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Mercurial-Induced Hydrogen Peroxide Generation in Mouse Brain Mitochondria: Protective Effects of Quercetin

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Plants of the genus Polygala have been shown to possess protective effects against neuronal death and cognitive impairments in neurodegenerative disorders related to excitotoxicity. Moreover, previous reports from our group have shown the neuroprotective effects of the plant *Polygala paniculata* against methylmercury (MeHg)-induced neurotoxicity. In this work, we have examined the potential protective effects of three compounds (7-prenyloxy-6-methoxycoumarin, quercetin, and 1,5-dihidroxi-2,3-dimethoxy xanthone) from *Polygala* species against MeHg- and mercuric chloride ($HgCl_2$)-induced disruption of mitochondrial function under in vitro conditions using mitochondrial-enriched fractions from mouse brain. MeHg and $HgCl_2$ (10–100 μM) significantly decreased mitochondrial viability; this phenomenon was positively correlated to mercurial-induced glutathione oxidation. Among the isolated compounds, only quercetin (100–300 μM) prevented mercurial-induced disruption of mitochondrial viability. Moreover, quercetin, which did not display any chelating effect on MeHg or $HgCl_2$, prevented mercurial-induced glutathione oxidation. The present results suggest that the protective effects of quercetin against mercurial-induced mitochondrial dysfunction is related to the removal of oxidant species generated in the presence of either MeHg or $HgCl_2$. Reinforcing this hypothesis, MeHg and $HgCl_2$ increased the production of hydrogen peroxide in the brain mitochondria, as well as the levels of malondialdehyde. These oxidative phenomena were prevented by co-incubation with quercetin or catalase. These results are the first to show the involvement of hydrogen peroxide as a crucial molecule related to the toxic effects of both organic and inorganic mercurials in brain mitochondria. In addition, the study is the first to show the protective effect of quercetin against mercurial-induced toxicity, pointing to its capability to counteract mercurial-dependent hydrogen peroxide generation as a potential molecular mechanism of protection. Taken together, these data render quercetin a promising molecule for pharmacological studies with respects to mercurials' poisoning.

Introduction

Methylmercury (MeHg) is a highly neurotoxic compound leading to neurological and developmental deficits in animals and humans (*1*). Although MeHg-induced neurotoxicity is an extensively reported phenomenon, the molecular mechanisms underlying its toxicity are still under debate. The major mechanisms involved in MeHg neurotoxicity currently being explored are the impairment of intracellular calcium homeostasis (*2*), the alteration of glutamate homeostasis (*3–6*), and oxidative stress (*7*). The last seems to be related to the direct oxidative properties of MeHg on endogenous thiols (*8*) and to its effects toward antioxidant enzymes (*9–11*). In addition, it is noteworthy that MeHg-induced oxidative stress and MeHg-induced glutamate dyshomeostasis appear to be connected phenomena affecting each other; for review, see Aschner et al (*12*).

Notwithstanding the massive efforts in the search for new drugs that counteract mercurial toxicity, there are no effective treatments available that completely abolish its toxic effects. In MeHg poisoning, supportive care is given when necessary to maintain vital functions. Moreover, the use of chelating agents assists the body's ability to eliminate mercury from the tissues. However, these drugs are of limited use because of their adverse side effects (*13*).

Several studies have focused their efforts on the protective effects of plants and their isolated compounds on diverse neuropathological conditions (*14, 15*). In this regard, plants of the genus *Polygala* have been shown to possess protective effects against neuronal death and cognitive impairments in neurodegenerative disorders related to excitotoxicity (*16, 17*).

A previous study from our group (*10*) demonstrated that the hydroalcoholic extract of *Polygala paniculata* protected against MeHg-induced neurotoxicity in mice. Although data examined in that study have showed that both behavioral and neurochemical changes induced by MeHg were prevented by *Polygala paniculata* extract co-administration, data about the potential compound(s) responsible for the observed neuroprotective effects as well as its/their potential molecular mechanisms were lacking.

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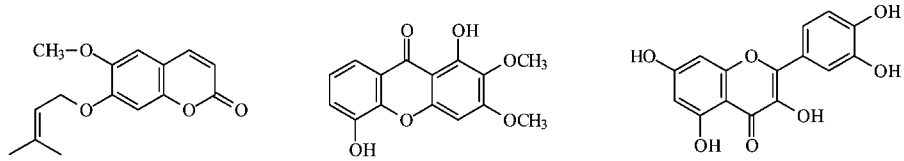

Figure 1. Chemical structures of *Polygala*-derived compounds.

As already mentioned, oxidative stress is a central phenomenon related to MeHg-induced neurotoxicity. In this regard, mitochondria appear to be important cellular organelles targeted by the pro-oxidative effects of MeHg (*18*). Apoptosis under mitochondrial control has been shown to have an important role in the neuronal death process (*19, 20*). It usually involves the reduction of mitochondrial transmembrane potential and the release of cytochrome C into the cytoplasm, followed by caspase activation (*19, 21*). Therefore, disturbances of mitochondrial integrity may initiate cellular death processes. Mercury is known to accumulate inside mitochondria, and it can thus change mitochondrial membrane permeability and cause reduction of mitochondrial transmembrane potential (*22–24*). Consequently, several studies have pointed to apoptosis as a critical phenomenon involved with MeHg-induced neurotoxicity (*25–27*).

Taking into account the protective effects of *Polygala paniculata* against MeHg-induced neurotoxicity in mice (*10*) and the involvement of mitochondrial dysfunction during MeHg-induced neurotoxicity, the aim of this study was to investigate the potential protective effects of three compounds (7-prenyloxy-6-methoxycoumarin, quercetin, and 1,5-dihidroxi-2,3-dimethoxy xanthone) isolated from two plants of genus *Polygala (Polygala paniculata* and *Polygala sabulosa)* against mercurial-induced mitochondrial dysfunction under in vitro conditions, using mitochondrial-enriched fractions from mouse brain. Mercuric chloride was also investigated as mitochondrial challenge because of the degradation of MeHg into inorganic mercury in mammalian tissues (*28*).

Materials and Methods

Materials. Adult Swiss Albino male mice (2 months old) were bred in the animal facilities of the Universidade Federal de Santa Catarina. The mice were maintained according to the Animal Care Guidelines from the National Institutes of Health of the United States of America, and all experiments were approved by our ethic committee for animal use (313/CEUA; 23080.026023/2004-39/UFSC). The animals were maintained at 23°C on a 12 h light/dark cycle with free access to water and food (Nuvital, PR, Brazil). Methylmercury (II) chloride, mercuric chloride, 5,5′-dithiobis-(2-nitrobenzoic acid), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO). All other chemicals were analytical grade.

Plant Material. *Polygala paniculata* L. (Polygalaceae) was collected in Florianópolis (SC, Brazil) and identified by comparison with the voucher UPCNB 26027 by Prof. Dr. Olavo de Araújo Guimarães of the Botany Department of the Universidade Federal do Paraná, Brazil. *Polygala sabulosa* A.W. Bennett was collected in Rancho Queimado (SC, Brazil) and was also identified by Dr. Guimarães by comparison with the voucher UPCNB 19640.

Extraction and Isolation. 1. *Polygala paniculata.* Air-dried whole plant (3.500 g) was degreased with hexane and extracted with ethanol:water (4:1, v/v). The hydroalcoholic extract was subjected to chromatography fractionation on silica gel column using mixtures of hexane:ethyl acetate:methanol of increasing polarity to give 75 fractions. Fractions 29–35 were combined on the basis TLC analysis in hexane:ethyl acetate (3:4, v/v) and purified by flash chromatography to give the 1,5-dihidroxi-2,3-dimethoxy xanthone.

2. *Polygala sabulosa.* The dried and powdered whole plant (500 g) was extracted exhaustively with ethanol (96%) at room temperature. The crude extract (135 g) obtained was partitioned into hexane, CH_2Cl_2, ethyl acetate (EtOAc), and water to give four distinct hexane-soluble (16.1 g), CH_2Cl_2-soluble (28 g), AcOEt-soluble (16.8 g), and aqueous (64.6 g) fractions. The CH_2Cl_2-soluble fraction (16 g) was chromatographed on a silica gel column eluted with increasing amounts of ethyl acetate in *n*-hexane to give 36 fractions. The combined fractions 21–26 were dissolved in EtOAc (50 mL) and treated under heating and shaking with activated charcoal (1 g). After filtration, drying (anhydrous $NaSO_4$), and removal of the solvent, the white residue was purified by recrystallization from hexane:EtOAc (3:1) to obtain 530 mg of the 7-prenyloxy-6-methoxycoumarin (*29*). The EtOAc-soluble fraction (10 g) was further subjected to column chromatography over silica gel eluted with a hexane:EtOAc gradient system. The polar fractions were further subjected to Sephadex LH-20 eluted with MeOH to yield quercetin (87 mg). The compounds isolated were identified by comparison of their physical and spectral (IV, ^1H, and ^{13}C NMR) data with authentic samples, and their chemical structures are depicted in Figure 1.

Preparation of Mitochondrial Enriched Fractions. Mitochondrial-enriched fractions were prepared essentially as described previously (*30*). Briefly, adult (2 months old) male Swiss mice were sacrificed by decapitation. The whole brains (less cerebellum) were 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, resulting in a myelin-rich supernatant and a pellet (P2) consisting of synaptosomes and free mitochondria. The supernatant was discarded, and the pellet was resuspended in the isolation medium but without albumin. The mitochondrial-enriched fractions were kept on ice for 10–15 min until the experiments were performed.

Incubations. P2 (approximately 2 mg of protein) was incubated with different concentrations of mercurials (0, 10, 30, and 100 μM) and/or the *Polygala*-derived compounds (7-prenyloxy-6-methoxy-coumarin, quercetin, and 1,5-dihidroxi-2,3-dimethoxy xanthone at 0, 100, and 300 μM) in a medium containing 10 mM HEPES buffer (pH 7.0), 220 mM mannitol, 68 mM sucrose, and 10 mM KCl (total incubation volume = 300 μL). Incubations were carried out for 30 min at 25 °C. *Polygala*-derived compounds were dissolved in dimethyl sulfoxide, of which the final concentration did not exceed 0.5% and did not display any effect itself (data not shown). After incubations, mitochondrial function or glutathione content were determined. Parallel experiments were also carried out in the presence of catalase (200 units) or hydrogen peroxide (100 μM) in order to elucidate molecular mechanisms of toxicity and protection.

Assessment of Mitochondrial Function. Mitochondrial function was assessed by the conversion of the dye methylthiazolyldiphenil-tetrazolium bromide (MTT) to formazan (*18*). This assay is based on the ability of the mitochondrial enzyme succinate dehydrogenase to metabolize MTT into formazan, a reaction that takes place only in functionally intact mitochondria. Briefly, after the preincubation of P2 with mercurials and/or *Polygala*-derived compounds (30 min

at 25 °C), the reaction medium (300 μL) was incubated with 300 μL of 1.2 mM MTT 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 at 550 nm. Data were expressed as percentage of control.

Assessment of Glutathione Content. Glutathione content was measured as nonprotein thiols according to a method previously described (*31*), with minor modifications. Briefly, after the preincubation of P2 with mercurials and/or *Polygala*-derived compounds (30 min at 25 °C), 300 μL of trichloroacetic acid 10% was added to the reaction medium (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 (*31*).

Assessment of Hydrogen Peroxide Content and Lipid Peroxidation. The levels of hydrogen peroxide produced by mitochondria were evaluated through the xylenol orange assay (*32*), with minor modifications. In short, after the pre-incubation of P2 with mercurials and/or quercetin/catalase (30 min at 25 °C), the reaction medium was centrifuged at 17 500 g for 10 min at 4°C and the supernatant was incubated for 30 min in a reaction medium containing 250 mM perchloric acid, 2.5 mM ammonium iron (II) sulfate hexahydrate, and 1 mM xylenol orange. Hydroperoxide levels were determined at 560 nm using a hydrogen peroxide curve as standard. Lipid peroxidation levels were measured as thiobarbituric acid reactive substances (TBARS) according to the method described by Ohkawa and collaborators (*33*). Briefly, samples were incubated in a reaction media containing 0.28 % 2-thiobarbituric acid, 1.2 % SDS, and 0.45 M/0.12 M acetic acid/HCl buffer (pH 3.4). After incubation at 95 °C for 60 min, TBARS were measured at 532 nm and compared to a standard curve of malondialdehyde.

In vitro Experiments To Detect Possible Chelating Effects of Quercetin. Taking into account the protective role of quercetin on mercurial-induced mitochondrial dysfunction and glutathione oxidation, the potential chelating effects of quercetin towards mercurials were analyzed. Such methodological approach was based on the indirect determination of free mercurial using reduced glutathione (GSH), where free mercurial is available to oxidize GSH. Briefly, different concentrations of MeHg or $HgCl_2$ (0, 10, 25, 50, and 100 μM) were incubated with GSH (100 μM) in the presence or in the absence of quercetin (100 μM) at 25 °C (volume total of reaction = 1 mL). After incubation for 30 min, the amount of GSH was determined by reaction with 55′-dithiobis-(2-nitrobenzoic acid) (*31*).

Assessment of Protein Content. Protein concentration was determined according to Bradford (*34*), using a bovine serum albumin as standard.

Statistical Analysis. Statistical differences among groups were analyzed by one-way or two-way analysis of variance, followed by Duncan's multiple range test when appropriate. Pearson analysis was used to correlate variables. Differences were considered statistically significant when $P < 0.05$.

Results

Figure 2 shows that both MeHg (A) and $HgCl_2$ (B) caused a significant and dose-dependent decrease in mitochondrial viability in mitochondrial-enriched fractions of mouse brain. In addition, both mercurials induced a significant oxidation of endogenous glutathione in a dose-dependent manner (Figure 3). Correlation analyses showed significant positive correlations between mitochondrial viability and glutathione content for MeHg (Pearson coefficient = 0.933; $P < 0.01$) and or $HgCl_2$ (Pearson coefficient = 0.854; $P < 0.01$). It is important to state that even the lowest mercurial concentration (10 μM) was able to significantly decrease mitochondrial viability and glutathione levels when either organic or inorganic mercury was used. This concentration is closely related to that (around 2 μg/g) found in the cortex of humans exposed to mercury because of the consumption of MeHg-contaminated food (*35*).

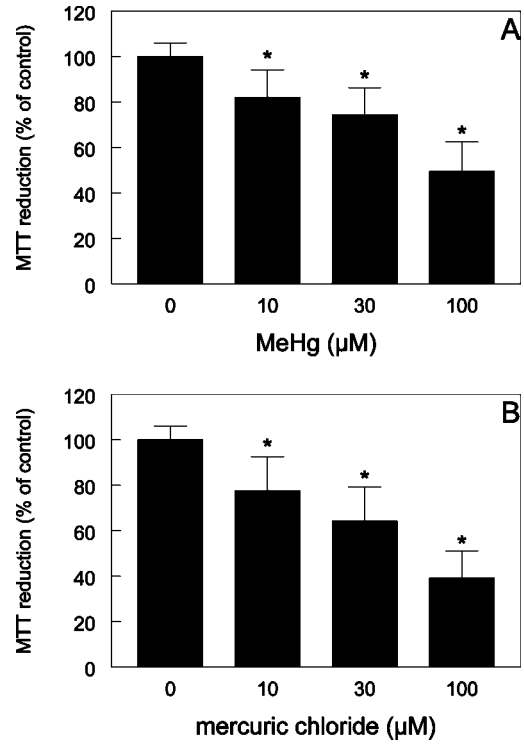

Figure 2. Effects of MeHg or $HgCl_2$ on mitochondrial viability. Mitochondrial-enriched fractions from mouse brain were incubated with different concentrations (0, 10, 30, and 100 μM) of (A) MeHg or (B) $HgCl_2$ in 10 mM HEPES buffer (pH 7.0) containing 220 mM mannitol, 68 mM sucrose, and 10 mM KCl (total incubation volume = 300 μL). After preincubation (30 min at 25 °C), mitochondrial viability was assessed by the MTT reduction assay (see Materials and Methods). Data are expressed as mean ± standard deviation and represented as percent control for four independent assays. *Statistically different from control ($P < 0.05$).

The potential protective effects of *Polygala*-derived compounds against mercurial-induced mitochondrial dysfunction are depicted in Figures 4 and 5. Figures 4A and 5A show that quercetin, at 100 and 300 μM, completely prevented MeHg- and $HgCl_2$-induced mitochondrial dysfunction, respectively. However, 7-prenyloxy-6-methoxycoumarin and 1,5-dihidroxi-2,3-dimethoxy xanthone did not display protective effects (Figures 4B, 4C, 5B, and 5C). Indeed, 7-prenyloxy-6-methoxycoumarin and 1,5-dihidroxi-2,3-dimethoxy xanthone alone decreased mitochondrial viability.

Taking into account the positive correlation between mitochondrial viability and glutathione content after mercurial exposure and the protective effects of quercetin against mercurial-induced mitochondrial dysfunction, we evaluated glutathione levels after simultaneous exposure to both MeHg/$HgCl_2$ and quercetin. The flavonoid completely prevented MeHg- and $HgCl_2$-induced decrease in reduced glutathione levels (Figure 6). In an attempt to discard the possible direct chemical interaction between quercetin and mercurials, we performed an in vitro assay in order to elucidate if this flavonoid is able to decrease the amount of "free" mercurial in the reaction medium. As expected, Table 1 showed that quercetin was unable to bind MeHg or $HgCl_2$. In fact, the presence of quercetin in the reaction medium did not change the oxidative capability of both MeHg and $HgCl_2$ to reduced glutathione.

Taking the aforementioned results into consideration, we hypothesized that quercetin could be capable of detoxifying reactive/oxidant molecules generated in the presence of mer-

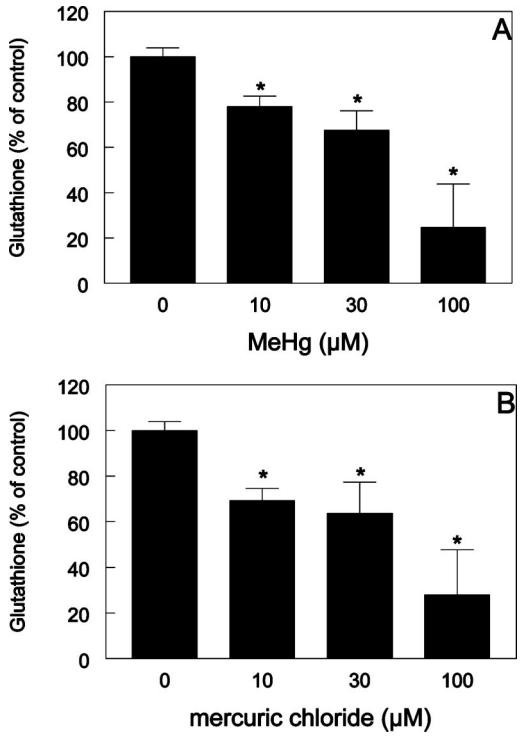

Figure 3. Effects of the MeHg or $HgCl_2$ on glutathione content. Mitochondrial-enriched fractions from mouse brain were preincubated with different concentrations of (A) MeHg or (B) $HgCl_2$ (0, 10, 30, and 100 μM) in 10 mM HEPES buffer (pH 7.0) containing 220 mM mannitol, 68 mM sucrose, and 10 mM KCl (total incubation volume = 300 μL). After preincubation (30 min at 25 °C), glutathione content was quantified using 5,5′-dithiobis-(2-nitrobenzoic acid) (see Materials and Methods). Data are expressed as mean ± standard deviation and represented as percent control for four independent assays. *Statistically different from control ($P < 0.05$). Glutathione content in basal condition was 1.0 ± 0.04 nmol/mg of protein.

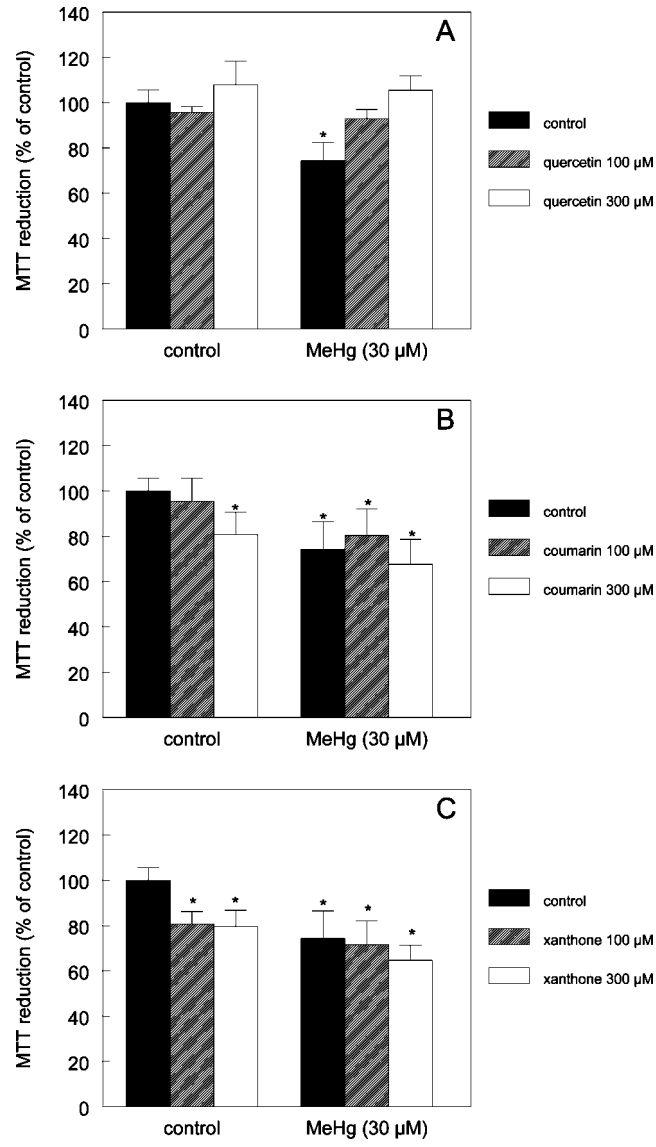

Figure 4. Effects of the *Polygala*-derived compounds on MeHg-induced mitochondrial dysfunction. Mitochondrial-enriched fractions from mouse brain were preincubated with MeHg (30 μM) and/or (A) quercetin, (B) 7-prenyloxy-6-methoxycoumarin, or (C) 1,5-dihidroxi-2,3-dimethoxy xanthone (0, 100, and 300 μM) in 10 mM HEPES buffer (pH 7.0) containing 220 mM mannitol, 68 mM sucrose, and 10 mM KCl (total incubation volume = 300 μL). After pre- (30 min at 25 °C), mitochondrial viability was assessed by the MTT reduction assay (see Materials and Methods). Data are expressed as mean ± standard deviation and represented as percent control for four independent assays. *Statistically different from control ($P < 0.05$).

curials in the brain synaptosomal medium. This idea was based on the study of Lund and collaborators (*36*), which points to increased hydrogen peroxide production in rat kidney mitochondria during mercuric chloride exposure. Figure 7 shows that catalase, an enzyme involved in the detoxification of hydrogen peroxide, completely prevented MeHg-induced mitochondrial dysfunction. In addition, catalase partially prevented $HgCl_2$-induced mitochondrial dysfunction.

Because the previous data (Figure 7) have pointed to hydrogen peroxide as an important molecule involved with mercurial-induced mitochondrial dysfunction, the levels of hydrogen peroxide generated during MeHg or $HgCl_2$ exposure were evaluated. Figure 8 shows that both MeHg (Figure 8A) and $HgCl_2$ (Figure 8B) enhanced the levels of hydrogen peroxide. Interestingly, such phenomena were abolished by the addition of either catalase (200 units) or quercetin (100 μM) in the reaction medium. Moreover, both MeHg (Figure 9A) and $HgCl_2$ (Figure 9B) enhanced the levels of thiobarbituric acid reactive substances (a marker for lipid peroxidation), and these phenomena were also abolished by the addition of catalase (200 units) or quercetin (100 μM) in the reaction medium.

Discussion

A previous study from our group (*10*) reported significant beneficial effects of the hydroalcoholic extract of *Polygala paniculata* against MeHg-induced neurotoxicity in mice. In that study, the hydroalcoholic extract of *Polygala paniculata* was able to prevent behavioral and neurochemical changes induced by the oral exposure to MeHg, pointing to such a plant as a potential therapeutic agent for the treatment of pathological conditions related to excitotoxicity and oxidative stress, including MeHg poisoning. In agreement, plants of the genus *Polygala* have been reported to display neuroprotective effects in several neuropathological conditions related to excitotoxicity and oxidative stress (*16, 17*).

In the present study, we sought potential compounds derived from *Polygala* that might be responsible for protection against mercurial-induced neurotoxicity, using mitochondrial-enriched fractions from mouse brain. Three molecules, from different chemical groups, a flavonoid (quercetin), a coumarin (7-prenyloxy-6-methoxycoumarin), and a xanthone (1,5-dihidroxi-2,3-dimethoxy xanthone), were selected for this proposal. Taking into account the possible degradation of MeHg into inorganic mercury in mammalian tissues (*28*), we investigated both

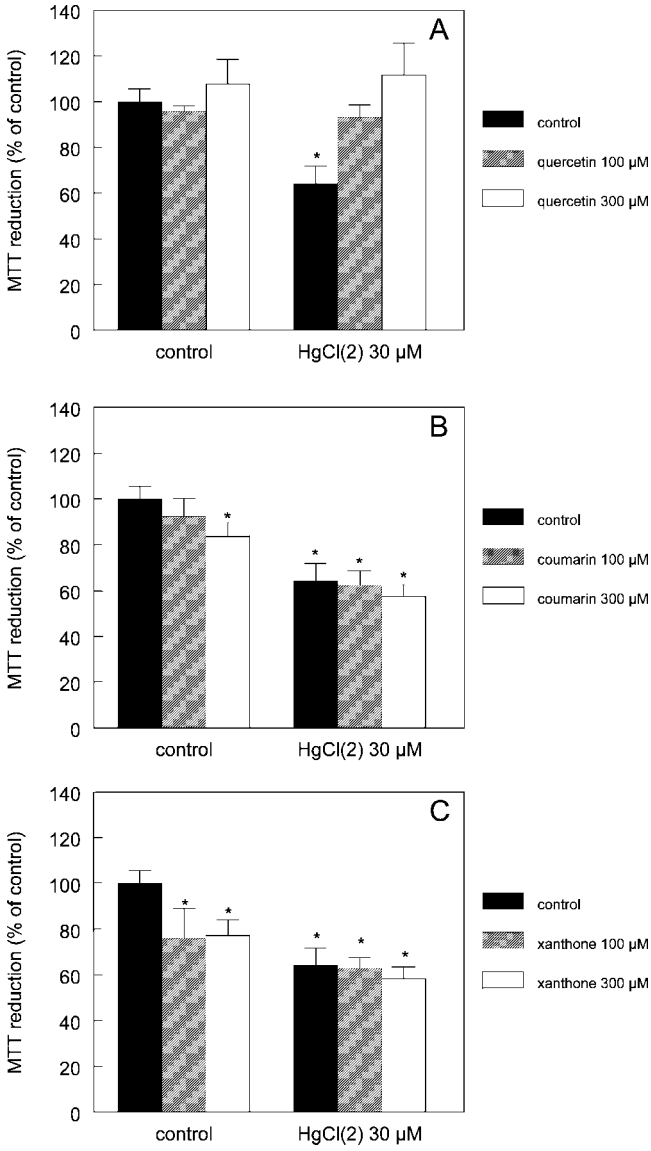

Figure 5. Effects of the *Polygala*-derived compounds on $HgCl_2$-induced mitochondrial dysfunction. Mitochondrial-enriched fractions from mouse brain were preincubated with $HgCl_2$ (0 or 30 μM) and/or (A) quercetin, (B) 7-prenyloxy-6-methoxycoumarin, or (C) 1,5-dihidroxi-2,3-dimethoxy xanthone (0, 100, and 300 μM) in 10 mM HEPES buffer (pH 7.0) containing 220 mM mannitol, 68 mM sucrose, and 10 mM KCl (total incubation volume = 300 μL). After preincubation (30 min at 25 °C), mitochondrial viability was assessed by the MTT reduction assay (see Materials and Methods). Data are expressed as mean ± standard deviation and represented as percent control for four independent assays. *Statistically different from control ($P < 0.05$).

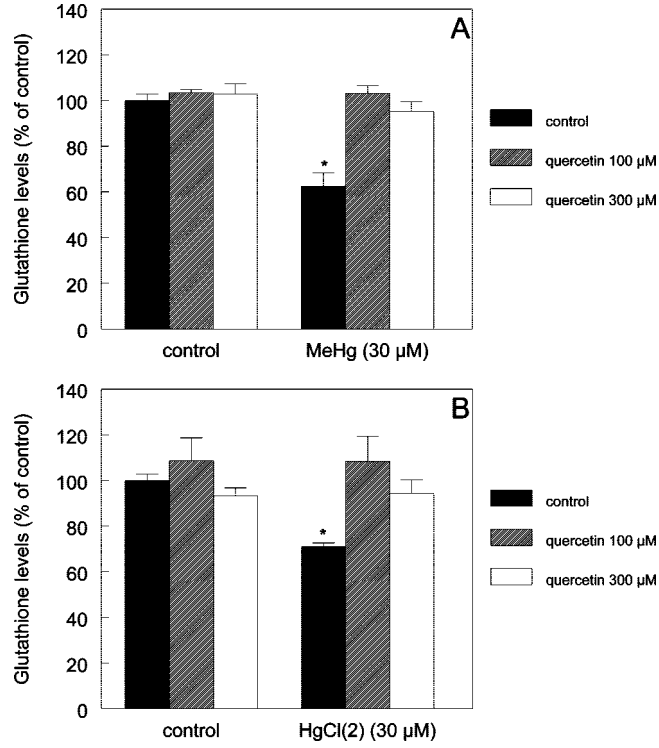

Figure 6. Effects of the quercetin on MeHg- or $HgCl_2$-induced glutathione oxidation. Mitochondrial-enriched fractions from mouse brain were preincubated with (A) MeHg or (B) $HgCl_2$ (30 μM) and/or quercetin (0, 100 or 300 μM) in 10 mM HEPES buffer (pH 7.0) containing 220 mM mannitol, 68 mM sucrose, and 10 mM KCl (total incubation volume = 300 μL). After preincubation (30 min at 25 °C), glutathione content was quantified using 5,5′-dithiobis-(2-nitrobenzoic acid) (see Materials and Methods). Data are expressed as mean ± standard deviation and represented as percent control for four independent assays. *Statistically different from control ($P < 0.05$). Glutathione content in basal condition was 1.0 ± 0.028 nmol/mg protein.

Table 1. Effects of Quercetin on the Mercurial-Induced Glutathione (GSH) Oxidation[a]

	remaining reduced GSH (nmol)	
	without quercetin	with quercetin
MeHg (nmol/mL)		
0	100 ± 3.9	96.8 ± 5.2
10	92.0 ± 2.5	88.7 ± 3.4
25	81.2 ± 1.7	81.7 ± 2.3
50	65.0 ± 8.4	60.7 ± 8.8
100	20.3 ± 0.6	18.2 ± 2.7
$HgCl_2$ (nmol/mL)		
0	98.9 ± 4.0	97.1 ± 2.2
10	87.7 ± 4.4	87.3 ± 6.5
25	62.1 ± 2.4	57.2 ± 5.2
50	18.5 ± 2.0	16.9 ± 2.1
100	1.3 ± 0.1	1.5 ± 0.3

[a] MeHg or $HgCl_2$ (0, 10, 25, 50, and 100 μM) were incubated with GSH (100 μM) in the presence or in the absence of quercetin (100 μM) at 25 °C (volume total of reaction = 1 mL). After incubation for 30 min, the amount of remaining GSH was determined using 55′-dithiobis-(2-nitrobenzoic acid). Data are expressed as mean ± standard deviation and represented as nmol of reduced glutathione for three independent assays. Two-way analysis of variance (5 mercurial doses vs quercetin) showed no significant interactions of quercetin with MeHg [$F(4,29) = 0.624$, $p = 0.651$] or with $HgCl_2$ [$F(4,29) = 0.695$, $p = 0.604$] toward glutathione oxidation.

organic (MeHg) and inorganic ($HgCl_2$) mercurials as potential mitochondrial challenges.

It was noteworthy that both mercurials (MeHg and $HgCl_2$) caused a similar and dose-dependent decrease in mitochondrial viability, which was positively correlated to glutathione oxidation. From a molecular point of view, these results represent important findings. In fact, considering the differential number of potential electrophilic regions of mercury atom in the organic-methylated form (one positive charge) and in the mercuric mercury form (two positive charges), when dissociated in aqueous medium, the similar profiles of mitochondrial glutathione oxidation and dysfunction observed after MeHg and $HgCl_2$ exposure were unexpected. Such results indicate that MeHg- and $HgCl_2$-induced mitochondrial dysfunction depend on phenomena other than the simple direct interaction between mercury and sulfhydryl groups from biomolecules. Taking into account the degradation of MeHg into inorganic mercury in mammalian tissues, one could suppose that the similar potency of MeHg and $HgCl_2$ toward mitochondrial viability and glutathione oxidation could be related, at least in part, to the

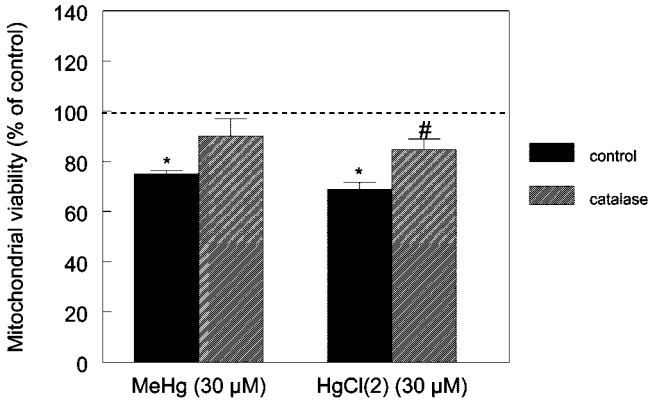

Figure 7. Effects of catalase on MeHg- or $HgCl_2$-induced mitochondrial dysfunction. Mitochondrial-enriched fractions from mouse brain were preincubated with MeHg or $HgCl_2$ (30 μM) and/or catalase (200 units) in 10 mM HEPES buffer (pH 7.0) containing 220 mM mannitol, 68 mM sucrose, and 10 mM KCl (total incubation volume = 300 μL). After preincubation (30 min at 25 °C), mitochondrial viability was assessed by the MTT reduction assay (see Materials and Methods). Data are expressed as mean ± standard deviation and represented as percent control (dotted line) for three independent assays. *Statistically different from the corresponding condition with catalase ($P < 0.05$). #Statistically different from control ($P < 0.05$).

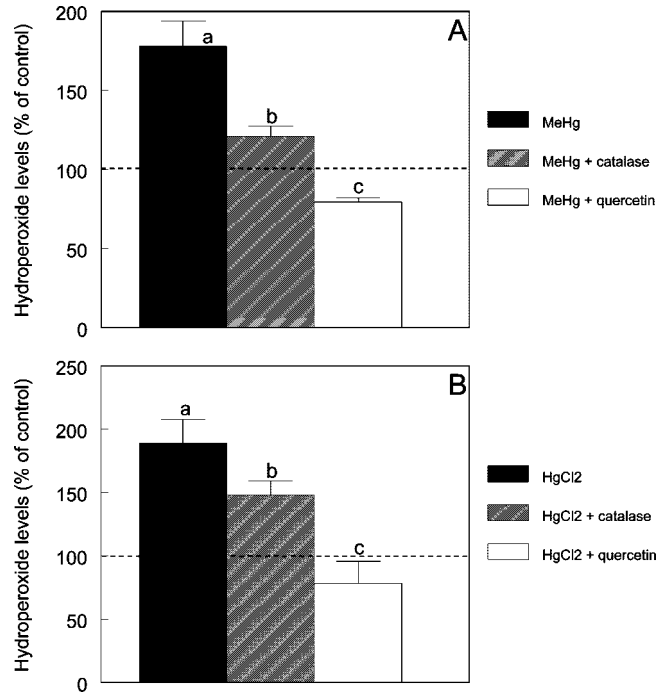

Figure 8. Effects of mercurials on hydrogen peroxide production. Mitochondrial-enriched fractions from mouse brain were preincubated with (A) 100 μM MeHg or (B) $HgCl_2$ and/or catalase (200 units) or quercetin (100 μM) in 10 mM HEPES buffer (pH 7.0) containing 220 mM mannitol, 68 mM sucrose, and 10 mM KCl (total incubation volume = 300 μL). After preincubation (60 min at 25 °C), hydrogen peroxide formation was assessed by the xylenol orange assay (see Materials and Methods). Data are expressed as mean ± standard deviation and represented as percent of control (dotted line) for four independent assays. Different letters indicate significant difference ($P < 0.05$) by one-way analysis of variance. Hydrogen peroxide level in basal condition was 0.14 ± 0.021 nmol/mg ofprotein.

instability of MeHg and its conversion to inorganic mercury. Although this hypothesis could not be ruled out, it is important to state that the degradation of MeHg into inorganic mercury depends on the activity of NADPH-cytochrome P-450 reductase, an enzyme present only in microsomal preparations (*28*).

Herein, the incubation of mitochondrial enriched fractions with MeHg or $HgCl_2$ enhanced by around 80% the hydrogen peroxide (H_2O_2) production and such effects were reduced by the addition of catalase, strongly pointing to H_2O_2 as a crucial molecule involved in mercurials-induced mitochondrial dysfunction. This result is confirmed by the fact that both mercurials increased the levels of thiobarbituric acid reactive substances (marker of lipid peroxidation) and such phenomena were also prevented by the addition of catalase, an enzyme that specifically detoxifies H_2O_2.

Therefore, the increase in H_2O_2 formation by mouse brain mitochondria exposed to mercurials may represent a significant finding of our study, considering the oxidative role of H_2O_2 toward sulphydryl groups. This observation is in agreement with Lund et al. (*36*), who effectively demonstrated that $HgCl_2$ is able to induce renal mitochondrial dysfunction and pointed to H_2O_2 as a key molecule in mercury toxicity to renal tissue. Furthermore, an increase in reactive oxygen species generation in brain preparations after mercurials (organic or inorganic mercury) exposure has been also demonstrated (*8, 18*). However, those studies were based on the 2′,7′-dichlorofluorescein methodology, which can not discriminate between hydroxyl radicals, peroxynitrite, or H_2O_2 (*37*).

Among the three Polygala-derived compounds, only quercetin displayed protective effects against mercurial-induced mitochondrial dysfunction. In this regard, several studies have shown the protective effects of quercetin in different models of chemical injury under in vitro and in vivo conditions (*38–40*). Particularly important: quercetin has been reported to display beneficial effects against metal-induced toxicity (*41, 42*). Moreover, two recent and interesting studies have reported the anti-peroxidative action of quercetin under in vitro conditions (*43, 44*). However, to the best of our knowledge, there are no data in the literature showing protective effects of quercetin against mercurial-induced neurotoxicity.

Even though quercetin prevented mercurial-induced mitochondrial dysfunction and glutathione oxidation, our results demonstrate that such protective effect is not directly related to chelating effects. Indeed, quercetin did not change the oxidative capability of both MeHg and $HgCl_2$ regarding reduced glutathione, indicating that the "free" amounts of both mercurials are not changed by the presence of quercetin. Because the mitochondrial dysfunction observed after mercurial exposure was related to H_2O_2 production, we hypothesized that quercetin could be capable of detoxifying the H_2O_2 generated in the presence of mercurials in the brain mitochondria. Our results indeed showed that quercetin decreased mercurial-induced H_2O_2 production and such data are in agreement with the fact that quercetin also abolished mercurial-induced lipid peroxidation in the brain mitochondrial-enriched fraction. In conformity, the protective effect of quercetin against H_2O_2-induced toxicity has been shown under in vitro conditions (*45*).

Even though quercetin prevented mercurial-induced mitochondrial toxicity under in vitro conditions, it is difficult to affirm that quercetin is directly and exclusively involved with the beneficial effects (*10, 16, 17*) of plants of the genus Polygala observed under in vivo conditions. Several compounds—aurapten, phebalosin, murrangatin, and 7-methoxy-8-(l,4-dihydroxy-3-methyl-2-butenyl)coumarin (*46*); 1-hydroxy-2,3,5-trimethoxyxanthone, 1-hydroxy-5-methoxy-2,3-methylenedioxyxanthone, 1,5-dihydroxy-2,3-dimethoxyxanthone, coumarin murragatin, rutin, spinasterol, and delta-25-spinasterol (*29*)—were isolated from *Polygala paniculata*, and the knowledge about their biological effects are scarce. So, it is possible that the beneficial effects displayed by *Polygala paniculata* under in vivo condi-

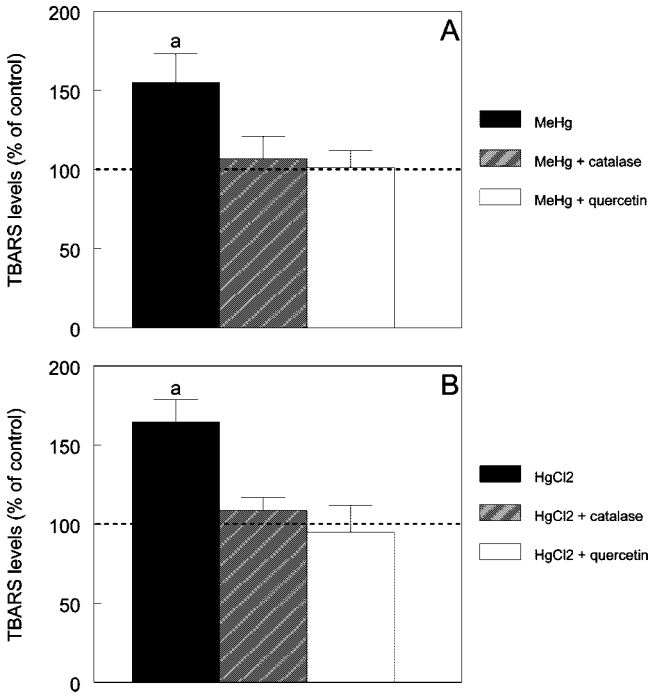

Figure 9. Effects of mercurials on lipid peroxidation. Mitochondrial-enriched fractions from mouse brain were pre-incubated with 100 μM (A) MeHg or $HgCl_2$ (B) and/or catalase (200 units) or quercetin (100 μM) in 10 mM HEPES buffer (pH 7.0) containing 220 mM mannitol, 68 mM sucrose, and 10 mM KCl (total incubation volume = 300 μL). After preincubation (60 min at 25 °C), thiobarbituric acid reactive substances (TBARS) levels were assessed (see Materials and Methods). Data are expressed as mean ± standard deviation and represented as percent control (dotted line) for four independent assays. [a]Statistically different from control ($P < 0.05$) by one-way analysis of variance. TBARS levels in basal condition were 7.8 ± 0.14 nmol MDA/mg of protein.

tions (*10*) are not necessarily related to a single compound. This hypothesis is reinforced by the fact that 100 and 300 μM are high quercetin concentrations that practically could not be reached under in vivo conditions.

In summary, this study shows that thiol oxidation is a crucial phenomenon involved with MeHg- and $HgCl_2$-induced mitochondrial dysfunction and that the protective effect of quercetin on such process is not directly related to chelating effects. Moreover, our results are the first to show that H_2O_2 is involved with the toxic effects of both organic and inorganic mercurials in brain mitochondria. The study also shows the protective effect of quercetin against mercurial-induced toxicity, pointing to its capability to counteract mercurial-dependent H_2O_2 generation as a potential molecular mechanism of protection. Taken together, these data add new information about molecular mechanisms associated to mercurial toxicity and render quercetin a promising molecule for pharmacological studies with respect to mercurials' poisoning.

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