Original Article

The Role of Phosphoinositide 3-Kinase/Akt Signaling in Low-Dose Mercury–Induced Mouse Pancreatic β-Cell Dysfunction In Vitro and In Vivo

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The relationship between oxidation stress and phosphoinositide 3-kinase (PI3K) signaling in pancreatic β-cell dysfunction remains unclear. Mercury is a well-known toxic metal that induces oxidative stress. Submicromolar-concentration $HgCl_2$ or methylmercury triggered reactive oxygen species (ROS) production and decreased insulin secretion in β-cell–derived HIT-T15 cells and isolated mouse islets. Mercury increased PI3K activity and its downstream effector Akt phosphorylation. Antioxidant *N*-acetyl-L-cysteine (NAC) prevented mercury-induced insulin secretion inhibition and Akt phosphorylation but not increased PI3K activity. Inhibition of PI3K/Akt activity with PI3K inhibitor or by expressing the dominant-negative p85 or Akt prevented mercury-induced insulin secretion inhibition but not ROS production. These results indicate that both PI3K and ROS independently regulated Akt signaling–related, mercury-induced insulin secretion inhibition. We next observed that 2- or 4-week oral exposure to low-dose mercury to mice significantly caused the decrease in plasma insulin and displayed the elevation of blood glucose and plasma lipid peroxidation and glucose intolerance. Akt phosphorylation was shown in islets isolated from mercury-exposed mice. NAC effectively antagonized mercury-induced responses. Mercury-induced in vivo effects and increased blood mercury were reversed after mercury exposure was terminated. These results demonstrate that low-dose mercury–induced oxidative stress and PI3K activation cause Akt signaling–related pancreatic β-cell dysfunction. *Diabetes* 55:1614–1624, 2006

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DCF, dichlorofluorescein; DCFH-DA, DCF diacetate; ELISA, enzyme-linked immunosorbent assay; KRB, Krebs Ringer buffer; MeHg, methylmercury; NAC, *N*-acetyl-L-cysteine; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species.

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Oxidative stress has been implicated in a wide variety of disease processes, including diabetes (1). Pancreatic β-cells are vulnerable to oxidative stress (2,3). It has been shown that chronic exposure to elevated free fatty acids or glucose levels increases apoptosis in rat pancreatic islets and that these cytotoxic effects could be mediated by oxidative stress (4). On the other hand, the phosphoinositide 3-kinase (PI3K) pathway is implicated in human diseases, including diabetes and cancer (5). A PI3K-dependent signaling pathway has been demonstrated to exist in β-cells, and it might function to restrain glucose-induced insulin secretion from β-cells (6,7). Moreover, various studies have shown that reactive oxygen species (ROS) could regulate the activation of Akt, a downstream effector of PI3K, in cultured cells such as fibroblasts (8), mesangial cells (9), or vascular smooth muscle cells (10). It has also been reported that activation of PI3K/Akt signaling during the cellular response to oxidant injury was important for survival (11). However, the relationship between oxidation stress and PI3K/Akt signaling in pancreatic β-cell dysfunction is still unclear.

Various studies (12–15) have indicated that heavy metals acted as catalysts in the oxidative reactions of biological macromolecules; therefore, the toxicities associated with these metals might be due to oxidative tissue damage. Mercury is a well-known toxic metal that induces oxidative stress (16–19). Among humans, the major source of exposure to methylmercury (MeHg) is the consumption of fish and sea mammals (20). 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 (20). Elemental and inorganic mercury are found in scientific instruments, electrical equipment, dental amalgams, disinfectants, production of caustic soda, disk batteries, and the atmosphere (21). These mercury sources are now usually related to accidental and occupational exposures (20,22). Recently, increased oxidative DNA damage and serum redox status have been detected in individuals exposed to mercury (23). Moreover, previous in vitro studies have shown that $HgCl_2$ altered intracellular Ca^{2+} homeostasis in pancreatic β-cells isolated from mouse islets (24) and decreased insulin secretion from secretion granules isolated from toadfish islets (25). It has also been shown that an increased incidence of diabetes existed in patients with documented Minamata disease

(MeHg poisoning) in Japan (26). Nevertheless, the precise action and mechanism of mercury-induced oxidative stress on the pancreatic β-cell function and blood glucose regulation remain unclear.

Taken together, in the current study, we try to explore the role of PI3K/Akt signaling in low-dose mercury–induced mouse pancreatic β-cell dysfunction. To this aim, we sought to investigate the in vitro effects of low concentrations of inorganic mercury ($HgCl_2$) and organic mercury (MeHg) (0.2–1 μmol/l) on ROS generation, investigate PI3K/Akt activation and insulin secretion in the isolated mouse islets and pancreatic β-cell line HIT-T15, and test whether low-dose exposure to $HgCl_2$ (50 and 500 μg/kg) or MeHg (20 μg/kg), which approaches the human-exposed mercury levels in blood, would generate lipid peroxidation, activate PI3K signaling, and alter the regulation of insulin secretion and blood glucose in mice. Moreover, the potential protective effects of *N*-acetyl-L-cysteine (NAC; ROS scavenger) on the pancreatic β-cell functions in vitro and in vivo were investigated. These observations may provide evidence that oxidative stress causes pancreatic β-cell dysfunction through a PI3K/Akt-related pathway and that mercury is a possible environmental risk factor to diabetes.

RESEARCH DESIGN AND METHODS

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-h light-dark cycles. Mice were randomly assigned to pretreatment groups, weighed, and administered $HgCl_2$, MeHg, or vehicle (oral application by gavage). These groups were given 0, 50, and 500 $\mu g \cdot kg^{-1} \cdot day^{-1}$ $HgCl_2$ or 20 $\mu g \cdot kg^{-1} \cdot day^{-1}$ MeHg (7 days/week) in the presence or absence of NAC (150 mg/kg) for 2–4 weeks. Each group contained 16 mice. All mice were observed daily for clinical signs. Following collection of blood samples, the animals were exsanguinated, while still under anesthesia, and necropsied. A necropsy examination was performed on all animals from each group. All pathological findings were recorded. A histopathological examination was performed on organs from groups treated with 0 and 500 μg/kg $HgCl_2$ and 20 μg/kg MeHg.

Plasma lipid peroxidation assay. Blood samples were collected from the peripheral vessels of mice under ketamine anesthesia (85 mg/kg i.p.). Whole blood was centrifuged at 3,000*g* for 10 min, and plasma was obtained and assayed immediately using the lipid peroxidation assay kit (Calbiochem). An absorbance of 586 nm was measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader.

Blood glucose measurement and glucose tolerance test. Blood samples were collected after an overnight fast. Blood glucose levels were determined using the SureStep blood glucose meter (Lifescan, Milpitas, CA). For oral glucose tolerance testing, D-glucose (1 g/kg) was administered by stomach tube after an overnight fast. Blood samples were collected before and 30, 60, 90, 120, and 180 min after delivery of the glucose load.

Pancreatic islet isolation. Islets of Langerhans were isolated by collagenase digestion from the mouse pancreas as previously described (27,28). After separation on a Ficoll gradient, the islets were further purified by handpicking to eliminate any remaining exocrine tissue. Whole islets were maintained in culture medium consisting of RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/amphotericin B at 37°C in an atmosphere of 95% air/5% CO_2 before experimentation.

HIT-T15 cell culture. Pancreatic β-cell–derived HIT-T15 cells were cultured in a humidified chamber with a 95% air/5% CO_2 mixture at 37°C and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and containing 11.1 mmol/l glucose as described (29). In some experiments, transfection in HIT-T15 cells was performed using the Lipofectin reagent (BD Clontech) according to the recommendations of the manufacturer with 2 μg total DNA (or vector) per sample. The efficiency of transfection (~80%) was determined by using an equal amount of a plasmid encoding the green fluorescent protein under the cytomegalovirus promoter. Plasmids containing the DN-p85 (δ-p85) or DN-Akt (Akt K179A) (30,31) were kindly provided by Dr. R.H. Chen and Dr. M.L. Kuo (Institutes of Molecular Medicine and Toxicology, National Taiwan University, Taiwan).

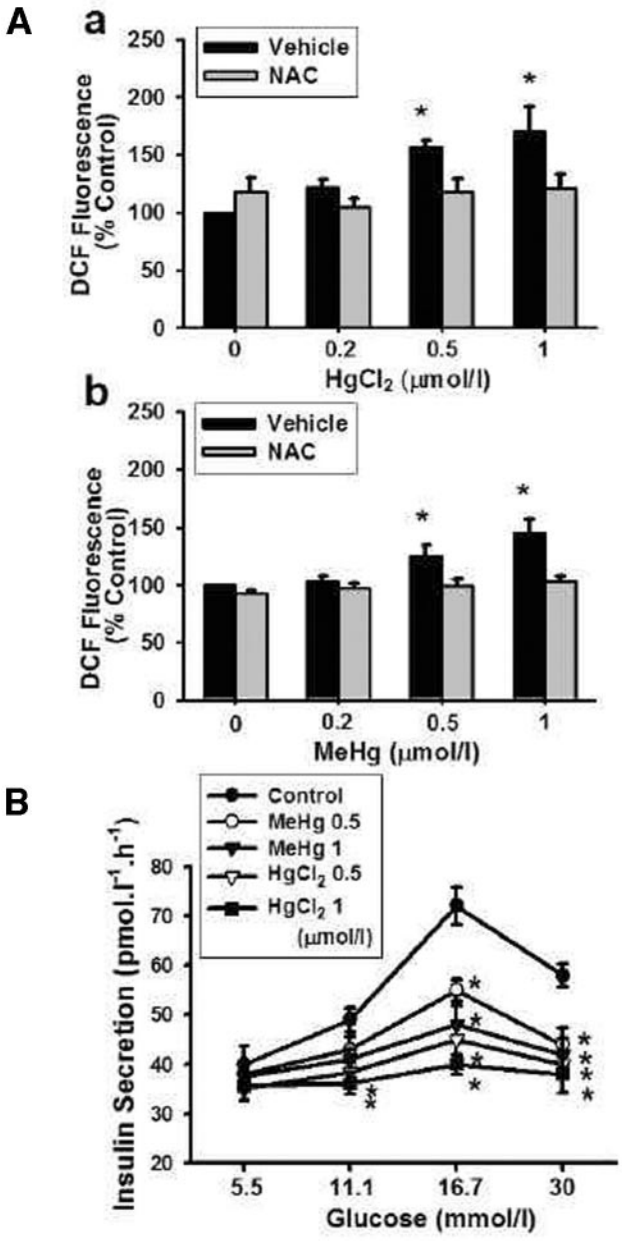

FIG. 1. Effects of mercury on ROS generation and insulin secretion in β-cell–derived HIT-T15 cells. *A*: Cells were treated with or without $HgCl_2$ (*a*) or MeHg (*b*) (0.2–1 μmol/l) for 1 h in the presence or absence of NAC (1 mmol/l). ROS was determined by flow cytometry as described in RESEARCH DESIGN AND METHODS. *B*: Insulin secretion from cells incubated in KRB containing various concentrations of glucose with or without $HgCl_2$ or MeHg (0.5 and 1 μmol/l) for 4 h. All data are presented as means ± SE for four independent experiments with triplicate determinations. *$P < 0.05$ compared with control.

Insulin secretion studies. HIT-T15 insulin secretion experiments were performed in Krebs Ringer buffer (KRB), and islet secretion studies were performed in islet culture media for a 1-h experimental incubation with a 95% air/5% CO_2 mixture at 37°C, as previously described (32). All experimental agents were mixed together in experimental solutions (KRB or media) and then added to the cells at the start of the experimental incubation. To measure the amount of insulin secreted, aliquots of samples were collected from the plasma or experimental solutions at indicated time points and subjected to insulin antiserum immunoassay according to the manufacturer's instruction (Mercodia, Uppsala, Sweden).

Cell viability. Cells were washed with fresh media from dishes and cultured in 96-well plates (2×10^5/well), then stimulated with $HgCl_2$ and MeHg. After

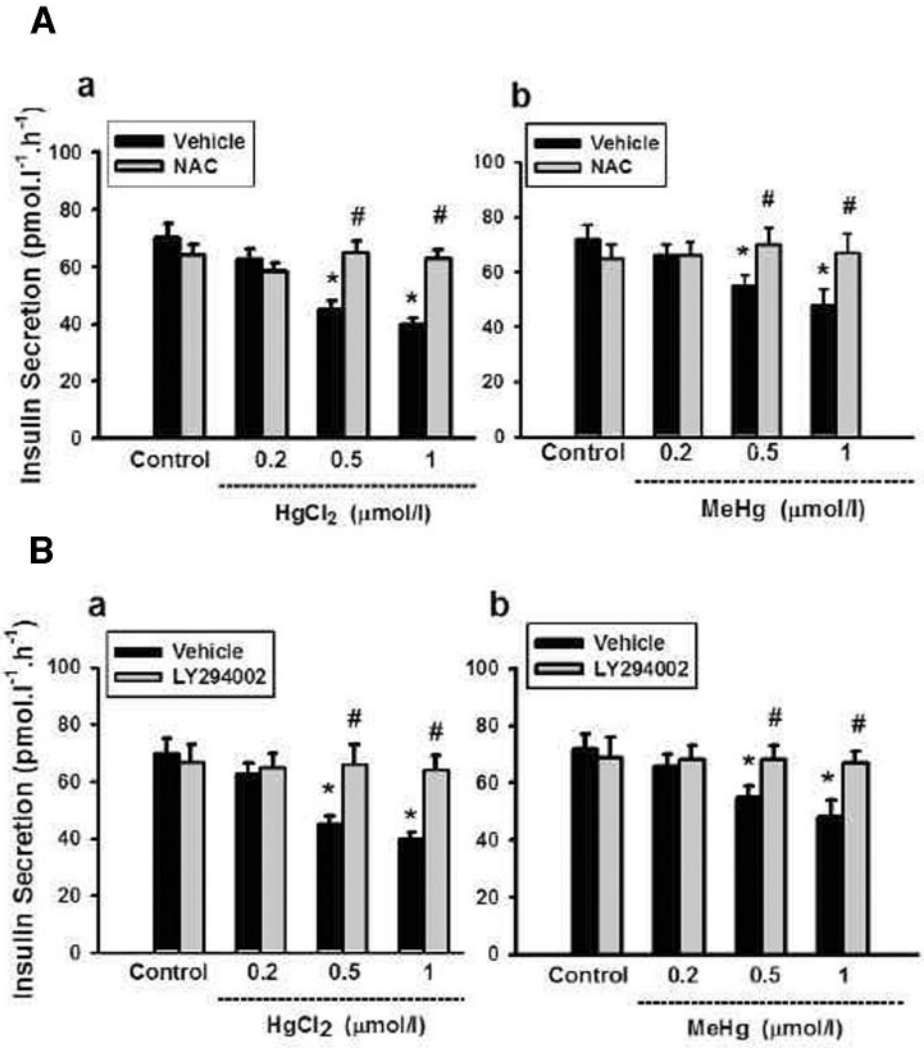

FIG. 2. Effects of antioxidant NAC and PI3K inhibitor LY294002 against mercury-induced insulin secretion inhibition in HIT-T15 cells. Insulin secretion from cells incubated in KRB containing 16.7 mmol/l glucose with or without $HgCl_2$ (*A*, *a* and *B*, *a*) or MeHg (*A*, *b* and *B*, *b*) (0.2–1 μmol/l) for 4 h in the presence or absence of NAC (*A*, 1 mmol/l) or LY294002 (*B*, 5 μmol/l). All data are presented as means ± SE for four independent experiments with triplicate determinations. *$P < 0.05$ compared with control; #$P < 0.05$ compared with mercury alone.

24 h, the medium was replaced with fresh medium containing 30 μl (2 mg/ml) 3-(4,5-dimethyl thiazol-2-yl-)-2,5-diphenyl tetrazolium bromide. After incubation for 4 h, the medium was removed and 1 ml dimethyl sulfoxide added to dissolve blue formazan crystal. An ELISA reader was used for fluorescence detection; absorption was 570 nm.

Detection of intracellular ROS. Intracellular ROS generation was monitored by flow cytometry using peroxide-sensitive fluorescent probe (2′,7′-dichlorofluorescin diacetate [DCFH-DA; Molecular Probes]). In brief, HIT-T15 cells or islets were coincubated with 20 μmol/l DCFH-DA for 15 min at 37°C. 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 PBS and placed on ice in darkness for flow cytometry analysis. Using trypsin, the dispersed islets and intracellular peroxide levels were measured with the FACScan flow cytometry device (Becton Dickinson, Franklin Lakes, NJ). More than 100 islets from at least three separate islet isolations were studied for each group. The results were expressed as the mean fluorescence intensity. In some experiments, after 15 min of incubation of DCFH-DA, the islets were washed twice and images captured by a Leica DMIL inverted microscope equipped with a charged-coupled device camera.

Analysis of cellular ATP levels. Cellular ATP concentration was determined by the ATP bioluminescent assay kit (Sigma), which measures the intracellular ATP level using the ATP-dependent luciferin-luciferase reaction.

Western blotting. Fifty micrograms of protein of each cell lysate were subjected to electrophoresis on 10% SDS-polyacrylamide gels. The samples were then electroblotted on polyvinylidene difluoride membranes. After blocking, blots were incubated with anti-Akt and anti–phospho-Akt (New England BioLabs) antibodies in PBS and 0.1% Tween 20 for 1 h followed by two washes (15 min each) in PBS and 0.1% Tween 20. The membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies for 30 min. Enhanced chemiluminescence reagents (Amersham) were used to depict the protein bands on membranes.

PI3K activity assay. PI3K activity was assayed as described previously (33). Cell extracts were incubated with 2 μg anti-p85 antibody overnight at 4°C. The immunocomplex was precipitated with protein A sepharose for 1 h at 4°C and washed three times with lysis buffer, twice with LiCl buffer, and twice with TNE buffer (10 mmol/l Tris, 100 mmol/l NaCl, and 1 mmol/l EDTA), and then the immunoprecipitated enzymes were subjected to a kinase assay by competitive ELISA, according to the manufacturer's instructions (Echelon Biosciences, Salt Lake City, UT).

Mercury contents. The levels of mercury in blood were determined by cold vapor atomic absorption spectrophotometer combined with the flow-injection analysis system (34), and analysis was performed with a PerkinElmer 5100PC AAS equipped with a PerkinElmer FIAS-200 flow-injection analysis system and AA WinLab software. The detection limit for mercury was ~0.1 ppb (μg/l).

Statistics. The values in the text are given as means ± SE. 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 ANOVA. Statistical difference was indicated by $P < 0.05$.

RESULTS

Effects of low-concentration mercury on ROS formation and insulin secretion and the involvement of PI3K/Akt in HIT-T15 cells. To understand whether mercury-induced oxidative stress could affect the function of pancreatic β-cells, we investigated the in vitro effects of low-concentration $HgCl_2$ and MeHg (0.2–1 μmol/l) on the β-cell–derived HIT-T15 cells. Exposure of cells to mercury for 24 h did not affect cell viability (data not shown). Measurement of DCF fluorescence as an indicator of ROS formation, after exposure of HIT-T15 cells to 0.5 and 1

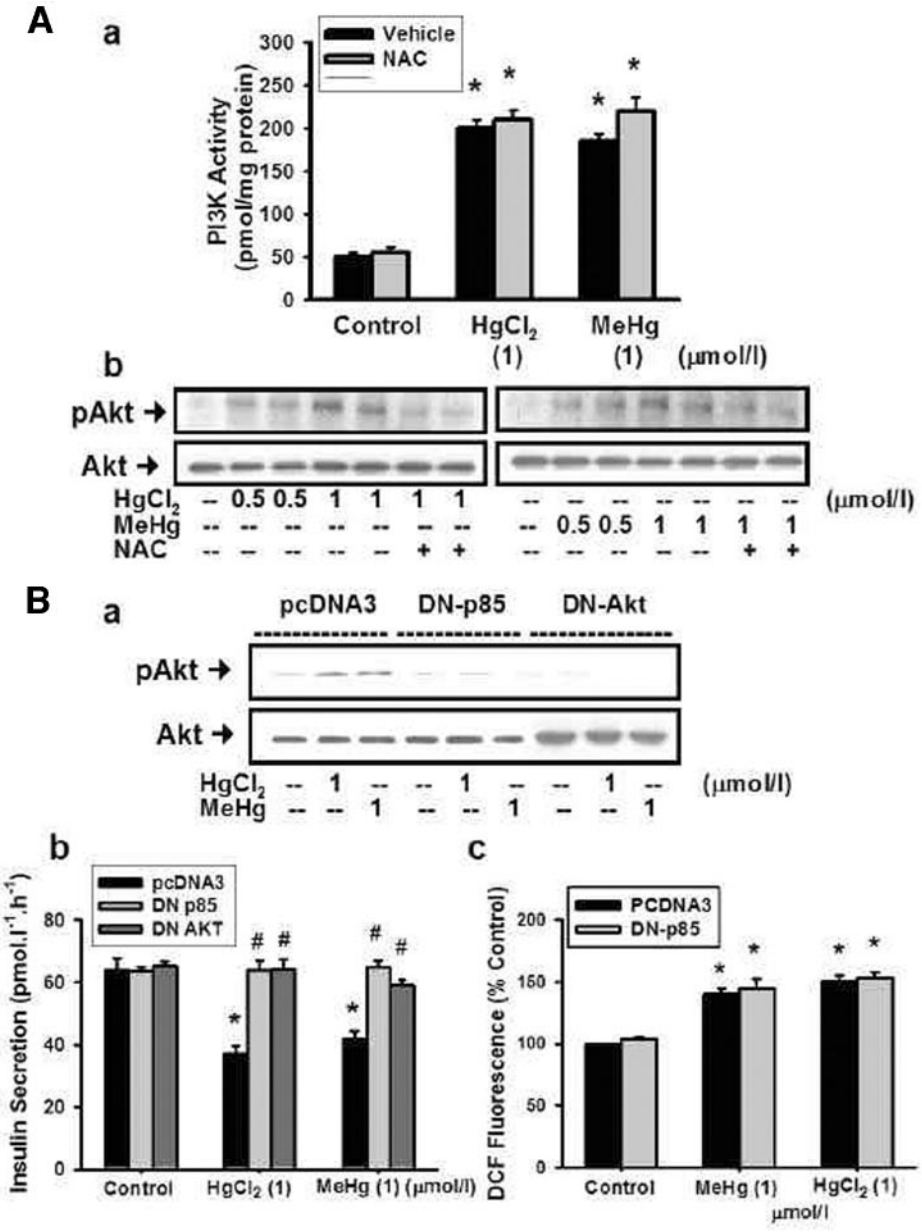

FIG. 3. Mercury-activated PI3K/Akt signaling pathway in HIT-T15 cells. *A*: Mercury-activated PI3K activity (*a*) and Akt phosphorylation (*b*). Cells were treated with $HgCl_2$ or MeHg (0.5 and 1 μmol/l, respectively) in the presence or absence of NAC (1 mmol/l) for 5 min (*a*) or 1 h (*b*). In *a*, data are presented as means ± SE for four independent experiments with triplicate determinations. **P* < 0.05 compared with control. In *b*, results shown are representative of four independent experiments. *B*: Mercury-induced insulin secretion inhibition requires the activation of PI3K/Akt signaling in HIT-T15 cells. Cells were transiently transfected with pcDNA3-control, DN-p85, or DN-Akt and then cells were treated with or without $HgCl_2$ (1 μmol/l) or MeHg (1 μmol/l) for 1h (*a* and *c*) or 4 h (*b*). In *a*, the phosphorylation of Akt protein was detected. Results shown are representative of four independent experiments. In *b*, insulin secretion in HIT-T15 cells under 16.7 mmol/l glucose. In *c*, ROS was determined by flow cytometry as described in RESEARCH DESIGN AND METHODS. Data are presented as means ± SE for four independent experiments with triplicate determinations. **P* < 0.05 compared with control; #*P* < 0.05 compared with mercury alone.

μmol/l mercury for 1 h, significantly increased ROS levels, which were inhibited by the antioxidant NAC (1 mmol/l) (Fig. 1*A*). Furthermore, measurement of insulin secretion from HIT-T15 cells after exposure to mercury under various concentrations of glucose (5.5–30 mmol/l) showed that $HgCl_2$ or MeHg (0.5 and 1 μmol/l, respectively) significantly inhibited insulin secretion (Fig. 1*B*). Cells treated with NAC effectively prevented the inhibition of insulin secretion in HIT-T15 cells exposed to mercury (Fig. 2*A*). The PI3K inhibitor LY294002 (5 μmol/l) also effectively prevented the mercury-induced inhibition of insulin secretion in HIT-T15 cells (Fig. 2*B*). In a 4-h incubation, 5 μmol/l LY294002 alone did not affect the insulin secretion; however, it was capable of increasing the insulin secretion in HIT-T15 cells at concentration of 10 μmol/l (131.6 ± 1.2% of control, $n = 5$).

To further evaluate the involvement of PI3K/Akt signaling in the responses triggered by mercury-induced oxidative stress, the PI3K and Akt activities in HIT-T15 cells were examined by determining the PI3K activity and phosphorylated status of Akt. Immunoprecipitates with the anti-p85 antibody revealed a substantial increase in PI3K activity 5 min after the exposure of mercury (1 μmol/l) (Fig. 3*A*, *a*). The significant activation of Akt was also shown in mercury-treated cells (Fig. 3*A*, *b*). LY294002 markedly reduced the PI3K activity and phosphorylation of Akt induced by mercury in HIT-T15 cells (data not shown). NAC effectively inhibited the mercury-induced Akt phosphorylation (Fig. 3*A*, *b*) but not the increased PI3K activity (Fig. 3*A*, *a*).

The next aim of the investigation was to ascertain whether inhibition of PI3K/Akt activities might affect the mercury-induced inhibition of insulin secretion in HIT-T15 cells. To address this issue, cells were transfected with a dominant-negative form of the regulatory subunit (p85) of PI3K (DN-p85) or a dominant-negative form of Akt (DN-Akt) (35). As shown in Fig. 3*B*, *a*, DN-p85 or DN-Akt transfection markedly decreased the phosphorylation of Akt. The levels of Akt protein expression in DN-Akt–transfected cells were increased compared with control. Blocking the PI3K and Akt

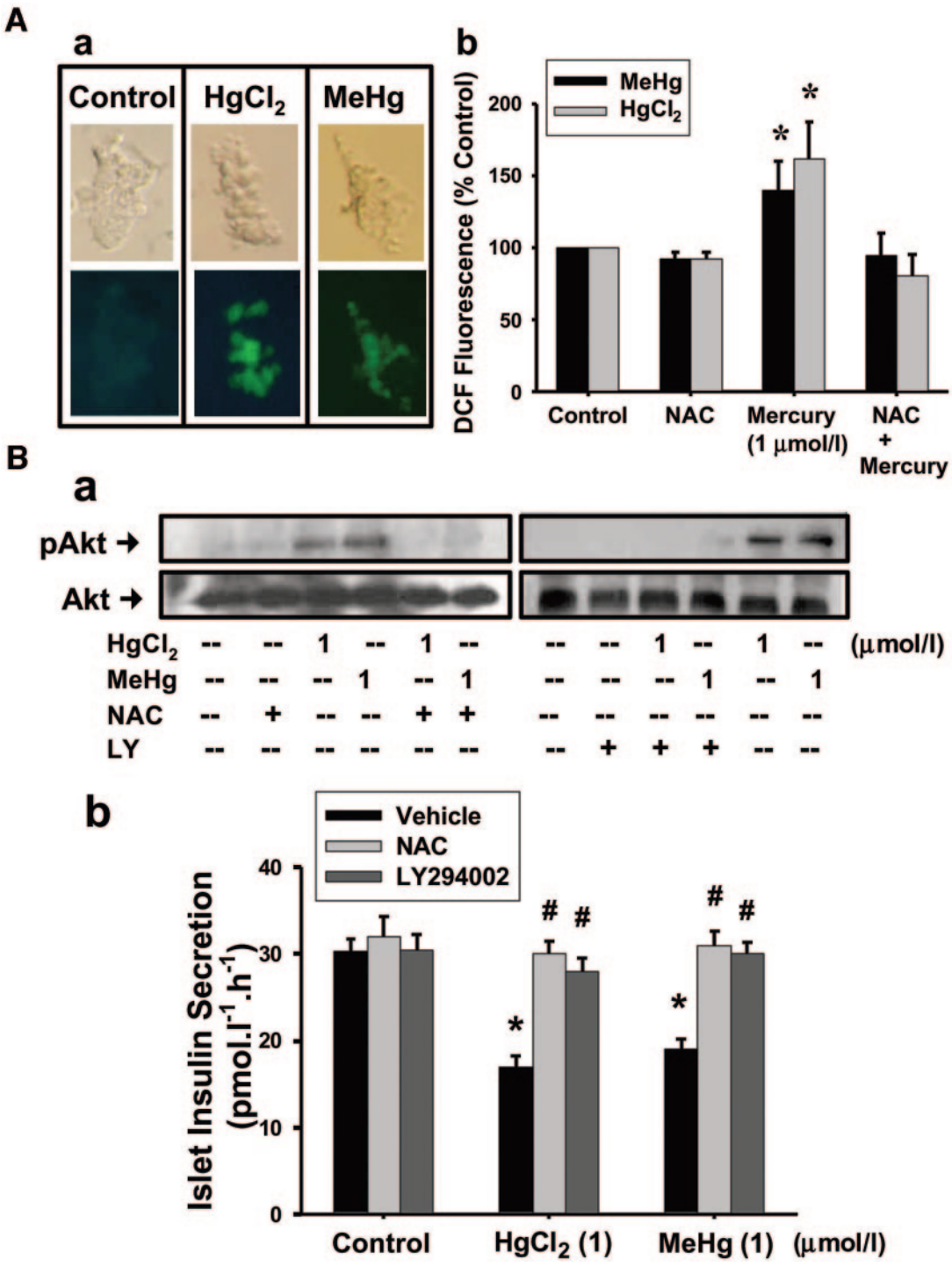

FIG. 4. Mercury-triggered ROS generation, Akt phosphorylation, and insulin secretion inhibition in isolated mouse islets. *A*: Islets were treated with or without $HgCl_2$ or MeHg (1 μmol/l) for 1 h in the presence or absence of NAC (1 mmol/l). *a*: Fluorescence of DCF-DA in islets 1 h after $HgCl_2$ or MeHg (1 μmol/l) treatment (*upper panel*, transmitted light images; *lower panel*, fluorescence images). Results shown are representative of three independent experiments. *b:* Intracellular peroxide levels in islets were determined by flow cytometry as described in RESEARCH DESIGN AND METHODS. Results are expressed as mean fluorescence intensity. Group data from at least three independent experiments are shown as means ± SE. *$P < 0.05$ compared with control. *B*: Islets were treated with or without $HgCl_2$ or MeHg (1 μmol/l) for 1 h (*a*) or 4 h (*b*) in the presence or absence of NAC (1 mmol/l) or LY294002 (5 μmol/l). *a:* Cellular lysates were prepared to perform electrophoresis and immunoblotting as described in RESEARCH DESIGN AND METHODS utilizing a specific anti–phospho-Akt antibody or anti-Akt antibody. Results shown are representative of four independent experiments. *b:* Mercury-induced insulin secretion inhibition in islets under 16.7 mmol/l glucose. Data are presented as means ± SE for four independent experiments with triplicate determinations. *$P < 0.05$ compared with control; #$P < 0.05$ compared with mercury alone.

activities with DN-p85 and DN-Akt greatly prevented the 1 μmol/l mercury–triggered insulin secretion inhibition (Fig. 3*B*, *b*). The control pcDNA3 transfection did not affect the mercury-induced responses. However, blocking the PI3K activity with DN-p85 did not affect ROS generation by mercury (Fig. 3*B*, *c*). Therefore, these results imply that both PI3K and ROS independently regulated Akt signaling–related mercury-induced insulin secretion inhibition.

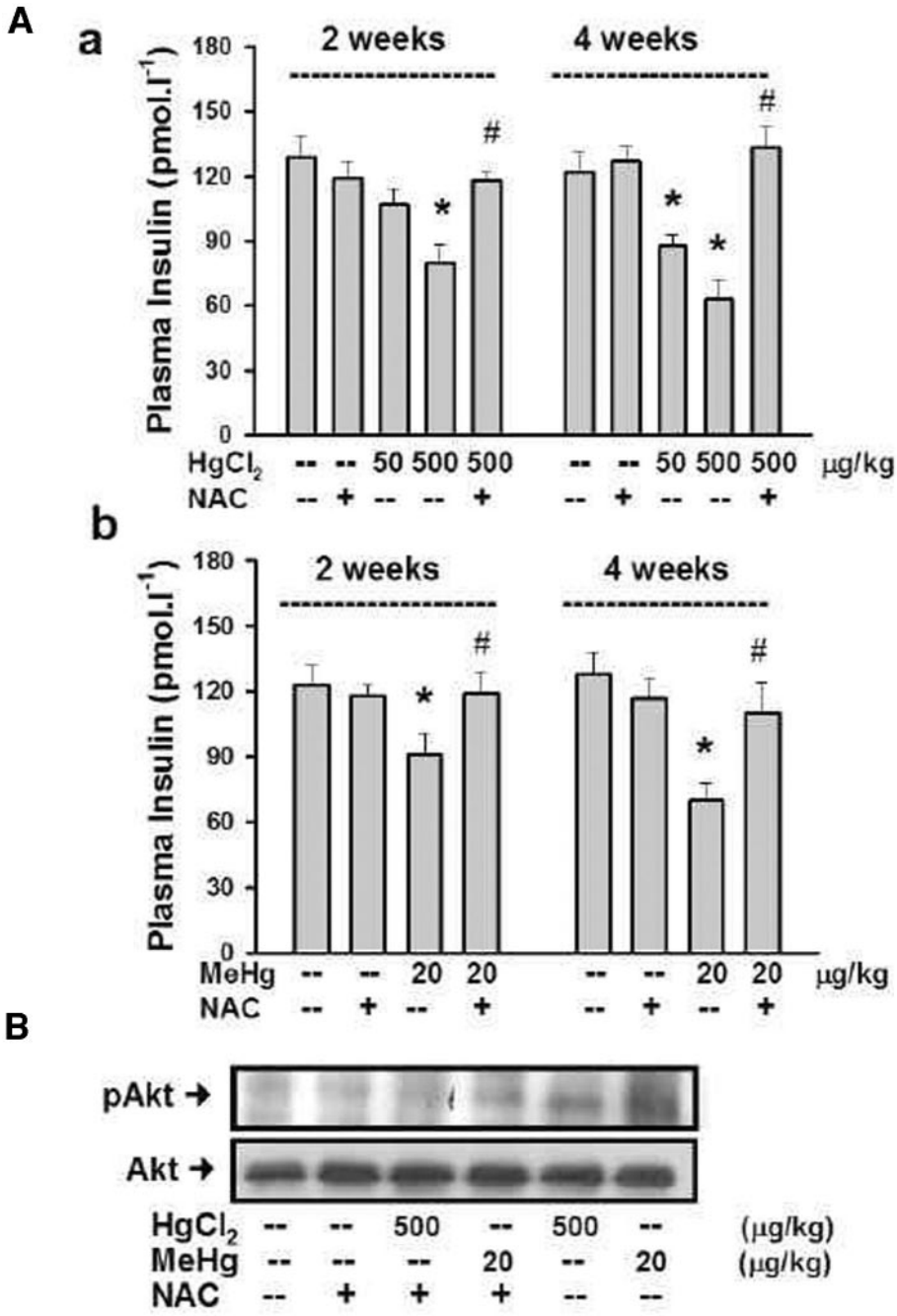

FIG. 5. Plasma insulin levels and islet Akt phosphorylation in mercury-exposed mice. *A*: Mice were orally gavaged with 50 or 500 μg · kg^{-1} · day^{-1} $HgCl_2$ or 20 μg · kg^{-1} · day^{-1} MeHg for 2–4 weeks. Plasma insulin levels were determined as described in RESEARCH DESIGN AND METHODS (*a*, $HgCl_2$; *b*, MeHg). All data are presented as means ± SE; $n = 16$ for all groups. *$P < 0.05$ compared with control; #$P < 0.05$ compared with mercury alone. *B*: Mercury-triggered phosphorylation of Akt protein in islets isolated from mercury-exposed mice. Mice were orally gavaged with 500 μg · kg^{-1} · day^{-1} $HgCl_2$ or 20 μg · kg^{-1} · day^{-1} MeHg for 2 weeks. Results shown are representative of three independent experiments. In some experiments, the effects of NAC (150 mg/kg) on the $HgCl_2$ (500 μg/kg)– or MeHg (20 μg/kg)–induced alteration of insulin secretion levels (*A*) and islet Akt phosphorylation (*B*) were detected.

Effects of low-concentration mercury on ROS formation and insulin secretion and the involvement of PI3K/Akt in isolated mouse islets. Exposure of isolated mouse islets to 1 μmol/l mercury for 1 h increased DCF-sensitive ROS formation, which was prevented by 1 mmol/l NAC (Fig. 4). The role of PI3K/Akt signaling was further investigated. A significant phosphorylation of Akt in isolated mouse islets was shown 1 h after the treatment of mercury (1 μmol/l) (Fig. 4*B*, *a*). NAC and LY294002 (5 μmol/l) markedly reversed the increase of Akt phosphorylation by mercury (Fig. 4*B*, *a*). Furthermore, mercury (1 μmol/l) was capable of inhibiting the insulin secretion in mouse islets, which could also be reversed by NAC and LY294002 (Fig. 4*B*, *b*).

Effects of low-dose mercury on blood glucose, plasma insulin secretion, plasma lipid peroxidation, and islet Akt phosphorylation in mice. The next aim of the investigation was to ascertain whether mercury-induced oxidative stress might affect the function of pancreatic β-cells in vivo and the involvement of PI3K/Akt signaling. A 2- or 4-week exposure to low-dose $HgCl_2$ (50 or 500 μg · kg^{-1} · day^{-1}) or MeHg (20 μg · kg^{-1} · day^{-1}) (oral application by gavage) to mice caused a significant decrease in plasma insulin levels (Fig. 5*A*). Moreover, a significant phosphorylation of Akt was shown in islets isolated from mice exposed to mercury for 2 weeks (Fig. 5*B*). Mercury-exposed mice also showed an elevation in blood glucose (Fig. 6*A*) and glucose intolerance (Fig. 6*B*). Moreover, the result of plasma lipid peroxidation assay after exposure to $HgCl_2$ or MeHg to mice showed that plasma malondialdehyde levels were significantly increased at 2–4 weeks (Fig. 7). These mercury-triggered

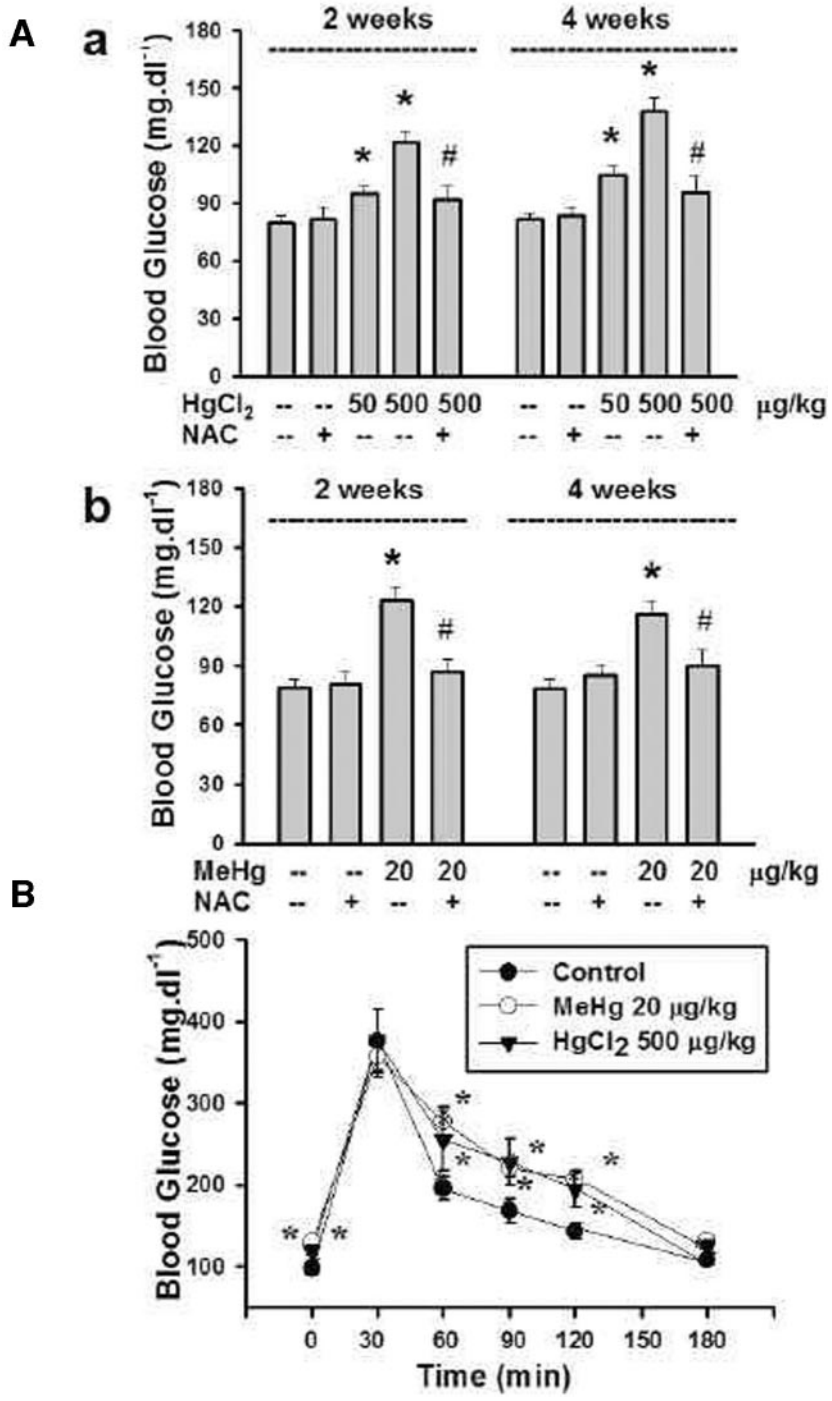

FIG. 6. Blood glucose and glucose tolerance test in mercury-exposed mice. *A*: Mice were orally gavaged with 50 and 500 μg · kg^{-1} · day^{-1} $HgCl_2$ (*a*) or 20 μg · kg^{-1} · day^{-1} MeHg (*b*) for 2–4 weeks. The blood glucose levels were determined as described in RESEARCH DESIGN AND METHODS. In some experiments, the effect of NAC (150 mg/kg) on the $HgCl_2$ (500 μg/kg)– or MeHg (20 μg/kg)–induced alteration of blood glucose levels was detected. *B*: Oral glucose tolerance was determined as described in RESEARCH DESIGN AND METHODS. Oral glucose tolerance tests were carried out in mice given vehicle or $HgCl_2$ (500 μg · kg^{-1} · day^{-1}) or MeHg (20 μg · kg^{-1} · day^{-1}) for 4 weeks. All data are presented as means ± SE; n = 16 for all groups. *P < 0.05 compared with control; #P < 0.05 compared with mercury alone.

responses in mice could also be reversed by the treatment of NAC (150 mg/kg) (Figs. 5–7).

Blood mercury and insulin secretion and blood glucose in mice after $HgCl_2$ or MeHg exposure was terminated. Mice exposed to mercury over a 2- to 4-week period had elevated blood mercury levels ranging from ~4.5 to 60 μg/l (Fig. 8*A*). There was a similar blood mercury range for treatment with 500 μg/kg $HgCl_2$ and 20 μg/kg MeHg. The elevated blood mercury and decreased plasma insulin and elevated blood glucose levels were gradually reversed after mercury withdrawal (Fig. 8*A*, blood mercury; Fig. 8*B*, plasma insulin; blood glucose [mg/dl]: $HgCl_2$ 4 weeks 144.2 ± 8.4, withdrawal 2 weeks 112.4 ± 5.8, withdrawal 4 weeks 89.7 ± 6.7, n = 8; MeHg 4 weeks 122.1 ± 7.7, withdrawal 2 weeks 93.6 ± 5.4, withdrawal 4 weeks 82.9 ± 4.2, n = 8; age-matched controls 81.6 ± 4.9, n = 8).

There were no clinical signs and grossly detectable lesions (including brain, liver, kidney, lung, heart, adrenal, gonad, and intestine) related to the administration of mercury for 2–4 weeks in mice, and there was no effect on whole-body weight (data not shown).

DISCUSSION

Oxidative stress has been demonstrated to be produced under diabetic conditions and was likely involved in the progression of pancreatic β-cell dysfunction found in

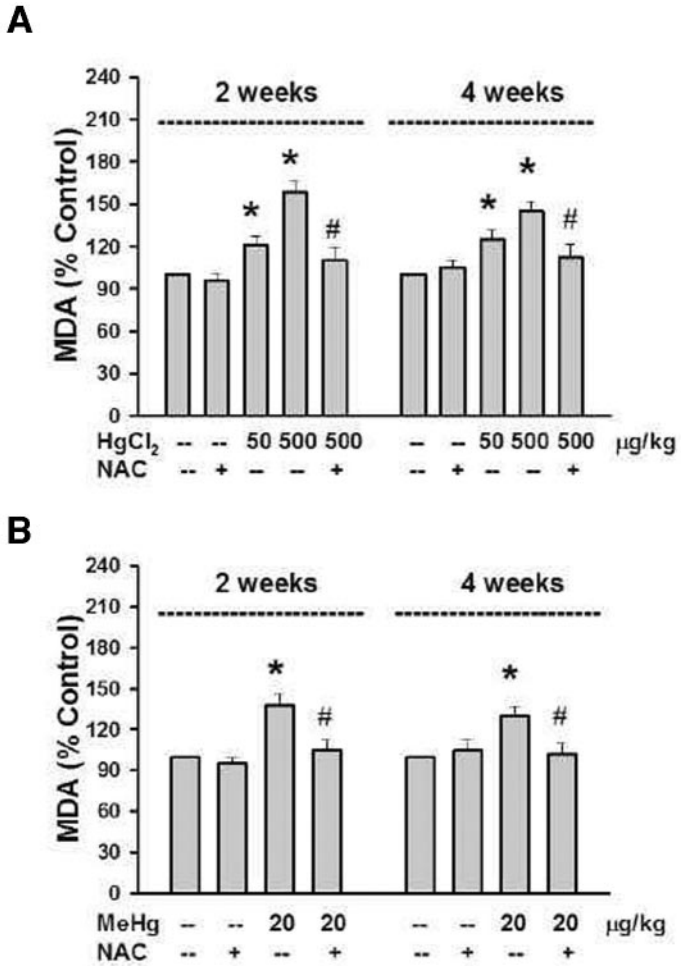

FIG. 7. Plasma lipid peroxidation in mercury-exposed mice. Mice were orally gavaged with 50 and 500 $\mu g \cdot kg^{-1} \cdot day^{-1}$ $HgCl_2$ or 20 $\mu g \cdot kg^{-1} \cdot day^{-1}$ MeHg for 2–4 weeks. Plasma malondialdehyde (MDA) levels in $HgCl_2$ (*A*)- or MeHg (*B*)-exposed mice were determined as described in RESEARCH DESIGN AND METHODS. In some experiments, the effects of NAC (150 mg/kg) on the $HgCl_2$ (500 μg/kg)– or MeHg (20 μg/kg)–induced alteration of MDA levels were detected. All data are presented as means ± SE; $n = 16$ for all groups. *$P < 0.05$ compared with control; #$P < 0.05$ compared with mercury alone.

diabetes (3,36). It has been demonstrated that mitochondrial ROS suppressed glucose-induced insulin secretion from β-cells (2). Despite several studies showing that mercury could induce oxidative stress with subsequent oxidative damage in several organs or systems (16–19,37), the precise action and mechanism of mercury-induced oxidative stress on the pancreatic β-cell function is unknown. To investigate the effects of mercury-induced oxidative stress on the function of pancreatic β-cells, we first investigated the in vitro effects of $HgCl_2$ and MeHg on the β-cell–derived HIT-T15 cells and isolated mouse islets. Our results showed that submicromolar-concentration exposure to mercury was capable of triggering ROS production and decreasing insulin secretion in HIT-T15 cells and isolated islets, which could be prevented completely by treatment of cells with antioxidant NAC, indicating that ROS was involved in mercury-induced β-cell insulin secretion inhibition. On the other hand, changes in glucose levels to insulin secretion are related to intracellular ATP levels. Following glucose entry into pancreatic β-cells, products of glucose metabolism enter the mitochondrial respiratory chain, which uses them to generate ATP. The increase in ATP inhibits ATP-sensitive potassium channels, which in turn stimulate insulin secretion (38). Here we found that increasing doses of mercury did not change the intracellular ATP levels in HIT-T15 cells after exposure to 0.2–1 μmol/l $HgCl_2$ or MeHg at 1–4 h (data not shown), and hence, it may not be a primary toxic mechanism in dysfunction of pancreatic β-cells induced by low-concentration mercury. Next, we investigated the in vivo effects of mercury-induced oxidative stress on the insulin secretion and blood glucose regulation in mice. The results showed that a 2- or 4-week oral exposure to low-dose mercury caused an increase in plasma lipid peroxidation levels, a decrease in plasma insulin levels, and an elevation in blood glucose and glucose intolerance. NAC could prevent these mercury-induced responses. These findings indicate that oxidative stress is involved in the toxic mechanism in mercury-induced hypoinsulinemia and hyperglycemia. In addition, it has been found that endogenous nitric oxide (NO) production could be stimulated by glucose and implicated this glucose-stimulated NO production in physiological glucose-stimulated insulin secretion (32). Whether dysfunction or deregulation of the NO system is involved in mercury-induced β-cell damage may need to be clarified in the future.

Some studies (6,7) have suggested that as insulin resistance developed in peripheral tissues, a potential result of impaired PI3K activation in β-cells promoted a linked increase in insulin secretion to maintain glucose homeostasis. Eto et al. (39) have also indicated that PI3K suppressed glucose-stimulated insulin secretion by affecting postcytosolic Ca^{2+} elevation signals. It has been shown that redox stress regulated cell proliferation and apoptosis of human hepatoma through PI3K/Akt pathway (40). Angiotensin II–induced Akt phosphorylation has been demonstrated to be mediated by ROS, which contributed to angiotensin II–induced hypertrophy in vascular smooth muscle cells (10). However, the role of PI3K/Akt signaling pathway in the oxidative stress–induced pancreatic β-cell dysfunction is still unclear. The present work showed that low-dose mercury could enhance the phosphorylation of Akt, which was reversed by NAC. Moreover, the inhibition of PI3K/Akt activity with the PI3K inhibitor LY294002, or by expressing the dominant-negative p85 or Akt, effectively attenuated the inhibition of insulin secretion induced by mercury. LY294002 was capable of increasing the insulin secretion in HIT-T15 cells at a concentration of 10 μmol/l. However, unexpectedly, we found that NAC could not inhibit mercury-increased PI3K activity, and the inhibition of PI3K activity by expressing the dominant-negative p85 also could not suppress mercury-induced ROS production in β-cells. Therefore, these results implicate that both PI3K and ROS independently regulate Akt signaling–related, mercury-induced insulin secretion inhibition and suggest that low-dose mercury–induced oxidative stress and PI3K activation cause pancreatic β-cell dysfunction through an Akt signaling pathway.

A recent study of mercury exposure in an urban pediatric population has shown that mean urinary mercury was 1.08 ± 1.82 μg/l (range 0.2–11.7) and found that 5% of subjects had unsuspected elevated urinary mercury levels (>5 μg/l) (41). The Food and Drug Administration advises women to limit the consumption of fish with up to 0.5 ppm (μg/ml) mercury to 14 oz/week or with up to 1.0 ppm mercury to 7 oz/week (42). Recently, the Wisconsin Department of Natural Resources lowered its mercury in fish

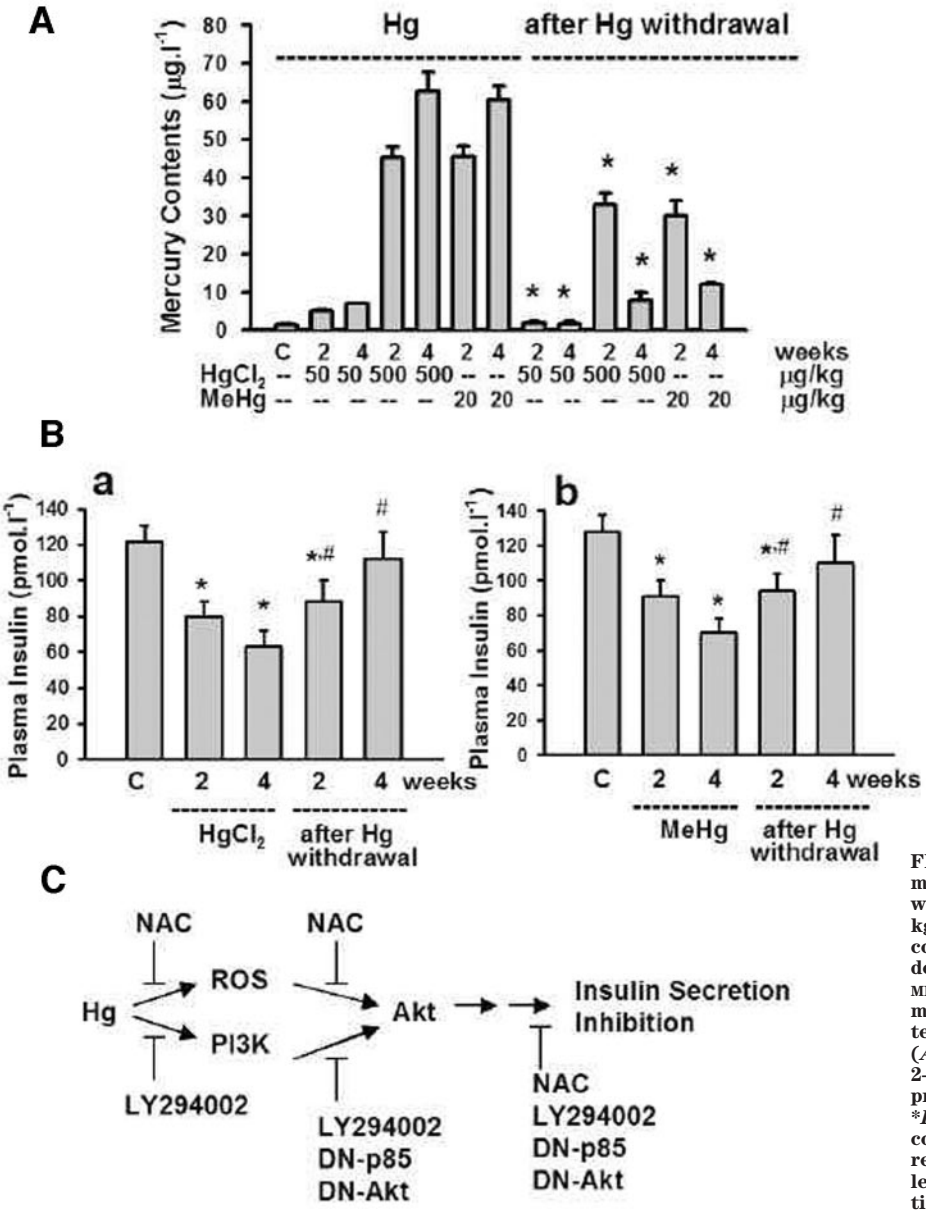

FIG. 8. Blood mercury and plasma insulin levels in mercury-exposed mice. Mice were orally gavaged with 50 and 500 μg · kg^{-1} · day^{-1} $HgCl_2$ or 20 μg · kg^{-1} · day^{-1} MeHg for 2–4 weeks. Blood mercury contents (*A*) and plasma insulin levels (*B*) were determined as described in RESEARCH DESIGN AND METHODS. In some experiments, mice were treated mercury for 4 weeks and then the exposure was terminated. The observations of mercury contents (*A*) and plasma insulin levels (*B*) were conducted 2–4 weeks after mercury withdrawal. All data are presented as means ± SE; *n* = 16 for all groups. **P* < 0.05 compared with control; #*P* < 0.05 compared with mercury alone. *C*: Schematic representation of proposed intracellular signaling leading to mercury-induced β-cell insulin secretion inhibition.

action level by a factor of 10× to 0.05 ppm based on subtle alterations in neurodevelopment in children born to mothers who eat large amounts of fish (43). A study (44) of blood mercury levels in workers exposed to the preparation of mercury cadmium telluride layers has shown that the mercury value was estimated at 1.60 ± 0.20 μg Hg/l in control and at 10.72 ± 1.34 μg Hg/l in phase I and 8.08 ± 1.15 μg Hg/l in phase II; an individual who met with a mercury accident showed 226 μg Hg/l of blood, which decreased to 25 μg Hg/l after 3 months. It has been reported that sensitive nonhuman mammals showed significant adverse effects of mercury when dietary levels were 1,100 μg/kg body wt (45). The study of Nakagawa has shown that the total mercury concentrations in the hair of ordinary diseased people, including diabetes, were from 2.08 to 36.5 ppm; those values were considerably higher than that of healthy people of the same age-groups (46). In the present study, we used low doses of inorganic mercury (50 and 500 μg/kg) and organic methylmercury (20 μg/kg) to expose mice. The blood mercury content ranges are ~3.9–5.8 and 38.4–72.2 μg/l in 50 and 500 μg · kg^{-1} · day^{-1} $HgCl_2$–treated mice, respectively, or 40.2–64.6 μg/l in 20 μg · kg^{-1} · day^{-1} MeHg–treated mice, which were exposed over a 2- to 4-week period (0.83–1.62 μg/l in age-matched controls). Moreover, no evidence of mercury-induced target organ toxicity was seen in either the clinical pathology parameters or histomorphologic evaluations. Thus, the increased blood mercury levels in the present study approach the human-exposed levels.

An increased incidence of diabetes has been found to exist in patients with documented Minamata disease (blood mercury >200 μg/l [20]) in Japan (26). Takeuchi and Eto (47) reported that disturbance of pancreatic islet cells were found in autopsy cases of Minamata disease. Nevertheless, a health-surveillance study in the population living in a methylmercury-polluted area ~30 years after the outbreak of Minamata disease by Futatsuka et al. (48) showed that the prevalence of liver disease, renal disease, and diabetes was not higher in this methylmercury-polluted area compared with other areas in Japan, contrary to what was expected based on standard mortality ratios and pathological findings. However, the results of Sakamoto et

al. (49) and Harada et al. (50) showed that no significant difference in the mercury concentrations in the erythrocytes and scalp hair was observed between the cases with Minamata disease and the controls about several decades after the outbreak of Minamata disease. Around 1960, marine products were heavily polluted by methylmercury in the Minamata area. When the cause of Minamata disease had already proved to be methylmercury-contaminated marine products, the residents on the local coast were hesitant to eat those marine products, so the patient's mercury levels were gradually reduced (50). These findings may explain why there are no positive correlations between those diseases and methylmercury exposure ~30 years after the outbreak of Minamata disease, as indicated by the study of Futatsuka et al. (48). These findings also imply that some diseases induced by methylmercury exposure may be reversible if the exposure was terminated. Our present results support this possibility; we found that the elevated blood mercury, decreased plasma insulin, and elevated blood glucose levels in mercury-treated mice were gradually reversed after mercury exposure was terminated. Thus, the present study indicates that low-dose mercury is capable of altering blood insulin and glucose homeostasis, although individuals may tend to ignore this risk of long-term exposure to low-dose mercury.

In conclusion, as indicated in Fig. 8*C*, our results strongly suggest an essential and specific role for the Akt signaling in low-dose mercury–induced, oxidative stress–caused pancreatic β-cell dysfunction. Moreover, low-dose mercury–induced oxidative stress causes pancreatic islet Akt activation and induces hypoinsulinemia and hyperglycemia in mice in the absence of overt mercury toxicity. These observations also give further evidence to confirm the possibility that mercury is an environmental risk factor for diabetes.

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