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Diphenyl diselenide administration enhances cortical mitochondrial number and activity by increasing hemeoxygenase type 1 content in a methylmercury-induced neurotoxicity mouse model

Viviane Glaser · Roberta de Paula Martins · Ana Julia Hoffmann Vieira · Eliana de Medeiros Oliveira · Marcos Raniel Straliotto · Jorge Humberto Mukdsi · Alicia Inés Torres · Andreza Fabro de Bem · Marcelo Farina · João Batista Teixeira da Rocha · Ana Lucia De Paul · Alexandra Latini

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Abstract Interest in biochemistry of organoselenium compound has increased in the last decades, mainly due to their chemical and biological activities. Here, we investigated the protective effect of diphenyl diselenide $(PhSe)_2$ (5 μmol/kg), in a mouse model of methylmercury (MeHg)-induced brain toxicity. Swiss male mice were divided into four experimental groups: control, $(PhSe)_2$ (5 μmol/kg, subcutaneous administration), MeHg (40 mg/L, in tap water), and MeHg + $(PhSe)_2$. After the treatment (21 days), the animals were killed and the cerebral cortex was analyzed. Electron microscopy indicated an enlarged and fused mitochondria leading to a reduced number of organelles, in the MeHg-exposed mice. Furthermore, cortical creatine kinase activity, a sensitive mitochondrial oxidative stress sensor, was almost abolished by MeHg. Subcutaneous $(PhSe)_2$ co-treatment rescued from MeHg-induced mitochondrial alterations. $(PhSe)_2$ also behaved as an enhancer of mitochondrial biogenesis, by increasing cortical mitochondria content in mouse-receiving $(PhSe)_2$ alone. Mechanistically, $(PhSe)_2$ (1 μM; 24 h) would trigger the cytoprotective Nrf-2 pathway for activating target genes, since astroglial cells exposed to the chalcogen showed increased content of hemeoxygenase type 1, a sensitive marker of the activation of this via. Thus, it is proposed that the $(PhSe)_2$-neuroprotective effect might be linked to its mitoprotective activity.

Keywords Diphenyl diselenide · Methylmercury · Mitochondrial morphology · Creatine kinase · Hemeoxygenase type 1

Viviane Glaser and Roberta de Paula Martins have contributed equally to this study.

V. Glaser · R. P. Martins · A. J. H. Vieira · M. R. Straliotto · A. F. de Bem · M. Farina · A. Latini (✉)
Laboratório de Bioenergética e Estresse Oxidativo, Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Campus Universitário - Córrego Grande, Bloco C-201/214, Florianópolis, SC 88040-900, Brazil
e-mail: alatini@ccb.ufsc.br

E. M. Oliveira
Laboratório Central de Microscopia Eletrônica, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil

J. H. Mukdsi · A. I. Torres · A. L. De Paul
Centro de Microscopía Electrónica, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Córdoba, Argentina

J. B. T. da Rocha
Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

Introduction

A large number of potential pharmaceutical agents derived from selenium have been studied, and in the last three decades, the interest in organoselenium biochemistry and pharmacology has increased due to the development of a variety of organoselenium compound that possess biological activities [1]. In this context, a promising organoselenium compound named diphenyl diselenide [$(PhSe)_2$] has been demonstrated to have antioxidant [2], antinociceptive [3], anti-inflammatory [4, 5], antihyperglycemic [6], antiatherogenic [7], hepatoprotective [8], antiulcer [9], and neuroprotective activities [9–13], that appears to be linked to the formation of the selenol intermediate, selenophenol

[1]. However, the exact mechanisms for the $(PhSe)_2$-induced afforded protection are still not completely defined.

Here, we hypothesize that enhanced mitochondrial activity is linked to $(PhSe)_2$ neuroprotective activity, since (i) mitochondria are essential organelles for the survival of eukaryotic cells, and (ii) it has been recently demonstrated that Se supplementation stimulates mitochondrial biogenesis [14, 15]. Therefore, in the present investigation, the effect of $(PhSe)_2$ on the activities of the key brain energy metabolism enzymes, adenylate, pyruvate, and mitochondrial creatine kinases (mCK), and on ultrastructure and content of mitochondria was evaluated in an experimental mouse model of severe neurotoxicity induced by long-term methylmercury (MeHg)-administration [16]. Additionally, the effect per se of $(PhSe)_2$ on the cytoprotective biomarker hemeoxygenase type 1 (HO-1) content in astroglial cells, the nerve cells where MeHg appears to preferentially accumulate [17], was investigated.

Experimental procedures

Animals

Adult male Swiss albino mice were obtained from the Central Animal House of the Universidade Federal de Santa Catarina, Florianópolis-SC, Brazil. The animals were maintained in a 12-h light/dark cycle in a constant temperature (22 ± 1 °C) colony room, with free access to water and protein commercial chow (Nuvital-PR, Brazil). The experimental protocol was approved by the Ethics Committee for Animal Research (PP00084/CEUA) of the Universidade Federal de Santa Catarina, Florianópolis-SC, Brazil, and followed the Guiding Principles in the Use of Animals in Toxicology, adopted by the Society of Toxicology in July 1989 (available in http://www.toxicology.org/gp/air6.asp).

Experimental protocol

The experimental protocol was performed on 28 animals randomly divided into four experimental groups as follows: control group (vehicle), MeHg 40 mg/L (40 ppm), $(PhSe)_2$ 5 μmol/kg, and MeHg plus $(PhSe)_2$. The animals remained in these conditions for 21 days. MeHg was diluted in tap water, and was freely available, and $(PhSe)_2$ was dissolved in dimethylsulfoxide (DMSO) and subcutaneously administrated [$(PhSe)_2$ daily injections of 5 μmol kg^{-1}]. Control animals received vehicle injections (DMSO, 1 mL/kg body weight). This experimental schedule has been reported to lead to mercury brain toxic concentrations of 3–5 μg g^{-1} tissue (3–5 ppm) and to induce systemic energy deficiency [16, 18, 19].

Biochemical analysis

The animals were killed by decapitation and the cerebral cortex was rapidly dissected and homogenized in appropriate buffer. Afterward, the activities of mCK, adenylate kinase, and pyruvate kinase were assessed as previously reported by our group [16, 20]. Enzyme activities were expressed as nmol/min/mg protein.

Morphological analysis

Animals were perfused with saline solution and afterward the cerebral cortex was rapidly dissected and immersed in the post fixation solution composed by 1.5 % glutaraldehyde, 4 % formaldehyde in 0.1 M cacodylate buffer, pH 7.35, and processed as previously described [21]. Afterward, thin brain sections were cut with a diamond knife on a JEOL JUM-7 ultramicrotome (Nikon, Tokyo, Japan) and examined using a Zeiss Leo 906 E electron microscope equipped with the digital camera Megaview III (Oberkochen, Germany). For quantitative analysis of mitochondrial content, three brains from each experimental condition were used and fifty random micrographs of the same brain region were acquired at 10,000× magnification. The data were expressed as the mean of mitochondrial content (50 micrograph/experimental condition/per animal). The software Image J was used and the relationship between mitochondria number/85 μm^2 of brain tissue was established with the aim to consider a constant area.

Maintenance and treatment of cell line

The astroglioma C6 cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA). The cells were seeded in flasks and cultured in Dulbecco's modified eagle's medium (pH 7.4) containing 5 % fetal bovine serum, sterile antimycotic solution 100×: penicillin 100 IU/mL, streptomycin 0.1 mg/mL, and amphotericin 0.25 μg/mL, in a 95 % O_2 and 5 % CO_2 humidified atmosphere, at 37 °C. Exponentially growing cells were detached from the culture flasks using 0.05 % trypsin/ethylene-diaminetetracetic acid and seeded in 6-well plates (2.5×10^4 cells/well) [22]. After cells reached confluence, the culture medium was removed by suction and the cells were exposed to 1 μM $(PhSe)_2$ for 24 h, and the hemeoxygenase type 1 content (HO-1) was measured.

Hemeoxygenase type 1 (HO-1) content

HO-1 content was assessed by western blot analysis. After treatment, cell lysates were prepared in lysis buffer containing 1 mM Tris-HCl (pH 7.4), 2.5 M NaCl, 0.25 M EDTA, 1 % Triton X-100, 1 mM dithiothreitol, 5 mM

Na_3VO_4, 1 mM β-glycerol phosphate, and 50 μL protease inhibitor cocktail (Roche, Mannheim, Germany). Supernatants obtained after centrifugation (15,000×*g*, 30 min; 4 °C) were recovered and the protein content was quantified [23]. An aliquot of 50 μg of total protein was size-separated by electrophoresis in 10 % SDS–polyacrylamide gel, under reducing conditions, and transferred to a nitrocellulose membrane. After washing and blocking, the membranes were incubated overnight with HO-1 primary antibody (1:500 dilution; Santa Cruz Biotech, Santa Cruz, CA). Afterward, membranes were exposed to the anti-rabbit secondary antibody (1:5,000; Santa Cruz Biotech, Santa Cruz, CA). The immunocomplexes were visualized using the ECL chemiluminescence detection system (GE Healthcare, São Paulo, SP, Brazil). Membranes were stripped and the content of β-actin (antibody anti- β-actin, 1:3000 dilution; Santa Cruz Biotech, Santa Cruz, CA) was performed for verifying loading evenness.

Statistical analysis

Results are presented as mean ± SD. Data from in vivo experiments were analyzed using one-way ANOVA followed by the post-hoc Tukey test, when F was significant. Data from the in vitro experiment were analyzed by the Student t test for paired samples. Only significant values are given in the text. Differences between the groups were rated significant at $P < 0.05$. Statistics were performed using SPSS® (Statistical Package for the Social Sciences software; version 16.0 for Windows).

Results

Figure 1a–f shows the effect of $(PhSe)_2$ on the mitochondrial ultrastructure and content in cerebral cortex from MeHg-poisoned mice. Figure 1a shows numerous cortical mitochondria with spherical or oval profiles, with delimited intermembrane space, and clear projections of the inner membrane protruding into the matrix to form the cristae (Fig. 1e). It is shown in Fig. 1c, f, g that MeHg exposure promoted several mitochondrial ultrastructural changes and reduction in the number of cortical organelle. The most prominent mitochondrial ultrastructure alteration consisted in organelle enlargement with greater volume (Fig. 1f), and the adoption of different forms: curved, S-shaped, and markedly elongated, a phenomenon observed when mitochondrial fusion is stimulated [24]. Moreover, the electron microscopy analysis also revealed a subtle presence of a fluffy material in mitochondrial matrix and a clear disorganization of mitochondrial crista in brain from MeHg-poisoned mice (Fig. 1f). In addition, Fig. 1g shows that the MeHg-induced mitochondrial content reduction (up to 60 % of reduction) was significantly prevented by $(PhSe)_2$ treatment [$F_{(3,146)} = 58.19$; $P < 0.0001$].

Furthermore, $(PhSe)_2$ alone or co-administered with MeHg significantly stimulated mitochondrial biogenesis as shown by the increased number of this organelle in the mouse-exposed brain (up to 69 % of increment induced by $(PhSe)_2$ per se, and up to 57 % of increment in MeHg + $(PhSe)_2$ treated animals) [Controls (mitochondrial content per field): 35.44 ± 7.88; $(PhSe)_2$: 55.28 ± 17.05; MeHg: 16.52 ± 5.93; MeHg plus $(PhSe)_2$: 51.64 ± 14.50]. Similarly, the MeHg-induced abolishment of the activity of the brain key energy enzyme, mCK, (up to 97 % of inhibition) was significantly prevented by $(PhSe)_2$ treatment (up to 69 % of prevention) [$F_{(3,8)} = 44.29$; $P < 0.001$] [Controls (nmol/min/mg protein): 7.31 ± 0.90; $(PhSe)_2$: 8.64 ± 1.65; MeHg: 0.20 ± 0.12; MeHg plus $(PhSe)_2$: 5.06 ± 0.37]. However, AK and PK activities were not altered by the treatments (Fig. 2).

Furthermore, the content of a Nrf-2 target heme-oxygenase-1 (HO-1) was analyzed to elucidate $(PhSe)_2$ mechanism of neuroprotection. It was observed that 1 μM of this compound for 24 h increased HO-1 content in C6 cells (up to 87 %) [$t_{(4)} = 5.355$; $P < 0.01$] (controls 0.77 ± 0.06; $(PhSe)_2$ 1.23 ± 0.13) (Fig. 3).

Discussion

It was here demonstrated that the simple organoselenium compound, $(PhSe)_2$, rescue from severe mitochondrial alterations depicted by enlarged and fused mitochondria with reduced mitochondrial content, and an almost abolished mCK activity, in the cerebral cortex of MeHg-poisoned mice. Additionally, this afforded neuroprotection appears to be related to a non-reported behavior of $(PhSe)_2$, as an enhancer of mitochondrial biogenesis, since the mitochondrial content in brain from mouse-receiving $(PhSe)_2$ alone was significantly increased. Furthermore, the underlying molecular mechanism involved in this effects appears to be linked to Nrf-2 activation, since $(PhSe)_2$ per se elicited the increase of HO-1 content in nerve cells.

Our group has previously described that $(PhSe)_2$ protects MeHg-exposed tissues by reducing the mercury availability [16, 25]. Due to its intrinsic thiol–peroxidase like activity that mediates its own reduction to form a selenol intermediate (reduced form; PhSeH; [1, 16, 25, 26], which can react with MeHg [27, 28]. The selenol can further react with the mercurial, facilitating the formation and further clearance of a stable PhSe-$HgCH_3$ complex [27, 28]. This complex would be even more easily excreted than others formed with endogenous thiolates, reducing i.e., the transport into the brain [25, 29–31]. The yielded protection induced by $(PhSe)_2$ have also been associated with the

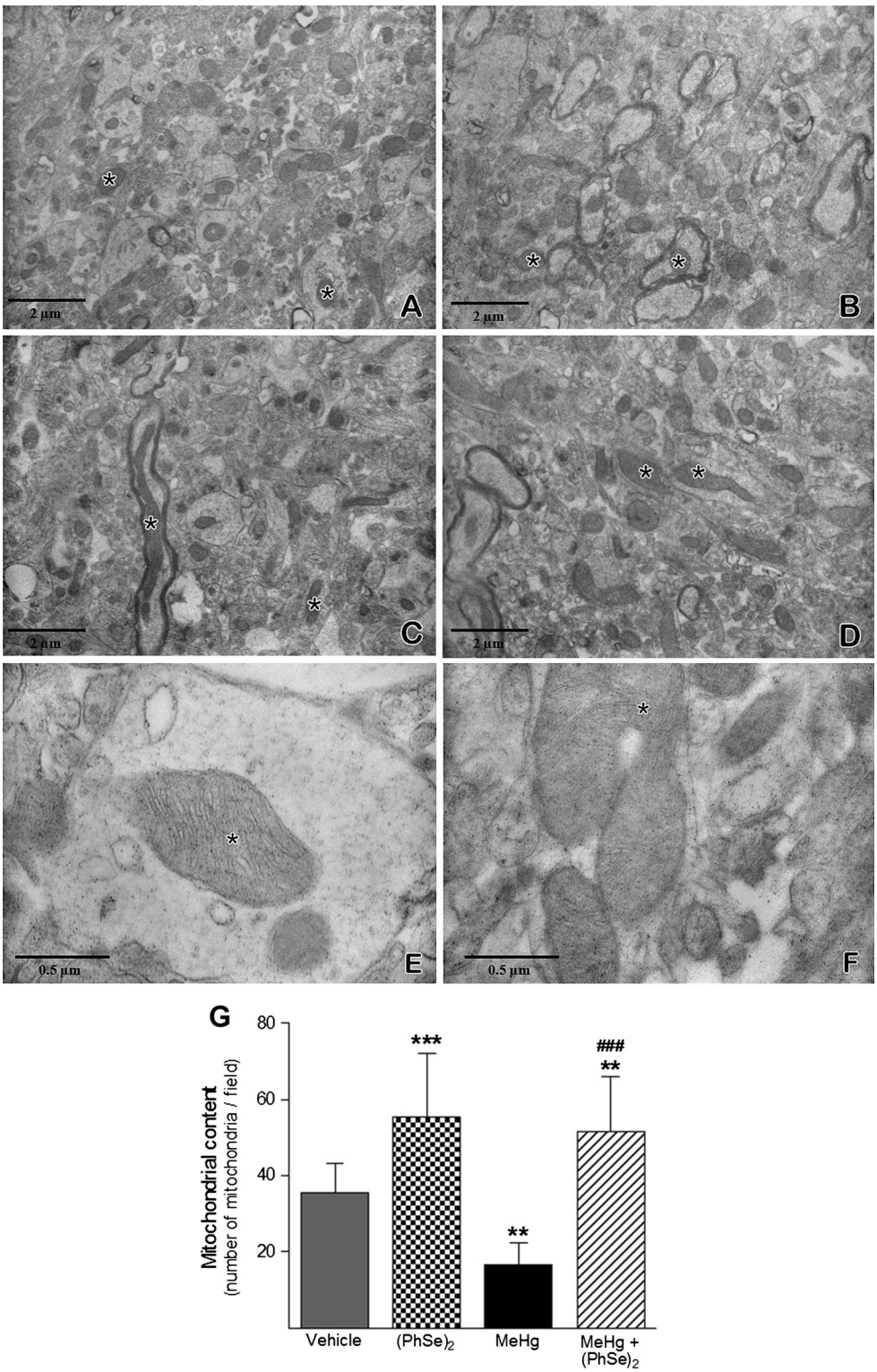

Fig. 1 Mitochondrial ultrastructure in cerebral cortex from adult mice controls (**a**); treated with diphenyl diselenide [$(PhSe)_2$; 5 µmol/kg; **b**]; exposed to methylmercury (MeHg; 40 mg/L; **c**) and to MeHg (40 mg/L) plus $(PhSe)_2$ (5 µmol/kg; **d**). **e**, **f** are enlarged photos from control and MeHg group. Control cortical sections show the presence of numerous round-shape mitochondria, some of them inside of unmyelinated axons (*asterisk*; ×10,000; **a**). Cortical sections from $(PhSe)_2$-treated mice show unchanged mitochondrial features axons (*asterisk*; ×10,000; **b**). Images from cortical preparations from MeHg-treated mice display reduced number of mitochondria, and some of them characterized by a significant increase in their size (*asterisk* elongated mitochondrion in a myelinated axon; ×10,000; **c**). Brain preparations from MeHg plus $(PhSe)_2$-treated mice show the predominance of elliptical and round-shape mitochondria (*asterisk*) similar to those observed in $(PhSe)_2$-treated mice. Also, some of them exhibit a significant increase in their volume (×10,000; **d**). Control cortical sections from unmyelinated axons (*asterisk*) show the inner membrane forming cristae (*asterisk*; ×47,000; **e**). An elongated and curved-shaped mitochondria (*asterisk*) with a slight internal disorganization, particularly in its cristae (×47,000; **f**). Cortical mitochondrial content were expressed as number of mitochondria/field (**g**). Values are mean ± SD from three animals. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$, compared to controls; $^{\#\#\#}P \leq 0.001$, compared to MeHg (One-way ANOVA followed by the Tukey multiple range test)

formation of selenophenol, another intermediates, by hepatic or cerebral thioredoxin reductase, including, the decomposition hydrogen peroxide, peroxynitrite and lipid peroxides [1, 25, 32]. In this scenario, these mechanisms appear to contribute to the reported beneficial properties of $(PhSe)_2$, including antioxidant, antinociceptive, antiinflammatory, antihyperglycemic, antiatherogenic, hepatoprotective, antiulcer, antidepressant-like and anxiolytic-like actions [2–6, 8, 9, 32, 33]. Furthermore, we have recently proposed that the main mechanism involved in

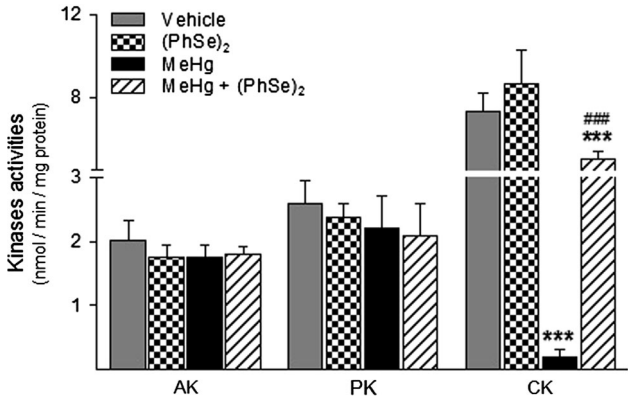

Fig. 2 Activities of mitochondrial creatine kinase (mCK), adenylate kinase (AK) and pyruvate kinase (PK) in brain from adult mice exposed to methylmercury (MeHg; 40 mg/L) and/or diphenyl diselenide [$(PhSe)_2$; 5 μmol/kg]. Values are mean ± SD from four animals. $^{***}P \leq 0.001$, compared to controls; $^{\#\#\#}P \leq 0.001$, compared to MeHg (One-way ANOVA followed by the Tukey multiple range test)

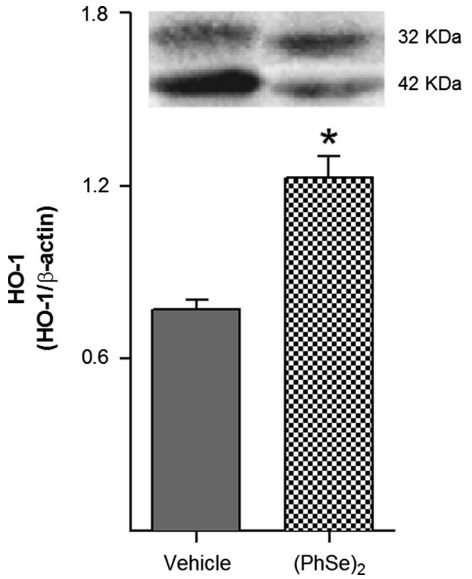

Fig. 3 In vitro effect of diphenyl diselenide ($(PhSe)_2$; 1 μM; 24 h) on hemeoxygenase type I (HO-I) content in astroglioma C6 cells. Values are mean ± SD of three independent experiments. $^{**}P < 0.01$; Student *t* test for paired samples

these effects would be also linked to the capacity of $(PhSe)_2$ of enhancing mitochondrial function [26]. Therefore, this study focused on the effects of MeHg and/or $(PhSe)_2$ treatment on the morphological profile of brain mitochondria. Although, some studies have previously shown that MeHg is toxic to mitochondria [16, 19, 24], this is the first one to evidence that the number and morphological mitochondrial profiles change after MeHg exposure in the cerebral cortex of adult rodents (Fig. 1). Since the number of mitochondria was reduced and the morphology depicted tubular mitochondria, it is possible, that mitochondrial fusion was induced by MeHg poisoning. In this context, fusion of mitochondria is a process required for the intramitochondrial exchange of metabolic substrates and maintenance of respiratory capacity [34]. Therefore, under situations of energetic impairment, as that elicited by MeHg poisoning, the increased fusion process might serve to mix and unify mitochondrial compartments, which would counteract respiratory deficiencies [35, 36]. This is also in agreement with previous results from our group, demonstrating several mitochondrial enzymes inhibitions in this animal model of mercurial-induced neurotoxicity [19, 26, 37, 38]. Thus, the enlarged mitochondria and the reduced number of the organelles might represent a compensatory mechanism in adult poisoned animals. On the other side, this compensation for reduced efficiency of aerobic metabolism might be different according to the rodent brain developmental stage, since it has been reported that neonatal animals that received subcutaneous MeHg administrations until adult life, showed increased number and reduced size of the cortical mitochondria, indicating induction of the fission process [19]. It should be remarked here that during neonatal brain development, there is a clear increase of mitochondrial fission that correlates with neuronal proliferation and synaptogenesis, while in the adult life the neuronal proliferative rates are diminished [39]. Therefore, the compensation for reduced efficiency of aerobic metabolism might be command by different mechanisms, according to the rodent development of the central nervous system, even when both processes are pointing out to energy deficits.

The altered mitochondrial dynamics induced by MeHg (increased of fused mitochondria) was fully counterbalanced by the co-administration of $(PhSe)_2$, by increasing the number of the organelles in the cerebral cortex of the poisoned animals. This finding strongly suggests that the underlying mechanism involved in the previous reported beneficial effects of $(PhSe)_2$ against pathological conditions [11, 25, 40–42] might be linked to its capacity of enhancing mitochondrial biogenesis. Indeed, we recently demonstrated that $(PhSe)_2$ administration rescued from cell death and mitochondrial dysfunction in brain of MeHg-exposed animals [19, 26]. In line with this, rescue from mitochondrial deficits by the activation of Nrf-2 (nuclear factor E2-related factor 2) has extensively demonstrated neuroprotection in many animal models [43–48]. The Nrf-2 pathway activation is an adaptive response to intrinsic and extrinsic cellular stresses [49], and it can be triggered by sulfhydryl reactive, small molecule pharmacological agents, provoking the inducible expression of an extended battery of cytoprotective genes, including those related to antioxidant defenses—i.e., glutathione metabolism linked enzymes and hemeoxygenase type 1 (HO-1) and mitochondrial biogenesis [49–51]. Since, HO-1 is a sensitive

biomarker of the Nrf-2 downstream signaling, and its expression is regulated essentially at the transcriptional level [52], it is feasible that $(PhSe)_2$ activated this pathway for promoting protection.

The inducible HO-1 degrades the intracellular pro-oxidant heme to free iron, carbon monoxide (CO), and biliverdin. Biliverdin is subsequently converted to bilirubin, a potent antioxidant, and the iron is sequestered by ferritin [53, 54]. In this scenario, it is known that CO activates the biogenesis of mitochondria, by promoting the activation of the mitochondrial transcription factors namely, nuclear respiratory factor-1 (NRF1) and -2 (NRF2), and mitochondrial transcription factor A, as well as the peroxisome proliferator-activated receptor-γ coactivator (PGC-1α). CO binds to the reduced a3 heme of cytochrome c oxidase, enhancing mitochondrial hydrogen peroxide production, which serves as signal transduction [50, 55] and contributes to retrograde activation of mitochondrial biogenesis [56, 57]. Furthermore, and since another selenium-containing compound, sodium selenite, also stimulates the biogenesis of the organelle by increasing the levels of nuclear mitochondrial biogenesis regulators, including PGC-1α and NRF1, as well as the mitochondrial proteins cytochrome *c* and cytochrome c oxidase IV [15], and also results in prevention of brain energy deficits [16], it appears that the selenium moiety of the $(PhSe)_2$ is determinant in controlling the mitochondrial dynamics, and the high lipophilicity of the compound would favor the intracellular distribution [34].

Similar protective effects of $(PhSe)_2$ were observed here, on the MeHg-induced almost abolished mitochondrial CK activity in the cerebral cortex of intoxicated mice. This inhibition induced by the mercurial has already been described by our group [16, 50], and it mostly occurs by oxidation of a cysteinyl residue at the active site of the enzyme that is critical for substrate binding [58]. On the other side, the effect of the chalcogen compound on CK activity appears to be independent of the $(PhSe)_2$-induced increased mitochondria, since AK activity, a ubiquitous enzyme involved in energy metabolism and nucleotide synthesis, which was also measured in the mitochondrial fraction, was not altered by the treatments. In line with this, PK was also not altered by the treatments in the mouse cerebral cortex, detonating the lack of critical thiol residues in the active site [59].

Besides the potential $(PhSe)_2$ ability of quenching the toxicant, its antioxidant activity, and the capacity of modulate mitochondrial function, $(PhSe)_2$ appears to be a promising cytoprotective molecule, based on its low toxicity, which has been already demonstrated in rabbits [60, 61] and rodents [62]. Moreover, $(PhSe)_2$ is less toxic than the related compound ebselen, which has been already used in clinical trials and consistently demonstrated to reduce brain damage in patients with delayed neurological deficits after aneurysmal subarachnoid hemorrhage and improved the outcome of acute ischemic stroke [63, 64]. Even when hepatic or renal toxicity was observed to be induced by $(PhSe)_2$ in rodents and non-rodent models, the effects were induced by 15-fold doses (75 μM). Moreover, the doses here used of $(PhSe)_2$ are several times lower than the ED_{50}; which was estimated in >500 μmol/kg in mice when administered by the subcutaneous route [65] and >1 mmol/kg after gastric gavage [3].

Summarizing the presented findings add new concepts in the field of MeHg-toxicity and $(PhSe)_2$ properties, evidencing that (i) the number of mitochondria is reduced in the cerebral cortex, (ii) the morphology of mitochondria is altered, suggesting the induction of the mitochondrial fusion process, (iii) $(PhSe)_2$ alone modulates mitochondrial homeostasis by increasing the number of mitochondria, which could be related to its previously reported beneficial effects; (iv) $(PhSe)_2$ increases the content of HO-1; and (v) $(PhSe)_2$ prevents against the inhibition on CK activity, either by reducing the MeHg content in brain or by exerting its antioxidant properties.

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