Probucol Increases Glutathione Peroxidase-1 Activity and Displays Long-Lasting Protection Against Methylmercury Toxicity in Cerebellar Granule Cells

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Short title: Probucol protects against MeHg toxicity

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Abstract

Methylmercury (MeHg) is an environmental neurotoxicant whose molecular mechanisms underlying toxicity remain elusive. Here we investigated molecular events involved in MeHg-induced neurotoxicity in cultured cerebellar granule cells (CGCs) as well as potential protective strategies for such toxicity. Glutathione peroxidase (GPx-1) activity was significantly (p = 0.0017) decreased at 24 h before MeHg-induced neuronal death (day in vitro 4). This event was related to enhanced susceptibilities to hydrogen- or tert-butyl-peroxides and increased lipid peroxidation. However, intracellular calcium levels, glutamate uptake and glutathione levels, as well as glutathione reductase and catalase activities, were not changed by MeHg exposure at this time-point. Probucol (PB), a lipid-lowering drug, displayed a long-lasting protective effect against MeHg-induced neurotoxicity. The beneficial effects of PB were correlated to increased GPx-1 activity and decreased lipid peroxidation. The protection afforded by PB was significantly higher when compared to the antioxidants ascorbic acid and trolox. In vitro studies with the purified GPx-1 proved that MeHg inhibits and PB activates the enzyme activity. Overexpression of GPx-1 prevented MeHg-induced neuronal death. These data indicate that (i) GPx-1 is an important molecular target involved in MeHg-induced neurotoxicity and (ii) PB, which increases GPx-1 activity in CGCs, induces enduring protection against such toxicity. The results bring out new insights on the potential therapeutic strategies for poisonings to MeHg and other pathological conditions related to increased production and/or decreased detoxification of peroxides.

Keywords: Methylmercury, glutathione peroxidase-1, probucol, cerebellar granule cells, neurotoxicity.
Introduction

Mercury is present in the environment due to natural events and anthropogenic sources. Within the environment, mercury exists mainly in three chemical forms: elemental mercury vapor, inorganic mercury salts, and organic mercury (Clarkson et al., 2003). Of particular importance, organic mercurials, such as methylmercury (MeHg), have received extensive attention due to its capability to induce enduring neuropsychological deficits in children exposed to this neurotoxicant during pre- and early post-natal periods (Grandjean and Landrigan, 2006).

The high affinity of mercurials for protein and nonprotein thiols (-SH), which has been suggested to be the basis of their toxicity, also represents an important phenomenon in MeHg-induced neurotoxicity. In fact, various reports have implicated a critical role for glutathione (GSH) (Stringari et al., 2008) and sulfhydryl-containing proteins (Rocha et al., 1993; Fonfria et al., 2005) on MeHg neurotoxicity. However, thiols are not the exclusive nucleophilic groups able to interact with mercurials in the biological systems. Selenols (-SeH), having a lower pKa (around 5.3) than thiols, are fully ionized to selenolates (-Se⁻) under physiological conditions and thus are more reactive toward mercurials (Sugiura et al., 1978). Although the amount of selenol groups in cells is lower when compared to thiols, selenocysteine (the unique selenol-containing amino acid in mammalian proteins) displays a critical role in the active site of selenoenzymes (Lu and Holmgren, 2009).

Glutathione peroxidases (Gpxs) represent a family of selenoproteins that catalyze the reduction of hydrogen peroxide as well as a large variety of hydroperoxides into water and alcohols, respectively. GPx-4 is a critical and multifunctional selenoprotein that reduces complex lipid hydroperoxides, acts as a structural protein in sperm
maturation, and regulates eicosanoid biosynthesis and cell signaling pathways
(Savaskan et al., 2007). In opposition to the well accepted central role of GPx-4 to the
homeostasis of several biological events, the significance of Gpx-1 isoenzyme under
physiological and oxidative stress conditions is still an unsolved and sometimes
controversial question. Some studies using knockout mice as a model have provided
unexpected data about the function of Gpx-1. In fact, mice deficient in Gpx-1 are
healthy and fertile and do not show any histopathologies up to 15 months of age, thus
suggesting a limited role for Gpx-1 during normal development and under physiological
conditions (Ho et al., 1997). Moreover, Gpx-1 knockout mice display no abnormalities
when exposed to an oxidative stressor such as hyperbaric oxygen (Ho et al., 1997),
suggesting a limited role for this enzyme under situations of oxidative stress.
Conversely, other studies have shown that Gpx-1 knockout mice show increased
susceptibility to the oxidative stress-inducing agents paraquat and hydrogen peroxide
(de Haan et al., 1998), bringing out the concept that Gpx-1 presents specific effects in
protection against different oxidative stressors and in different disease states. GPx-1 has
also been reported to be crucial in protecting astrocytes from organic peroxides (Liddell
et al., 2006a) and iron-mediated hydrogen peroxide toxicity (Liddell et al., 2006b),
reinforcing the hypothesis that GPx-1 is essential in protecting against particular
oxidative challenges. In this regard, recent studies have shown that GPx-1 activity is
significantly decreased after MeHg-exposure in vivo (Stringari et al., 2006; Carvalho et
al., 2007; Stringari et al., 2008; Franco et al., 2009). Although MeHg-exposed animals
displayed increased oxidative stress in the CNS and alterations in behavioral parameters
related to the motor function (Stringari et al., 2006; Carvalho et al., 2007), the
involvement of the decreased GPx-1 activity in these outcomes remains elusive.
Probucol (PB) is a phenolic, lipid-lowering prototype agent with anti-inflammatory and antioxidant properties. It has a long history of clinical application with established efficacy and safety profiles and has been in clinical use during the past few decades for the treatment and prevention of cardiovascular diseases (Yamashita and Matsuzawa, 2009). Of particular importance, a previous study showed that a two weeks PB treatment increased the GPx activity in the heart of rats, preventing adriamycin-induced myocardial toxicity (Li and Singal, 2000). Although the stimulatory effect of PB on hearth GPx activity was confirmed by additional studies (El-Demerdash et al., 2005), the molecular mechanisms related to this phenomenon remain elusive. Studies on the effect of PB toward the activity of GPx derived from cerebral structures or cells are lacking in the literature.

Hydrogen peroxide is continuously generated during aerobic cerebral metabolism, reaching extracellular concentrations around 20 µM under basal conditions and up to 100 µM under certain pathological/pro-oxidative conditions (Hyslop et al., 1995). Increased hydrogen peroxide levels are responsible for disrupting neuronal intracellular calcium homeostasis (Santos et al., 2005) and glutamate transport in cells (Allen et al., 2001), which represent important molecular events involved in MeHg-induced neurotoxicity (Aschner et al., 2007). Taking into account (i) the role of GPx-1 in detoxifying peroxides in the CNS (de Haan et al., 1998; Liddell et al., 2006a); (ii) the reduced GPx-1 activity in the cerebral cortex and cerebellum of MeHg-exposed animals (Carvalho et al., 2007; Stringari et al., 2008; Franco et al., 2009); (iii) the potential involvement of hydrogen peroxide in MeHg-induced neurotoxicity (Allen et al., 2001; Franco et al., 2007); and (iv) the modulatory effects of PB on GPx activity (Li and Singal, 2000; El-Demerdash et al., 2005), the aim of the present study was to perform an evaluation of the potential molecular events involved in the neurotoxicity induced by
low-dose MeHg exposure in cultured cerebellar granule cells (CGCs) with particular emphasis on the systems involved in peroxide detoxification (glutathione peroxidase and catalase activities and glutathione levels), intracellular calcium homeostasis and glutamate uptake. We hypothesized that, due to the high affinity of MeHg by selenols, GPx-1 could represent a primary molecular target involved in MeHg-induced CGC toxicity. The potential protective effects of PB against MeHg-induced neurotoxicity were also investigated and compared to the antioxidant compounds ascorbic acid and trolox. Cultured CGCs were used as neuronal model due to their vulnerability to MeHg neurotoxicity in humans. Because of the developmental neurotoxicity of MeHg, CGCs were exposed to such compound during their differentiation period in vitro.

Materials and Methods

Materials- Rabbit polyclonal IgG anti-Glutathione Peroxidase (GPx-1/2 – sc-30147) was from Santa Cruz (Santa Cruz, CA, USA). Monoclonal anti-Actin primary antibody was from Sigma and protein A/G horseradish peroxidase conjugate secondary antibody was from Pierce Biotechnology (Rockford, IL, USA). Glutathione peroxidase-1 from bovine erythrocytes was from Sigma (St. Louis, MO, USA). Methylmercuric (II) chloride was from ICN (Cleveland, OH, USA). Calcein-AM, fluo-3/AM, pluronic F-127, and propidium iodide were from Molecular Probes (Eugene, OR). L-[3H]glutamic acid (52 Ci/mmol) was from Amersham Life Sciences (Buckinghamshire, UK). Fetal calf serum was from Gibco (Invitrogen, Barcelona, Spain) and culture medium from Biochrom KG (Berlin, Germany). The other reagents were purchased from Sigma (St. Louis, MO, USA).

Animals and neuronal culture- Seven-day-old NMRI mice were from Iffa Credo (St. Germain-sur-l’Arbreste, France) and were handled in compliance with the protocol
number 1582 of the University of Barcelona, approved by the Generalitat de Catalunya, Spain, following the EU guidelines. The primary cultures of cerebellar granule cells were performed as previously described (Fonfria et al., 2005). In brief, the cerebellum tissue was trypsinized at 37°C followed by trituration in a DNase solution (0.005 % w/w) containing a trypsin inhibitor from soybeans (0.05% w/v). Cells were suspended in a Dulbecco’s Modified Eagle Medium (DMEM) (25 mM KCl, 31 mM glucose and 0.2 mM glutamine) supplemented with p-aminobenzoate, insulin, penicillin and 10% fetal calf serum. The cell suspension (1.6 x 10^6 cells/mL) was seeded in 96, 24 or 6-multiwell plates precoated with poly-D-lysine and incubated during different days in a humidified 5% CO_2/95% air atmosphere at 37°C. For the studies on GPx-1 overexpression, cellular suspensions of 3.2 x 10^6 cells/mL were used. A mixture of 5 µM 5-fluoro-2’-deoxyuridine and 20 µM uridine was added at 24 h after seeding to prevent glial proliferation.

Cell treatments- Cerebellar granule cells (CGCs) were exposed to methylmercury, ascorbic acid, hydrogen peroxide, tert-butyl hydroperoxide, trolox and/or probucol by adding concentrated solutions of these compounds directly to the bathing medium. Cells were exposed to MeHg, ascorbic acid, trolox or probucol at 24 h after seeding (day in vitro 1) and kept in the same medium until 4-8 day in vitro (div). So, MeHg exposure was simultaneous to the treatment with these antioxidants. Trolox and probucol were dissolved in dimethylsulfoxide (DMSO), whose concentration did not exceed 0.1%. The others compounds were dissolved in deionised water. In the experiments to evaluate the susceptibility of MeHg-exposed cells to peroxides, they were exposed to MeHg at 24 h after seeding (div 1) and kept in the same medium until 4 div. Thereafter, cells were exposed to hydrogen peroxide or tert-butyl hydroperoxide and kept in the same medium during 12 h.
Cell viability assays- Cell viability was measured by three different assays, which evaluate different events of the cellular homeostasis. All the three assays were performed in 96-well plates. (i) The reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT assay), which assesses the activity of labile mitochondrial dehydrogenases, was conducted as previously described (Vendrell et al., 2005). (ii) The lactate dehydrogenase (LDH) release, which evaluates plasma membrane integrity, was measured as previously described (Petegnief et al., 2003). Although some studies have shown that oxidizing agents may inhibit LDH activity (Kendig and Tarloff, 2007), making difficult to interpret results based on its measurement in the culture medium, we observed that 10 µM MeHg were necessary to present significant inhibitory effects toward LDH (data not shown). (iii) Propidium iodide (PI), which is excluded by living cells, but rapidly enters cells with damaged membranes and binds to DNA, rendering them brightly fluorescent, was evaluated essentially as previously described (Rosa et al., 1997; Petegnief et al., 2003). Results of MTT assays were expressed as percentage of control values after subtracting the blanks. Results of LDH assays were expressed as percent of cell death, where the 100% value represents control cells treated with 2% triton X-100 during 30 min. Results of PI assays were expressed as fluorescence (arbitrary units).

Glutathione levels- Total (GSx) and oxidized (GSSG) glutathione levels were estimated by the 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) recycling method (Tietze, 1969). The absolute GSx and GSSG levels in each sample were calculated based on standards (0 to 25 nmol) and normalized per milligram protein.

Glutathione related enzymes and catalase activity- For enzyme activity determinations, cultures derived from 6-well plates were rinsed and harvested in 200 µL of PE buffer (100 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA). Catalase
activity was measured by the method of Aebi (1974). GPx-1 activity was performed based on Handy et al., (2009), using tert-butyldihydroperoxide as substrate. For the assays with purified GPx-1 from bovine erythrocytes, the reaction medium did not contain EDTA. Glutathione reductase (GR) activity was performed based on Calberg and Mannervik (1985).

Intracellular Ca\(^{2+}\) - Fluo-3/AM was used to determine \([\text{Ca}^{2+}]_i\), essentially following the method of Rosa et al. (1997). The free cytosolic calcium concentration was calculated as \([\text{Ca}^{2+}]_i = K_D(F - F_{\text{min}})/(F_{\text{max}} - F)\), where \(K_D\) is 450 nM (Rosa et al., 1997), \(F\) the fluorescence of the well, \(F_{\text{max}}\) the maximal fluorescence obtained after further incubating with the calcium ionophore A23187 (10 \(\mu\)M), and \(F_{\text{min}}\) the minimal fluorescence signal obtained after quenching the fluorescence with 5 mM CuSO\(_4\).

Glutamate uptake- Glutamate uptake was assessed using L-[\(^3\)H]glutamic acid based on the protocols previously standardized (Fonfria et al., 2005).

Lipid peroxidation- Lipid peroxidation was determined by measuring the 8-isoprostane levels (Vendrell et al., 2007) by using an ELISA kit (Cayman Chemicals, Ann Arbor, MI, USA). Samples were collected in the presence of 0.005\% 2,6-di-tert-butyl-4-methylphenol, immediately stored at -80\(^{\circ}\)C and processed according to the manufacturer’s instructions.

Western blot analyses- For western blot analyses, cultured cells were rinsed with PBS and collected in ice-cold lysis buffer containing 62.5 mM Tris-HCl, pH 6.8, 2\% SDS, 10\% glycerol and 50 mM DTT, sonicated for 15 seconds, boiled for 5 min and cooled immediately on ice. Sample extracts were centrifuged at 14,000 \(x\) \(g\) for 5 min at 4\(^{\circ}\)C and protein concentration was quantified in the supernatant. Forty micrograms of protein extract were subjected to SDS-PAGE on 15\% polyacrylamide gels. Gels were run at 20 mA per gel for about 90 min, with the electrophoresis tank placed in an iced water bath.
Separated proteins were electroblotted onto nitrocellulose membranes at a constant current intensity of 300 mA for 60 min. The membranes were blocked for 60 min at room temperature in blocking buffer containing 5% non-fat dry milk. Blots were incubated overnight at 4°C with either the primary polyclonal antibody against GPx (GPx-1/2 (H-151):sc-30147, Santa Cruz, Inc., Heidelberg, Germany) or against actin (fragment 20 - 33, Sigma-Aldrich) diluted 1/100 and 1/1000, respectively, in TBS-Tween-BSA buffer (20 mM Tris base, 140 mM NaCl, 0.1% Tween-20). After washing, the blots were incubated for 60 min at room temperature with protein A/G-horseradish peroxidase conjugate (Research Labs; West Grove, USA) diluted 1/1000 in TBS-Tween buffer. Then, membranes were washed and developed with Immun-Star HRP Chemiluminescent reagents (Bio-Rad) and chemiluminescence was viewed with the Versadoc Imaging system (Bio-Rad). Actin was used as loading control. Band intensity was quantified by using the Quantity one 1D-analysis software (Bio-Rad). Densitometric values from GPx-1 bands were normalized with respect to actin bands.

**Glutathione peroxidase overexpression**- The construct hGPX198LEU, which consists of the retroviral vector pLNCX and the MDR321 human breast carcinoma GPx-1 198 LEU, was kindly gifted by Dr. Alan Diamond (Hu and Diamond, 2003). This construct was used to overexpress human GPx-1 in CGCs. In short, at 0 div, the plasmids (50 ng/µL) were transfected into CGCs (3 x 10^7 cells/mL of cell medium) using a Pipette type Electroporator (Model MP100, Digital Bio Technology, Suwon, Korea). Electroporations consisted of a single pulse of 1700 Volts and 20 milliseconds. Overexpression efficiency was evaluated by GPx-1 enzymatic assays and western blotting analysis.

**Statistical analyses**- Data were analyzed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). Student’s t-test and one-way or two-
way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test were performed for statistical analyses. Results are expressed as mean ± SEM.

Results

*MeHg-induces time- and concentration-dependent cytotoxicity.* The initial question motivating this study was to detect molecular events that precede MeHg-induced cell death in cerebellar granule cells (CGCs). For such propose, it was necessary to detect specific concentrations/time-points in which cells were still alive. Figure 1 depicts a concentration-response study where CGCs were exposed to different concentrations of MeHg (0-300 nM) from day in vitro (div) 1 to div 6. The three different assays for evaluating cell viability [MTT (Figure 1A), PI (Figure 1B) and LDH (Figure 1C)] showed that significant cell death was first observed at 300 nM. Therefore, such concentration was used in a temporal study, where cells were exposed to 300 nM MeHg from div 1 to div 4-8. Figure 2 shows that cell death became significantly different from controls at div 5. Based on these results, it was possible to affirm that no significant cell death was observed at 4 div when CGCs were exposed to 300 nM MeHg.

*Biochemical changes preceding MeHg-induced neuronal death.* Since no significant cell death was observed at 4 div when CGCs were exposed to 300 nM MeHg, this condition was used to determine potential biochemical changes that occur before MeHg-induced cell death. Biochemical parameters that are usually affected by relatively short time and high dose MeHg exposures, such as intracellular calcium (Atchison, 2005), glutamate (Fonfria et al., 2005) and glutathione (Stringari et al., 2008) homeostasis, were evaluated. Table 1 shows that total (GSx) and oxidized (GSSG) glutathione levels, as well as glutathione reductase activity, glutamate uptake and [Ca^{2+}]_{i}, were not changed by 300 nM MeHg treatment at 4 div (when MeHg-exposed
cells presented similar viability when compared to controls; see Figure 2). However, a significant (p = 0.0017) decrease in GPx-1 activity was observed at this time-point. MeHg-exposure did not affect neuronal catalase activity (Table 1), which is also important to detoxify hydrogen peroxide in the CNS.

**MeHg exposure renders neurons more susceptible to peroxides.** Because neuronal GPx-1 is crucial for detoxifying peroxides (Liddell et al., 2006a) and its activity was the unique evaluated parameter that changed in MeHg-treated cells before significant cell death (Table 1), we asked whether MeHg-exposed cells would be more susceptible to exogenously added peroxides. In agreement with the decreased GPx-1 activity, MeHg-treated cells were more vulnerable to both hydrogen peroxide (Figure 3A) and tert-butyl hydroperoxide (Figure 3B). In fact, although no significant differences in the cellular viability were observed between control and MeHg-treated cells in the absence of peroxides at 4 div, a 12 h exposure to both hydrogen peroxide (Figure 3A) and tert-butyl hydroperoxide (Figure 3B) induced a significantly higher cell death in MeHg-treated cells when compared to controls. In agreement, two-way ANOVA showed significant interactions between MeHg and hydrogen peroxide \([F_{5,24} = 6.667; P = 0.0005]\) and between MeHg and tert-butyl hydroperoxide \([F_{5,24} = 3.948; P = 0.0094]\) concerning cell viability. This indicate that MeHg exposure renders neurons more susceptible to the toxicity elicited by either hydrogen or tert-butyl hydroperoxides.

**Antioxidants decrease MeHg-induced neuronal toxicity.** Because MeHg exposure decreased GPx-1 activity, rendering neurons more susceptible to exogenously added peroxides, one could speculate that the observed MeHg-induced neurotoxicity should be related, at least in part, to oxidative events. Since PB has been reported to increase GPx activity in the heart of rats (Li and Singal, 2000; El-Demerdash et al., 2005), MeHg-treated cells were co-incubated with the antioxidant PB in an attempt to detect possible
neuroprotective effects. The potential neuroprotective effects of the well known antioxidants ascorbic acid (AA) or trolox (Tx) were also investigated and compared to PB. The choice of these particular antioxidants was based on the fact that AA and Tx, as well as PB, are able to neutralize ROS that are formed downstream to H$_2$O$_2$ after Fenton’s reaction, such as hydroxyl and lipoperoxyl radicals (Cabelli and Bielski, 1983; Defraigne et al., 1994; Naguib, 1998). Figure 4A shows that the cell death induced by exposure to 300 nM MeHg from div 1 to div 5 was prevented by co-exposure to AA (200 µM), Tx (10 µM) or PB (10 µM). Similar results were observed by using MTT assay (data not shown). However, in a parallel experiment, only PB prevented MeHg-induced lipid peroxidation at this time-point (Figure 4B). So, we performed a time-course study on the potential neuroprotective effects of AA, Tx or PB for longer periods of co-exposure to both MeHg and the antioxidants (from div 1 up to div 8). Figure 5 shows that only PB displayed an enduring neuroprotection, which was maintained even at 8 div. Similar results were observed by using MTT assay (data not shown). These findings are in agreement with data on lipid peroxidation (Figure 4B) and were also evidenced by calcein-AM/propidium iodide fluorescence microscopy, where only PB prevented MeHg-induced cell death until 8 div (Supplementary data 1).

**PB increases GPx-1 activity in CGCs.** Because the decrease of GPx-1 activity appears to be an important event related to MeHg-induced CGCs death and PB displayed a complete and long-lasting protection against such phenomenon, we sought for a potential relationship between MeHg-induced decrease of neuronal GPx-1 activity and the protective effect of PB. Figure 6A shows that the exposure of CGCs to 300 nM MeHg from div 1 to div 4 decreased neuronal GPx-1 activity and that PB hindered such phenomenon. In addition, PB, but neither AA acid nor Tx, increased neuronal GPx-1 activity per se. These data reinforce the idea that the decrease of GPx-1 activity is a
central event of MeHg-induced neurotoxicity and indicate that the neuroprotective
effects elicited by PB are related, at least in part, to the maintenance of GPx-1 activity in
MeHg-exposed cells. Interestingly, western blot analyses (Figure 6B) showed that the
protein levels of neuronal GPx-1 were not modified by either MeHg or PB treatment.
Since MeHg or PB exposure did not change the levels of neuronal GPx-1, the
occurrence of direct inhibitory (MeHg) and activator (PB) effects of these compounds
toward GPx-1 was hypothesized and further investigated with the purified enzyme.

*Effects of MeHg and PB on purified GPx-1 activity.* Figure 7A shows that purified
GPx-1 was inhibited by MeHg in a concentration-dependent manner (EC$_{50}$ = 1.9 ± 0.5
µM, N=5), suggesting that the decreased GPx-1 activity observed in MeHg-exposed
cells is related to direct inhibitory effects of MeHg. Interestingly, PB significantly
increased purified GPx-1 activity (Figure 7B). It is important to state that PB did not
show thiol-peroxidase activity *per se* in the absence of the purified GPx-1 (data not
shown), indicating that it is not a substrate for the glutathione reductase used in the
GPx-1 couple assay. These data indicate that PB positively modulates the GPx-1
activity. Although MeHg and PB alone displayed significant main effects (P<0.0001),
two-way ANOVA showed a non-significant [F$_{4,18}$ = 0.9599; P = 0.4532] interaction
between both compounds toward purified GPx-1 activity. This indicates that MeHg
inhibits and PB stimulates GPx-1 activity; however, both phenomena are disconnected
and do not affect each other. Nevertheless, the stimulatory effect of PB on neuronal
GPx-1 (Figure 6A) was enough to avoid MeHg-induced lipid peroxidation and neuronal
death in our experimental protocol.

*GPx-1 overexpression prevents MeHg-induced neurotoxicity.* Because our data indicate
that GPx-1 is an important molecular target for MeHg and strongly suggest that its
inhibition is a critical event in MeHg-induced neurotoxicity, experiments with CGCs
overexpressing human GPx-1 were performed. GPx-1 activity (Figure 8A) and expression levels (Figure 8B and C) were significantly higher (two-fold) in cells transfected with the pLNCX-GPx1 plasmid when compared to cells transfected with the empty plasmid (pLNCX). GPx-1-overexpressing cells were significantly more resistant to MeHg-induced cell death when compared to sham-transfected cells (Figure 8D and Supplementary data 2). In fact, GPx-1 overexpression completely prevented cell death induced by 300 nM MeHg at 5 div.

Discussion

Methylmercury (MeHg) is a ubiquitous environmental contaminant that has received significant concern due to its ability to induce developmental neurotoxicity, which is evidenced by impairments in memory, attention, language, and visuospatial perception in exposed children (Grandjean and Landrigan, 2006). The main events observed during acute MeHg exposure, namely, impairment of intracellular calcium and glutamate homeostasis, as well as oxidative stress generation, represents connected phenomena affecting each other (Aschner et al., 2007), making complex to elucidate initial events and primary molecular targets concerning its neurotoxicity. In the present study, the reduction of glutathione peroxidase-1 (GPx-1) activity was an early event observed after the low-dose and prolonged exposure of cerebellar granular cells (CGCs) to MeHg. In fact, this phenomenon, which preceded MeHg-induced cell death, was observed in the absence of significant changes in other biochemical parameters that are usually affected by relatively short time and high dose MeHg exposure, such as intracellular calcium levels (Atchison, 2005), glutamate uptake (Fonfria et al., 2005; Aschner et al., 2007) and glutathione levels (Stringari et al., 2008). Because GPx-1 is a crucial enzyme involved with the neuronal detoxification of hydrogen peroxide (Liddell
et al., 2006a), which has been reported to disrupt neuronal intracellular calcium homeostasis (Santos et al., 2005) and glutamate transport in MeHg-exposed cells (Allen et al., 2001), one could speculate that the observed decrease in GPx-1 activity might represent a decisive event responsible for MeHg-induced glutamatergic and calcium dyshomeostasis.

Although our study shows no changes in the GSH levels of MeHg-exposed CGCs at 4 div, the present results do not necessarily contradict our previous in vivo studies showing decreased GSH levels in the central nervous system of MeHg-exposed animals (Stringari et al., 2008). In fact, decreased peroxide detoxification induces hydroxyl radical formation that, in turn, leads to lipoperoxyl radical generation in a chain reaction (Reiter, 1995), culminating in lipid peroxidation and GSH depletion. So, decreased GSH levels can represent a consequence of increased peroxide levels. Of particular importance, it is notable that MeHg-induced decrease of brain glutathione peroxidase activity also preceded MeHg-induced brain GSH depletion under in vivo conditions (Stringari et al., 2008), making both in vivo (Stringari et al., 2008) and in vitro (this work) data in close agreement.

In this study, CGCs were exposed to low concentrations of MeHg, at the nanomolar range (up to 300 nM). Based on the mercury levels found in the CNS of infants prenatally exposed to MeHg - around 300 ppb $\approx$ 1.5 µM (Lapham et al., 1995) - our experimental protocol presents toxicological relevance from an environmental point of view. Based on the relatively high affinity of MeHg for thiols (Rocha et al., 1993; Stringari et al., 2008) and on the direct and equimolar interaction between MeHg and GSH, it is difficult to presume how these low MeHg concentrations (at the nanomolar range) could induce neurotoxicity in cultured CGCs, whose GSH levels are at the millimolar range (Giordano et al., 2008). This intriguing phenomenon could be
explained by the higher affinity of mercurials for selenol groups. In fact, due to the
greater nucleophilicity of the selenol (-SeH) (pKa around 5.7) compared to that of the
thiol (-SH) (pKa around 8.5), the former is fully ionized to selenolates (-Se⁻) under
physiological conditions, and thus are extremely more reactive toward mercurials
(Sugiura et al., 1978). This phenomenon corroborates our findings, which show that the
selenohydryl-containing enzyme GPx-1, whose catalytic activity depends upon its
selenol group, is inhibited by MeHg exposure in CGCs without changes in the GSH
levels. Moreover, western blot analyses and in vitro studies with purified GPx-1 clearly
indicate that MeHg-induced decrease in GPx-1 activity was related to direct enzyme
inhibition, with no effects on the protein levels. In agreement, Carvalho and
collaborators (2008) showed that selenoproteins of the thioredoxin system also
represent molecular targets of mercury compounds. These authors also showed that the
remarkable mercury affinity for the selenol group of these proteins was a major
molecular mechanism of toxicity.

The hypothesis that the reduction in GPx-1 activity represents a key event leading to
neurotoxicity was investigated by exposing MeHg-treated cells to increasing
concentrations of either hydrogen- or t-butyl hydroperoxide. MeHg-treated cells
displayed a significantly lower resistance to both peroxides, which is in agreement with
the fact that GPx-1 detoxifies organic- and hydro-peroxides. Since GSH levels and
catalase activity were not changed at this time-point (div 4; see Table 1), it is possible to
assume that the decrease of GPx-1 activity in MeHg-treated cells is the main
responsible to such event. These data are in agreement with the fact that MeHg also
induced lipid peroxidation (8-isoprostane levels) in MeHg-treated CGCs.

The significant higher cell death observed in MeHg-treated CGCs after acute
exposure to peroxides indicates an increased susceptibility to oxidative damage.
Consequently, it is not surprising that MeHg-induced neurotoxicity was partially decreased by the co-administration of the antioxidant agent ascorbic acid (AA), trolox (Tx) or probucol (PB). However, it should be noted that only PB circumvented MeHg-induced lipid peroxidation after 4 days of exposure to 300 nM MeHg. In agreement, only PB displayed enduring protective effects against MeHg-induced neurotoxicity in the time-course study (till div 8). This event might be related to the positive modulatory effects of PB toward GPx-1 activity. In fact, the decreased GPx-1 activity observed in MeHg-exposed cells at 4 div was completely restored to control levels by PB co-exposure, but not by AA or Tx. Moreover, PB per se increased GPx-1 activity in cultured CGCs, suggesting that it does not prevent MeHg-induced decrease of GPx-1 activity, but modulates the enzyme activity through direct activating affects. This idea was confirmed by western blotting analyses, which showed that PB and MeHg exposures did not change GPx-1 levels in CGCs. Moreover, enzymatic studies with purified GPx-1 showed that MeHg is a direct inhibitor and that PB is a direct activator of GPx-1. The direct inhibitory effect of MeHg toward the selenoprotein GPx-1 represents a novelty of our study. Moreover, the data presented herein are also the first to show that PB increases GPx-1 activity due to direct activating effects (Figure 7), with no changes in the levels of the neuronal enzyme (Figure 6). Of particular importance, increasing attention has been devoted to developing GPx mimetics as a way to treat overt inflammation associated with the pathophysiology of many human disorders, including Alzheimer’s disease and stroke (Day, 2009). However, there are no data in the literature reporting drugs with positive modulatory effects toward the endogenous GPx activity. So, our data bring out a novel molecular mechanism related to the beneficial antioxidant effects of PB (Kalyanaraman et al., 1992), which opens a new window for pharmacological studies on potential therapeutic strategies for pathological conditions.
related do increased peroxides levels, such as Alzheimer disease (Huang et al., 1999) and amyotrophic lateral sclerosis (Yim et al., 1996). The present findings appear to be extremely relevant taking into consideration the actual scenario of the pharmacological studies with PB, in which human data have shown its promising antiatherogenic and anti-diabetic (Yamashita and Matsuzawa, 2009) properties. Taking into account the relationship of diabetes and dyslipidemia with neurodegenerative disorders (Helzner et al., 2009) and the absence of epidemiological evidence that can support a neuroprotective role for PB in humans, the association of its use with lower incidence of chronic neurodegenerative diseases should be investigated.

It is important to mention that GPx-1 inhibition occurred before any changes on potential targets of MeHg toxicity. These data indicate that the inhibition of GPx-1 may trigger “peroxide toxicity”, which possibly will latter trigger changes in other biochemical parameters (i.e., calcium, glutamate and glutathione dyshomeostasis). This idea is in agreement with previous data (Allen et al., 2001, Franco et al., 2007), supporting the hypothetical sequence in MeHg neurotoxicity: MeHg ⇒ GPx-1 inhibition ⇒ increase in peroxide toxicity ⇒ cell death. Such hypothesis was validated by studies on GPx-1 overexpression. In fact, GPx-1 overexpression in CGCs completely prevented MeHg-induced cell death, confirming that the decreased GPx-1 activity is an important event responsible for MeHg-induced neurotoxicity in our experimental protocol. These results corroborate previous in vivo data on the beneficial effects of the peroxidase-mimetics ebselen (Farina et al., 2003) and diphenyl diselenide (de Freitas et al., 2009) against MeHg-induced neurotoxicity in mice. Although some lines of evidence suggest a limited role for Gpx-1 under physiological conditions and when exposed to certain oxidative stressors (Ho et al., 1997), our data corroborate further studies showing that GPx-1 is essential in protecting against specific oxidative
challenges (de Haan et al., 1998; Liddell et al., 2006a; 2006b). With particular emphasis to MeHg, the direct hydrogen peroxide generation (Mori et al., 2007) likely represents an additional event that contributes to the observed toxicity to cells with decreased GPx-1 activity.

Regarding the sensitivity of purified or neuronal GPx-1 to MeHg, it is important to acknowledge that about 2 µM MeHg were needed for 50% inhibition of purified enzyme (Figure 7A), but 0.3 µM inhibited about 30% of GPx-1 activity in cultured neurons (Table 1). These intriguing results could be explained as a consequence of the high MeHg uptake into CGCs, which might result in intracellular MeHg levels higher than 0.3 µM. In addition, since the commercial purified GPx-1 contains 2.5% dithiothreitol, the direct interaction between MeHg and this thiol compound could not be ruled out.

In addition to GPx-1, GPx-4 is also an important selenoprotein involved in the reduction of peroxides in the CNS (Savaskan et al., 2007). Thus, in addition to catalase and peroxiredoxins, the peroxide tone in the CNS is modulated by both GPx-1 and GPx-4. Since GPx-4 is a selenoprotein whose catalytic activity also depends upon its selenol group, one could speculate that this GPx isozyme might also represent a molecular target of MeHg. Further studies are necessary to confirm such hypothesis. Anyway, it is important to acknowledge that the GPx-1 overexpression completely prevented MeHg induced neuronal death under our experimental conditions, which is in agreement with the increased production of hydrogen peroxide (a main GPx-1 substrate) after MeHg exposure (Franco et al., 2007).

Concluding, this study shows that GPx-1 is an important molecular target involved in MeHg-induced neurotoxicity, pointing to a direct inhibitory effect of MeHg toward this selenoenzyme, leading to enhanced susceptibility to peroxides, increased lipid
peroxidation and cell death. The findings also show that PB induces an enduring *in vitro* protection against MeHg-induced neurotoxicity by increasing GPx-1. Altogether, the results presented herein introduce the concept that GPx-1 inhibition is a decisive event leading to MeHg-induced neurotoxicity. Moreover, the results render PB a promising molecule for further pharmacological studies on the search for therapeutic strategies to counteract the neurotoxicity elicited by short-term mercurial exposure, as well as to treat other pathological conditions related to increased production or decreased detoxification of peroxides.

**Acknowledgements**

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**List of Abbreviations**

MeHg (methylmercury); CGCs (cerebellar granule cells); GPx (glutathione peroxidase); GPx-1 (glutathione peroxidase, isozyme 1); GR (glutathione reductase); PB (probucol); GSH (glutathione); GSSG (oxidized glutathione); CAT (catalase); AA (ascorbic acid); Tx (trolox); t-BuOOH (*tert*-butyl hydroperoxide); Glu (glutamate); GSx (total glutathione: oxidized plus reduced); MTT( 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-
tetrazolium bromide); PI (propidium iodide); LDH (lactate dehydrogenase); DTT (dithiothreitol); div (day in vitro); CNS (central nervous system).
References


Legends

Fig. 1. Concentration-dependent MeHg-induced cytotoxicity in primary cultures of cerebellar granule cells. Cerebellar granule cells (CGCs) were exposed to different methylmercury (MeHg) concentrations from day in vitro (div) 1 to div 6. Cell viability was evaluated by the reduction of MTT (A), propidium iodide (PI) staining (B) or lactate dehydrogenase (LDH) release (C). Results of MTT assays are expressed as percent of survival cells compared to control values (dotted line) after subtracting the blanks. Results of PI assay are expressed as fluorescence (arbitrary units – A.U.). Results of LDH assays are expressed as percent of cell death, where the 100% value represents control cells treated with 2% triton X-100 during 30 min. Data are represented as mean ± SEM (N = 3-4 independent experiments). * P<0.05 when compared to respective controls.

Fig. 2. Time-dependent MeHg-induced cytotoxicity in primary cultures of cerebellar granule cells. CGCs were exposed to vehicle (white bars/dotted line) or to 300 nM MeHg (black bars) from div 1 to div 4-8. Cell viability was evaluated by the reduction of MTT (A), PI staining (B) or LDH release (C) according to Figure 1. Data are represented as mean ± SEM (N = 3-4 independent experiments). *P<0.05, **P<0.01, and ***P<0.001 when compared to controls at the same time-point.

Fig. 3. MeHg exposure renders neurons more susceptible to peroxides. CGCs were exposed to vehicle (■) or to 300 nM MeHg (▲) from div 1 to div 4. Thereafter, cells were exposed to different concentrations (10-500 µM) of either hydrogen peroxide (A) or tert-butyl hydroperoxide (B) during 12 h. Cell viability was evaluated by PI staining.
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**Fig. 4.** (A) Antioxidants decrease MeHg-induced toxicity in primary cultures of cerebellar granule cells. CGCs were exposed to 300 nM MeHg and the antioxidant compounds ascorbic acid (AA - 200 µM), trolox (Tx - 10 µM) or probucol (PB - 10 µM) from div 1 to div 5. Cell viability was evaluated by PI staining according to Figure 1. Results are expressed as fluorescence (arbitrary units – A.U.). (B) Probucol decreases MeHg-induced lipid peroxidation. CGCs were exposed to 300 nM MeHg and the antioxidant compounds ascorbic acid (AA - 200 µM), trolox (Tx - 10 µM) or probucol (PB - 10 µM) from div 1 to div 5. 8-Isoprostane levels are expressed as percent of control. Data are represented as mean ± SEM (N = 3-4 independent experiments). *P<0.05 when compared to respective control.

**Fig. 5.** Probucol elicits long-lasting protection against MeHg-induced toxicity in primary cultures of cerebellar granule cells. CGCs were exposed to 300 nM MeHg and the antioxidant compounds ascorbic acid (AA - 200 µM), trolox (Tx - 10 µM) or probucol (PB - 10 µM) from div 1 to div 4-8. Cell viability was evaluated by PI staining according to Figure 1. Symbols: ▼ - control; ⊕ - MeHg; ⊗ - MeHg + AA; □ - MeHg + Tx; Δ - MeHg + PB. Results are expressed as fluorescence (arbitrary units – A.U.) and represented as mean ± SEM (N = 3-4 independent experiments). *P<0.05, **P<0.01 ***P<0.001 when compared to respective controls (same day in vitro – DIV)) by two-way ANOVA with repeated measures, followed by Bonferroni post-test.
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Fig. 7. Modulatory effects of probucol and MeHg in GPx-1 activity. (A) Purified glutathione peroxidase (GPx-1) from bovine erythrocytes (750 mU/ml) was incubated with different MeHg concentrations (1 nM to 10 µM) in EDTA-free HEPES-balanced salt solution (HBSS) for 30 min at 30ºC. Thereafter, enzyme activity was determined (see experimental section). Data are expressed as percent of control. *P<0.05 when compared to control. (B) Purified glutathione peroxidase (GPx-1) from bovine erythrocytes (750 mU/ml) was incubated with vehicle (white bars), 100 nM MeHg (black bars) or 300 nM MeHg (horizontal bars) and/or probucol (PB, 0, 10 or 30 µM) in EDTA-free HBSS for 30 min at 30ºC. Enzyme activity is expressed as percent of control. Different letters indicate significant difference (P<0.05) when compared to respective control (PB effect); and different numbers indicate significant difference (P<0.05) when compared to respective control (MeHg effect) by two-way ANOVA.
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Table 1

<table>
<thead>
<tr>
<th></th>
<th>GSx</th>
<th>GSSG</th>
<th>GR</th>
<th>GPx</th>
<th>CAT uptake</th>
<th>[Ca^{2+}]_i</th>
</tr>
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<tr>
<td>Control</td>
<td>93.84 ± 15.28</td>
<td>0.13 ± 0.03</td>
<td>52.66 ± 12.62</td>
<td>10.84 ± 0.65</td>
<td>0.34 ± 0.03</td>
<td>343.1 ± 69.8</td>
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<tr>
<td>MeHg</td>
<td>102.4 ± 17.61</td>
<td>0.19 ± 0.03</td>
<td>52.61 ± 12.45</td>
<td>7.07 ± 0.44 **</td>
<td>0.40 ± 0.02</td>
<td>360.2 ± 57.7</td>
</tr>
</tbody>
</table>

Biochemical changes preceding MeHg-induced neurotoxicity. CGCs were exposed to 300 nM MeHg from div 1 to div 4. Total (GSx) and oxidized (GSSG) glutathione are expressed as nmol/mg protein. Glutathione reductase (GR) and glutathione peroxidase (GPx) activities are expressed as nmol NADPH/min/mg protein. Catalase (CAT) activity is expressed as k/min/mg of protein, where k is the rate constant of a first-order reaction. Glutamate (Glu) uptake is expressed as DPM/µg protein. Free cytosolic calcium concentration - [Ca^{2+}]_i - is expressed as nM. Data are represented as mean ± SEM (N = 3-4 independent experiments). ** P = 0.0017 when compared to control.
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Farina et al., Figure 2

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88x196mm (600 x 600 DPI)
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