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Protective effects of organoselenium compounds against methylmercury-induced oxidative stress in mouse brain mitochondrial-enriched fractions

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Abstract

We evaluated the potential neuroprotective effect of 1-100 µM of four organoselenium compounds: diphenyl diselenide, 3'3-ditrifluoromethyldiphenyl diselenide, *p*-methoxy-diphenyl diselenide, and *p*-chloro-diphenyl diselenide, against methylmercury-induced mitochondrial dysfunction and oxidative stress in mitochondrial-enriched fractions from adult Swiss mouse brain. Methylmercury (10-100 µM) significantly decreased mitochondrial activity, assessed by MTT reduction assay, in a dose-dependent manner, which occurred in parallel with increased glutathione oxidation, hydroperoxide formation (xylenol orange assay) and lipid peroxidation end-products (thiobarbituric acid reactive substances, TBARS). The co-incubation with diphenyl diselenide (100 µM) completely prevented the disruption of mitochondrial activity as well as the increase in TBARS levels caused by methylmercury. The compound 3'3-ditrifluoromethyldiphenyl diselenide provided a partial but significant protection against methylmercury-induced mitochondrial dysfunction (45.4 ± 5.8% inhibition of the methylmercury effect). Diphenyl diselenide showed a higher thiol peroxidase activity compared to the other three compounds. Catalase blocked methylmercury-induced TBARS, pointing to hydrogen peroxide as a vector during methylmercury toxicity in this model. This result also suggests that thiol peroxidase activity of organoselenium compounds accounts for their protective actions against methylmercury-induced oxidative stress. Our results show that diphenyl diselenide and potentially other organoselenium compounds may represent important molecules in the search for an improved therapy against the deleterious effects of methylmercury as well as other mercury compounds.

Key words: Methylmercury; Mitochondria; Oxidative stress; Organoselenium compounds; Diphenyl diselenide

Introduction

Reactive oxygen/nitrogen species (ROS/RNS) such as superoxide anion, hydrogen peroxide and nitric oxide induce damage to key biological components and cell membranes. In order to counteract the deleterious effects of reactive species, cells developed a specialized machinery of antioxidant defense. Cellular defense against ROS involves enzymes such as catalase, superoxide dismutase and glutathione peroxidase, which play a central role in the detoxification of reactive species (1,2).

Seleno-organic compounds such as ebselen and diphenyl diselenide (DD) (3,4) have a catalytic activity similar to that of the enzyme glutathione peroxidase involving the reduction of peroxides at the expense of thiol compounds (2,5) and represent important molecules whose protective and antioxidant properties against experimental oxidative stress conditions have been reported (6-8). These studies stimulated the search for new organoselenium compounds with catalytic properties similar to those of ebselen and DD, which could provide antioxidant and protective effects in biological systems.

Methylmercury (MeHg) has been recognized as a ubiquitous environmental toxicant whose toxicity is associated with neurological and developmental deficits in animals and humans (9). Nowadays, especially in the Amazon Region,

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gold mining activity has been associated with intense environmental and human contamination with mercury compounds (10,11). Among the mechanisms involved in MeHg neurotoxicity, oxidative stress (12,13) appears to play a central role. MeHg-induced oxidative stress seems to be related to the direct oxidative properties of MeHg toward endogenous thiols (14) and to its inhibitory effects toward antioxidant enzymes like glutathione peroxidase (6,15,16). Mitochondria appear to be important cellular organelles targeted by MeHg (17), which is known to accumulate in mitochondria, where it can change mitochondrial membrane permeability and cause disruption of mitochondrial membrane potential (18,19). Considerable efforts have been made in the search for new drugs that counteract mercury toxicity. However, until now, no effective treatments are available to completely abolish the toxic effects of MeHg (20).

In previous studies, the organoselenium compounds DD and ebselen demonstrated potential protective effects against MeHg toxicity (6,21). Moreover, in *in vivo* studies, DD demonstrated lower toxicity than ebselen (5) and DD reversed MeHg-induced oxidative stress *in vivo* and in cortical slices (22,23). These data support the potential use of organoselenium compounds against MeHg poisoning.

The aim of the present study was to investigate the potential protective effects of DD and three novel selenium compounds: 3'3-ditrifluoromethyldiphenyl diselenide (DFD), *p*-methoxy-diphenyl diselenide (MD) and *p*-chloro-diphenyl diselenide (CLD) against MeHg-induced mitochondrial dysfunction.

Material and Methods

Chemicals

Glutathione reductase (G3664), reduced glutathione (GSH), oxidized glutathione (GSSG), *t*-butyl-hydroperoxide (*t*-bOOH), 5,5'-dithiobis(2-nitrobenzoic acid (DTNB), methylmercury (II) chloride, xylenol orange salt, and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (USA). All other chemicals used in this study were of the highest analytical grade available.

Synthesis and preparation of organoselenium compounds

All organoselenium compounds (Figure 1) tested in the present study were prepared as previously described (4). Analysis of the hydrogen-1 nuclear magnetic resonance (^{1}H NMR) and carbon-13 NMR (^{13}C NMR) spectra showed that all compounds presented analytical and spectroscopic data in full agreement with their assigned structures (data not shown). The diselenides were purified by flash chromatography on silica gel (hexane) and identified by (^{1}H NMR), ^{13}C NMR and gas chromatography-mass spectrometry (GCMS), which revealed homogeneous product (data not shown). Compounds were dissolved in ethanol immediately prior to use in each assay. The final ethanol in each experiment was 0.1%, and did not affect any of the parameters analyzed when compared to a control sample without ethanol (data not shown). We also investigated the basal activity of the compounds tested for the parameters analyzed in this study. We did not find significant changes when comparing the effects of compounds alone in all experiments performed (data not shown).

Preparation of mouse brain mitochondrial-enriched fractions

All procedures involving animals were performed according to the Animal Care Guidelines from the National Institutes of Health of the United States of America, and all procedures were approved by the Universidade Federal de Santa Catarina Ethics Committee for animal use (313/CEUA; 23080.026023/2004-39/UFSC). Mouse brain mito-

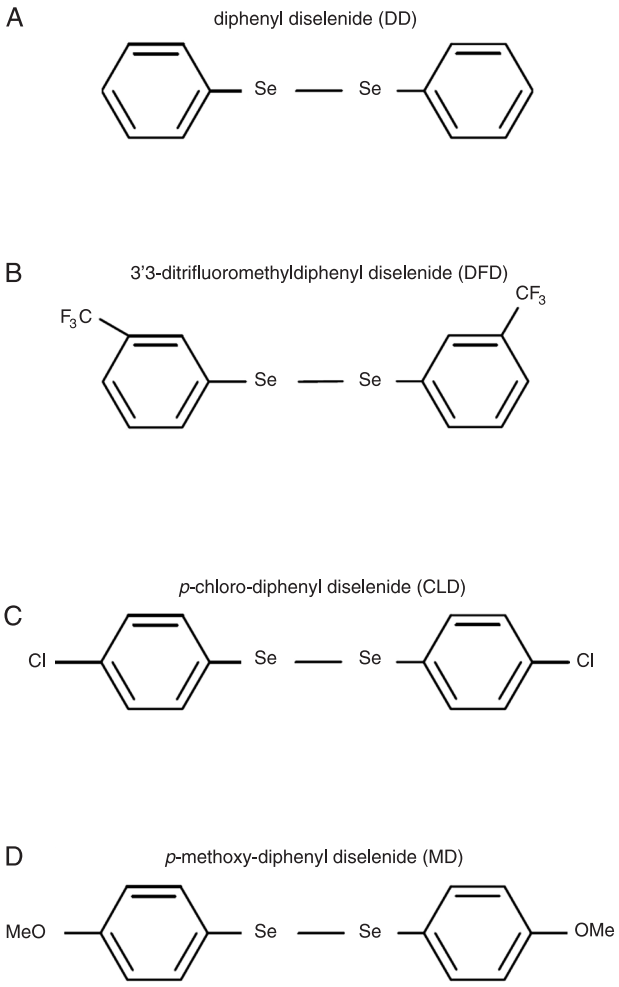

Figure 1. Structures of the organoselenium compounds tested in the present study.

chondrial-enriched fractions were prepared as described previously (24). Briefly, adult (8-10 weeks) male Swiss mice were sacrificed by decapitation. The whole brain (minus the cerebellum) was removed and homogenized on ice in 10 volumes 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, providing a myelin-rich supernatant and a pellet (P2) consisting of synaptosomes and free (extra-synaptosomal) mitochondria. The supernatant was discarded, and the pellet was suspended in the isolation medium without albumin. The samples were kept on ice until the experiments were performed, usually within 10-15 min.

Incubations

P2 (2 mg protein) was incubated with different concentrations of MeHg (0, 10, 30, and 100 µM) diluted in incubation buffer, and/or selenium compounds (1, 10, 30, and 100 µM) in a incubation medium containing 10 mM HEPES buffer, pH 7.0, 220 mM mannitol, 68 mM sucrose, and 10 mM KCl (total incubation volume = 300 µL). In previous studies, we found a concentration of about 10 µM Hg in the brain of mice treated orally with 40 mg/L MeHg in drinking water (24). Considering the high amount of protein (2 mg), in the present study we used concentrations of MeHg up to 100 µM in order to obtain clear detection of all parameters tested during the *in vitro* assays. Incubations were carried out at 25°C. After incubation, mitochondrial dehydrogenase activity, GSH content, total hydroperoxides, and lipid peroxidation (TBARS) were determined. Parallel experiments with the presence of catalase (200 U) were also carried out in order to test the role of hydrogen peroxide (H_2O_2) in the mechanisms of toxicity and protection of MeHg and organoselenium compounds, respectively.

Assessment of mitochondrial activity

Mitochondrial activity was assessed by the conversion of the MTT dye to formazan (17). This assay is based on the ability of mitochondrial enzymes to metabolize MTT into formazan, a reaction that takes place only in functionally intact mitochondria. Briefly, samples (300 µL) were incubated for 30 min at 25°C. The purple formazan crystals were pelleted by centrifugation, and the supernatant was discarded. The pellets were dissolved in DMSO and the formazan was quantified spectrophotometrically by absorbance measurements at 550 nm. Data are reported as percentage of control. Selenium compounds alone did not interfere with the MTT method described here (data not shown).

Assessment of glutathione and hydroperoxide content and lipid peroxidation

Glutathione content was measured as nonprotein thiols according to a method previously described (25). After treatment with different concentrations (10, 30, and 100 µM) of MeHg for 30 min, 300 µL 10% trichloroacetic acid was added to the samples (300 µL). After centrifugation (4000 *g* at 4°C for 10 min), the protein pellet was discarded and free thiol groups were determined in the clear supernatant (which was neutralized with 0.1 M NaOH) by the method of Ellman (25). The total hydroperoxide content was assessed using the xylenol orange method (24) that allows the detection of hydrogen peroxide as well as lipid hydroperoxides. Briefly, samples were incubated in the medium described above for 60 min at 25 ± 1°C in the presence or absence of MeHg and/or selenium compounds. Then, the xylenol orange reagent, containing 0.25 mM $Fe(NH_4)_2(SO_4)_2$, 0.25 mM xylenol orange, and 110 mM perchloric acid, was added to the incubation medium. After 30 min, absorbance was recorded at 560 nm and compared to a hydrogen/cumene peroxide standard curve. Selenium compounds alone did not interfere with the method described here (data not shown).

The lipid peroxidation end-products were determined by the TBARS assay originally described by Ohkawa et al. (26). After 60 min of incubation as described for the xylenol orange method, samples were incubated with 0.45 M acetic acid/HCl buffer, pH 3.4, 0.28% thiobarbituric acid, 1.2% SDS, and thereafter at 95°C for 60 min to promote color reaction, measured at 532 nm. Malondialdehyde (0 to 3 nmol/mL) was used as a standard. Protein concentration was determined by the method of Bradford (27) using bovine serum albumin as standard.

Assessment of glutathione peroxidase (GPx)-like activity of organodiselenides

The GPx-like activity of the organoselenium compounds was determined using the coupled assay described by Wendel (28), which indirectly monitors the consumption of NADPH at 340 nm. The GPx/GPx-like compounds use GSH to reduce tert-butylhydroperoxide, producing GSSG, which is readily reduced to GSH by excess glutathione reductase (GR), thus consuming NADPH.

Statistical analysis

Statistically significant differences among groups were analyzed by one-way ANOVA followed by the Duncan multiple range test when appropriate. Differences were considered to be statistically significant when $P < 0.05$.

Results

MeHg induces oxidative stress and reduction of mitochondrial metabolic activity

The toxicity of MeHg has been demonstrated in *in vitro* and *in vivo* models and is generally associated with increased oxidative stress, disruption of oxidative defenses by inhibition of antioxidant enzymes as well as reduction

of GSH levels in the cell (15,16). Moreover, mitochondria are an important cellular target for MeHg toxicity (17). In the present study, as shown in Figure 2A, using the MTT reduction test, MeHg decreased the mitochondrial activity in a concentration-dependent manner. This effect was significantly different from control at concentrations as low as 10 µM and a reduction of about 50% in cell viability was verified at the highest concentration (100 µM). This effect was followed by a concentration-dependent decrease in mitochondrial GSH levels (Figure 2B). In parallel, a significant increase in total-hydroperoxide production (Figure 2C) and increased lipid peroxidation were observed with 100 µM MeHg (Figure 2D). The following experiments were performed using 100 µM MeHg, which affected all parameters analyzed.

Protective effects of organoselenium compounds against MeHg-induced mitochondrial oxidative stress

We investigated the potential protective effects of organoselenium compounds against the decrease in mitochondrial activity promoted by MeHg, using the MTT reduction assay. As observed in Figure 3A, DD at concentrations of 30 and 100 µM was able to partially and totally reverse the effect of 100 µM MeHg on mitochondrial activity, respectively. Only the highest concentration of DFD (100 µM) caused a slight reversal of the MeHg-induced reduction of mitochondrial activity (Figure 3B), while CLD and MD did not prevent the effects of MeHg on mitochondrial function (Figure 3C and D).

Considering the significant decrease in GSH levels promoted by MeHg treatment, we investigated whether co-incubation of MeHg in the presence of organoselenium compounds would be able to modulate the decrease in GSH levels caused by this metal. As observed in Figure 4 (A-D), none of the compounds tested was able to protect against the depletion of GSH levels caused by MeHg. In fact, when MD and MeHg were co-administered (Figure 4D), the decrease of GSH levels was greater than in the presence of MeHg alone, although this effect was not statistically significant (P = 0.08).

The antioxidant potential of the different organoselenium compounds against lipid peroxidation induced by MeHg was investigated by determining TBARS levels. As observed in Figure 5A, DD totally blocked the increase in lipid peroxidation induced by MeHg at a concentration as low as 10

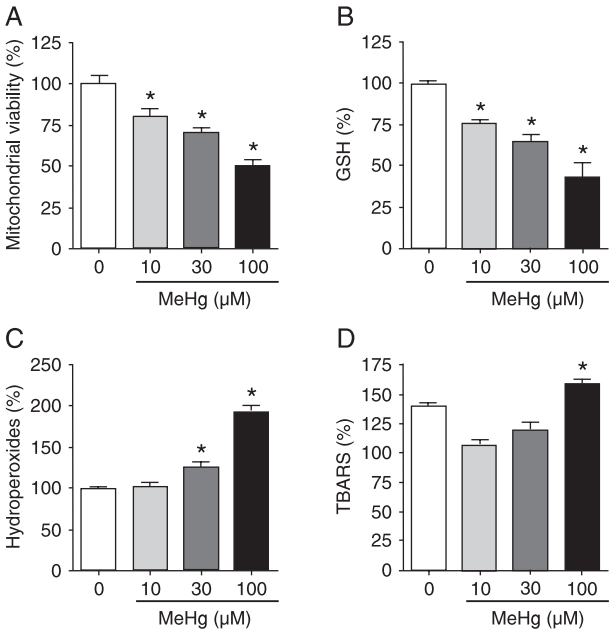

Figure 2. Effects of MeHg on brain mitochondria. Mouse brain mitochondrial-enriched fractions were isolated and incubated with different concentrations (0, 10, 30, and 100 µM) of MeHg for 30 min (MTT test and GSH measurement) or 60 min (total-hydroperoxide and TBARS content). Data are reported as means ± SD of percent of control for 4-6 separate assays carried out in duplicate. *P < 0.05 compared to control (without MeHg; ANOVA and Duncan *post hoc* test). *A*, Mitochondrial activity; *B*, GSH levels; *C*, total-hydroperoxides content; *D*, production of thiobarbituric acid reactive substances (TBARS).

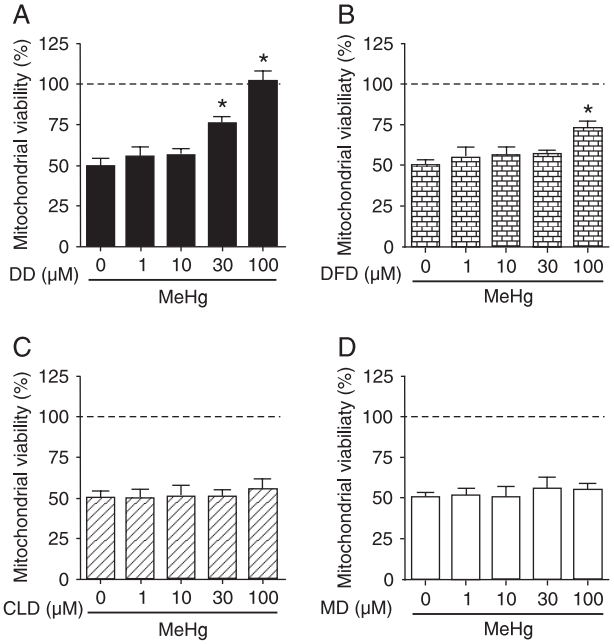

Figure 3. Effects of organoselenium compounds on MeHg-induced decrease in mitochondrial activity. Mouse brain mitochondrial-enriched fractions were incubated with 100 µM MeHg in the presence or absence of different concentrations (1-100 µM) of diphenyl diselenide (DD; *A*); 3'3-ditrifluoromethyldiphenyl diselenide (DFD; *B*); *p*-chloro-diphenyl diselenide (CLD; *C*), and *p*-methoxy-diphenyl diselenide (MD; *D*). Data are reported as means ± SD of percent of control for 4-6 separate assays carried out in duplicate. *P < 0.05 compared to MeHg alone (ANOVA and Duncan *post hoc* test). The dashed line indicates the control without MeHg (100%).

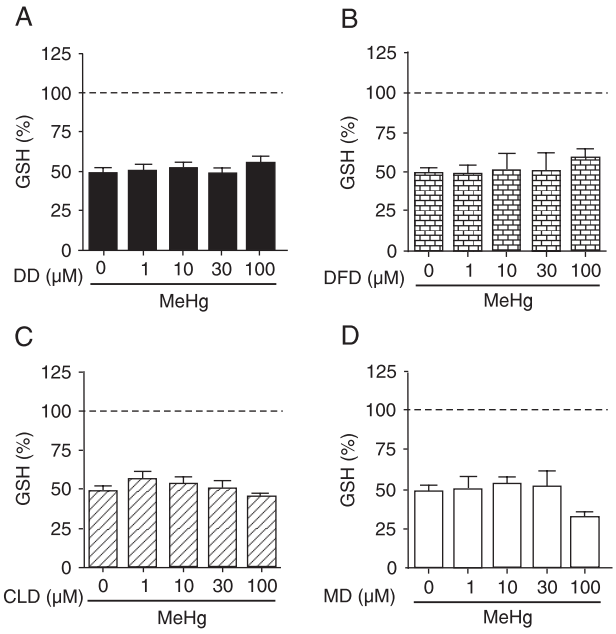

Figure 4. Effects of organoselenium compounds on MeHg-induced decrease in reduced glutathione (GSH) levels. Mouse brain mitochondrial-enriched fractions were incubated with 100 μM MeHg in the presence or absence of different concentrations (1-100 μM) of diphenyl diselenide (DD; *A*); 3'3-ditrifluoromethyldiphenyl diselenide (DFD; *B*); *p*-chloro-diphenyl diselenide (CLD; *C*), and *p*-methoxy-diphenyl diselenide (MD; *D*). Data are reported as means ± SD of percent of control for 4-6 separate assays carried out in duplicate. The dashed line indicates the control without MeHg (100%).

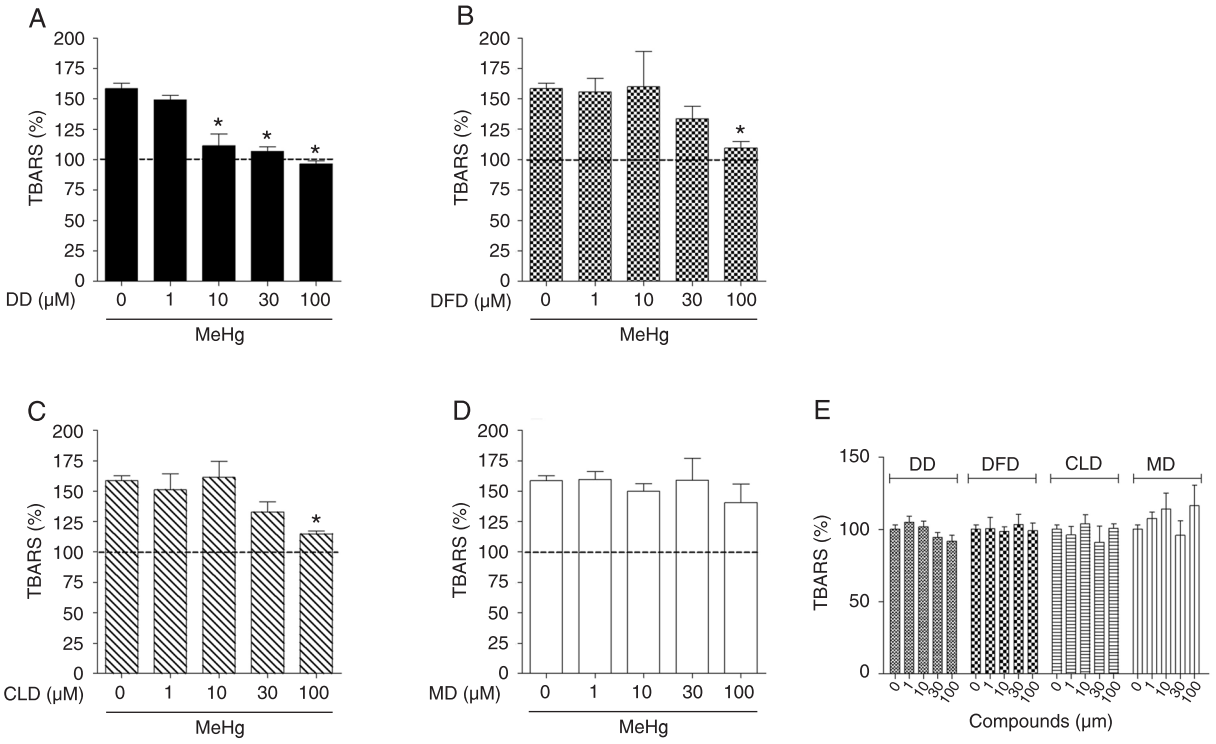

Figure 5. Effects of organoselenium compounds on MeHg-induced increase in the content of thiobarbituric acid reactive species (TBARS) levels. Mouse brain mitochondrial-enriched fractions were incubated with 100 μM MeHg in the presence or absence of increasing concentrations (1-100 μM) of diphenyl diselenide (DD; *A*); 3'3-ditrifluoromethyldiphenyl diselenide (DFD; *B*); *p*-chloro-diphenyl diselenide (CLD; *C*), and *p*-methoxy-diphenyl diselenide (MD; *D*). Effect of compounds alone on TBARS induction (*E*). Data are reported as means ± SD of percent of control for 4-6 separate assays carried out in duplicate. *P < 0.05 compared to MeHg alone (ANOVA and Duncan *post hoc* test). The dashed line indicates the control without MeHg (100%).

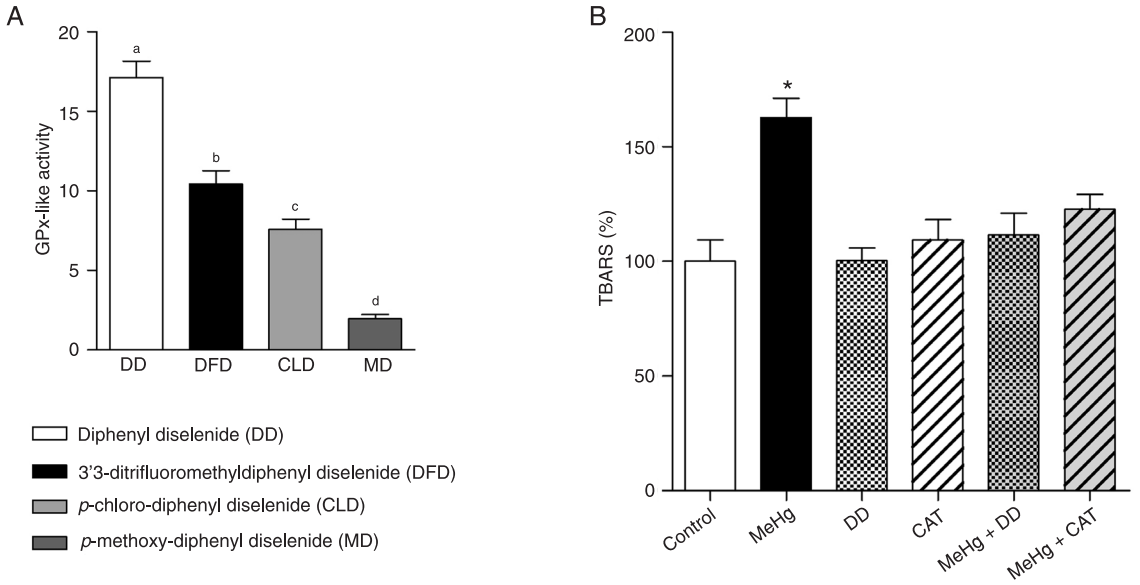

Figure 6. *A*, Glutathione peroxidase (GPx)-like activity of organodiselenides. A solution of each compound was added to cuvettes and GPx activity was determined as described in the Material and Methods section. Small letters indicate statistical differences between compounds, considering GPx-like activity ($P < 0.05$; ANOVA and Duncan *post hoc test*). *B*, Effect of DD and catalase (CAT) on MeHg-induced lipid peroxidation (thiobarbituric acid reactive species, TBARS). *$P < 0.05$ compared to control and to all other groups tested (ANOVA and Duncan *post hoc test*).

μM. However, DFD and CLD (Figure 5B and C) blocked the increase in lipid peroxidation promoted by MeHg only at the highest concentration (100 μM), while MD did not demonstrate a protective potential against lipid peroxidation induced by MeHg (Figure 5D). There was no significant TBARS induction by compounds alone (Figure 5E).

Glutathione peroxidase-like activity of organoselenium compounds

In a previous report (24), our group demonstrated that H_2O_2 generation represents a relevant event in MeHg-mediated oxidative stress in mouse brain mitochondria. GPx (EC 1.11.1.9) is a main cellular antioxidant responsible for the removal of peroxides in the brain (29). Thus, considering that GPx-like activity of organoselenium compounds is potentially involved in their antioxidant properties, we investigated the *in vitro* GPx-like activity of each compound. As observed in Figure 6A, DD demonstrated a higher GPx-like activity when compared to the other compounds tested. The order of magnitude for GPx-like activity was DD > DFD > CLD > MD. In parallel experiments, in order to confirm whether the anti-peroxidative activity of DD is linked to its peroxide removal ability, we incubated mouse brain mitochondrial-enriched fractions with MeHg in the presence or absence of DD and catalase, an enzyme involved in the clearance of H_2O_2. As shown in Figure 6B, MeHg-induced lipid peroxidation was completely reversed by catalase (200 U) as well as 100 μM DD, confirming that removal of peroxides is an important mechanism responsible for the protective effects of DD in our study model.

Discussion

In the present study, we used isolated brain mitochondria as a model to investigate MeHg toxicity, since this organelle represents a major target for MeHg in cells and plays a pivotal role in the initiation of biochemical cascades that lead to cell death (30,31). The effects of MeHg on mitochondrial function are associated with loss of the regular organization of the cristae (32) and dissipation of mitochondrial membrane potential (18,17,33).

The acute treatment of mitochondrial-enriched fractions from mouse brain with MeHg caused a decrease in mitochondrial activity, in agreement with previously reported results for kidney, brain and striatal mitochondrial fractions (17,24,34). This effect occurred in parallel to an increase in lipid peroxidation and GSH depletion. The relationship between ROS formation and mitochondrial damage after MeHg exposure is not fully understood. ROS can cause oxidative damage to mitochondria, leading to compromised mitochondrial function (35,36). On the other hand, ROS can also be produced by the mitochondria via leakage of electrons from the electron transport chain to molecular O_2, forming superoxide anion radicals ($O_2^{\cdot -}$). The $O_2^{\cdot -}$ is converted to H_2O_2 by the mitochondrial enzyme manganese superoxide dismutase (MnSOD) (37). Although this process occurs normally at a low rate in intact mitochondria, $O_2^{\cdot -}$ production can be dramatically increased if mitochondria

are challenged by toxicants (38). It was previously demonstrated that treatment with MeHg causes an increase in H_2O_2 generation as well inhibition of GPx activity (15,16,39), which could be responsible in part for the increase in lipid peroxidation observed in our model. We confirmed the participation of H_2O_2 formation in MeHg toxicity when we incubated the brain mitochondria with catalase. This H_2O_2 detoxifying enzyme was able to ameliorate the increase in lipid peroxidation promoted by MeHg, which points to an involvement of H_2O_2 in the lipid peroxidation promoted by MeHg and suggests that the GPx-like activity of DD is involved in the antioxidant effect against MeHg.

DD (30 and 100 µM) protected mouse brain mitochondria against MeHg toxicity by reversing the MeHg-induced loss of mitochondrial activity/viability. Among the novel organoselenium compounds tested here, only DFD partially reversed the effect of MeHg on mitochondrial activity at the highest concentration (100 µM). The co-treatment with DD completely blocked TBARS production by MeHg. This effect was observed from 10 µM up to 100 µM of this compound. The novel organodiselenides DFD and CLD were able to reverse the increase in lipid peroxidation promoted by MeHg only at 100 µM, emphasizing a higher efficiency of DD as a protective antioxidant, as demonstrated in previous studies (6-8).

GPx mimetic compounds can degrade hydroperoxides, consuming thiol reserves. Such ability confers to these compounds the capacity of protecting cells against oxidative stress conditions (7). The GPx-like activity of DD was significantly higher than the activity of DFD, CLD and MD, an effect possibly related to the higher antioxidant and protective effects of this compound. This result was confirmed by the fact that incubation of samples with catalase, which removes H_2O_2, avoided TBARS formation induced by MeHg exposure. These data suggest that the protective and antioxidant actions of DD are linked to its ability to remove peroxides. MeHg is known to increase H_2O_2 formation by mitochondria (24,34). In this regard, the GPx mimetic activity of DD may represent a promising tool against the cytotoxic effects of this environmental neurotoxin. The lack of protective effect of the organodiselenides DFD, MD and CLD may be related to their lower peroxidase-like activity compared to DD. A recent study from our group has shown the central role of GPx in the toxicity of MeHg (40). In that study, we showed that MeHg was able to decrease GPx activity in cell and animal models. In addition, the inhibition of GPx activity with mercaptosuccinic acid increased cell susceptibility to the toxic effects of methylmercury. On this basis, it seems plausible that DD, which showed higher thiol peroxidase activity than the other three substituted diselenides tested here, had the most prominent protective effects against methylmercury-induced oxidative stress and loss of mitochondrial activity *in vitro*. In addition to the antioxidant properties of selenium compounds, the ability to bind Hg ions may represent an important mechanism for cytoprotection. In fact, a recent study from our group showed that DD is able to remove Hg from tissues (22), reinforcing the therapeutic potential of selenium compounds against Hg intoxication.

The data reported here reinforce the antioxidant and protective potential of DD when comparing to other substituted organodiselenides. Our findings support the fact that compounds with glutathione peroxidase-like activity are potent blockers of mercurial-induced neurotoxic actions. In addition, our data indicate that depending on the chemical substitutions made on DD, its GPx-like activity may be impaired, which is crucial for the protective capacity of the compound. Considering that oxidative stress has been implicated in MeHg toxicity and that there are no effective treatments available to counteract the toxic effects of MeHg, the use of DD may represent an important therapeutic approach.

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