Toxicology and Applied Pharmacology 243 (2010) 323–331

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Inorganic mercury causes pancreatic β-cell death via the oxidative stress-induced apoptotic and necrotic pathways

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ARTICLE INFO

Article history:
Received 4 September 2009
Revised 18 November 2009
Accepted 26 November 2009
Available online 16 December 2009

Keywords:
$HgCl_2$
Pancreatic β-cell
ROS
Apoptosis
Necrosis

ABSTRACT

Mercury is a well-known highly toxic metal. In this study, we characterize and investigate the cytotoxicity and its possible mechanisms of inorganic mercury in pancreatic β-cells. Mercury chloride ($HgCl_2$) dose-dependently decreased the function of insulin secretion and cell viability in pancreatic β-cell-derived HIT-T15 cells and isolated mouse pancreatic islets. $HgCl_2$ significantly increased ROS formation in HIT-T15 cells. Antioxidant *N*-acetylcysteine effectively reversed $HgCl_2$-induced insulin secretion dysfunction in HIT-T15 cells and isolated mouse pancreatic islets. Moreover, $HgCl_2$ increased sub-G1 hypodiploids and annexin-V binding in HIT-T15 cells, indicating that $HgCl_2$ possessed ability in apoptosis induction. $HgCl_2$ also displayed several features of mitochondria-dependent apoptotic signals including disruption of the mitochondrial membrane potential, increase of mitochondrial cytochrome *c* release and activations of poly (ADP-ribose) polymerase (PARP) and caspase 3. Exposure of HIT-T15 cells to $HgCl_2$ could significantly increase both apoptotic and necrotic cell populations by acridine orange/ethidium bromide dual staining. Meanwhile, $HgCl_2$ could also trigger the depletion of intracellular ATP levels and increase the LDH release from HIT-T15 cells. These $HgCl_2$-induced cell death-related signals could be significantly reversed by *N*-acetylcysteine. The intracellular mercury levels were markedly elevated in $HgCl_2$-treated HIT-T15 cells. Taken together, these results suggest that $HgCl_2$-induced oxidative stress causes pancreatic β-cell dysfunction and cytotoxicity involved the co-existence of apoptotic and necrotic cell death.

Introduction

Uncontrolled industrialization has resulted in a very wide segment of the human population being exposed to agents that have the potential to cause or exacerbate disease. Mercury (Hg) is widespread and persistent in the environment. Mercury has become an important public health concern of our day because of growing evidence of its presence in some components of the human food chain. There are numerous sources of mercury for human exposure including seafood, seeds, foodstuffs, disinfectant, disk batteries and dental amalgam (Aschner and Walker, 2002; Clarkson et al., 2003). Mercury is a notorious heavy metal and has serious toxicity in various mammalian cells and organ systems (Guo et al., 1998; Silbergeld et al., 2000). Shaffi (1981) has reported that mercuric compounds, about 200 tons, were introduced into the environment annually as effluents from industries. A cross-sectional analysis has also shown that people in the highest quartile of fish consumption had median mercury levels 1.82 times above the levels in the lowest quartile (Latshaw et al., 2006). In past experiments using mice and fish, it has found that $HgCl_2$ altered intracellular Ca^{2+} homeostasis and decreased insulin secretion in pancreatic β-cells or islets (Bloom et al., 1972; Liu and Lin-Shiau, 2002). However, the cytotoxic mechanism of $HgCl_2$ on the pancreatic β-cells still remain unclear.

Reactive oxygen species (ROS) has been implicated in a wide variety of undesirable biological reaction and functional cell damage, including the induction of pancreatic β-cell dysfunction or death by autoimmune attack in type 1 diabetes (Finkel and Holbrook, 2000; Hotta et al., 2000). Mercury has been shown to induce toxic effects by oxidative stress induction that caused the alteration of cellular function and eventually resulted in cell death and pathological injury,

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doi:10.1016/j.taap.2009.11.024

which were accompanied with the damage of antioxidant enzymes (Sarafian, 1999; Valko et al., 2005). It has been shown that methyl mercury triggers the early acute necrosis and delayed apoptosis in murine peritoneal neutrophils (Kuo and Lin-Shiau, 2004). An apoptotic death of pancreatic β-cells has also been found to be induced by methyl mercury (Chen et al., 2006a). A recent *in vivo* study has also shown that inorganic mercury could induce the apoptosis and proliferative reactions in renal cells (Fouda et al., 2008). However, the effect of inorganic mercury on pancreatic β-cell survival still remains unclear. Taken together, in the current study, we try to explore the cytotoxic effect and mechanism of inorganic mercury ($HgCl_2$) on pancreatic β-cells. To this aim, we attempt to investigate the *in vitro* effects of $HgCl_2$ on ROS generation, insulin secretion and apoptotic and necrotic cell death in pancreatic β-cell-derived HIT-T15 cells and isolated mouse islets. Moreover, the potential protective effects of antioxidant *N*-acetylcysteine on the pancreatic β-cell dysfunction and death *in vitro* and *in vivo* were also investigated.

Materials and methods

Pancreatic β-cell-derived HIT-T15 cell culture. HIT-T15 is a hamster pancreatic β-cell line, showing an increase in insulin secretion as a function of stimulation (glucose-stimulated insulin secretion). Cells were cultured in a humidified chamber with a 5% CO_2–95% air mixture at 37 °C and maintained in RPMI 1640 medium (Gibco BRL, Life Technologies) supplemented with 10% fetal bovine serum (FBS) and containing 11.1 mM D-glucose as previously described (Chen et al., 2006a).

Mouse pancreatic islet isolation. Islets of Langerhans were isolated by collagenase digestion of the mouse pancreas as previously described (Chen et al., 2006a). We purchased 18–25 g male ICR mice from the Animal Center of the College of Medicine, National Taiwan University (Taipei, Taiwan). The Animal Research Committee of College of Medicine, National Taiwan University, conducted the study in accordance with the guidelines for the care and use of laboratory animals. Mice were housed in a room at a constant temperature of 22 ± 2 °C with 12:12-h light and dark cycles. In each experiment, the pancreases from three mice were used. After collagenase digestion and separation on a Ficoll gradient, the islets were further purified by hand picking to eliminate any remaining exocrine tissue. Whole islets were maintained in culture medium consisting of RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin/amphotericin B at 37 °C in an atmosphere of 95% air and 5% CO_2 before experimentation.

Cell viability. Cells were washed with fresh media and cultured in 96-well plates (2×10^4/well) and then stimulated with $HgCl_2$ (2–20 μM) for 24 h. After incubation, the medium was aspirated and fresh medium containing 30 μL of 2 mg/mL 3-(4, 5-dimethyl thiazol-2-yl-)-2,5-diphenyl tetrazolium bromide (MTT) was added. After 4 h, the medium was removed and replaced with blue formazan crystal dissolved in dimethyl sulfoxide (100 μL; Sigma, St. Louis, MO). Absorbance at 570 nm was measured using an enzyme linked immunosorbent assay microplate reader (Bio-Rad, model 550, Hercules, CA).

ROS production. Intracellular ROS generation was monitored by flow cytometry using the peroxide-sensitive fluorescent probe [2′,7′-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes, Inc)], as described by Chen et al. (2006b). In brief, HIT-T15 cells were coincubated with 20 μM DCFH-DA for 15 min at 37 °C. DCFH-DA was converted by intracellular esterases to 2′,7′-dichlorofluorescin (DCFH). In the presence of a proper oxidant, DCFH was oxidized into the highly fluorescent 2′,7′-dichlorofluorescein (DCF). After incubation with the dye, cells and islets were resuspended in ice-cold phosphate-buffered saline (PBS) and placed on ice in a dark environment for flow cytometer (FACScalibur, Becton Dickinson, Sunnyvale, CA). Each group was acquired more than 10,000 individual cells.

Insulin secretion. To measure the amount of insulin secretion in HIT-T15 cells and primary mouse islets after exposure to $HgCl_2$, HIT-T15 cells and mouse islets were performed in Krebs Ringer buffer (KRB), as previously described (Chen et al., 2006a). Aliquots of samples were collected from the experimental media at indicated time points and subjected to insulin antiserum immunoassay according to the manufacturer's instructions (Mercodia AB, Sweden).

Determination of mitochondrial membrane potential. The mitochondrial membrane potential was analyzed using the fluorescent probe [3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$, Molecular Probes, Inc)]. HIT-T15 cells, treated with $HgCl_2$ (5 and 20 μM) or vehicle for 8 h, were harvested and loaded with 40 nM $DiOC_6$ for 30 min and analyzed in a FACScan flow cytometer (Becton Dickinson).

Western blot analysis. Western blotting was performed using standard protocols, as previously described (Chen et al., 2006a). In brief, equal amounts of proteins (50 μg per lane) were subjected to 10% (w/v) SDS–polyacrylamide gels and transferred to nitrocellulose membrane. The membrane was blocked for 1 h in PBST (PBS, 0.05% Tween-20) containing 5% nonfat dry milk and incubated with antibodies for cytochrome *c*, pro-caspase 3, α-tubulin (Santa Cruz Biochemicals) and poly (ADP-ribose) polymerase (PARP) (Oncogene). After they were washed in 0.1% PBST, the respective secondary antibodies conjugated to horseradish peroxidase were applied for 1 h. The antibody-reactive bands were revealed by the enhanced chemiluminescence reagent kit (Perkin-Elmer™, Life Sciences) and were used to expose them to Kodak radiographic film.

Flow cytometric analysis of apoptotic and necrotic cells. (1) *Measurement of sub-G1 DNA content*. HIT-T15 cells were detached and washed with PBS, then resuspended in 1 mL of cold 70% (v/v) ethanol and stored at 4 °C for 24 h. After they were washed with PBS, the cells were stained with propidium iodide (PI; Sigma-Aldrich) [50 μg/mL PI and 10 μg/mL ribonuclease (Rnase) in PBS] at 4 °C for 30 min in dark conditions. The cells were washed and subjected to flow cytometry analysis of DNA content (FACScalibur, Becton Dickinson). Nuclei displaying hypodiploid, sub-G1 DNA contents were identified as apoptotic. The sample of each group was collected more than 10,000 individual cells. (2) *Determination of phosphatidyl serine externalization: annexin-V fluorescein isothiocyanate (FITC) and PI binding assay*. The externalization of phosphatidyl serine is an early event in apoptosis (Anderson et al., 2002). Flow cytometry analysis was performed to determine this event using the annexin-V-FITC and PI binding assay kit (BioVision). HIT-T15 cells were treated with or without $HgCl_2$ (5 and 20 μM) for 24 h and then washed twice with PBS and stained with annexin-V-FITC and PI for 20 min at room temperature. The cells were washed twice PBS, and the apoptosis level was determined by measuring the fluorescence of the cells by flow cytometry analysis. More than 10,000 individual cells were collected for each group. (3) *Determination of apoptotic and necrotic cells by acridine orange/ethidium bromide dual staining.* The dual staining of acridine orange and ethidium bromide was ability to differentiate live cells from apoptotic and necrotic cells (Johnson et al., 2005). Briefly, HIT-T15 cells were treated with or without $HgCl_2$ (5 and 20 μM) for 24 h, and the floating and adherent cells were collected and combined. Cells were centrifuged at 200×*g* for 10 min at 4 °C, washed twice PBS and resuspended in 1 mL PBS, and 2 μl of acridine orange and 2 μl of ethidium bromide (final concentration 200 ng/mL for each) were added to each tube, and the cells were stained for 5 min at a dark environment. Two-parameter fluorescence was acquired

from more than 10,000 individual cells per sample using a flow cytometer (FACScalibur, Becton Dickinson). Green emissions from acridine orange and red emissions from ethidium bromide were captured at 525 and 620 nm, respectively. Live, apoptotic and necrotic cell populations were analyzed using CellQuest software.

Analysis of cellular ATP levels. The intracellular ATP content in the HIT-T15 cells was determined by adenosine 5′-triphosphate bioluminescent assay kit (FL-AA, Sigma-Aldrich, USA), as previously described (Yen et al., 2007). Bioluminescent luciferase-luciferin reactions provide the basis of simple, rapid and highly sensitive assay for ATP. The 2×10^5 cells/mL were grown in 24-well culture plates; after exposure to $HgCl_2$, cells were washed twice with PBS and then lysed by RIPA buffer. Cell lysate were harvested and centrifuged at 10,000×*g* for 10 min at 4 °C, and then 50 μl supernatant and ATP assay buffer FL-AAB (pH 7.8) were mixed and immediately measured the amount of light with a luminometer (Berthold Detection Systems, BET001).

Lactate dehydrogenase (LDH) release assay. The amount of LDH leaked from the cytosol of damaged cells into the medium after exposure of β-cells to the extracts for 24 h was detected. The amount of LDH released from cells was quantified using the LDH cytotoxicity kit II (Biovision) according to the manufacturer's instructions. The absorbance was measured with a plate reader equipped with 450 nm filter.

Detection of mercury content in β-cells. The HIT-T15 cells were treated with $HgCl_2$ (5 and 20 μM) for 24 h. Cells were harvested and washed with PBS three times followed by addition of 0.15% nitric acid; the mixture was vortexed and frozen at −20 °C for 2 h or overnight.

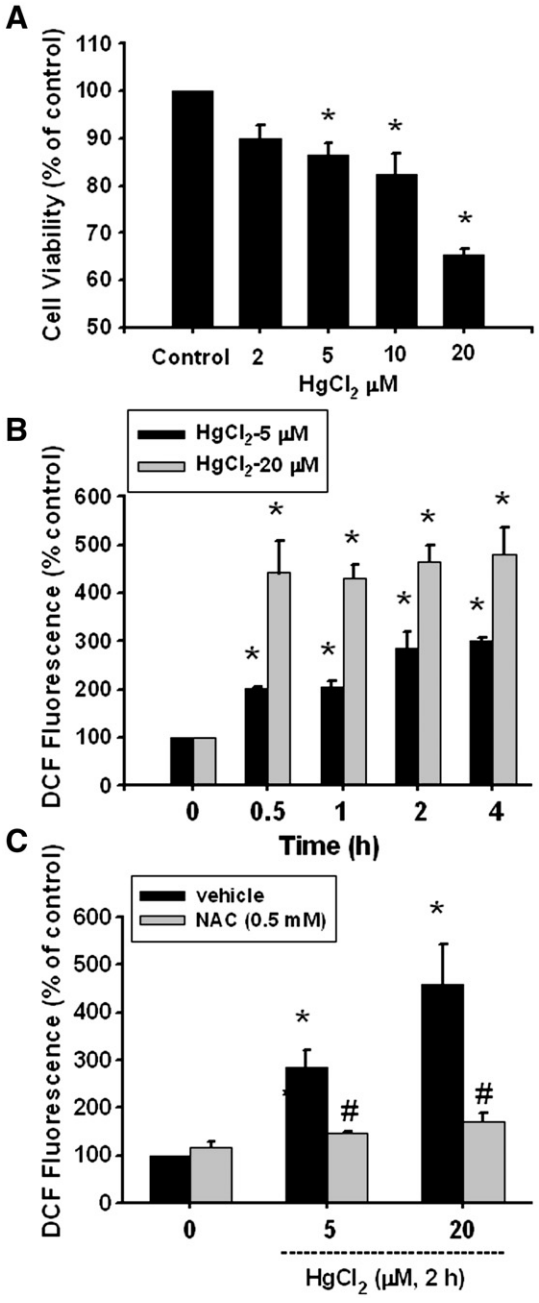


Fig. 1. Effects of $HgCl_2$ on cell viability and ROS generation in β-cell-derived HIT-T15 cells. (A) Cells were treated with $HgCl_2$ (2–20 μM) for 24 h, and cell viability was determined by MTT assay. (B) Cells were treated with $HgCl_2$ (5 and 20 μM) for various time courses; (C) cells were treated with $HgCl_2$ (5 and 20 μM) in presence or absence of NAC (0.5 mM) for 2h, and ROS was determined by flow cytometry as described in the Materials and methods section. All data are presented as mean ± SEM for four independent experiments with triplicate determination. *P<0.05 as compared with control. #P<0.05 as compared with mercury alone.

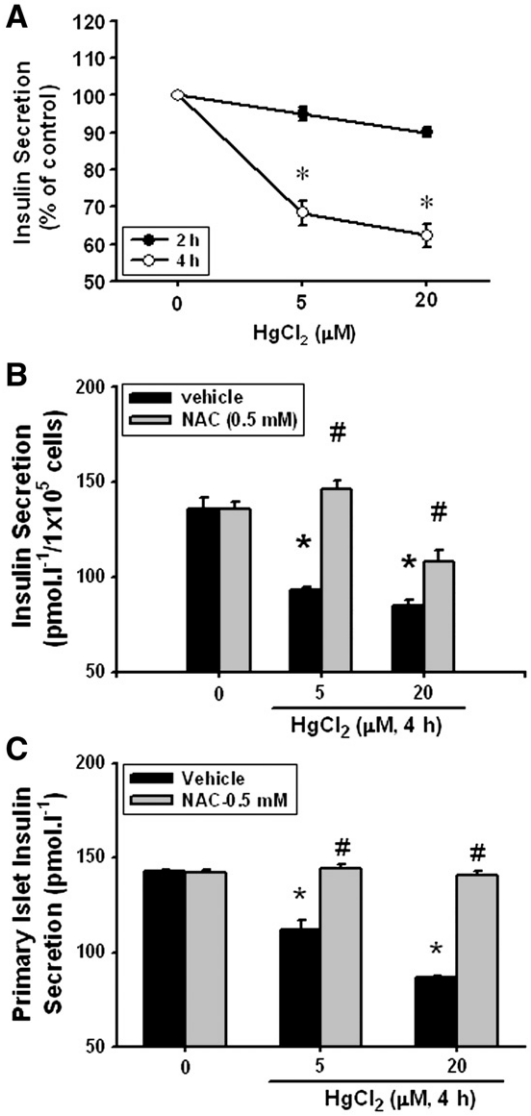


Fig. 2. Effect of $HgCl_2$ on insulin secretion. β-Cell-derived HIT-T15 cells (A and B) and isolated mouse islets (C) were treated with or without $HgCl_2$ (5 and 20 μM) in the presence or absence of *N*-acetylcysteine (NAC, 0.5 mM) for 2 or 4 h. The insulin secretion was detected under 16.7 mM glucose-stimulated condition. Data are presented as means ± SEM for four independent experiments with triplicate determinations. *P<0.05 as compared with control. #P<0.05 as compared with mercury alone.

Tubes were thawed at 37 °C for 20 min and centrifuged at 1000 rpm at 4 °C for 10 min. The supernatant was taken and analyzed for mercury contents. The levels of mercury in cells were determined by cold vapor atomic absorption spectrophotometer (AAS) combined with the flow-injection analysis system (FI-CVAAS) as described previously (Yen et al., 2002). FI-CVAAS analysis was performed with a Perkin-Elmer 5100PC AAS equipped with a Perkin-Elmer FIAS-200 flow-injection analysis system and AA WinLab software. The detection limit for mercury was approximately 0.1 ppb (μg/L).

Statistics. The values in the text are given as means ± SEM. The significance of difference was evaluated by the paired Student's *t*-test. When more than one group was compared with one control, significance was evaluated according to one way analysis of variance (ANOVA); the Duncan's post hoc test was applied to identify group differences. Probability values of <0.05 were considered statistically significant.

Results

$HgCl_2$ decreases insulin secretion and induces cell death and ROS production in β-cell-derived HIT-T15 cells

The cell viability of HIT-T15 cells was significantly reduced by the treatment of $HgCl_2$ (2 to 20 μM) for 24 h in a dose-dependent manner (Fig. 1A). After exposure of HIT-T15 cells to $HgCl_2$ (5 and 20 μM) for 0.5–4 h, the intracellular ROS levels were significantly increased (Fig. 1B). *N*-acetylcysteine (NAC, 0.5 mM), an antioxidant and glutathione (GSH) precursor (Bernard, 1991), could effectively reduce $HgCl_2$-induced ROS generation (Fig. 1C). The $HgCl_2$-induced cell viability reduction in HIT-T15 cells could also be reversed by NAC (5 μM $HgCl_2$, 82.31 ± 3.22, +NAC, 98.42 ± 4.34% of control; 20 μM $HgCl_2$, 64.51 ± 4.18, +NAC, 88.73 ± 5.41% of control, $n = 4$, $P<0.05$ as compared with $HgCl_2$ alone).

To examine the effect of $HgCl_2$ on insulin secretion function, we detected the short-term (2 or 4 h) response of $HgCl_2$ (5 and 20 μM) on insulin secretion from β-cells. After 4 h treatment, $HgCl_2$ (5 and 20 μM) effectively inhibited insulin secretion in HIT-T15 cells and isolated mouse islets (Fig. 2). NAC could effectively reverse $HgCl_2$-induced insulin secretion inhibition in HIT-T15 cells (Fig. 2B) or isolated mouse islets (Fig. 2C).

$HgCl_2$ induces apoptosis via a mitochondria-dependent pathway in HIT-T15 cells

We next investigated the involvement of apoptosis in $HgCl_2$-induced β-cell cytotoxicity. We firstly analyzed the sub-G1 hypodiploid cell population by flow cytometry. As shown in Fig. 3, cells treated with $HgCl_2$ (5 and 20 μM) for 24 h triggered the increase in sub-G1 hypodiploid cell population. Moreover, the externalization of phosphatidyl serine (PS), an early event in apoptosis (Zurgil et al., 2002), was determined using annexin-V-FITC binding assay by flow cytometry. As shown in Fig. 4A, the cells in the lower right quadrant were the early apoptotic cells. Cells treated with $HgCl_2$ (5 and 20 μM) for 24 h increased the annexin-V-positive cell population (Fig. 4A). Antioxidant NAC (0.5 mM) effectively prevented the $HgCl_2$-induced β-cell apoptosis (Fig. 4B). These results indicate that exposure of $HgCl_2$ to β-cells could induce apoptosis.

We next investigated whether $HgCl_2$ induced apoptosis through the mitochondria-dependent pathway. To address this issue, we analyzed the mitochondrial membrane potential (MMP) by use of the cationic dye $DiOC_6$ to show that $HgCl_2$ affects the mitochondrial permeability transition. As shown in Fig. 5A, exposure of HIT-T15 cells to $HgCl_2$ (5 and 20 μM) for 8 h significantly induced the decrease in MMP. We also investigated the release of cytochrome *c* from the mitochondria into the cytosol of $HgCl_2$-treated HIT-T15 cells. Treatment with $HgCl_2$ (5 μM) to HIT-T15 cells for 8 h effectively increased the cytochrome *c* level in the cytosol fraction (Fig. 5B). These $HgCl_2$-induced responses could be reversed by NAC (0.5 mM) (Figs. 5A and B).

Moreover, the PARP degradation and caspase-3 protease activation were detected. As shown in Fig. 5C, the exposure of HIT-T15 cells to

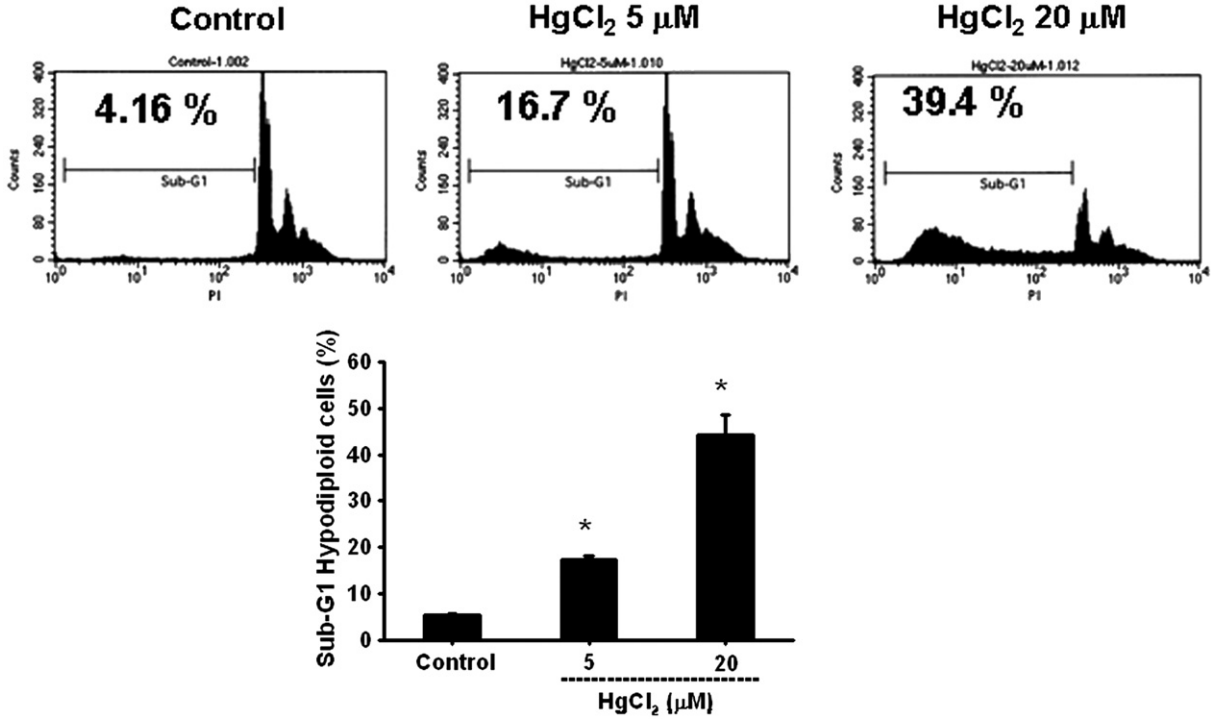
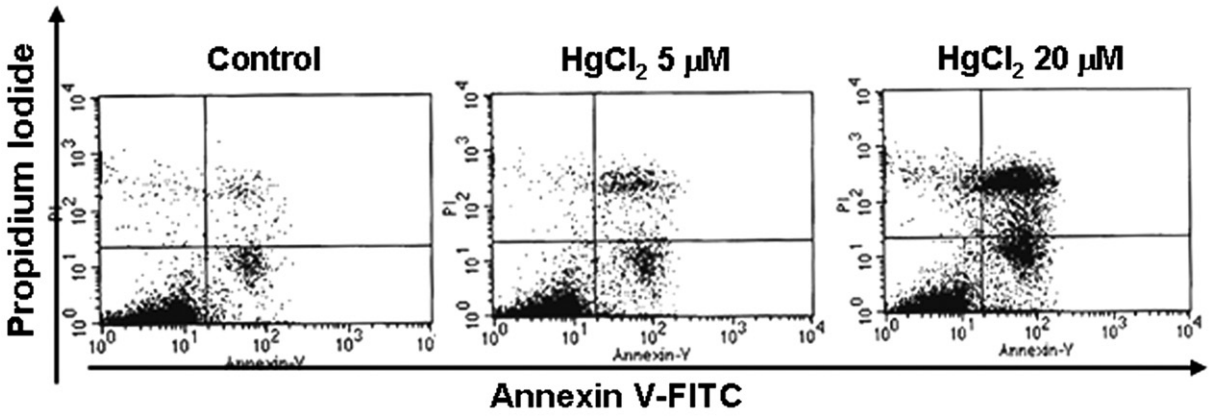


Fig. 3. Sub-G1 hypodiploid cell population analysis showing $HgCl_2$-induced apoptosis in HIT-T15 cells. Cells were treated with or without $HgCl_2$ (5 and 20 μM) for 24 h. Cells with genomic DNA fragmentation (sub-G1 DNA content) were analyzed by flow cytometry. Data are presented as mean ± SEM for four independent experiments with triplicate determination. *$P<0.05$ as compared with control.

A



Annexin V	---	+	+	---
Propidium iodide	---	---	+	+
(Groups, %)				
Control	95.57±0.56	1.44±0.29	1.90±0.26	1.10±0.14
$HgCl_2$ 5 μM	72.75±0.58 *	16.1±1.67 *	8.32±0.74 *	1.67±0.61 *
$HgCl_2$ 20 μM	50.44±1.27 *	24.16±2.78	18.67±1.17 *	7.68±1.17 *

B

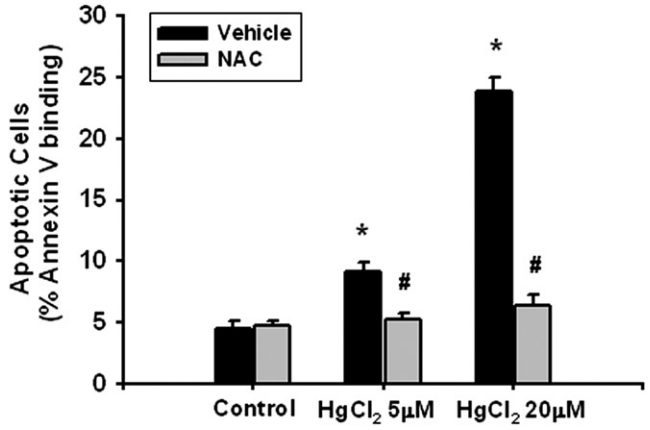


Fig. 4. $HgCl_2$ induced apoptosis in HIT-T15 cells determined by annexin-V-FITC/propidium iodide staining. Cells were treated with or without $HgCl_2$ (5 and 20 μM) for 24 h. Measurement of phosphatidylserine exposure on the outer cellular membrane leaflets by staining with annexin-V-FITC and live gating on annexin-V-FITC-positive, propidium iodide-negative cells (A). In some experiments, the effect of NAC (0.5 mM) on $HgCl_2$-induced apoptosis was examined (B). Data are presented as mean ± SEM for four independent experiments with triplicate determinations. *$P<0.05$ as compared with control. # $P<0.05$ as compared with mercury alone.

5 μM $HgCl_2$ for 12–18 h caused the marked degradation of 116-kDa full-length PARP and 32-kDa full-length pro-caspase-3 levels.

Necrosis is also involved in $HgCl_2$-induced cytotoxicity

In order to investigate whether $HgCl_2$-induced cytotoxicity in HIT-T15 cells was also associated with necrotic cell death, cell necrosis was analyzed by flow cytometric examination with acridine orange/ethidium bromide staining. It has been shown that dual staining with cell-permeant acridine orange and cell-impermeant ethidium bromide is an effective flow cytometric method for discriminating apoptotic from necrotic cell death (Lecoeur, 2002; Johnson et al., 2005). Exposure of HIT-T15 cells to $HgCl_2$ (5 and 20 μM) for 24 h significantly increased both apoptotic cells (apoptotic (Ap) population: 9.78 ± 0.79% and 18.81 ± 0.38% for 5 and 20 μM $HgCl_2$, respectively, vs. 3.82 ± 0.69% for control) and necrotic cells (necrotic (N) population: 7.41 ± 0.28% and 16.27 ± 0.68% for 5 and 20 μM $HgCl_2$, respectively, vs. 1.88 ± 0.58% for control) (Fig. 6).

Moreover, it has been found that annexin-V can bind to PS on the inner leaflet when the integrity of the plasma membrane is compromised and is indicative of necrosis or secondary necrosis following apoptosis (Johnson et al., 2005). As shown in Fig. 4A, the upper right quadrant represented cells that have undergone necrotic cell death, which is staining for PI and annexin-V. The amount of cells

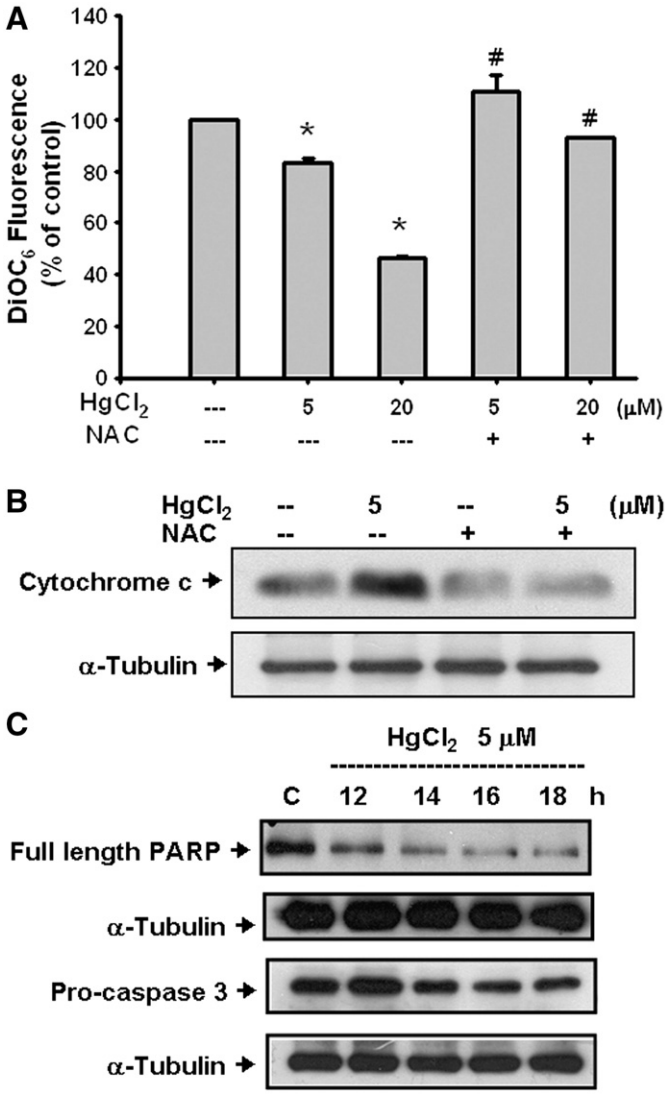
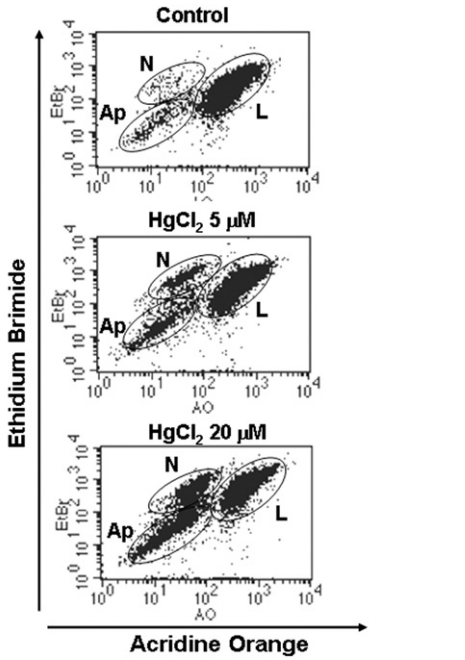


Fig. 5. $HgCl_2$ induced mitochondrial membrane potential depolarization, cytochrome *c* release and cleavages of poly (ADP-ribose) polymerase (PARP) and pro-caspase-3 in HIT-T15 cells. Cells were treated with or without $HgCl_2$ (5 or 20 μM) for 8 h (A, mitochondrial membrane potential; B, cytochrome *c*) or 12–18 h (C, PARP and pro-caspase-3 cleavage) in the presence or absence of NAC (0.5 mM). Data in A are presented as means ± SEM for four independent experiments with triplicate determinations. $^*P<0.05$ as compared with control. $^{\#}P<0.05$ as compared with mercury alone. Results shown in B and C are representative of three independent experiments.



group	N	Ap	L
control	1.88±0.48	3.82±0.39	93.28±0.43
$HgCl_2$ 5 μM	7.41±0.28*	9.78±0.79*	80.91±0.99*
$HgCl_2$ 20 μM	16.27±0.68*	18.81±0.38*	61.78±0.84*

Fig. 6. $HgCl_2$ induced apoptosis and necrosis in HIT-T15 cells. Cells were treated with or without $HgCl_2$ (5 and 20 μM) for 24 h. Discrimination of live, apoptotic and necrotic cell populations was determined using the acridine orange/ethidium bromide staining by flow cytometry analysis as described in the Materials and methods section. L, Live cells; Ap, apoptotic population; N, necrotic population. Data are presented as means ± SEM for four independent experiments with triplicate determinations. $^*P<0.05$ as compared with control.

in the upper right quadrants was increased by $HgCl_2$ (5 and 20 μM) (annexin-V-positive and PI-positive cells (%): control: 1.90 ± 0.26; 5 and 20 μM $HgCl_2$: 8.32 ± 0.74 and 18.67 ± 1.17, respectively; $n = 4$, $P<0.05$).

Necrotic cell death has been associated with an early loss of ATP (Kim et al., 2003). We next examined the intracellular ATP levels in HIT-T15 cells following exposure to $HgCl_2$. Treatment of cells with $HgCl_2$ (5 and 20 μM) for 4 h effectively decreased the intracellular ATP levels, which could be reversed by NAC (0.5 mM) (Fig. 7A). Moreover, we measured LDH leakage as a marker of cell membrane damage and cell death due to necrosis (Mangipudy1 and Vishwanatha1, 1999; De La Peña et al., 2007). The LDH release from HIT-T15 cells was significantly increased by treatment with $HgCl_2$ (5 and 20 μM) for 24 h, which could be reversed by NAC (0.5 mM) (Fig. 7B).

On the other hand, after 4 h of exposure to $HgCl_2$ (5 and 20 μM), HIT-T15 cells had a slight but significant increase in intracellular Hg levels; there was a marked increase in intracellular Hg levels 24 h after $HgCl_2$ (5 and 20 μM) treatment. These results indicated that $HgCl_2$ application could enter into the cells (Fig. 8).

Discussion

Many studies have reported that mercuric compounds potently induce oxidative stress, which played a key role for cascade activation during mercury-induced cellular injury (Gatti et al., 2004; Shenker et al., 2002; Yin et al., 2007). The deleterious effect of ROS has been found to be induced in the progression of glucose toxicity-triggered pancreatic β-cell dysfunction under diabetic condition (Kajimoto and Kaneto, 2004; Robertson et al., 2007). Despite several studies showing that mercury could induce ROS with subsequent oxidative damage in several kinds of cells and organs, the precise action and mechanism of inorganic mercury $HgCl_2$-induced oxidative stress on the pancreatic β-cell dysfunction and cell death is still unclear, especially to discriminate between $HgCl_2$-induced apoptosis and necrosis and elucidate the underlying signaling pathways involved in these processes. In the present work, we investigate the $HgCl_2$-induced cytotoxicity and its cellular mechanism on pancreatic β-cells. We found that exposure to low-concentration of $HgCl_2$ significantly decreases cell viability and insulin secretion and markedly increase ROS generation in HIT-T15 cells or isolated mouse islets. $HgCl_2$-induced β-cell cytotoxicity could be reserved by antioxidant NAC. Therefore, these findings indicate that oxidative stress is involved in the $HgCl_2$-induced pancreatic β-cell cell death and dysfunction.

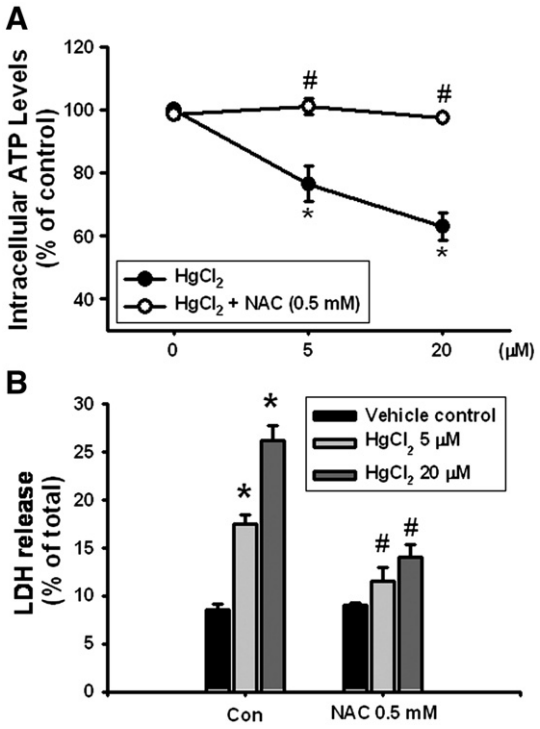


Fig. 7. Intracellular ATP levels and LDH release in HIT-T15 cells treated with $HgCl_2$. Cells were exposed to $HgCl_2$ (5 and 20 μM) for 4 h (ATP assay) and 24 h (LDH assay) in the presence or absence of NAC (0.5 mM). The intracellular ATP level was measured by adenosine 5′-triphosphate bioluminescent assay kit (A) and LDH release was detected by LDH cytotoxicity kit (B) as described in the Materials and methods section. Data are presented as means ± SEM for three independent experiments with triplicate determinations. $^{*}P<0.05$ as compared with control. $^{\#}P<0.05$ as compared with mercury alone.

ROS generation can serve as a trigger for cell death, which causes oxidative modification of DNA and gene mutation in a lot of cell types (Buttke and Sandstrom, 1995; Inoue et al., 2004). Oxidative stress could also trigger the inductions of apoptosis-related proteins (for examples, c-Jun-N-terminal kinase (JNK), extracellular signal-regulated kinase-1/2 (ERK-1/2) and p38 mitogen-activated protein kinase (MAPK) cascades), Bcl-2 family of pro- and anti-apoptosis proteins, p53, mitochondria-associated proteins (for examples, cytochrome *c*, Apaf-1 and apoptosis-inducing factor), cleavage of poly (ADP-ribose) polymerase (PARP) and caspase cascades (Kim et al., 2009; Kroemer et al., 1997; Ueda et al., 2002). PARP has been shown to be activated at

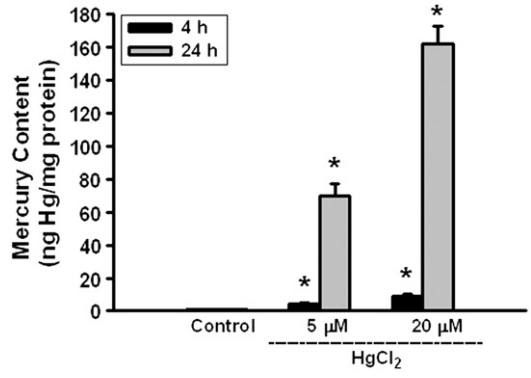


Fig. 8. Intracellular mercury content in HIT-T15 cells treated with $HgCl_2$. Cells were exposed to $HgCl_2$ (5 and 20 μM) for 4 and 24 h. Intracellular mercury content was detected by atomic absorption spectrophotometer as described in the Materials and methods section. Data are presented as means ± SEM for three independent experiments with triplicate determinations. $^{*}P<0.05$ as compared with control.

an intermediate stage of apoptosis and is then cleaved and inactivated at a late stage by apoptotic proteases, namely caspase-3/CPP-32/Yama/apopain and caspase-7 (Decker and Muller, 2002). However, several reports have also mentioned that PARP is involved in either necrosis and subsequent inflammation or apoptosis (Gobeil et al., 2001; Decker and Muller, 2002). Furthermore, the important role of ROS in death of several kinds of cells induced by mercury has been documented; its deteriorated effects included the DNA damage, DNA and RNA synthesis inhibition and disruption of mitochondrial functions (Baskin et al., 2003; Stohs and Bagchi, 1995; Xiang and Shao, 2003). Mitochondria are a sensitive organelle to the effects of oxidative stress (Kowaltowski et al., 1996 and 2001; Orrenius et al., 2003). Recent studies have demonstrated that organic mercury could induce cell death by activating mitochondrial caspase-dependent apoptotic pathway in several types of cells (Humphrey et al., 2005; Nishioku et al., 2000; Sutton and Tchounwou, 2006). There were a few reports showing that inorganic mercury (4–100 μM $HgCl_2$) could cause cytotoxicity by ROS generation in lymphocytes, macrophages, and renal proximal tubule cells (Kim and Sharma, 2004; Sarmento et al., 2004; Sutton and Tchounwou, 2007); however, the precise action and cellular mechanism of $HgCl_2$-induced cytotoxic effects on pancreatic β-cell cells are still unclear. In the current study, we found that $HgCl_2$ was capable of inducing apoptosis by triggered mitochondrial membrane depolarization and cytochrome *c* release in β-cell-derived HIT-T15 cells, which could be prevented by treatment of cell with antioxidant NAC. In addition, we also found that 5 μM $HgCl_2$ caused a significant degradation of 116-kDa PARP, and it was associated with the activation of caspase 3 protease. These results implicate that $HgCl_2$ induced an oxidative stress-regulated β-cell apoptosis through a mitochondria-dependent apoptotic pathway.

The characterizations of apoptosis are cell shrinkage, chromatin condensation, compaction of organelles and systematic DNA cleavage, which is genetically regulated (Arends and Wyllie, 1991; Bursch et al., 1992). In contrast, necrosis is characterized by cell swelling and ultimate rupture of cells in which plasma membrane is the major site of damage (Schwartz et al., 1993; Wyllie et al., 1980). Oxidative stress has been shown to be capable of inducing both apoptotic and necrotic cell death in several kinds of cells (Higuchi, 2004; Tan et al., 1998). Several studies have also shown that mercury-induced cell death was through both apoptosis and necrosis in macrophages, neutrophils and proximal tubular cells (Kim and Sharma, 2004; Kuo and Lin-Shiau, 2004; Lash et al., 2007). In the present study, we performed dual staining with fluorescence probes of acridine orange (cell-permeant) and ethidium bromide (cell-impermeant) for discriminating apoptotic from necrotic cell death by flow cytometry (Lecoeur, 2002; Johnson et al., 2005). In early stage of apoptosis, cells were impermeable to ethidium bromide and their nuclei stained green, which had condensed and/or fragmented nuclei; this ability was lost and their nuclei stained red during the later stage of apoptosis. Necrotic cells appeared a red nuclear stain, which was no nuclear condensation. Viable cells were impermeable to ethidium bromide and their nuclei stained green. Here, our results showed that exposure to $HgCl_2$ (5 and 20 μM) effectively induced both apoptosis and necrosis in HIT-T15 cells. On the other hand, it has been suggested that high ATP release accompanied by the decrease in the ATP levels under the cell plasma membrane damage are responsible for the cell death by necrosis (Lopez et al., 2003; Kim et al., 2003; Kon et al., 2004). Moreover, LDH release from cells has been shown to be a marker of cell membrane damage and cell death due to necrosis (Mangipudy1 and Vishwanatha1, 1999; De La Peña et al., 2007). In the present work, we found that treatment with $HgCl_2$ for 4 and 24 h remarkably caused the intracellular ATP depletion and LDH release increase in HIT-T15 cells, respectively. These results indicated that the necrotic pathway is involved in the $HgCl_2$-triggered pancreatic β-cell damage. Taken together, these findings indicate that $HgCl_2$ triggers pancreatic β-cell damage through both apoptotic and necrotic pathway.

GSH is the most abundant intracellular thiol-based antioxidant in living cells. GSH has been shown to function as a direct hydrogen peroxide scavenger (Nordberg and Arner, 2001). NAC is a low-molecular-weight thiol and a precursor of glutathione. Our unpublished data have shown that the glutathione levels in β-cells treated with $HgCl_2$ (5 and 20 μM) are not decreased at 4 h, but slightly decreased at 16 h (about 10–20% inhibition) and markedly decreased at 24 h (about 40–60% inhibition). Therefore, the antagonized effect of NAC on insulin secretion inhibition in β-cells by short-term (4 h) $HgCl_2$ exposure may be caused by its thiol reducing action but not glutathione formation action. Nevertheless, the antagonized effect of NAC on cell viability inhibition in β-cells by $HgCl_2$ treatment for 24 h may be caused by its glutathione formation action or both of glutathione formation action and thiol reducing action.

In conclusion, from this *in vitro* study, we found that $HgCl_2$ is capable of inducing the ROS-related insulin secretion suppression and cell death in pancreatic β-cells. The further evidences indicate that $HgCl_2$ enters β-cells and triggers oxidative stress to induce cell death through both apoptotic and necrotic pathways.

Conflict of interest statement
The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by a research grant from the National Science Council of Taiwan (NSC93-2314-B-002-178), and the China Medical University (CMU97-293).

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