ρ^0 cells. JC-1 staining demonstrated that mitochondrial membrane depolarization was a prelude to apoptosis. On the other hand, the intracellular calcium concentration increased 12.5-fold after a 2-h exposure to Cd. More importantly, the apoptogenic activity of Cd was almost abolished by ruthenium red, a mitochondrial calcium uniporter blocker. This led us to conclude that mtDNA-depleted cells provide an alternative pathway for Cd to conduct caspase-independent apoptosis through a mitochondria-calcium mechanism.

KEYWORDS: cadmium; caspase; apoptosis; miotochondria

[e]Y-L.S. and C-J.L. contributed equally to this work.

Address for correspondence: Dr. Chwen-Ming Shih, Department of Biochemistry, School of Medicine, Taipei Medical University, 250 Wu-Hsing Street, Taipei, Taiwan 110, ROC. Voice: +886-2-27361661 ext. 3151; fax: +886-2-86421158.
cmshih@tmu.edu.tw

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INTRODUCTION

Apoptosis, so-called programmed cell death, is important in development and tissue homeostasis of multicellular organisms. Apoptosis is associated with cell shrinkage, plasma membrane blebbing, chromatin condensation, DNA fragmentation, and formation of apoptotic bodies that can be taken up and degraded by neighboring cells without producing an inflammatory response.[1] Furthermore, the strong relationship between a dysfunction in apoptosis and diseases such as AIDS, neurodegenerative diseases, and oncogenesis is well known. In the apoptotic signaling pathway, mitochondria, in addition to their respiratory function, play a crucial role. It has been demonstrated that apoptotic proteins (cytochrome *c*, AIF, endonuclease G, and Smac/DIABLO) are released from the mitochondrial intermembrane space to their new destination to complete the apoptotic process.[2]

Cadmium (Cd) is a toxic metal with an extremely long biological half-life of 15~30 years in humans.[3] It has been known for decades that Cd exposure can cause a variety of adverse health effects, among which kidney dysfunction, lung diseases, disturbed calcium metabolism, and bone effects are most prominent.[4] Exposure to Cd causes loss of bone mass and increased incidence of bone fractures, leading to osteoporosis and osteomalacia as observed in itai-itai patients and laboratory animals.[5,6] The mechanisms of Cd-induced damage include the production of free radicals that alter mitochondrial activity and trigger apoptosis.[7–9] Therefore, the toxicity of cadmium is thought to occur through the induction of apoptosis. However, the apoptotic signaling induced by this toxicity is still unclear. In this report, using mitochondrial DNA (mtDNA)-depleted cells (rho zero cells, ρ^0 cells)[10–11] as a cell model, we suggest that Cd induces a caspase-independent apoptosis through the mitochondria–calcium pathway. It was noted, however, that no ROS were produced and no pro-apoptotic factors were released from mitochondria, such as cytochrome *c,* apoptosis-inducing factor (AIF), endonuclease G (Endo G).

MATERIALS AND METHODS

Cell Culture

The ρ^0 cells derived from a human osteosarcoma cell line, 143BTK (ATCC CRL 8303), were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 50 μg/mL uridine, 100 μg/mL pyruvate, 50 ng/mL ethidium bromide, 100 units/mL penicillin, and 100 units/mL streptomycin in 5% CO_2, and 95% air at 37°C in an incubator with a humidified atmosphere.[12] Serum starvation was achieved by incubation in DMEM containing 1% FBS for at least 16 h. Following this, unless otherwise stated, ρ^0 cells were treated with 25 μM Cd for the indicated time periods.

Assessment of Cell Death

As described previously, cell death was determined using a Becton Dickinson (San Jose, CA) FACSCalibur flow cytometer using propidium iodine (PI) single staining and Annexin V/PI double staining for assessment of hypodiploid DNA content and phosphatidylserine (PS) externalization, respectively.[13]

Analysis of Caspase 3 Activity

Caspase 3 activity was measured using a Caspase-3/CPP32 Fluorometric Assay Kit according to the manufacturer's instructions (BioVision, Mountain View, CA). In brief, 5×10^6 cells were incubated with 50 μL cell lysis buffer for 10 min on ice, harvested, and centrifuged at $16{,}000 \times g$ for 1 min at 4°C. The supernatant (200 μg proteins) was incubated with 50 μL of 2X reaction buffer and 5 μL of the 1 mM DEVD-AFC (benzyloxy-carbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl-coumarin) substrate for 1 h at 37°C in a 96-well plate. The fluorescence of the cleaved product, AFC, was measured at 405-nm excitation and 535-nm emission wavelengths on a fluorescence reader, Fluoroskan Ascent (Thermoelectron, Waltham, MA). Cleaved AFC was quantified by a calibration curve using known AFC concentrations.

Detection of the Mitochondrial Membrane Potential ($\Delta\Psi_m$)

The dual emission dye, JC-1, was used as a measure of $\Delta\Psi_m$ according to the methods described previously.[14] In brief, cells were incubated with 2.5 mg/mL JC-1 (dissolved in DMSO) for 15 min at room temperature in darkness. After centrifugation for 5 min at $200 \times g$, cells were washed twice with PBS at 4°C, resuspended in 0.5 mL PBS, and analyzed on a FACSCalibur flow cytometer. JC-1 is a lipophilic cationic fluorescence dye and is capable of selectively entering mitochondria, which will change color from red (FL-2) to greenish (FL-1) once the $\Delta\Psi_m$ declines.

Measurement of Intracellular Calcium

Cells were treated with or without Cd and harvested at indicated time periods. Before data acquisition, cells were incubated with 3 μM Fluo-3 AM dye for a total of

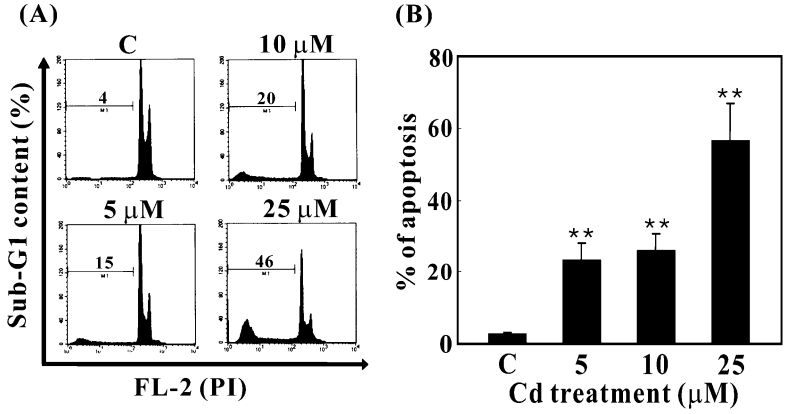

FIGURE 1. Dose-response and time course of cell death induced by Cd in ρ^0 cells. (**A**) Cells were treated with 5–25 μM $CdCl_2$ for 24 h and then analyzed by PI staining using flow cytometry to determine their hypodiploid DNA (sub-G1) proportion. The percentage of M1 indicates the cell proportion of the sub-G1 peak. Data presented in **A** are representative of three independent experiments, and their statistical results for the apoptosis are presented in **B**. **, $P < 0.01$.

30 min at 37°C and then immediately analyzed on a flow cytometer using FL1 as a detector.[15] For relative intracellular calcium concentrations, the geographic mean values of the FL-1 peak generated from Cd-treated cells over each one's own negative control group (without Cd treatment) were calculated.

Statistics

Data are expressed as the mean ± standard deviation (SD) from a minimum of three independent experiments, unless otherwise indicated. Statistical analysis was performed using Student's *t*-test, with $P < 0.01$ as a criterion of significance.

RESULTS AND DISCUSSION

Cd Induced Caspase-Independent Apoptosis in ρ^0 Cells

As shown in FIGURE 1, Cd induced apoptosis in a dose-responsive manner and did not seem to alter the cell cycle based on the consistent ratio of G1 (FL-1 = 200) versus G2 (FL1 = 400) using PI staining for assessing hypodiploid DNA content with a flow cytometer. To reveal the involvement of caspase, Z-VAD-fmk [Z-Val-Ala-DL-Asp(OMe)-fluoro-methylketone], a broader spectrum of caspase inhibitor, was employed to examine its ability to prevent apoptosis by Cd toxicity (FIG. 2). In the left panel of FIGURE 2A, we assumed that the Z-VAD-fmk was able to prevent HL-60 apoptosis suffered from H_2O_2 treatment, which has been demonstrated to be a caspase-dependent apoptotic system.[18] In ρ^0 cells, pretreatment of Z-VAD-fmk failed to reduce the apoptotic percentage after exposure to different concentrations of Cd, suggesting that Cd might trigger ρ^0 cells to undergo caspase-independent apoptosis (see right panel of FIG. 2A and B). Furthermore, in contrast to H_2O_2-treated HL-60 cells, Cd treatment did not activate caspase 3 activity in ρ^0 cells (TABLE 1), which obviously demonstrated that the apoptogenic activity of Cd is caspase-independent in mtDNA-depleted cells

The apoptotic pathway of ρ^0 cells is still being debated. Most of them are caspase dependent, such as anoxia- and TNFα-treated ρ^0 cells derived from human A549 lung epithelial cells,[19] saturosporine-treated ρ^0 cells derived from human D238 medulloblastoma cells,[20] saturosporine-treated ρ^0 cells derived from human WAL-3A lymphocytes,[21] and saturosporine-treated ρ^0 cells derived from human 143BTK osteosarcoma cells.[22] However, consistent with our observations, C2- and C8-ceramide–induced human D238 medulloblastoma-derived ρ^0 cells underwent caspase-independent apoptosis.[23] Therefore, further investigation is warranted to reveal the apoptotic mechanism of ρ^0 cells.

Mitochondrial Membrane Depolarization Is a Prelude to Apoptosis

Emerging evidence has suggested that Cd might exert its cell toxicity through induction of an ROS burst that accompanies collapse of the mitochondria.[7–9] Using JC-1 as an indicator of $\Delta\Psi_m$, we demonstrated that after 8 h of Cd exposure, cells with normal $\Delta\Psi_m$ dropped from 97% to 76% to 57% after 16 h of Cd treatment (see upper-left quadrant in FIG. 3A and the statistical results in FIG. 3B). Nevertheless, we failed to detect any ROS burst after Cd treatment, including hydrogen peroxide,

TABLE 1. Caspase 3 activity of Cd-treated cells[a]

Cell line	Cd treatment	Caspase 3 activity (pmole/mg/min)
HL-60	Control	1.00 ± 0.49
	100 μM, 12 h	63.66 ± 11.51
ρ^0	Control	1.00 ± 0.13
	25 μM, 8 h	0.46 ± 0.19
	25 μM, 16 h	0.99 ± 0.20
	25 μM, 24 h	0.52 ± 0.17

[a]HL-60 and ρ^0 zero cells were treated with 100 μM and 25 μM Cd, respectively, for the indicated time periods and then collected for the capase 3 activity assay as described in Materials and Methods. Data were calculated from three independent experimants and represented as mean ± SD.

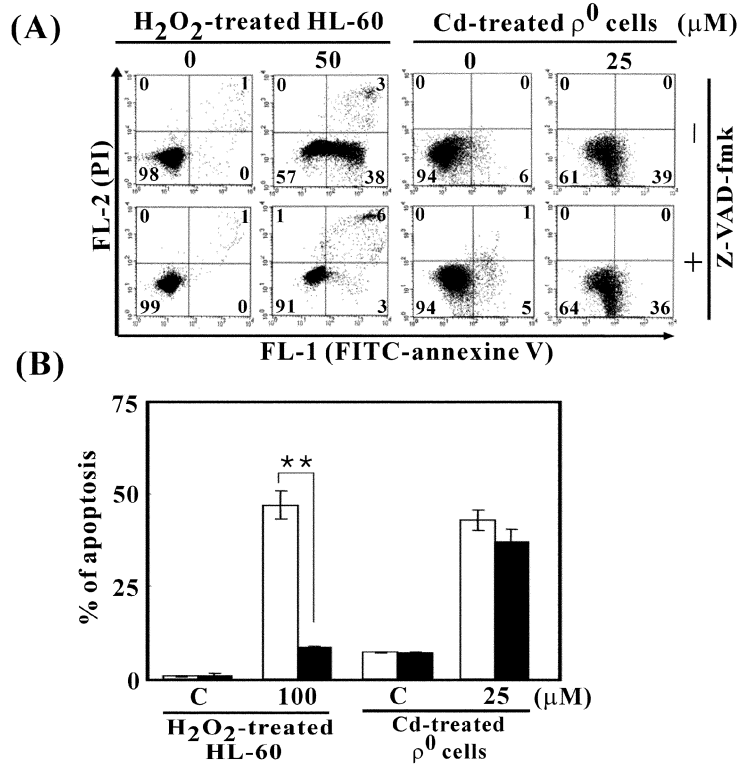

FIGURE 2. Inability of the broad-spectrum caspase inhibitor, Z-VAD-fmk, to prevent apoptosis. (**A**) Pretreatment with 40 M Z-VAD-fmk could rescue HL-60 cells from the effects of 50 μM H_2O_2 treatment. However, Z-VAD-fmk was unable to protect ρ^0 cells from Cd toxicity. Three independent experiments were performed, and their statistical results are presented in **B**. **, $P < 0.01$.

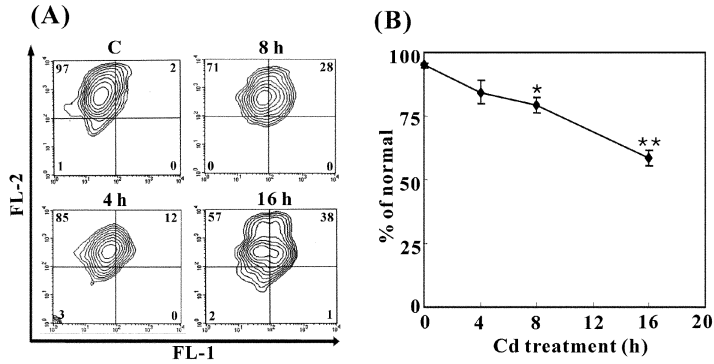

FIGURE 3. Mitochondrial membrane potential declined after Cd treatment. Cells were incubated with 2.5 μg/mL JC-1 dye for 15 min before the end of Cd treatment and were subsequently analyzed using flow cytometry. Red fluorescence (FL-2 channel) emitted from the J-aggregate form of JC-1 and green fluorescence (FL-1 channel) emitted from its monomer form increased when $\Delta\Psi_m$ was normal and depolarized, respectively. Percentages in the-upper-left quadrant and right two quadrants indicate proportions of cells with normal and depolarized mitochondria, respectively. Data presented in **A** are representative of three independent experiments, and their statistical results are presented in **B**. Asterisks (*, **) indicate a significant difference from control at $P < 0.05$ and < 0.01, respectively.

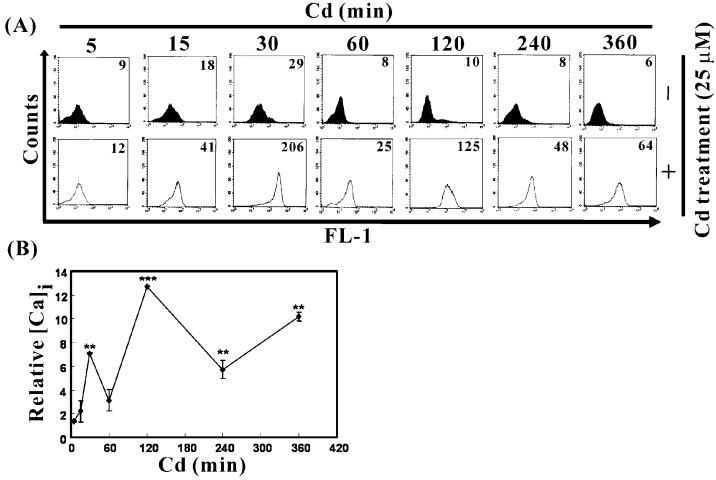

FIGURE 4. Cd induced intracellular calcium oscillation in ρ^0 cells. Cells were treated with Cd for the indicated time periods and incubated with 0.4 μM Fluo-3 AM dye for a total of 30 min before analysis by flow cytometry. The fluorescence of Fluo-3 AM (FL-1 channel) increased as the intracellular calcium increased. Data presented in **A** are representative of three separate experiments. The Arabic numeral in the upper-right conner of each cytogram indicates the geographic (GEO) mean of each peak. Panel **B** was generated from the GEO mean of the Cd-treated cytogram over its respective control. Asterisks (**, ***) indicate a significant difference from control at $P < 0.01$ and < 0.001, respectively.

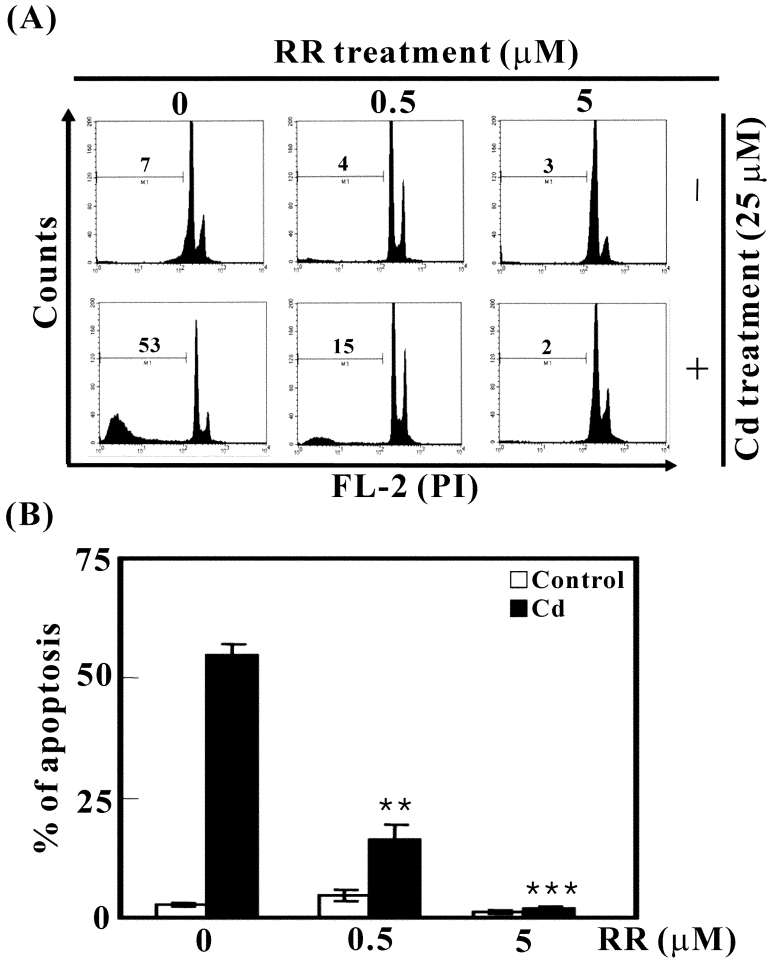

FIGURE 5. The apoptogenic activity of cadmium was suppressed by an inhibitor of the mitochondrial calcium uniporter. Cells were pretreated with various concentrations of RR, an inhibitor of the mitochondrial calcium uniporter, for 1 h, followed by treatment with Cd for another 24 h, and then were analyzed by PI staining to examine their hypodiploid DNA (sub-G1) proportion. M1 denotes the percentage of cells at the sub-G1 proportion. Three independent experiments were performed, and their statistical results are presented in **B**. Asterisks (**, ***) indicate a significant difference from control at $P < 0.01$ and < 0.001, respectively.

superoxide anions, and hydroxyl radicals (data not shown). Also, using confocal microcopy, we were unable to detect the translocation of AIF, Endo G, and cytochrome *c* from mitochondria to their destination (data not shown). This result suggested that the Cd-induced decline in $\Delta\Psi_m$ was irrelevant to the oxidative stress and the release of pro-apoptotic factors from mitochondria.

Elevation of Intracellular Calcium Is Involved in Apoptosis

Calcium signals have been identified as one of the major signals that converge on mitochondria to trigger mitochondria-dependent apoptosis.[24] In this report, using Fluo-3-AM as an indicator of intracellular calcium ($[Ca^{2+}]i$), Cd induced a $[Ca^{2+}]i$ oscillation which made it apparent that calcium signaling was a crucial mediator of Cd-triggered caspase-independent apoptosis in ρ^0 cells (FIG. 4). Calpains, Ca^{2+}-dependent cysteine proteases, are associated with both caspase-dependent and -independent apoptosis and are located downstream of calcium.[25] We are currently investigating the role of calpain in this system.

Ruthenium red (RR) is one of the most potent inhibitors of the mitochondrial calcium uniporter. As shown in FIGURE 5, RR can totally abolish Cd-induced apoptosis. The most likely hypothesis assumes that uptake of calcium by mitochondria might affect the mitochondrial permeability transition pore (MPTP), leading to a decline in mitochondrial $\Delta\Psi_m$, and then apoptosis. Alternatively, Cd might directly damage mitochondria through the calcium uniporter. Further investigation is required to examine mitochondrial Cd concentrations after Cd treatment.

CONCLUSIONS

In this report, our results support the notion that Cd induces caspase-independent apoptosis through induction of $[Ca^{2+}]i$ oscillation and disruption of mitochondrial ΔY_m. In this process, there is no ROS burst or release of proapoptotic factors from the mitochondria.

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