Methylmercury Induces Pancreatic β-Cell Apoptosis and Dysfunction

Ya Wen Chen,[†,‡] Chun Fa Huang,[†,‡] Keh Sung Tsai,[‡,§] Rong Sen Yang,[‡,∥] Cheng Chieh Yen,[†] Ching Yao Yang,[†,⊥,#] Shoei Yn Lin-Shiau,[†] and Shing Hwa Liu*[,†,#,▽]

Institute of Toxicology, Department of Laboratory Medicine, and Department of Orthopaedics, College of Medicine, National Taiwan University, Taipei, Taiwan, and Departments of Traumatology, Surgery, and Emergency Medicine, National Taiwan University Hospital, Taiwan

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Mercury is a well-known toxic metal, which induces oxidative stress. Pancreatic β-cells are vulnerable to oxidative stress. The pathophysiological effect of mercury on the function of pancreatic β-cells remains unclear. The present study was designed to investigate the effects of methylmercury (MeHg)-induced oxidative stress on the cell viability and function of pancreatic β-cells. The number of viable cells was reduced 24 h after MeHg treatment in a dose-dependent manner with a range from 1 to 20 μM. 2′,7′-Dichlorofluorescein fluorescence as an indicator of reactive oxygen species (ROS) formation after exposure of HIT-T15 cells or isolated mouse pancreatic islets to MeHg significantly increased ROS levels. MeHg could also suppress insulin secretion in HIT-T15 cells and isolated mouse pancreatic islets. After 24 h of exposure to MeHg, HIT-T15 cells had a significant increase in mercury levels with a dose-dependent manner. Moreover, MeHg displayed several features of cell apoptosis including an increase of the sub-G1 population and annexin-V binding. Treatment of HIT-T15 cells with MeHg resulted in disruption of the mitochondrial membrane potential and release of cytochrome c from the mitochondria to the cytosol and activation of caspase-3. Antioxidant N-acetylcysteine effectively reversed the MeHg-induced cellular responses. Altogether, our data clearly indicate that MeHg-induced oxidative stress causes pancreatic β-cell apoptosis and dysfunction.

Introduction

Methylmercury (MeHg) is a highly lipophilic environmental contaminant. Among humans, the major source of exposure to MeHg is the consumption of fish and sea mammals (*1*). The industrial release of MeHg into Minamata Bay and the Agano River in Japan resulted in the accumulation of the toxicant in fish and, subsequently, in two large epidemics related to fish consumption (*1*, *2*). Many studies have shown that MeHg causes cytotoxicity in various cells including rat cerebellar granule cells (*3*, *4*), human oligodendroglial cells (*5*), human and rat lung cells (*6*), mouse peritoneal neutrophils (*7*), and human lymphocytes and monocytes (*8*, *9*). Further studies demonstrated that MeHg killed cells by inducing apoptosis (*3*, *5*, *7*−*9*).

It has been shown that an increased incidence of diabetes existed in patients with documented Minamata disease (MeHg poisoning) in Japan (*10*). Takeuchi et al. reported that the disturbance of pancreatic islet cells was found in autopsy cases of Minamata disease (*11*). In experiments using rats, Shigenaga has found that pancreatic islets were injured by MeHg and that a high level of blood glucose was induced by repeated administration of MeHg (*12*). However, the cytotoxic effect and toxicological mechanism of MeHg on pancreatic β-cells and insulin secretion function have not been established. On the other hand, reactive oxygen species (ROS) induce undesirable biological reactions, including programmed cell death (*13*). MeHg has been shown to induce the formation of ROS that resulted in the induction of apoptosis in C6 glioma cells and human T-cells and monocytes (*8*, *14*, *15*). It has been suggested that pancreatic β-cells are vulnerable to oxidative stress (*16*). The formation of ROS such as superoxide anion (O_2^-), hydrogen peroxide, hydroxyl radicals, and the concomitant generation of nitric oxide have been implicated in β-cell dysfunction or cell death caused by autoimmune attack and actions of cytokines in type 1 diabetes (*17*). Taken together, in the current study, we hypothesized that MeHg-induced oxidative stress activates a pathway leading to apoptosis and pancreatic β-cell dysfunction. We therefore designed experiments to investigate the in vitro effects of MeHg on pancreatic β-cells and isolated mouse pancreatic islets and examined the possibility that generation of ROS contributes to MeHg-induced pancreatic β-cell dysfunction.

Materials and Methods

HIT-T15 Cell Culture. β-cell-derived HIT-T15 cells (CRL-1777, ATCC) 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 (*18*).

Pancreatic Islet Isolation. Islets of Langerhans were isolated by collagenase digestion of the mouse pancreas as previously described (*19*, *20*). 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

* To whom correspondence should be addressed. Tel: (+886)-2-23123456 ext. 8605. Fax: (+886)-2-23410217. E-mail: shliu@ha.mc.ntu.edu.tw.
† Institute of Toxicology, National Taiwan University.
‡ These authors contributed equally to this work.
§ Department of Laboratory Medicine, National Taiwan University.
∥ Department of Orthopaedics, National Taiwan University.
⊥ Department of Traumatology, National Taiwan University Hospital.
Department of Surgery, National Taiwan University Hospital.
▽ Department of Emergency Medicine, National Taiwan University Hospital.

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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 h light and dark cycles. In each experiment, the pancreases from three mice were used. After 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.

Insulin Secretion. To measure the amount of insulin secreted, aliquots of samples were collected from the plasma or experimental media at indicated time points and subjected to insulin antiserum immunoassay according to the manufacturer's instructions (Mercodia AB, Sweden).

Cell Viability. Cells were washed with fresh media and cultured in 96 well plates (2×10^5/well) and then stimulated with MeHg (1−20 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 (1 mL; Sigma, St. Louis, MO). Following mixing, 150 μL was applied to each of the 96 wells. An enzyme-linked immunosorbent assay reader (Bio-Rad, model 550, Hercules, CA) was used for fluorescence detection; the absorption was 570 nm.

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)]. In brief, HIT-T15 cells or islets 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 cytometry analysis. Islets then were dispersed using trypsin, and intracellular peroxide levels were measured with a flow cytometer (FACScalibur, Becton Dickinson, Sunnyvale, CA). More than 100 islets from at least three separate islet isolations were studied for each group.

Western Blot Analysis. Western blotting was performed using standard protocols. 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 3% nonfat dry milk and incubated with antibodies for procaspase-3, poly(ADP-ribose), cytochrome c (Santa Cruz Biochemicals), and polymerase (PARP) (Oncogene Research Products). After they were washed in PBST, the respective secondary antibodies conjugated to horseradish peroxidase were applied for 1 h. The antibody-reactive bands were revealed by the enhanced chemiluminescence kit (Amersham, United Kingdom) and were used to expose them to Kodak radiographic film.

Flow Cytometric Analysis of Apoptotic 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 cytometric analysis of DNA content (FACScalibur, Becton Dickinson). Nuclei displaying hypodiploid, sub-G1 DNA contents were identified as apoptotic.

2. Annexin-V-Fluorescein Isothiocyanate (FITC) and PI Staining. HIT-T15 cells or islets were washed twice with PBS and stained with annexin-V-FITC (BioVision) 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 cytometric analysis. More than 100 islets from at least three pancreases were studied for each group.

Measurement of Caspase-3 Activity. Caspase-3 activity was determined using the CaspACETM fluorometric activity assay (Promega Corporation, Madison, WI) as previously described (*21*). In brief, cell lysates were incubated at 37 °C with 10 μM Ac-DEVD-AMC, a caspase-3/CPP32 substrate. The fluorescence of the cleaved substrate was measured by a spectrofluorometer (Spectramax, Molecular devices) with an excitation wavelength at 380 nm and an emission wavelength at 460 nm.

Determination of Mitochondrial Membrane Potential. The mitochondrial membrane potential was analyzed using the fluorochrome stain $DiOC_6$ (Molecular Probes). HIT-T15 cells, treated with MeHg (2 and 5 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).

Detection of Mercury in β-Cells. The HIT-T15 cells were treated with MeHg (2 and 5 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. 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 combined with the flow-injection analysis system (FI-CVAAS) as described previously (*22*). 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. Results are expressed as means ± standard errors of the mean (SEM), and data were analyzed using Student's *t*-test. For multiple comparisons, results were analyzed using one-way analysis of variance followed by Fisher's test. $P < 0.05$ was considered statistically significant.

Results

Effects of MeHg on Cell Viability, ROS Production, and Insulin Secretion in HIT-T15 Cells. To examine the MeHg-induced pancreatic β cell cytotoxicity, cell viability was determined in HIT-T15 cells using the MTT assay. The number of viable cells was reduced 24 h after MeHg treatment in a dose-dependent manner with a range from 1 to 20 μM (Figure 1A).

To investigate the effect of mercury on the ROS production, we treated cells with MeHg (2 and 5 μM) and measured ROS production. After exposure of HIT-T15 cells to MeHg for 0.5−8 h, significantly increased ROS levels were observed using DCF fluorescence as an indicator of ROS formation (Figure 1B). This mercury-induced response could be inhibited by antioxidant N-acetylcysteine (NAC, 0.5 mM) (data not shown). Moreover, after 24 h of treatment, MeHg (2 and 5 μM) effectively suppressed insulin secretion in HIT-T15 cells, which could be reversed by NAC (0.5 mM) (Figure 2).

MeHg Induces Apoptosis, Mitochondrial Depolarization, Cytochrome c Release, and Activation of Caspase-3 in HIT-T15 Cells. To investigate the cytotoxicity of MeHg to HIT-T15 cells from the point of view of apoptosis, we analyzed the sub-G1 hypodiploid cell population by flow cytometry (*23*) and detected the externalization of phosphatidyl serine by annexin-V staining (*24*). As shown in Figure 3, cells that were treated with MeHg (2 and 5 μM) for 24 h triggered the increase in sub-G1 hypodiploid cell population and annexin-V staining. Antioxidant NAC (0.5 mM) could prevent the MeHg-induced cell apoptosis (Figure 3). Therefore, it is apparent that treatment of the pancreatic β-cell line with MeHg induces apoptosis.

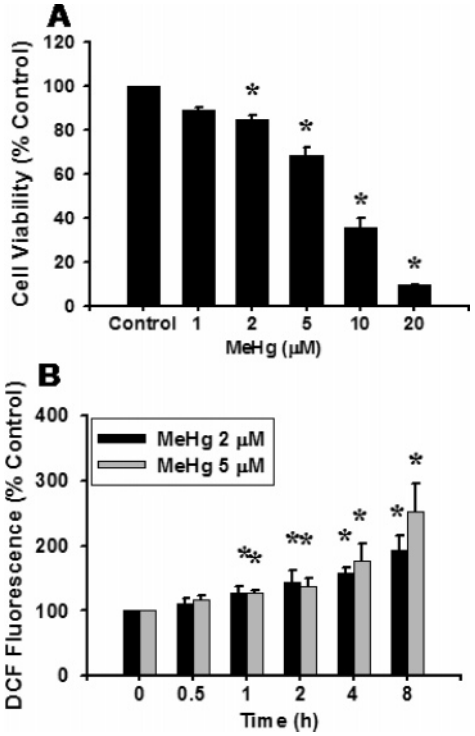

Figure 1. Effects of MeHg on cell viability and ROS generation in β-cell-derived HIT-T15 cells. (A) Cells were treated with or without MeHg (1−20 μM) for 24 h, and cell viability was determined by MTT assay. (B) Cells were treated with or without MeHg (2 and 5 μM) for various time courses, and ROS was determined by flow cytometry as described in the Materials and Methods. All data are presented as means ± SEM for four independent experiments with triplicate determinations. *P < 0.05 as compared with control.

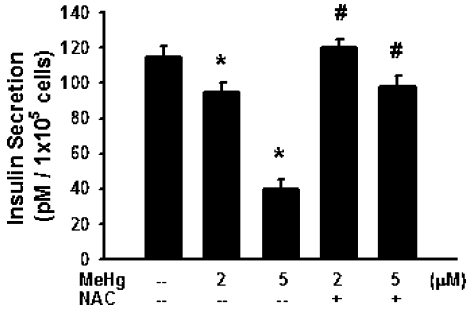

Figure 2. Effects of MeHg on insulin secretion in β-cell-derived HIT-T15 cells. Insulin secretion from cells with or without MeHg (2 and 5 μM) in the presence or absence of NAC (0.5 mM) for 24 h was detected under 16.7 mM glucose condition. Data are presented as means ± SEM for four independent experiments with triplicate determinations. *P < 0.05 as compared with control. $^{\#}P$ < 0.05 as compared with mercury alone.

To further investigate the mechanism of MeHg-induced apoptosis, we analyzed the mitochondrial pathway. Measurement of the mitochondrial membrane potential by use of the cationic dye $DiOC_6$ showed that MeHg affects the mitochondrial permeability transition. Exposure of HIT-T15 cells to MeHg (2 and 5 μM) for 8 h significantly depolarized the mitochondrial membrane potential (Figure 4A). Thus, mitochondrial damage preceded the induction of apoptosis indicating a role in MeHg-triggered cytotoxicity. Furthermore, to examine whether cytochrome c is released from the mitochondria into the cytosol of MeHg-treated HIT-T15 cells, we investigated the expression of cytochrome c by Western blot analysis. Treatment with MeHg (2 μM) to HIT-T15 cells for 8 h effectively increased the cytochrome c in the cytosol of cells, which could be reversed by NAC (0.5 mM) (Figure 4B).

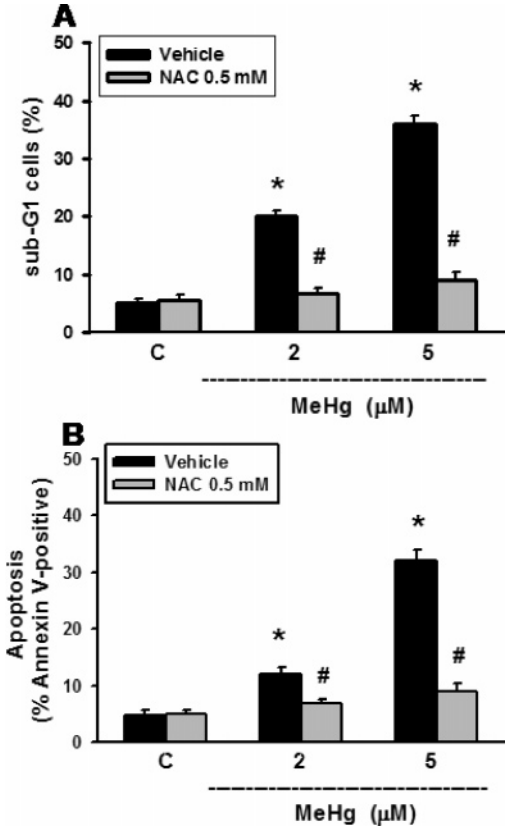

Figure 3. Flow cytometric analysis showing effects of MeHg-induced apoptosis in HIT-T15 cells. Cells were treated with or without MeHg (2 and 5 μM) for 24 h in the presence or absence of NAC (0.5 mM). (A) Cells with genomic DNA fragmentation (sub-G1 DNA content). (B) Measurement of phosphatidylserine exposure on the outer cellular membrane leaflets by staining with annexin-V-FITC and life gating on annexin-V-FITC-positive, propidium iodide negative cells. All 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.

To further evaluate the apoptotic signaling by MeHg, the caspase-3 activity was measured. The caspase-3 activity is an integral step in the majority of apoptotic events. Treatment of cells with MeHg at 2 and 5 μM induced caspase-3 activation (Figure 4C).

Moreover, after 24 h of exposure to MeHg, HIT-T15 cells had a significant increase in Hg levels in a dose-dependent manner (Figure 5).

Effects of MeHg on the Insulin Secretion, ROS Production, and Apoptosis in Isolated Mouse Pancreatic Islets. To examine the effects of MeHg on the pancreatic islets, we measured insulin secretion, ROS production, and apoptosis in primary cultures of isolated mouse islets. The exposure of islets to 2 μM MeHg for 1 h increased ROS production (Figure 6A). MeHg (2 μM)-treated islets for 24 h triggered the increase in apoptosis determined by annexin-V staining (Figure 6B). Moreover, MeHg-treated islets for 24 h could also inhibit the insulin secretion (Figure 7). These MeHg-induced responses could be prevented by NAC (0.5 mM).

Discussion

ROS can elicit oxidative stress and affect a wide variety of physiological and pathological processes (*25*). The deleterious effect of ROS is a function of activation of intracellular cell death circuitry. The involvement of ROS at different phases of the apoptotic pathway, such as induction of mitochondrial

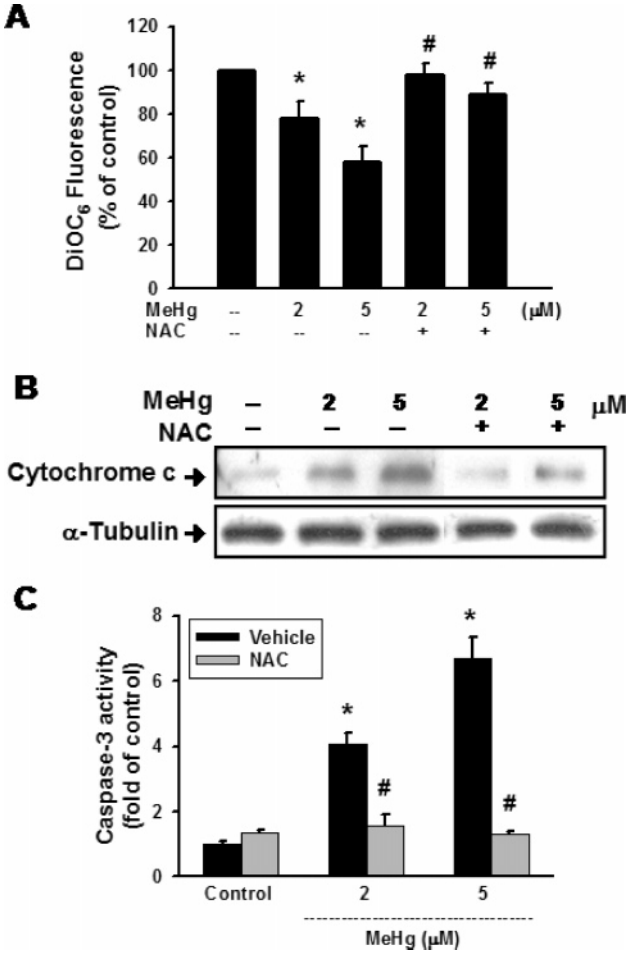

Figure 4. Analysis of mitochondrial membrane potential, cytochrome c, and caspase-3 activity in MeHg-treated HIT-T15 cells. Cells were treated with or without MeHg (2 and 5 μM) for 8 (A, mitochondrial membrane potential; B, cytochrome c) or 24 h (C, caspase-3 activity) in the presence or absence of NAC (0.5 mM). Data in A and C 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. Results shown in B are representative of three independent experiments.

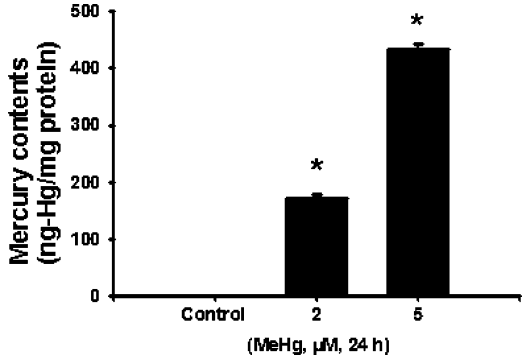

Figure 5. Detection of mercury in β-cells. HIT-T15 cells were treated with MeHg (2 and 5 μM) for 24 h. Mercury contents were determined as described in the Materials and Methods. Data are presented as means ± SEM for three independent experiments with triplicate determinations. $^{*}P < 0.05$ as compared with control.

permeability transition and release of mitochondrial death amplification factors and activation of intracellular caspases and DNA damage, has been clearly established (*25*, *26*). On the other hand, many studies have shown that ROS played a key role for cascade activation during apoptosis that was induced by mercuric compounds (*8*, *27*). However, the role of ROS in

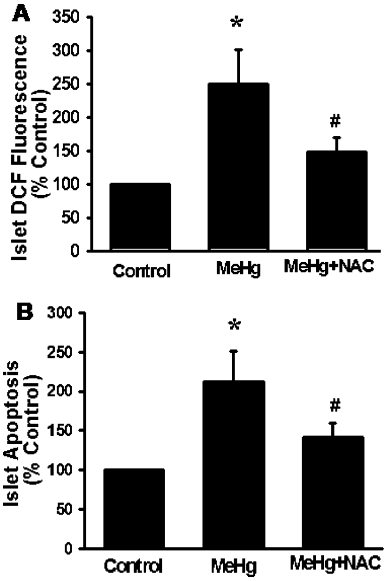

Figure 6. Effects of MeHg on ROS generation and apoptosis in isolated mouse islets. (A) Fluorescence of DCF-DA in islets at 1 h after MeHg (2 μM) treatment in the presence or absence of NAC (0.5 mM). (B) Flow cytometric analysis of phosphatidylserine exposure on the outer cellular membrane leaflet by staining with annexin-V-FITC in islets at 24 h after MeHg (2 μM) treatment in the presence or absence of NAC (0.5 mM). All 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.

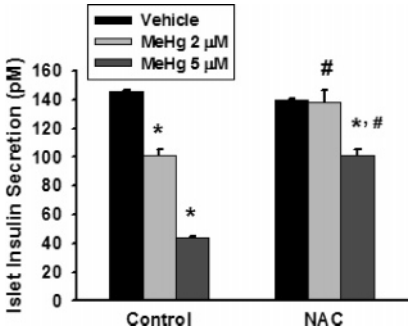

Figure 7. Effect of MeHg on insulin secretion in isolated mouse islets. Mouse islets were treated with or without MeHg (2 and 5 μM) for 24 h in the presence or absence of NAC (0.5 mM) under 16.7 mM glucose condition. All data are presented as means ± SEM for three independent experiments with triplicate determinations. $^{*}P < 0.05$ as compared with control. $^{\#}P < 0.05$ as compared with mercury alone.

the cytotoxic effects of mercury on pancreatic β-cells has not been established. In the present study, we used the β-cell-derived HIT-T15 cells and isolated mouse islets to analyze the production of ROS after exposure of low concentrations of MeHg for 0.5−8 h. The results showed that mercury significantly increased the production of ROS in HIT-T15 cells and isolated mouse islets. We also found that antioxidant NAC could reverse the MeHg-induced ROS production. These findings imply that oxidative stress is produced under MeHg exposure and it may be involved in the pancreatic β-cell dysfunction induced by MeHg.

It has been suggested that pancreatic β-cells might be rather sensitive to ROS attack when they are exposed to oxidative stress (*16*), because of the relatively low expression of antioxidant enzymes such as catalase and glutathione peroxidase (*28*). Kajimoto and Kaneto have further mentioned that oxidative stress and consequent activation of the c-Jun N-terminal kinase (JNK) pathway were involved in the progression of pancreatic β-cell dysfunction found in diabetes (*16*). In fact, diabetes is

typically accompanied by an increased production of free radicals and/or impaired antioxidant defense capabilities, indicating a central contribution for ROS in the onset, progression, and pathological consequences of diabetes (*29*). In this study, we found that MeHg (2 and 5 μM) effectively suppressed insulin secretion in HIT-T15 cells and isolated mouse islets, which could be reversed by antioxidant NAC. These results indicate that MeHg is capable of suppressing insulin secretion of β-cells through a ROS-triggered pathway.

ROS are the major factors that induce oxidative modification of DNA and gene mutation (*25*). Oxidative stress induces cytochrome c release from mitochondria and activation of caspases, p53, and kinases (*30*). Shenker and colleagues have shown that mercury could induce apoptosis in human T lymphocytes and proposed that the target organelle was the mitochondrion and that induction of oxidative stress led to activation of death-signaling pathways (*15*, *27*). Here, we found that MeHg was capable of inducing apoptosis in HIT-T15 cells and isolated mouse islets. MeHg could also trigger mitochondrial membrane depolarization, cytochrome c release, and caspase-3 activation in HIT-T15 cells. The antioxidant NAC prevented the MeHg-induced β-cell apoptosis. These results suggest that MeHg induces oxidative stress-regulated pancreatic β-cell cytotoxicity through a mitochondrial apoptosis pathway that caspase-3 activation in response to cytochrome c release from mitochondria.

Mercury is a well-known toxic agent that produces various types of cell and tissue damage. It has been shown that MeHg (2.5−5 μM) could induce cell death by activating apoptosis in human T lymphocytes (*15*). A previous study has shown that organic mercurials stimulated insulin release at concentrations of 10 μM or above for short-term exposure in pancreatic islets isolated from obese−hyperglycemic mice; they also found that no significant differences with respect to β-cell fine structure were noted between control islets and organic mercurial (100 μM, 60 min)-treated islets (*31*). Nevertheless, the cytotoxic effect and toxicological mechanism of MeHg on pancreatic β-cells and insulin secretion function are still unclear. In the present study, our observations showed that 2 μM MeHg significantly decreased cell viability in the pancreatic β-cell line, HIT-T15 cells, for 24 h. Treatment with MeHg decreased insulin secretion and initiated apoptosis in HIT-T15 cells and isolated mouse islets. These results indicate that MeHg is capable of inducing pancreatic β-cell damage and dysfunction.

Collectively, we present evidence that MeHg triggers ROS production, suppresses insulin secretion, and induces apoptosis in β-cell-derived HIT-T15 cells and isolated mouse pancreatic islets. The generation of oxidative stress and caspase-3 activation in response to cytochrome c release from mitochondria is involved in MeHg-induced apoptosis. These results indicate that MeHg-induced oxidative stress causes pancreatic β-cell apoptosis and dysfunction. Whether MeHg-induced oxidative stress causes pancreatic β-cell apoptosis and dysfunction in vivo remains to be elucidated.

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