Arch Toxicol (2010) 84:89–97
DOI 10.1007/s00204-009-0482-3

INORGANIC COMPOUNDS

Comparative study of quercetin and its two glycoside derivatives quercitrin and rutin against methylmercury (MeHg)-induced ROS production in rat brain slices

Caroline Wagner · Alessandra P. Vargas · Daniel H. Roos · Ademir F. Morel · Marcelo Farina · Cristina W. Nogueira · Michael Aschner · João B. Rocha

Received: 6 October 2009 / Accepted: 15 October 2009 / Published online: 10 November 2009

Abstract The hypothesis that methylmercury (MeHg) potently induces formation of reactive oxygen species (ROS) in the brain is supported by observations on the neuroprotective effects of various classes of antioxidants. Flavonoids have been reported to possess divalent metal chelating properties, antioxidant activities and to readily permeate the blood–brain barrier. They can also provide neuroprotection in a wide array of cellular and animal models of neurological diseases. Paradoxically, in vivo administration of quercetin displays unexpected synergistic neurotoxic effect with MeHg. Considering this controversy and the limited data on the interaction of MeHg with other flavonoids, the potential protective effect of quercetin and two of its glycoside analogs (i.e., rutin and quercitrin) against MeHg toxicity were evaluated in rat cortical brain slices. MeHg (100 μM) caused lipid peroxidation and ROS generation. Quercitrin (10 μg/mL) and quercetin (10 μg/mL) protected mitochondria from MeHg (5 μM)-induced changes. In contrast, rutin did not afford a significant protective effect against MeHg (100 μM)-induced lipid peroxidation and ROS production in cortical brain slices. MeHg-generated ROS in cortical slices was dependent upon an increase in intracellular Ca^{2+} levels, because the over-production of MeHg-induced H_2O_2 in mitochondria occurred with a concomitant increase in Ca^{2+} transient. Here, we have extended the characterization of mechanisms associated with the neuroprotective effects of quercetin against MeHg-induced toxicity in isolated mitochondria, by performing an array of parallel studies in brain slices. We provide novel data establishing that (1) Ca^{2+} plays a central role in MeHg toxicity and (2) in brain slices MeHg induces mitochondrial oxidative stress both via direct interaction with mitochondria (as previously reported in in vitro studies) as well as via mitochondria-independent (or indirect) mechanisms.

Keywords MeHg toxicity · Quercetin · Quercitrin · Rutin · ROS · Mitochondria

Electronic supplementary material The online version of this article (doi:10.1007/s00204-009-0482-3) contains supplementary material, which is available to authorized users.

C. Wagner · A. P. Vargas · D. H. Roos · A. F. Morel · C. W. Nogueira · J. B. Rocha (✉)
Centro de Ciências Naturais e Exatas,
Departamento de Química,
Universidade Federal de Santa Maria,
Santa Maria, RS 97105-900, Brazil
e-mail: jtbrocha@yahoo.com.br

M. Farina
Departamento de Bioquímica,
Centro de Ciências Biológicas,
Universidade Federal de Florianópolis,
Florianópolis, SC, Brazil

M. Aschner
Department of Pediatrics and Pharmacology,
Vanderbilt University School of Medicine,
Nashville, TN 37232-2495, USA

Introduction

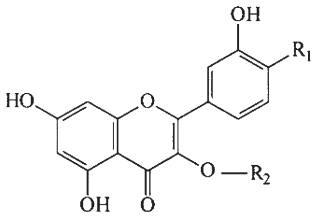
Methylmercury (MeHg) is a highly toxic environmental pollutant that causes neurological and developmental deficits in animals and humans (Clarkson et al. 2003). MeHg-induced neurotoxicity is an extensively reported phenomenon, but the precise molecular mechanisms underlying its cytotoxicity have yet to be fully clarified. The central nervous system (CNS) is considered the primary target for MeHg toxicity (Clarkson et al. 2003) and currently the

major mechanisms involved in its neurotoxicity are believed to be related to impairment of intracellular calcium homeostasis (Sirois and Atchison 2000), alterations of glutamate homeostasis (Allen et al. 2001; Aschner et al. 2000; Fonfria et al. 2005) and oxidative stress (Aschner et al. 2007; Franco et al. 2007).

Mercury is known to induce the formation of reactive oxygen species (ROS), cell death and DNA damage (Aschner et al. 2007; Grotto et al. 2009; Reichl et al. 2006a, b; Schmid et al. 2007). There are a number of reports concerning oxidative stress and the protective role of antioxidant enzymes against MeHg neurotoxicity (Allen et al. 2001, 2002; Shanker and Aschner 2003). In vitro studies have consistently demonstrated that MeHg can disrupt neuronal and astrocytic functions (Allen et al. 2001, 2002; Aschner et al. 2000; Yee and Choi 1994). Exposure of cortical rat brain slices and cortical astrocytes to MeHg have been shown to increase the ROS formation (Dreiem and Seegal 2007; Roos et al. 2009; Yee and Choi 1994).

Several in vitro studies have indicated that mitochondria are critical subcellular target for MeHg toxicity (Mori et al. 2007; Shenker et al. 1999; Verity et al. 1975). For example, MeHg decreases mitochondrial function and increases ROS levels in striatal synaptosomes (Dreiem and Seegal 2007). These effects may reflect the intra-mitochondrial accumulation of MeHg, leading to the collapse of the mitochondrial transmembrane potential (Araragi et al. 2003; InSug et al. 1997; Shenker et al. 1999) or can be related to an indirect effect of MeHg on glutamate homeostasis at the synaptic level (Allen et al. 2002; Aschner et al. 2000, 2007). MeHg can also induce hydrogen peroxide generation and decrease mitochondrial viability in isolated mouse brain mitochondria (Franco et al. 2007).

Flavonoids are widely found in vegetables, fruits, juices and tea and represent important components of the human diet (Hollman and Katan 1999; Rice-Evans et al. 1996). It has been proposed that phenolic phytochemicals exert a positive health effects in chronic diseases, such as cancer and neurodegenerative disorders. Many physiological benefits of flavonoids have been attributed to their antioxidant and free radical scavenging properties (Rice-Evans et al. 1996). Flavonoids possess divalent metal chelation properties, antioxidant and anti-inflammatory activities, readily permeate the blood–brain barrier (BBB) and afford neuroprotection in a wide array of cellular and animal models of neurological diseases (Mande et al. 2006; Youdim et al. 2004). In vitro quercetin administration has been reported to exhibit cytoprotection in different pro-oxidant models of apoptotic death (Dajas et al. 2003). However, contradictory results can also be found in the literature (Kaariaien et al. 2008; Zbarsky et al. 2005), where quercetin fails to provide protection against model of Parkinson's disease. Quercitrin (a glycoside rhamnose of quercetin) and rutin (a glycoside rutinose from quercetin) can also exhibit in vitro antioxidant properties in different pro-oxidant models (Pereira et al. 2009; Spanos and Wrolstad 1992; Wagner et al. 2006). Notably, recent reports have indicated that the pro-oxidant effect of MeHg can be counteracted by plant extracts rich in flavonoids and by purified quercetin (Farina et al. 2005; Franco et al. 2007). Paradoxically, simultaneous in vivo exposure to MeHg and quercetin caused synergistic neurotoxic effects in mice (Martins et al. 2009).



Flavonol	R_1	R_2
Rutin	OH	Rutinose
Quercitrin	OH	Rhamnose
Quercetin	OH	H

Fig. 1 Flavonoids structures

Given the lack of efficacious treatments that fully abolish MeHg-induced toxicity and that natural and synthetic antioxidants compounds afford protection in a variety of in vitro and in vivo models associated with oxidative stress (Gugliucci and Stahl 1995; Gupta et al. 2003; Sudati et al. 2009), the present study was designed to test the potential protective effects of antioxidant compounds against MeHg toxicity. Furthermore, the discrepant effects of quercetin against MeHg neurotoxicity after in vitro and in vivo studies indicate that the effect of structurally related quercetin flavonoids should be investigated in order to determine their potential toxic or protective effects. In this study, the potential protective effect of quercetin and its two glycoside structurally related flavonoids (rutin and quercitrin) (Fig. 1) against MeHg toxicity were evaluated in cerebral cortical slices, brain isolated mitochondria directly exposed to MeHg.

Materials and methods

Chemicals

Thiobarbituric acid, malonaldehyde bis-(dimethyl acetal) (MDA), dichlorofluorescin diacetate (DCFHDA), dichlorofluorescein (DCF), rutin, ethylene glycol tetraacetic acid (EGTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), succinic acid and ionophore A23187 were obtained from Sigma (St. Louis, MO, USA). Trichloroacetic acid, sodium phosphate, sucrose, mannitol and L-glutamate were obtained from Vetec (Rio de Janeiro, RJ,

Brazil). MeHg was obtained from Merck (Rio de Janeiro, RJ, Brazil). Quercitrin and quercetin were isolated from *Solidago microglossa* D.C., and the purity of the isolated compounds was >99.3% (Morel et al. 2006).

Animals

Male Wistar rats (2–4 months) maintained under standard conditions (12-h light/dark, 22 ± 2°C) with food and water ad libitum were used. The Animal Care Committee approved all handling and experimental conditions.

Preparation of brain cortical slices

Rats were decapitated and the two cerebral hemispheres were used for preparation of coronal slices (0.4 mm) from the parietal cortical area using a McIlwain tissue chopper.

Determination of lipid peroxidation

Lipid peroxidation was measured in the cortical slices by detection of TBA-reactive substances, according to previous reports (Santamaría et al. 1997).

The slices (5 slices per tube) were pre-incubated in a PBS buffer in the presence or absence of MeHgCl (100 μM) and three concentrations (5, 10 or 25 μg/mL) of each flavonoids (quercetin, quercitrin and rutin) were tested. The time of pre-incubation was 2 h. After exposure to the pre-incubation conditions, the slices were removed from the media and homogenized in PBS buffer. The homogenates were used for measurement of peroxidation by addition of 2 mL of the color reagent (0.375 g of thiobarbituric acid (TBA) + 7.5 g of trichloroacetic acid + 2.5 mL of HCl in 100 mL of water). The final solution was heated in a boiling water bath for 30 min. After cooling the samples on ice, they were centrifuged at 3,000*g* for 15 min, and the absorbance of the respective supernatants was measured spectrophotometerically at 532 nm. The results were calculated as nmol of thiobarbituric reactive substances (TBARS) per mg of protein and expressed as percentage of control (unstimulated).

Determination of ROS formation in slices

The levels of ROS in cortical slices were measured by the oxidation of 2,7-dichlorofluorescein diacetate (DCFHDA) (Wang and Joseph 1999). Slices were maintained in ice in a PBS medium containing (in mM) 10 glucose, 124 NaCl, 10 $NaHPO_4$, 5 NaH_2PO_4, 5 KH_2PO_4, pH 7.4. The slices were pre-incubated in the PBS buffer for 10 min containing DCFHDA (5 μM) and then exposed to flavonoids and 100 μM MeHg for 2 h. To quantify the extracellular ROS level, an aliquot (400 μL) of the incubation medium was mixed with 2.1 mL of buffer and the formation of the oxidized fluorescent derivative 2′,7′-dichlorofluorescein (DCF) was monitored using excitation and emission wavelengths of 488 and 525 nm, respectively (fluorescence spectrophotometer, Hitachi F-2000). For quantification of intracellular ROS, the slices were washed three times in ice-cold buffer and homogenized in a PBS buffer. Aliquots of 400 μL were mixed with 2.1 mL of PBS buffer for fluorescence quantification. The results were expressed as percentage of control.

Isolation of fresh rat brain mitochondria

Brain mitochondria were isolated as previously described by Brustovetsky and Dubinsky (2000) with minor modifications. Wistar rats were killed by decapitation. The brains were rapidly removed and placed on ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.1% bovine serum albumin (BSA; free fatty acid) and 10 mM HEPES pH 7.2. The tissue was then homogenized and the resulting suspension centrifuged for 7 min at 2,000×*g*. Next, the supernatant was centrifuged for 10 min at 12,000×*g*. The pellet was resuspended in isolation buffer II containing 225 mM mannitol, 75 mM sucrose, 1 mM EGTA and 10 mM HEPES pH 7.2 and centrifuged at 12,000×*g* for 10 min. The supernatant was discard and the final pellet gently washed and resuspended in buffer III containing 50 μM of EGTA, 10 mM sucrose, 65 mM KCl and 10 mM HEPES, pH 7.2, to a protein concentration of 0.5 mg/mL.

Determination of ROS production in brain mitochondria

Experiments were carried out in a standard reaction medium containing 10 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), 50 μM EGTA and 100 μg of mitochondrial protein. The DCFHDA (2 μM) was added to monitor ROS formation in the presence or absence of $CaCl_2$ (60 μM), which can induce ROS generation (Hansson et al. 2008). The formation of the oxidized fluorescent derivative (DCF) was monitored using excitation and emission wavelengths of 488 and 525 nm, respectively.

Protein estimation

Protein concentration was measured second method described by Lowry et al. (1951), using bovine serum albumin (BSA) as standard.

Statistical analysis

The results are expressed as means ± standard deviations and were analyzed by one-way analysis of variance (ANOVA). The Duncan's multiple range test was applied

post hoc to determine the significance of the difference between the various groups. Differences were considered statistically significant at $P < 0.05$. Data from DCFHDA oxidation were obtained directly from the spectrofluorimeter software and for sake of clarity part of the continuous reading data were not included in the statistical analysis.

Results

Lipid peroxidation in cortical brain slices

MeHg (100 μM) caused a statistically significant increase in lipid peroxidation when compared to the control (Fig. 2a–c). Quercitrin did not change basal levels of lipid peroxidation. However, it caused a statistically significant decrease in MeHg-induced lipid peroxidation at 25 μg/mL (Fig. 2a).

Under basal conditions, a concentration-dependent increase in TBARS production was observed after rutin treatment; however, the changes were statistically indistinguishable from the control group (Fig. 2b). Rutin also failed to protect against MeHg-induced oxidative stress (Fig. 2b).

Quercetin caused a significant reduction both in the basal and in the MeHg-induced TBARS levels in a concentration-dependent manner (Fig. 2c).

ROS production in cortical brain slices

MeHg (100 μM) had no significant effect on DCFHDA oxidation (ROS production; $P = 0.063$) in the supernatant of the incubation medium (data not shown). Quercitrin, rutin or quercetin did not modify DCFHDA oxidation in the slices' incubation medium in the presence or absence of MeHg (data not shown).

In contrast to the results obtained with the incubation medium, MeHg caused a significant increase (~60%) in DCFHDA oxidation (ROS production) in brain cortical slices (Fig. 3a–c). Quercitrin and quercetin counteracted the pro-oxidant effect of MeHg (Fig. 3a and c, respectively). Quercitrin caused a significant protective effect against MeHg-induced ROS generation at the highest concentrations (25 μg/mL) and quercetin caused a decrease in ROS production at all tested concentrations (Fig. 3a and c, respectively). Rutin did not attenuate the pro-oxidant effects of MeHg, levels remaining statistically indistinguishable from cortical slices treated with MeHg alone (Fig. 3b).

ROS production in brain mitochondria

Mitochondrial oxidation of DCFHDA was markedly stimulated by calcium (Fig. 4a–c). MeHg (5 μM) alone (Fig. 4a–c) did not cause an increase in ROS production when compared to the control. However, when MeHg was added in the presence of calcium (60 μM) (Fig. 4a–c), it caused a rapid increase in ROS production that was followed by a reduction in the rate of DCFHDA oxidation.

Quercetin (10 μg/mL) by itself caused a decrease in the rate of DCFHDA oxidation and significantly counteracted the pro-oxidant effect of calcium and that of calcium plus MeHg (Fig. 4c).

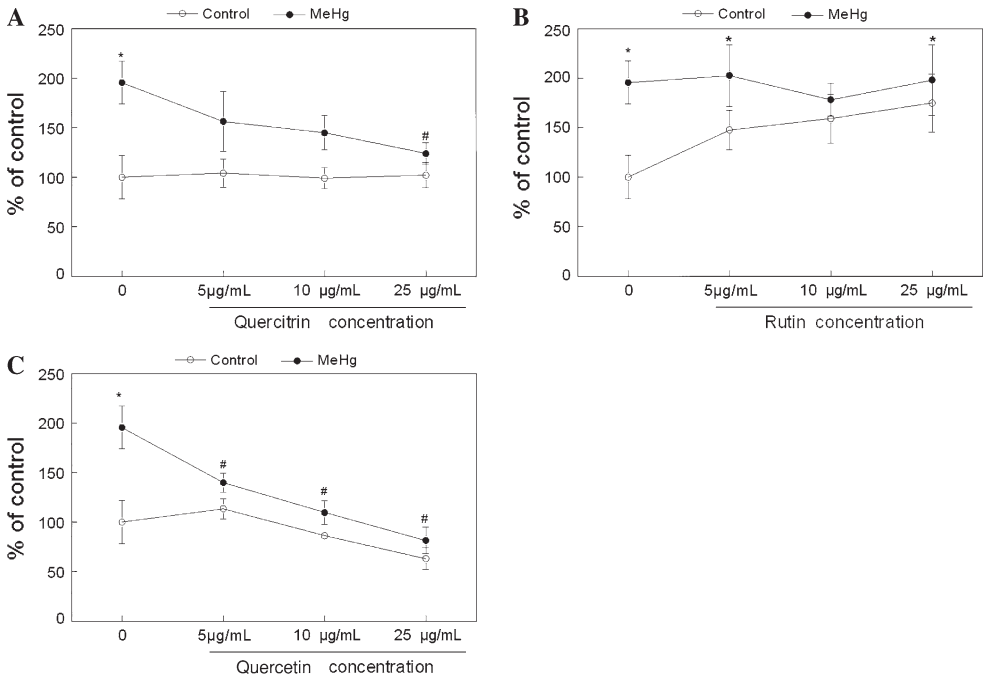


Fig. 2 Effect of different concentrations of flavonoids on MeHg (100 μM)-induced TBARS production in cortical slices. The slices were incubated for 2 h with MeHg in the presence or absence of quercitrin (**a**), rutin (**b**) and quercetin (**c**). Data are expressed as means ± SEM ($n = 4$ independent experiments performed in duplicates).*Represent a significantly difference from control; # statistically different from MeHg exposure (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $P < 0.05$ were considered statistically significant)

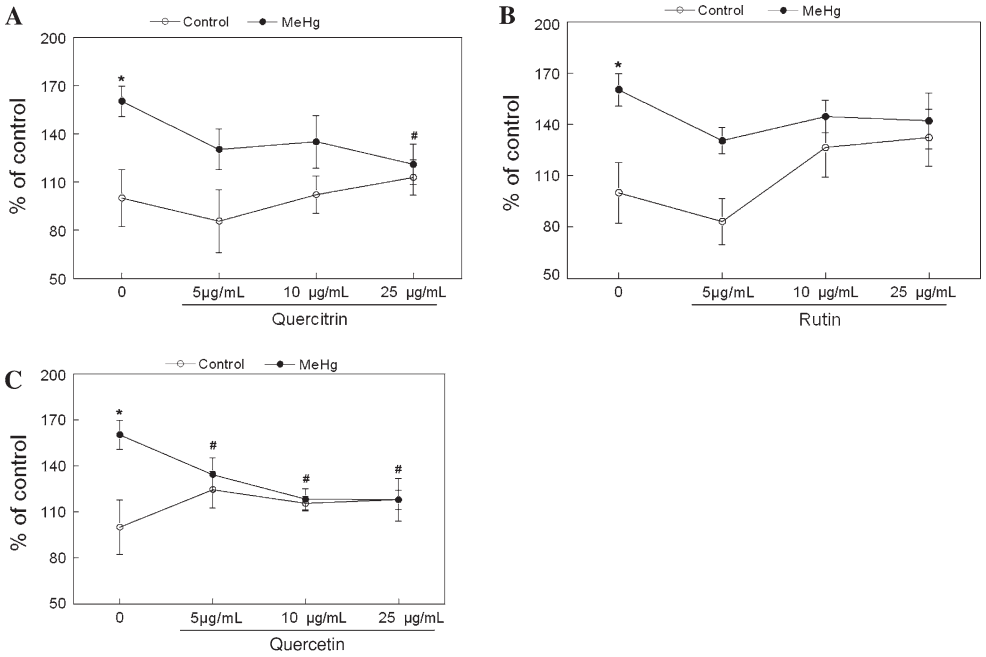


Fig. 3 Effects of the quercitrin (**a**), rutin (**b**), and quercetin (**c**) on MeHg-induced ROS generation in cortical slices. The brain cortical slices were incubated with DCFHDA in the presence or absence of MeHg (100 μM). Data are expressed as mean ± SEM and are calculated as percent control for five independent assays. *Statistically different from control; #statistically different from MeHg exposure (data were analyzed statistically by one-way ANOVA, followed by Duncan's multiple range tests when appropriate, $P < 0.05$ were considered statistically significant)

Quercitrin (10 μg/mL) had a partial protective effect against calcium-induced ROS formation and also protected against calcium plus MeHg-induced DCFHDA oxidation. In the absence of Ca^{2+}, quercitrin reduced the oxidation of DCFHDA determined in the presence of MeHg (Fig. 4a).

Rutin (10 μg/mL) did not afford a significant protective effect against MeHg-generated ROS formation either under basal condition or in the presence of calcium ion. (Fig. 4b).

In order to establish whether the rapid burst of DCFHDA oxidation was a consequence of fast Ca^{2+} entry into mitochondria and to exclude a possible inhibition of esterases by MeHg in the presence of Ca^{2+} (i.e., the intense increase in fluorescence that was followed by a significant slowdown in the rate of DCFHDA oxidation), we tested the effect of the calcium ionophore (A23187) on DCFHDA oxidation (Fig. 5). Addition of the calcium ionophore generated a trace that is similarly shaped to that observed after addition of MeHg to a medium containing Ca^{2+}(Fig. 4 and supplementary material), i.e., it produced an intense stimulation in the rate of DCFHDA oxidation that was followed by a significant slowdown in the DCFHDA oxidation. In the absence of Ca^{2+}, A23187 caused no change in the rate of DCFDA oxidation (data not shown), which indicated that DCFDA oxidation is Ca^{2+}-dependent.

Discussion

In agreement with earlier reports (Allen et al. 2001; Aschner et al. 2007; Shanker and Aschner 2003), we have established that MeHg causes increased lipid peroxidation and DCFHDA oxidation in brain rat cortical slices (Figs. 2, 3). MeHg-induced lipid peroxidation has been invoked to occur secondarily to elevations in intracellular Ca^{2+} concentrations (Denny et al. 1993; Komulainen and Bondy 1987), which can trigger the generation of ROS (Hansson et al. 2008). Here, we have obtained novel evidence in support of the critical role for Ca^{2+} in MeHg-induced ROS production in mitochondria. Our data established that MeHg increase the rate of DCFHDA oxidation only in the presence of Ca^{2+} (Fig. 4) suggesting that fast entry of Ca^{2+} into mitochondria is a primary event and a prerequisite for MeHg-induced ROS formation. In addition, MeHg has been shown to inhibit astrocyte glutamate transporter function resulting in increases glutamate concentrations in the extracellular fluid. The ensuing activation of NMDA (*N*-methyl D-aspartate) glutamate receptors leads to increased Na^+ and Ca^{2+} influx into neurons (Choi 1992). Accordingly, MeHg-induced lipid peroxidation likely reflects over-stimulation of the glutamatergic system with sustained elevation in intracellular free Ca^{2+} levels (Limke et al. 2004; Marty and Atchison 1997).

The exposure of cortical slices to the flavonoids, quercetin and quercitrin (Fig. 1), was associated with a protective effect against lipid peroxidation induced by MeHg (Fig. 2). These results are analogous to those obtained for quercetin in isolated brain mitochondria (Fig. 4), and this protective effect is correlated with the capacity of this flavonoid to detoxify the H_2O_2 generated in the presence of mercurials (Franco et al. 2007). In agreement with this data, flavonoids have been widely reported as effective scavengers of H_2O_2 (Cai et al. 1997). Furthermore, myrecitryn, a flavonoid structurally related to quercetin and quercitrin, has been shown to block Ca^{2+} influx into brain slices (Meotti et al.

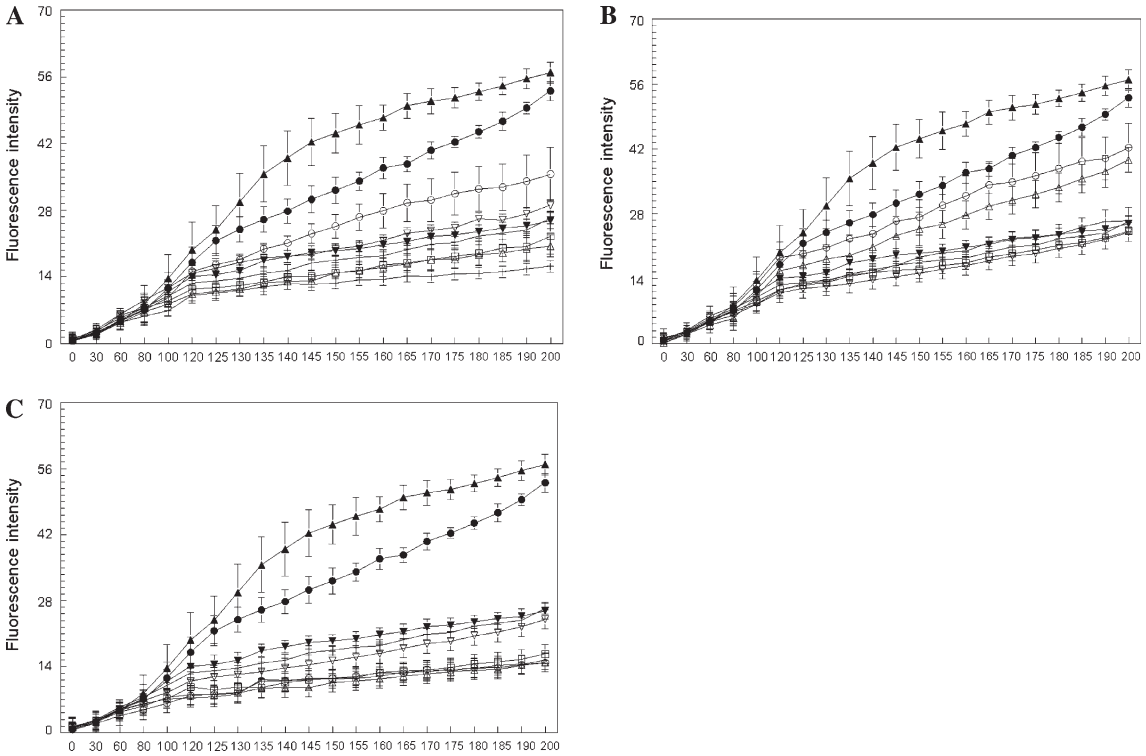


Fig. 4 **a** Effects of quercitrin on brain mitochondrial DCFHDA oxidation: MeHg and Ca^{2+} interactions. Mitochondria were incubated in a medium containing 50 μM EGTA 10 mM sucrose, 65 mM KCl, 5 mM glutamate, 5 mM succinate and 10 mM HEPES, pH = 7.2. The symbols represent the following: (*solid line*) control, (*filled circle*) $CaCl_2$ (60 μM), (*filled inverted triangle*) MeHg (5 μM), (*filled triangle*)$CaCl_2$ (60 μM) plus MeHg (5 μM), (*open square*) quercitrin (10 μg/mL), (*open inverted triangle*) ethanol (0.2%), (*dagger*) quercitrin (10 μg/mL) plus MeHg (5 μM), (*open circle*) quercitrin (10 μg/mL) plus MeHg (5 μM) plus $CaCl_2$ (60 μM) and (*open triangle*) quercitrin (10 μg/mL) plus $CaCl_2$ (60 μM). $CaCl_2$, MeHg and quercitrin were added at 50, 70 and 120 s, respectively. The assays were performed in triplicates using independent mitochondrial preparation. **b** Effects of rutin on brain mitochondrial DCFHDA oxidation: MeHg and Ca^{2+} interactions. Mitochondria were incubated in a medium containing 50 μM EGTA 10 mM sucrose, 65 mM KCl, 5 mM glutamate, 5 mM succinate and 10 mM HEPES, pH = 7.2 The symbols represent the following: (*solid line*) control, (*filled circle*) $CaCl_2$ (60 μM), (*filled inverted triangle*) MeHg (5 μM), (*filled triangle*) $CaCl_2$ (60 μM) plus MeHg (5 μM), (*open square*) rutin (10 μg/mL), (*open inverted triangle*) ethanol (0.2%), (*dagger*) rutin (10 μg/mL) plus MeHg (5 μM), (*open circle*) rutin (10 μg/mL) plus MeHg (5 μM) plus $CaCl_2$ (60 μM) and (*open triangle*) rutin (10 μg/mL) plus $CaCl_2$ (60 μM). $CaCl_2$, MeHg and rutin were added at 50, 70 and 120 s, respectively. The assays were performed in triplicates using independent mitochondrial preparation. **c** Effects of quercetin on brain mitochondrial DCFHDA oxidation: MeHg and Ca^{2+} interactions. Mitochondria were incubated in a medium containing 50 μM EGTA, 10 mM sucrose, 65 mM KCl, 5 mM glutamate, 5 mM succinate and 10 mM HEPES, pH = 7.2. Data were obtained in a Simatuzu Spectrofluorimeter (readings were made at every 0.1, i.e., continuous traces were obtained, but for sake of clarity an interval of 30 s was used for statistical data analysis. The symbols represent the following: (*solid line*) control, (*filled circle*) $CaCl_2$ (60 μM), (*filled inverted triangle*) MeHg (5 μM), (*filled triangle*) $CaCl_2$ (60 μM) plus MeHg (5 μM), (*open square*) quercetin (10 μg/mL), (*open inverted triangle*) ethanol (0.2%), (*dagger*) quercetin (10 μg/mL) plus MeHg (5 μM), (*open circle*) quercetin (10 μg/mL) plus MeHg (5 μM) plus $CaCl_2$ (60 μM) and (*open triangle*) quercetin (10 μg/mL) plus $CaCl_2$ (60 μM). $CaCl_2$, MeHg and quercetin were added at 50, 70 and 120 s, respectively. The assays were performed in triplicates using independent mitochondrial preparation

2007). Thus, in addition to a direct interaction with H_2O_2, flavonoids could reduce H_2O_2 production via inhibition of Ca^{2+} influx into brain slices or mitochondria. Flavonoids may also form redox inactive complexes with iron (Fe), rendering this pro-oxidant unavailable for Fenton reaction. Corroborating this hypothesis, both quercitrin and quercetin effectively block Fe-induced TBARS production in brain homogenates (Pereira et al. 2009; Wagner et al. 2006).

In contrast to quercetin and quercitrin, the third flavonoid tested, rutin, failed to protect against MeHg-induced lipid peroxidation. The antioxidant activities of flavonoids are influenced by their chemical structure (Rice-Evans et al. 1996). A comparison of quercetin with rutin (Fig. 1) indicates the possible influence of the 3-OH in combination with the adjacent double bond in the C ring. If one is dispensed with, the other apparently loses its impact on the antioxidant activity (Rice-Evans et al. 1996). Thus, the absence or low antioxidant effect of rutin is possibly related to its structure. Moreover, the glycoside group present in rutin increases its hydrophilicity (Saija et al. 1995), thus

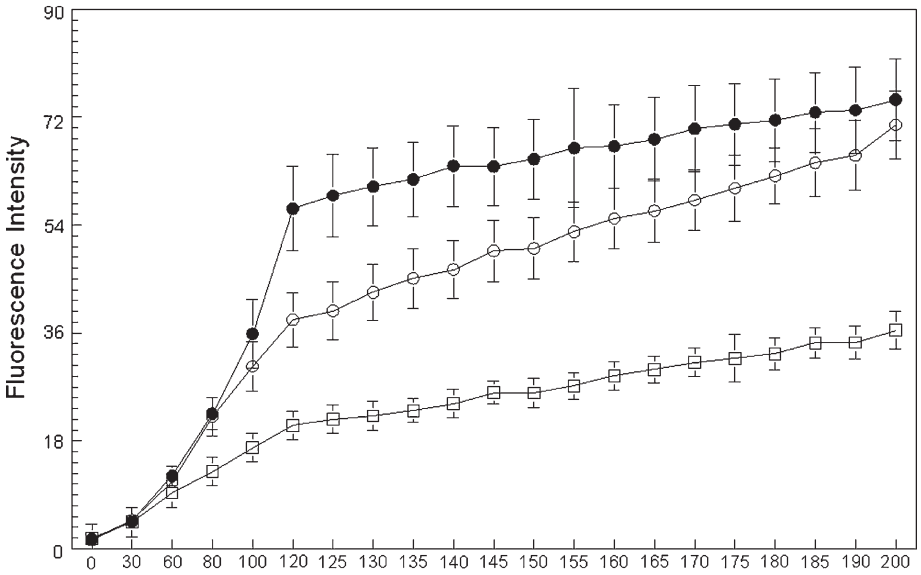


Fig. 5 Effects of the calcium ionophore, A23187, on DCFADA oxidation. Mitochondria were incubated in a medium containing 50 μM EGTA 10 mM sucrose, 65 mM KCl, 5 mM glutamate, 5 mM succinate and 10 mM HEPES, pH = 7.2. The symbols represent the following: (*open square*) control, (*open circle*) $CaCl_2$ (60 μM), (*filled circle*) $CaCl_2$ (60 μM) plus ionophore. $CaCl_2$ and ionophore was added at 60 and 80 s, respectively. The assays were performed in triplicates using independent mitochondrial preparation

decreasing its permeability across membranes. As shown, rutin failed to protect both the cortical slices and the brain mitochondria from MeHg-induced ROS generation (Figs. 3, 4). Rutin also tended to exhibit a pro-oxidant effect by itself (Fig. 2b), which is consistent with earlier data from literature (Cotelle 2001).

In contrast, quercetin and quercitrin were protective against MeHg-induced ROS production (Figs. 2, 3, 4). This effect likely reflects the presence of the *o*-dihydroxy group in the B ring of their structures (Fig. 1). This confers higher stability to the radical form and participates in electron delocalization; the 2,3 double bond in conjugation with a 4-oxo bond in the C ring are responsible for electron delocalization from the B ring. Thus, the antioxidant potency reflects electron delocalization of the aromatic nucleus. When these compounds react with free radicals, the phenoxyl radicals produced are stabilized by the resonance effect of the aromatic nucleus and the 3- and 5-OH groups with 4-oxo function in the A and C rings are required for maximal radical scavenging potential (Rice-Evans et al. 1996).

ROS cause cell injury by compromising the integrity of cell membrane, proteins and by cleaving the DNA (Valko et al. 2005). Moreover, ROS impair mitochondrial energy metabolism by inducing oxidative structural changes and the ensuing loss of activity in a number of mitochondrial enzymes that play critical roles in ATP production (Fiskum et al. 2004). Additionally, the direct action of ROS on mitochondrial membrane lipids and proteins results in the activation of apoptotic cascades, through both opening of the mPTP (mitochondrial permeability transition pore) and mPTP-independent mechanisms (Lifshitz et al. 2004). Accordingly, an important objective of the present study was to better understand the pro-oxidant effect of MeHg. As a first step to achieve this goal, we investigated the effect of MeHg on ROS generation in freshly isolated brain mitochondria and mitochondria isolated from brain cortical slices. MeHg alone did not cause an increase in ROS production in brain mitochondria, but in the presence of Ca^{2+} it led to an over-stimulation in ROS production (Fig. 4). Notably, we found the same effects with the Ca^{2+} ionophore, A23187. In fact, the responses to MeHg and to the ionophore in the presence of Ca^{2+} were qualitatively similar (compare Figs. 4, 5 and supplementary material). Dubinsky and Levi (1998) have demonstrated that in the presence of an ionophore, large Ca^{2+} loads lead to immediate mitochondrial depolarization and Ca^{2+} sequestration. These observations are consistent with our hypotheses that both the Ca^{2+} ionophore, A23187 and MeHg lead to rapid and massive increase in mitochondrial Ca^{2+} influx, which secondarily triggers the over-stimulation of ROS production. The latter, in turn, facilitates mPT induction, loss of the electron transport chain and the ensuing mitochondrial death.

In summary, our results establish that MeHg caused lipid peroxidation and ROS generation in mitochondria and brain slices. The flavonoids quercetin and quercitrin afforded protective effects with the following rank order: quercetin > quercitrin. Rutin failed to attenuate MeHg-induced ROS formation. Our results establish that quercetin and quercitrin offer possible therapeutic potential in MeHg toxicity; however, more in vivo experiments are needed to validate the possible use of these flavonoids against MeHg-induced injuries, particularly in view of the recently published synergistic toxic effect of quercetin and MeHg in adult mice.

Acknowledgments The support by CAPES, CNPq, and FAPERGS is gratefully acknowledged. MA was supported by grants from NIEHS ES07331 and 10563.

Conflict of interest statement The authors declare that they have no conflict of interest.

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