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Original Contribution

Methylmercury neurotoxicity is associated with inhibition of the antioxidant enzyme glutathione peroxidase

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ABSTRACT

In this study, we investigated the involvement of glutathione peroxidase—GPx in methylmercury (MeHg)-induced toxicity using three models: (a) in mouse brain after treatment with MeHg (40 mg/L in drinking water), (b) in mouse brain mitochondrial-enriched fractions isolated from MeHg-treated animals, and (c) in cultured human neuroblastoma SH-SY5Y cells. First, adult male Swiss mice exposed to MeHg for 21 days showed a significant decrease in GPx activity in the brain and an increase in poly(ADP-ribose) polymerase cleavage, an index of apoptosis. Second, in mitochondrial-enriched fractions isolated from MeHg-treated mice, there was a significant reduction in GPx activity and a concomitant decrease in mitochondrial activity and increases in ROS formation and lipid peroxidation. Incubation of mitochondrial-enriched fractions with mercaptosuccinic acid, a GPx inhibitor, significantly augmented the toxic effects of MeHg administered in vivo. Incubation of mitochondrial-enriched fractions with exogenous GPx completely blocked MeHg-induced mitochondrial lipid peroxidation. Third, SH-SY5Y cells treated for 24 h with MeHg showed a significant reduction in GPx activity. There was a concomitant significant decrease in cell viability and increase in apoptosis. Inhibition of GPx substantially enhanced MeHg toxicity in the SH-SY5Y cells. These results suggest that GPx is an important target for MeHg-induced neurotoxicity, presumably because this enzyme is essential for counteracting the pro-oxidative effects of MeHg both in vitro and in vivo.

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Methylmercury (MeHg) is a major environmental contaminant and a highly neurotoxic agent, which causes neurological and developmental deficits in animals and humans [1,2]. Serious poisoning episodes, including those in Japan and Iraq during the 1950s and 1970s, led to acute MeHg intoxication, causing severe nervous system damage to thousands of local inhabitants [3]. Environmental incidents like those in Japan and Iraq have not been reported recently; however, chronic exposure to methylmercury, due to industrial, agricultural, and mining activity, still raises concerns about the risks to human health of MeHg [4,5]. In particular, in the Amazon river and the surrounding regions in northern Brazil, gold mining activity causes intense environmental and human contamination with both organic and inorganic forms of mercury. This is considered to be a significant health problem [6]. In addition, the release of mercuric compounds into marine and freshwater ecosystems leads to bioaccumulation in fish, and MeHg-contaminated fish can be a major source of human intoxication [1]. Understanding the exact molecular mechanisms of MeHg toxicity is therefore of fundamental interest and will contribute to the development of new treatment strategies.

The main target for MeHg toxicity is the central nervous system (CNS), where it causes alterations in the ultrastructural and biochemical machinery of both neurons and astrocytes [7–9]. There is substantial evidence demonstrating the adverse effects of MeHg in a wide range of cellular processes, including neurochemical, neuroendocrine, and electrophysiological changes in cell function [3]. MeHg has been demonstrated to alter glutamatergic [8,10], GABAergic [11], monoaminergic [12–14], and cholinergic [15] neurotransmission systems. It is also known that MeHg leads to an impairment of intracellular calcium homeostasis [16,17].

Generation of reactive oxygen species (ROS) is a major factor in MeHg toxicity [18,19]. Oxidative stress elicited by MeHg is related to its interaction with intracellular thiols, such as glutathione (GSH) [20,21]. Previous reports from our group suggested that the glutathione antioxidant system could be a possible target for MeHg neurotoxicity [22–24].

The glutathione antioxidant system includes reduced glutathione (the most important low-molecular-weight sulfhydryl-containing antioxidant) and the GSH-related enzymes glutathione peroxidase (GPx; EC 1.11.1.9) and glutathione reductase (GR; EC 1.8.1.7) [25]. Mammalian cells contain five isoforms of selenium-dependent GPx's: cytosolic GPx (GPx1), gastrointestinal GPx (GPx2), plasma GPx (GPx3),

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phospholipid hydroperoxide GPx (GPx4), and, in humans, GPx6, expressed only in the olfactory system [26]. GPx1, also called cytosolic or cellular GPx, is the most prominent GPx isoform and it is able to reduce hydrogen peroxide and a range of organic peroxides, including phospholipid hydroperoxide and long-chain fatty acid peroxides, by expending GSH [27]. GPx1 is very specific for GSH as a reducing substrate. It utilizes the combined action of GPx and GR to convert oxidized glutathione (GSSG) to its reduced form via a β-nicotinamide adenine dinucleotide phosphate sodium salt, reduced form (NADPH)-dependent mechanism. This is a major antioxidant system in the CNS [25]. There is increased susceptibility to oxidative stress in animals lacking GPx1 [28,29], whereas overexpression of GPx1 is shown to have beneficial affects against pro-oxidative conditions, both in animals [30–32] and in cell culture models [33,34].

A decrease in GPx activity renders cells susceptible to oxidative stress. GPx can be inhibited by nitric oxide [35], homocysteine [36], and a range of mercaptans [37]. In particular, our group has documented the inhibition of GPx by mercury compounds in vivo [22–24]. Because GPx represents a major enzyme in the cellular machinery leading to peroxide detoxification and defense against oxidative stress, and MeHg is known to induce peroxide generation by cells, we aimed to investigate the participation of GPx in the toxicity induced by MeHg using both in vivo and in vitro approaches. In this study, we show that MeHg inhibits the activity of GPx in the mouse brain, in isolated mouse brain mitochondrial-enriched fractions, and in cultured SH-SY5Y cells. This phenomenon is concomitant with alterations in oxidative stress and apoptotic markers.

Materials and methods

Chemicals

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) reagents were from Bio-Rad Laboratories (Hercules, CA, USA). Molecular-weight PAGE standards, nitrocellulose membrane (Hybond ECL), ECL Plus kit, and anti-rabbit immunoglobulin (horseradish peroxidase-linked whole antibody from donkey) were from GE HealthCare (Little Chalfont, UK). MeHg, mercaptosuccinic acid (MS), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), β-actin antibody, MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide), GSH, NADPH, 5-5′-dithiobis-(2-nitrobenzoic) acid, GR from baker's yeast, catalase, superoxide dismutase, GPx, *tert*-butylhydroperoxide (TBOOH), Triton X-100, and xylenol orange were from Sigma Chemical Co. (St. Louis, MO, USA). Poly(ADP-ribose) polymerase 1 (PARP-1) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). GSSG was obtained from Fluka (Steinheim, Switzerland). Tissue culture reagents were from Sigma Chemical Co. and were of analytical or tissue culture grade.

Animals and treatments

Male Swiss mice (2–3 months of age) were treated with a dose of 40 mg/L MeHg diluted in tap water and they drank this MeHg solution for 21 days. This protocol increases the level of MeHg in mouse brain and induces locomotor deficits on the rotarod and open-field tasks [38,22,24].

Isolation of mitochondrial-enriched fractions

Mitochondrial-enriched fractions were prepared essentially as described previously [39]. Twenty-four hours after MeHg treatment was finished, mouse brains were removed and homogenized on ice in 10 vol of isolation medium (10 mM Hepes buffer, pH 7.0, containing 220 mM mannitol, 68 mM sucrose, 10 mM KCl, and 0.1% serum albumin) and the homogenate was centrifuged at 4°C for 10 min at 1000 *g*. The supernatant was then centrifuged at 17,500 *g* for 10 min at 4°C, resulting in a myelin-rich supernatant and a pellet consisting of synaptosomes and free mitochondria. The supernatant was discarded, and the pellet was resuspended in the isolation medium without albumin. The mitochondrial-enriched fractions were kept on ice for no longer than 1 h until the experiments were performed.

Cell culture

The human neuroblastoma SH-SY5Y cell line (CRL-2266) was obtained from the American Type Culture Collection (http://www.atcc.org). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 g/ml streptomycin, 4 mmol/L l-glutamine, 15 mmol/L Hepes (pH 7.4). Cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C in complete medium that was changed every 3–4 days. After reaching about 80% confluence the cells were plated, 48 h before treatment, on plastic culture plates at a density of 4×10^4 (96-well plates), 40×10^4 (12-well plates), or 80×10^4 cells/well (6-well plates). The complete medium was changed to serum-free medium 1.5 h before treatment. After this period in serum-free medium, the cells were exposed for 24 h to concentrations of MeHg solubilized in sterile 0.1 mM phosphate-buffered saline (PBS). MS and BCNU (GPx and GR inhibitors) were added 24 h before the MeHg treatment. Cell were washed five times with PBS, harvested, and prepared for biochemical analysis. The MS concentration used (500 μM) did not change cell viability in any of the protocols used. The BCNU concentration used (10 μM) also did not change cell viability.

Cell viability and mitochondrial activity

The viability of the SH-SY5Y cells was measured using the CellTiter-Blue cell viability assay kit. The method uses the indicator dye resazurin to measure the metabolic capacity of cells, which correlates with cell viability. Viable cells will reduce resazurin into resorufin, a highly fluorescent compound. Nonviable cells have reduced ability to reduce the indicator dye and do not produce the fluorescent signal. Cells were plated into 96-well plated and subjected to various treatments. Subsequently, an aliquot of CellTiter-Blue was added to the cells, according to the manufacturer's instructions. After 1 h, the fluorescence was recorded using a fluorescence plate reader at 544 nm_{exc}/590 nm_{em}. Mitochondrial activity was assessed by incubation of the mitochondrial-enriched fraction with the metabolic probe MTT as previously described [39]. When viable, mitochondria convert the MTT to a colorful formazan, which can be detected at 550 nm.

Apoptosis assays

The apoptotic changes caused by MeHg were assessed by two procedures: analysis of PARP cleavage and the propidium iodide assay.

PARP cleavage

After treatment, samples were solubilized by addition of 2% SDS, 2 mM EDTA, 1% dithiothreitol, 10 mM glycerol, 50 mM Tris (pH 6.8). The proteins were separated by SDS–PAGE using 10% gels and then electrotransferred to nitrocellulose membranes. The membranes were washed in Tris-buffered saline with Tween (TBST) containing 100 mmol/L Tris–HCl, 0.9% NaCl, 0.1% Tween 20, pH 7.5, and incubated overnight (4°C) with PARP-1 primary antibody. Subsequently, membranes were washed in TBST and incubated for 1 h at room temperature with horseradish peroxidase-linked anti-IgG secondary specific antibody. The immunoblots were visualized on the Fuji LAS 3000 imaging system using ECL Plus detection reagents. β-actin immunocontent was used as protein loading control.

Propidium iodide assay

Apoptotic nuclei were determined according to Nicoletti et al. [40]. After treatment, cells were washed with PBS and collected into 12 × 75-mm polystyrene tubes and centrifuged at 200 *g*. Subsequently, 0.5 ml of a hypotonic buffer (50 μg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100) was added to the cell pellet and the tubes were kept in the dark at 4°C overnight before flow-cytometric analysis. The propidium iodide fluorescence of individual nuclei was measured in the red fluorescence using a FACScan flow cytometer (Becton–Dickinson, Mountain View, CA, USA). At least 10^4 cells were analyzed for each sample. Apoptotic nuclei appeared as a broad hypodiploid DNA peak, which was distinguishable from the narrow hyperdiploid peak of nuclei in the control untreated cells.

Antioxidant activity

The ability of SH-SY5Y cells to detoxify peroxides was determined as described by Dringen et al. [41] with minor modifications. Cells were cultured in six-well plates. A solution of 100 μM hydrogen peroxide was applied into the cell culture wells. Straight after peroxide was applied, an aliquot (10 μl) of medium was collected and peroxide was measured using the Amplex red assay kit according to the manufacturer's instructions. The peroxide amount present from this first measurement was used as the control for each well, representing the amount of peroxide at time 0 (zero). After 1 h, a second aliquot was collected and peroxide concentration was measured. The rate of decrease in peroxide concentration was normalized as the concentration of H_2O_2 degraded per minute per milligram of total protein.

To analyze the antioxidant enzyme activity, SH-SY5Y cells were harvested from six-well culture plates and lysed in 20 mM Hepes buffer (pH 7.0) by passing through a 26-gauge needle (Terumo Corp., Tokyo, Japan). Mouse brain was homogenized in 20 mM Hepes, pH 7.0 and mitochondrial fractions were lysed by freeze–thawing cycles in 20 mM Hepes, pH 7. The lysates were centrifuged at 20,000 *g* for 30 min at 4°C. The supernatants were then immediately assayed for GR and GPx activity as described previously [23]. Briefly, GR reduces GSSG to GSH, expending NADPH, the disappearance of which can be measured at 340 nm. GPx activity was measured indirectly by the NADPH consumption at 340 nm. GPx uses GSH to reduce the *tert*-butylhydroperoxide-producing GSSG, which is readily reduced to GSH by excess GR, consuming NADPH. The disappearance of NADPH in this reaction reflects the GPx activity. The enzyme activities were expressed in milliunits per milligram of total protein content, which was quantified using the BCA protein assay kit.

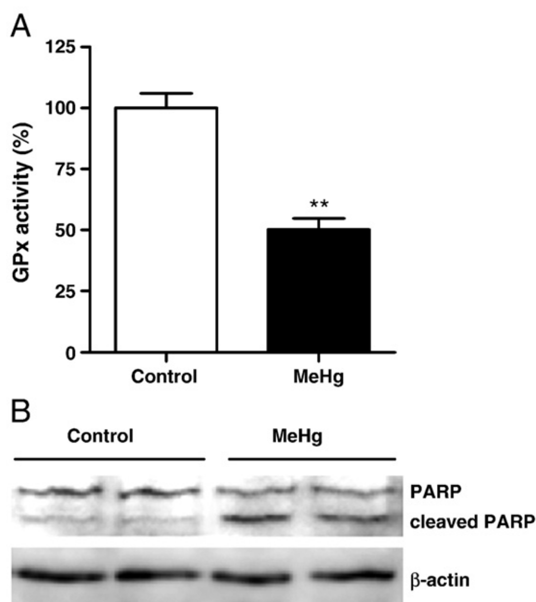

Fig. 1. Effects of MeHg exposure on GPx activity and apoptosis in mouse brain. Animals were orally treated with MeHg (40 mg/L in drinking water) for 21 days as described under Materials and methods. (A) When treatment was finished GPx activity was measured in mouse brain extracts. Activity is expressed as a percentage of control (tap water with no MeHg). Data are expressed as the means ± SD ($n = 6$). ** $p < 0.01$. (B) Apoptotic changes in mouse brain were assessed by poly(ADP-ribose) polymerase proteolysis. PARP cleavage can be observed in the MeHg-treated groups. The image is a representative blot of two separated experiments ($n = 4$). β-Actin was used as a protein content control.

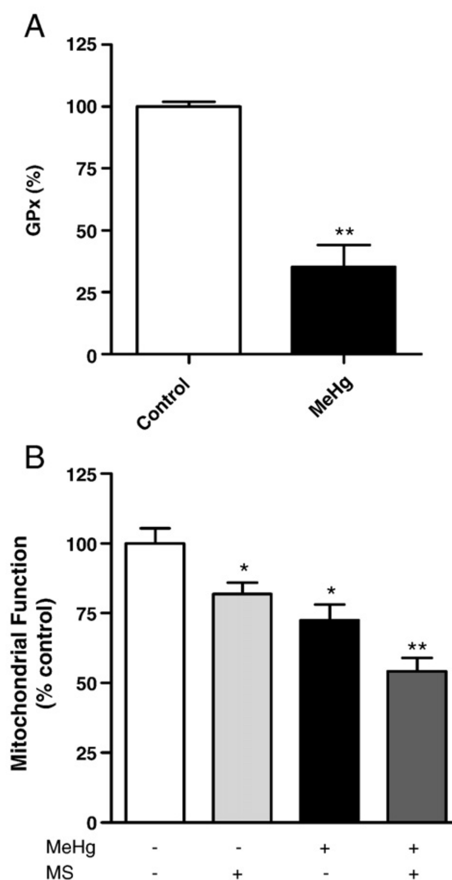

Fig. 2. Effects of MeHg on GPx activity and mitochondrial function. Mice were orally treated with MeHg (40 mg/L in drinking water) for 21 days, and 24 h after treatment was finished a brain mitochondrial-enriched fraction was isolated. Controls were prepared from the brains of animals drinking tap water with no MeHg. Before measurement of mitochondrial function, except for GPx activity, mitochondria were incubated for 30 min (25°C) with chemicals as indicated. Results are expressed as a percentage of control. Data are means ± SD ($n = 6$); * $p < 0.05$, ** $p < 0.01$ compared to control. (A) Mitochondrial GPx activity. (B) Mitochondrial activity.

Reactive oxygen species, total hydroperoxides, and lipid peroxidation

ROS produced by mouse brain mitochondria was detected using the fluorescent dye 2,7-dichlorofluorescein diacetate (DCFDA) as described [42]. The total hydroperoxide content was assessed using the xylenol orange method [40]. This approach allows the detection of hydrogen peroxide as well as other hydroperoxides, including lipid hydroperoxides. The lipid peroxidation end products were determined by the TBARS assay originally described by Ohkawa et al. [43].

Data analysis

Data are expressed as the percentage of controls or enzyme activity (means ± SE), as indicated in the figure legends. Each experiment was performed at least three times in duplicate. Statistical analysis was

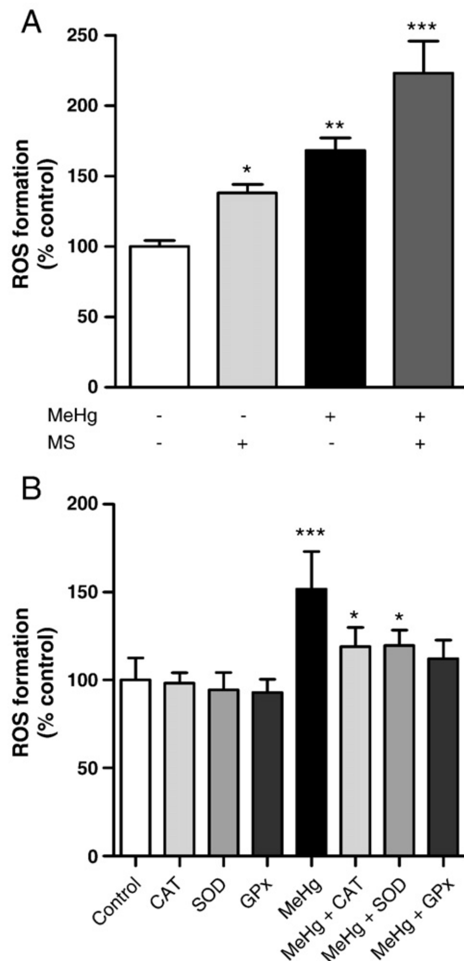

Fig. 3. Effects of MeHg on mitochondrial ROS formation. Mice were orally treated with MeHg (40 mg/L in drinking water) for 21 days, and 24 h after treatment was finished a brain mitochondrial-enriched fraction was isolated. Controls were prepared from the brains of animals drinking tap water with no MeHg. Samples were incubated with DCFDA (10 μM) and changes in DCF fluorescence were recorded over 15 min (25°C) in the presence or absence of chemicals as indicated. Results are expressed as a percentage of control. Data are means ± SD ($n = 6$); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control. (A) Mitochondrial ROS formation in the presence or absence of GPx inhibitor. (B) Mitochondrial ROS formation in the presence or absence of antioxidant enzymes. MS, mercaptosuccinic acid (GPx inhibitor; 300 μM); CAT, catalase (200 U); SOD, superoxide dismutase (50 U); GPx, purified glutathione peroxidase (5 U + GSH 100 μM).

performed by one-way analysis of variance followed by Duncan's post hoc test. Values were considered statistically significant when $p < 0.05$.

Results

MeHg inhibits mouse brain GPx and causes PARP cleavage

We investigated whether the treatment of adult male Swiss mice with MeHg (40 mg/L in drinking water) for 21 days causes inhibition of GPx activity (Fig. 1A). Oral administration of MeHg caused a significant reduction in GPx activity in the mouse brain. In parallel, we observed an increase in PARP cleavage, an index of apoptotic cell death (Fig. 1B). Glutathione levels were unchanged in the brains of mice after MeHg exposure (data not shown).

Inhibition of GPx increases MeHg toxicity toward mouse brain mitochondria

Previous studies from our group have demonstrated that GPx activity is decreased after MeHg exposure [22–24]. To investigate a

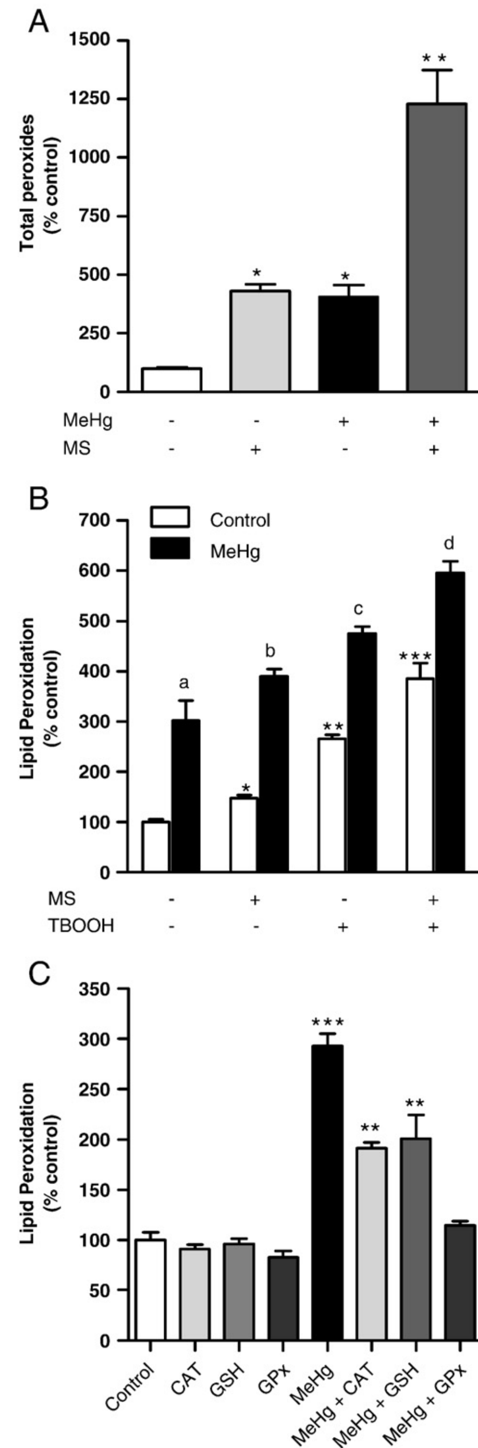

Fig. 4. Effects of MeHg on mitochondrial peroxide production and lipid peroxidation. Mice were orally treated with MeHg (40 mg/L in drinking water) for 21 days, and 24 h after treatment was finished a brain mitochondrial-enriched fraction was isolated. Controls were prepared from the brains of animals drinking tap water with no MeHg. Before measurement of peroxide levels and lipid peroxidation end products, mitochondria were incubated for 60 min (25°C) with chemicals as indicated. Results are expressed as a percentage of control. Data are means ± SD ($n = 6$); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control. Letters represent $p < 0.05$ between all bars (groups). (A) Total hydroperoxide formation. (B and C) Lipid peroxidation (TBARS). MS, mercaptosuccinic acid (GPx inhibitor, 300 μM); TBOOH, *tert*-butylhydroperoxide (100 μM); GSH, glutathione (100 μM); CAT, catalase (200 U); GPx, purified glutathione peroxidase (5 U + GSH 100 μM).

possible role of GPx in MeHg toxicity, we used a model coupling in vivo exposure to MeHg (40 mg/L in drinking water during 21 days) and subsequent in vitro incubations of mitochondria-enriched fractions with MS, a potent inhibitor of GPx, and TBOOH, an organic hydroperoxide that is preferably removed by GPx, without interference by catalase [41]. Fig. 2A shows that GPx activity was significantly decreased in mitochondria isolated from MeHg-treated animals. After MeHg treatment for 21 days, brain mitochondrial-enriched fractions were isolated and incubated for 1 h at room temperature with MS (300 μM) and/or TBOOH (100 μM). There was a significant decrease in the activity of mitochondria isolated from the MeHg-treated animals. A similar decrease occurred in mitochondria from control animals after 1 h in vitro exposure to MS. When mitochondria from MeHg-treated animals were subjected to 1 h incubation with MS, a significant further decrease in mitochondrial function was detected, which was statistically different from that of MeHg- or MS-alone groups (Fig. 2B). ROS formation was also increased in the MeHg and MS groups and an additive effect was observed in the MeHg plus MS group (Fig. 3A). The incubation of samples with the antioxidant enzyme SOD, a superoxide scavenger, as well as catalase and GPx, which are peroxide detoxifying enzymes, blocked MeHg-induced increase in ROS formation (Fig. 3B). A significant increase in total peroxides was observed with mitochondria isolated from MeHg-treated animals when they were incubated with MS (Fig. 4A). To determine the role of GPx on MeHg-induced lipid peroxidation, brain mitochondria were incubated with MS and/or TBOOH (Fig. 4B). Under these conditions, we created a strong and specific pro-oxidative condition favorable for investigating a potential protective effect of GPx against MeHg-driven oxidative damage. This is a result of the inhibition of GPx by MS and the peroxidative action of TBOOH, which can be detoxified preferably by GPx. MS and TBOOH alone elevated TBARS levels in the controls as well as in the MeHg-treated group. These effects were increased when MS and TBOOH were added simultaneously to mitochondria from both control and MeHg-treated groups.

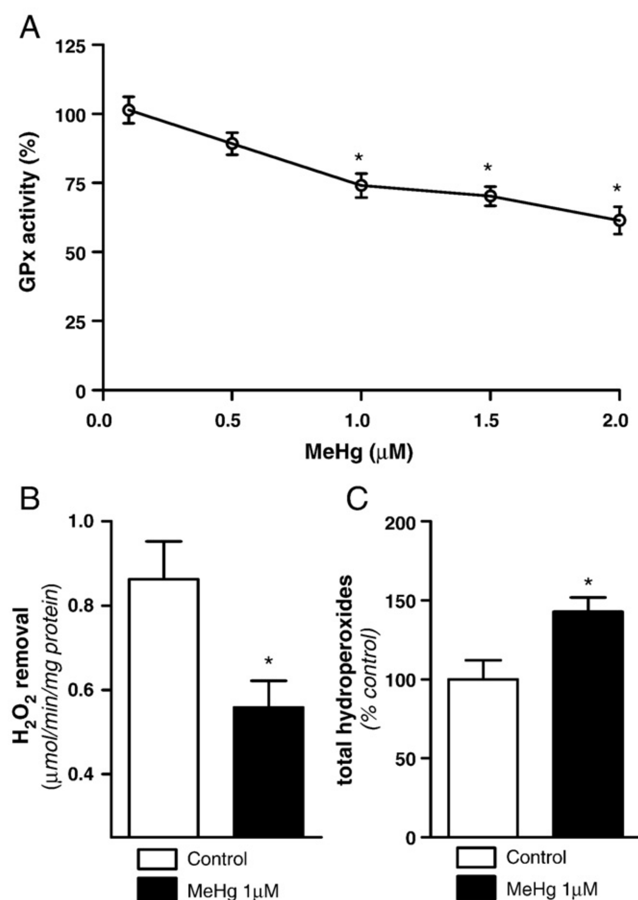

Fig. 5. Oxidative stress induced by MeHg in SH-SY5Y cells. Cells were incubated with increasing concentrations of MeHg for 24 h. Data are means ± SD ($n = 4$); $*p < 0.05$. (A) Concentration-dependent effect of MeHg on GPx activity of SH-SY5Y cells. (B) The rate of H_2O_2 removal in SH-SY5Y cells was decreased after incubation with MeHg 1 μM for 24 h. (C) The production of total hydroperoxides was increased in SH-SY5Y cells treated with MeHg 1 μM for 24 h.

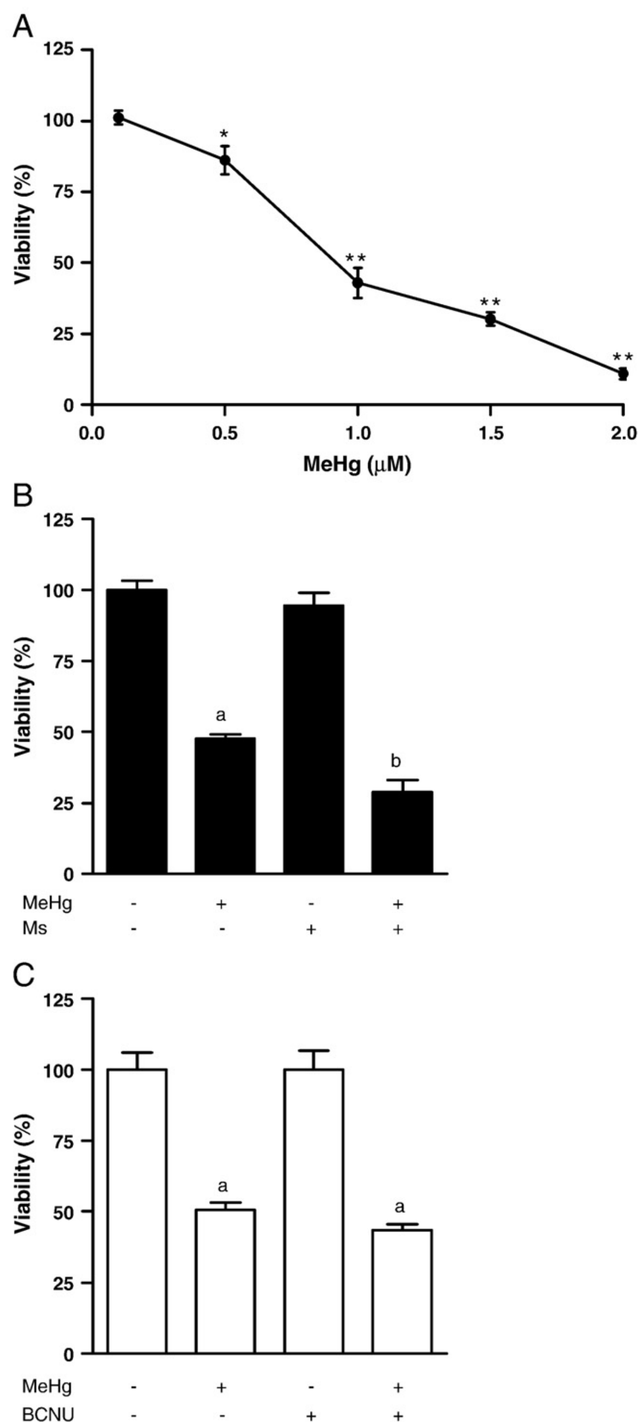

Fig. 6. Cell viability changes after incubation of SH-SY5Y cells with MeHg for 24 h. Data are expressed as means ± SD ($n = 4–10$); $*p < 0.05$, $**p < 0.01$. Letters represent $p < 0.05$ between each other. (A) MeHg causes a concentration-dependent loss of cell viability. (B) Inhibition of GPx with MS significantly increases the loss of cell viability caused by incubation of SH-SY5Y cells with MeHg 1 μM for 24 h. (C) Inhibition of GR with BCNU has no effect on MeHg-driven loss of cell viability in SH-SY5Y cells.

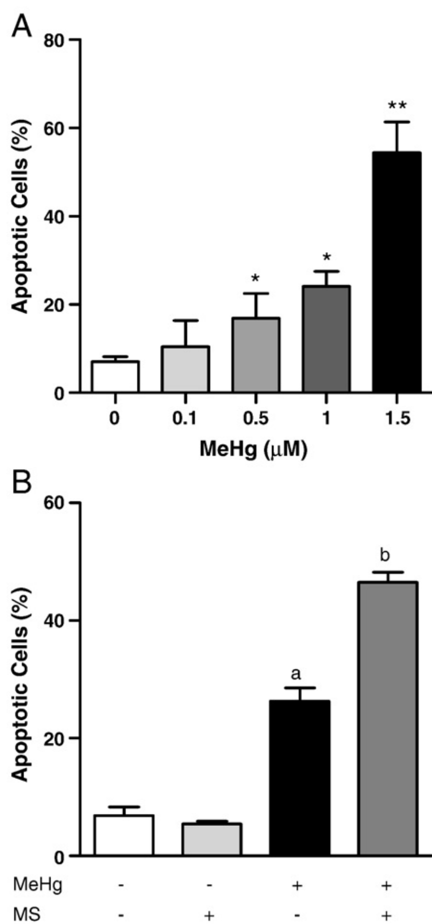

Fig. 7. Apoptosis induced by MeHg in SH-SY5Y cells. After incubation of cells with MeHg for 24 h, cells were harvested and processed for flow-cytometric analysis of apoptosis using the propidium iodide assay. Data are expressed as means ± SD ($n = 4$); *$p < 0.05$, **$p < 0.01$. Letters represent $p < 0.05$ between each other. (A) MeHg caused a concentration-dependent increase in the number of apoptotic cells. (B) Inhibition of GPx with MS significantly increased MeHg-induced apoptosis in SH-SY5Y cells.

We also tested whether antioxidants could block mitochondrial lipid peroxidation caused by MeHg treatment. Fig. 4C shows that MeHg strongly increased TBARS content. This effect was significantly reduced by incubation with GSH (100 μM) or catalase (200 U/ml). However, GSH and catalase were not able to completely block lipid peroxidation. On the other hand, a complete blockade of lipid peroxidation levels was observed after incubation with purified GPx (5 U/ml plus GSH 100 μM). GSH levels were unchanged in mitochondria isolated from MeHg-treated mice (data not shown).

Inhibition of GPx increases MeHg toxicity in SH-SY5Y cells

Incubation of SH-SY5Y cells with MeHg for 24 h caused a significant decrease in GPx activity at concentrations of 1, 1.5, and 2 μM (Fig. 5A). The observed decrease in GPx activity in cells treated with MeHg was correlated to impaired ability of cells to remove hydrogen peroxide from the medium and increased production of total hydroperoxides. As shown in Fig. 5B, the removal of H_2O_2 added to the incubation medium was significantly decreased in cells treated with MeHg (1 μM) for 24 h. Fig. 5C shows that incubation of cells with MeHg for 24 h significantly increased the amount of total hydroperoxides. Parallel to the observed impaired antioxidant activity, cell viability was also reduced after treatment of SH-SY5Y cells with MeHg for 24 h at concentrations ranging from 0.5 to 2 μM (Fig. 6A). Incubation of SH-SY5Y cells with MS, an inhibitor of GPx activity, followed by MeHg elicited a greater loss of cell viability than exposure to MeHg alone (Fig. 6B). Incubation of cells with BCNU, an inhibitor of GR, did not change the effects of MeHg on the viability of SH-SY5Y cells (Fig. 6C). GSH levels remained unaltered in SH-SY5Y cells incubated for 24 h with MeHg (data not shown).

The number of apoptotic nuclei, measured by the propidium iodide assay, was increased after treatment of SH-SY5Y cells with MeHg 0.5 to 1.5 μM (Fig. 7A). When cells were previously incubated with MS and MeHg, a substantial increase in apoptotic nuclei was observed compared to the control and MeHg-alone groups (Fig. 7B). To confirm the apoptotic changes caused by MeHg on SH-SY5Y cells, we utilized PARP cleavage as a second index of apoptosis. Fig. 8 shows that PARP cleavage became apparent in cells incubated with MeHg for 24 h at concentrations of 0.5 μM and was more obvious at 1 μM (Fig. 8A). Incubation of cells with MS followed by MeHg (0.5 μM) markedly increased PARP cleavage in SH-SY5Y cells (Fig. 8B).

Discussion

There is substantial evidence showing that oxidative stress acts as a major mechanism of MeHg toxicity [18,44,19,2]. The mechanisms of MeHg-induced oxidative stress involve the overproduction of ROS [42,45,39] and the impairment of cellular antioxidant defenses [20,21]. Studies from our group [22–24] and others [46,47] have demonstrated that MeHg is able to reduce the activity of the antioxidant enzyme GPx in vitro and in vivo. However, a detailed investigation of the role of GPx in MeHg neurotoxicity is unavailable.

MeHg dose and the time of exposure utilized in this study were based on previous studies from our group [48,38]. Indeed, in a time study on oral exposure to MeHg in adult mice [38], we observed that a 3-week MeHg exposure (40 mg/L, diluted in drinking water) induced a significant neurotoxicity in adult male mice, evaluated by behavioral parameters (motor performance). Moreover, based on a previous dose–response study [48], we observed that MeHg at 10 mg/L (diluted in drinking water, for 2 weeks) did not induce evident neurotoxicity, but levels higher than 40 mg/L led to a decreased liquid ingestion, probably due to the unpleasant metallic taste of MeHg. So, in this study, we selected a single MeHg level (40 mg/L, dissolved in drinking water) [48] with an exposure period of 21 days. With respect to the Hg levels in the CNS of animals after such exposure schedules, we observed that the oral exposure to MeHg at the aforementioned levels (via drinking water) led to cerebellar Hg levels of 2.6 ppm in adult mice [23], which correspond to around 10 μM. The significant

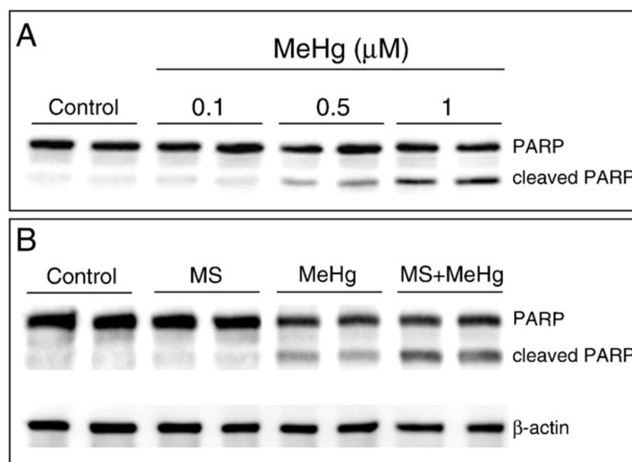

Fig. 8. Apoptosis induced by MeHg in SH-SY5Y cells. After incubation of cells with MeHg for 24 h, cells were harvested and processed for immunoblot analysis of apoptosis using PARP antibody. Images are representative of blots from at least three experiments ($n = 6$). (A) MeHg caused a concentration-dependent increase in PARP cleavage. (B) Cells were treated with MeHg 0.5 μM and MS 500 μM. Inhibition of GPx with MS increased MeHg-induced PARP cleavage in SH-SY5Y cells.

toxicity in cultured neuronal cell lines (present study)—at levels 10-fold lower (1 μM) than those found under in vivo conditions [23]—might be related, at least in part, to the absence of astrocytes in the culture. In fact, it is well known that the toxicity elicited by MeHg toward neurons is significantly decreased in cocultures containing astrocytes [49].

The long-term exposure of adult Swiss mice to a concentration of 40 mg/L (diluted in drinking water) MeHg caused a significant reduction in GPx activity in the brain (about 50%). The vulnerability of cells to the toxic effects of mercury compounds relies on a functional defense system [50]. Considering the role of ROS in MeHg toxicity [18], an impaired antioxidant defense apparatus can result in a critical susceptibility to the deleterious effects of MeHg. A reduced GPx activity impairs cellular defense systems against a range of pro-oxidative conditions and increases ROS formation and oxidative damage [35,51,52]. Because of the well-known capacity of MeHg to generate ROS, including hydrogen peroxide, GPx may act as a central protective enzyme for the removal of H_2O_2 induced by MeHg. In addition, inhibition of GPx by MeHg can be considered an important factor in the H_2O_2 production caused by this agent.

Decreased GPx activity has been associated with apoptosis in models of ischemic injury [53] and diabetes [54] as well as in metal intoxication studies [55]. In our study, the treatment of mice with MeHg for 21 days in drinking water caused an increase in PARP cleavage, indicating that in addition to the observed inhibition of GPx activity, MeHg also caused apoptosis in mouse brain. In previous reports, we have demonstrated that the treatment regimen adopted in this study is able to increase MeHg levels in the brain and induce locomotor deficits [38,22,24]. Thus, the present data together with our previously published results suggest that the deleterious effects of MeHg may be related to increased oxidative stress caused by inhibition of GPx and increased apoptotic cell death in the brain of treated animals.

Mitochondria have been suggested to be important targets for MeHg pro-oxidative effects [56,39,47]. Apoptosis driven by mitochondrial disturbances usually involves alterations of the mitochondrial transmembrane potential ($\Delta\psi_m$) and the release of cytochrome *c* into the cytoplasm, followed by caspase activation [57,58]. Mercury is known to accumulate inside mitochondria, and it can change mitochondrial membrane permeability and cause reduction of the mitochondrial transmembrane potential [59–61]. Consequently, several studies have suggested that apoptosis is a critical phenomenon involved in MeHg-induced neurotoxicity [62,63].

To evaluate the role of mitochondrial GPx in MeHg-induced mitochondrial dysfunction, we utilized an experimental model in which mouse brain mitochondria isolated from MeHg-treated animals were incubated in vitro with MS, a GPx inhibitor, and/or TBOOH, a peroxide preferably cleared by GPx. Mitochondrial GPx was substantially inhibited after MeHg treatment (about 65%). These data support the literature findings showing that mercury accumulates in mitochondria and add new information about MeHg-induced impairment of the mitochondrial redox balance, placing GPx as a central target for MeHg toxicity. In the study of Mori and colleagues [47], it was also shown that MeHg can decrease mitochondrial GPx activity, potentiating mitochondrial dysfunction. The inhibition of GPx activity in mouse brain mitochondria after MeHg poisoning was consistent with the increased production of ROS, total hydroperoxides, and lipid peroxidation end products. In line with these observations, we also observed a significant decrease in mitochondrial activity. The inhibition of GPx with MS substantially elicited mitochondrial oxidative stress and loss of viability caused by MeHg intoxication. When MS and TBOOH were incubated alone or in combination, an additive effect on MeHg-induced lipid peroxidation was always apparent (Fig. 4). Mitochondrial GSH levels were unchanged after MeHg treatment (data not shown). These observations strongly suggest that MeHg-induced lipid peroxidation is a consequence of GPx inhibition. To confirm this hypothesis, we incubated mouse brain mitochondria with exogenous GPx and then measured lipid peroxidation as TBARS levels. Addition of GPx to the incubation medium completely blocked MeHg-induced lipid peroxidation (Fig. 4C), indicating that deficient peroxide detoxification due to reduced thiol-peroxidase activity is a major event in MeHg-induced lipid peroxidation. The addition of catalase also reduced TBARS content, but to a lesser extent, confirming the major role of mitochondrial GPx in the protection against MeHg-induced lipid peroxidation. Concomitant with this result, the addition of catalase also partially blocked ROS formation in MeHg-exposed mouse brain mitochondria (Fig. 3B), whereas incubation with GPx completely prevented ROS production. The incubation with SOD also partially blocked ROS production in the MeHg-exposed samples, indicating that superoxide anions were being induced by MeHg. Superoxide can be converted to hydrogen peroxide by catalase and GPx in the mitochondria [64], which in turn could be, at least in part, responsible for the increase in DCF fluorescence in our model. The formation of ROS by mitochondria is strongly related to changes in the mitochondrial transmembrane potential [65]. Taking into account that MeHg is able to disturb $\Delta\psi_m$, an inhibition of GPx can cause severe deleterious effects to mitochondrial function via exacerbated ROS formation.

We also investigated the role of GPx in MeHg toxicity in human neuroblastoma SH-SY5Y cells. Similar to the observed decrease in GPx activity in mouse brain and mouse brain mitochondria, MeHg also caused an inhibition of GPx in SH-SY5Y cells (about 25%). This effect was correlated to a loss of cell viability and increased apoptosis. When cells were incubated with the GPx inhibitor MS, MeHg-induced loss of viability and apoptosis were substantially stimulated. Importantly, the MeHg-induced GPx inhibition correlates with the impaired ability of MeHg-treated cells to detoxify H_2O_2, suggesting that GPx inhibition by MeHg contributes to an increased oxidative condition.

The apoptotic changes caused by MeHg in SH-SY5Y cells may be linked to a disruption of mitochondrial function in these cells. MeHg was showed to potently disrupt mitochondrial $\Delta\psi_m$ [19]. It was demonstrated that loss of $\Delta\psi_m$ causes cytochrome *c* release into the cytosol and initiates apoptotic cascades involving caspase activation [57,58]. In our study we found that incubation of SH-SY5Y cells caused proteolysis of PARP, a substrate of multiple caspases. This is in line with literature data showing that mercury chloride induced apoptosis in HL-60 cells via mitochondrial dysfunction, cytochrome *c* release, and PARP cleavage [61]. In addition, it was shown that thimerosal, an organic form of mercury used as a preservative in vaccines, causes PARP cleavage by means of cytochrome *c* release and caspase activation in the SK-N-SH cell line [66].

Studies using genetic deletion of cellular GPx showed that animals lacking this enzyme are highly susceptible to oxidative challenge [31]. It is also evident that treatment with antioxidants such as vitamin E is not able to replace the GPx protective effects against acute oxidative damage [51]. However, treatment with GPx mimics known to increase GPx activity can protect cells against oxidative stress [67,68]. Considering MeHg as an environmental contaminant that is known to kill cells by mechanisms involving an overproduction of ROS, including hydrogen peroxide, GPx could be a major antioxidant and protective enzyme against MeHg toxicity. Impairment of antioxidant defenses seems to be a relevant issue in terms of human exposure to MeHg [69]. In this regard, our data show that MeHg is able to block GPx activity and cause deleterious effects to both cultured cells (SH-SY5Y) and mitochondrial-enriched fractions from mouse brain. In addition, our results also show that inhibition of GPx results in a potentiation of MeHg neurotoxicity. These results contribute positively to a better understanding of MeHg mechanisms of toxicity.

In conclusion, this study provides substantial evidence that suggests that GPx is an important target of and a major cellular defense against MeHg-induced neurotoxicity. Our results show that MeHg is able to reduce GPx activity in three different models and that

inhibition of this antioxidant enzyme causes a significant augmentation of MeHg-induced impairment of cell viability and oxidative stress.

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