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Organochalcogens inhibit mitochondrial complexes I and II in rat brain: possible implications for neurotoxicity

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Abstract

Organochalcogens, such as organoselenium and organotellurium compounds, can be neurotoxic under experimental conditions. Since mitochondrial dysfunction plays a pivotal role in neurological disorders, the present study was designed to test the hypothesis that rat brain mitochondrial complexes (I, II, I–III; II–III and IV) are molecular targets of organochalcogens. The results show that organochalcogens caused a statistically significant inhibition in mitochondrial complex I activity, which was prevented by pre incubation with NADH. Mitochondrial complex II activity remained unchanged in response to (PhSe)₂ treatment, whereas Ebs and (PhTe)₂ caused a concentration-dependent significant inhibition in this complex. Mitochondrial complex IV activity in response to organochalcogens treatments was indistinguishable from controls. In conclusion, Ebs, (PhSe)₂ and (PhTe)₂ were more effective inhibitors of brain mitochondrial complex I than complex II; complex IV activity in response to organochalcogens treatments was almost indistinguishable from controls. These observations are consistent with organochalcogens–induced mitochondrial complexes I and II inhibition via their thiol oxidase activity with Ebs, (PhSe)₂ and (PhTe)₂ oxidizing critical thiol groups on these complexes.

Keywords

Thiol oxidase; thiol oxidation; molecular target; brain mitochondria

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Introduction

Organochalcogenides such as organoselenium and organotellurium compounds are promising pharmacological agents (Nogueira and Rocha 2010; Nogueira et al. 2004). They have been widely studied because of their potential antioxidant capacity (Arteel and Sies 2001; Barbosa et al. 2008; Barbosa et al. 2006; de Bem et al. 2009; de Freitas et al. 2009; Hort et al. 2011; Moretto et al. 2007; Nogueira and Rocha 2011; Nogueira and Rocha 2010; Parnham and Graf 1991; Prauchner et al. 2011; Prigol et al. 2008; Puntel et al. 2007; Rossato et al. 2002). However, at high dosages, both organoselenium and organotellurium compounds are neurotoxic (Jacques-Silva et al. 2001; Maciel et al. 2000; Nogueira et al. 2003; Nogueira et al. 2001; Stangherlin et al. 2005).

The documented antioxidant activity of the organochalcogens has been partially attributed to their glutathione- and thioredoxin reductase-like activities (de Freitas and Rocha 2011; Maiorino et al. 1988; Sausen de Freitas et al. 2010; Sies 1993; Sies 1995; Zhao and Holmgren 2002; Zhao et al. 2002). In order to reduce peroxides, the selenium containing compounds must first form selenol/selenolate intermediates, a reaction which is catalyzed via reduction of the Se moiety by various thiols (Nogueira and Rocha 2010; Nogueira et al. 2004; Wendel et al. 1984). For organotellurium compounds, the chemistry is more complex due to the extreme instability/reactivity of the tellurol intermediate. Hence, it has been postulated that the antioxidant activity of organotellurium compounds is linked to changes in the oxidation state of the Te atom ($\text{Te(II)} \leftrightarrow \text{Te(IV)}$) (Engman et al. 1995; You et al. 2003).

Notably, excessive oxidation of thiols by organochalcogens in the absence of concomitant reduction in peroxides (named thiol-oxidase activity) may be potentially (neuro)toxic (Nogueira and Rocha 2011; Nogueira et al. 2004; Puntel et al. 2010). Indeed, exaggerated oxidation of thiols, including those in mitochondrial membranes has been linked to neuronal apoptotic cell death (Morin et al. 2003; Zhao et al. 2006). In support to this view, our group recently showed that the organoselenium compounds, Ebselen (Ebs) and diphenyl diselenide $[(\text{PhSe})_2]$, induce mitochondrial depolarization, swelling and NAD(P)H oxidation via interaction with critical mitochondrial thiols (Puntel et al. 2010). However, the effect of organochalcogens on mitochondrial complexes activity has yet to be determined. So, studies on altered mitochondrial complexes activity are highly relevant to neurological disorders as many of these disorders are characterized by mitochondrial electron transport chain inhibition, energy metabolism impairment and elevated levels of oxidative stressors (Beal 1998; Cadenas and Davies 2000; Caspersen et al. 2005; Cortopassi and Wong 1999; Hagen et al. 1998; Hauptmann et al. 2006; Navarro and Boveris 2007b; Nicholls 2002; Nicholls and Budd 2000; Saris and Eriksson 1995; Sastre et al. 2003).

Given that mitochondrial complexes play a key role in integrating cell death stimuli and executing apoptotic cell death (Navarro and Boveris 2007b), we sought to determine whether these complexes could be considered a toxicological molecular target for the thiol-oxidase activity of Ebs, $(\text{PhSe})_2$ and diphenyl ditelluride $[(\text{PhTe})_2]$. Specifically, we aimed to determine whether Ebs, $(\text{PhSe})_2$ and $(\text{PhTe})_2$ cause mitochondrial complex I, II, I–III, II–III and IV inhibition.

Materials and Methods

Chemicals

NADH, mannitol, rotenone, succinic acid, malonate, potassium cyanide (KCN), sucrose, HEPES and cytochrome *c* were obtained from Sigma Chemical Company (St Louis, MO, USA). All other reagents were of the highest purity grade available.

Animals

Adult male Wistar rats (250–350 g) from our breeding colony were used in this study. The animals were housed in plastic cages with water and food *ad libitum*, at 22–23°C, 56% humidity, and 12 hours (hs) light cycle. The diet of the rats contained (in g/100g): 52 carbohydrate, 20 crude protein, 5 fat, 6 crude fiber, 5 minerals and 11 mixture of other nutrients. The diet contained 0.1 mg/kg of Se and 30 IU/kg of vitamin E (for complete mineral and vitamin contents, see (Puntel et al. 2010). The animals were used in accordance to guidelines of the Committee on Care and use of Experimental Animal Resources, Federal University of Santa Maria, Brazil.

Preparation of rat brain mitochondria enriched fraction and mitochondrial membranes

Enriched rat brain mitochondrial fractions were prepared from rat whole brains. The brains were homogenized in a Potter homogenizer with a Teflon pestle in 0.23 M mannitol, 0.07 M sucrose, 15 mM HEPES (pH 7.2), at a ratio of 1 g of tissue/9 ml of homogenization medium. The homogenate was centrifuged at 700 *g* for 10 min, and the supernatant centrifuged at 8,000 *g* for 10 min to precipitate the mitochondria (which were washed in an identical condition). Total proteins were adjusted to 20 mg/ml (Peterson 1977) and the samples were immediately frozen and kept at –80°C. Mitochondria were disrupted and homogenized by freezing and thawing (x2) and passing through 15/10 tuberculin needles (Navarro et al. 2002).

Mitochondrial complexes activities assay

The activities of complexes I, I–III, II, II–III and IV were determined spectrophotometrically at 30°C with mitochondrial membranes (0.5 mg/ml) suspended in 100 mM phosphate buffer (pH 7.4) as previously described (Navarro et al. 2004; Navarro et al. 2002). For all experiments, the brain mitochondrial membranes were pre-incubated for 10 min in phosphate buffer with organochalcogens (Ebs 0–50 µM; [(PhSe)₂] 0–100 µM; [(PhTe)₂] 0–100 µM), vehicle (DMSO) or the respective classical inhibitor. After the pre-incubation, the reaction was initiated with the addition of the corresponding substrate.

For the NADH:ubiquinone oxidoreductase (complex I) activity assay, mitochondrial membranes (0.5 mg/ml) in 100 mM phosphate buffer were incubated for 10 min with organocompounds or rotenone (100 µM). The reaction was initiated after 10 min by addition of NADH at a final concentration of 100 µM. The enzymatic activity was determined by following the decrease in absorbance at 340 nm for 180 s.

For the NADH-cytochrome *c* (complexes I–III) activity assay, we carried out the experiments at two different conditions. In condition 1, mitochondrial membranes were

supplemented with 200 μM NADH as substrate and 1 mM KCN, and incubated for 10 min with organocompounds (in the presence of NADH) or 100 μM rotenone (complex I inhibitor). The reaction was initiated by the addition of 100 μM cytochrome *c3* (oxidized cytochrome). In condition 2, mitochondrial membranes were supplemented with 100 μM cytochrome *c3* (oxidized cytochrome) and 1 mM KCN, and pre-incubated for 10 min with organocompounds (in the absence of NADH) or rotenone. The reaction was initiated by adding 200 μM NADH to the reaction mixture. In both conditions, the enzymatic activity was determined at 550 nm ($\epsilon = 19 \text{ mM}^{-1} \text{ cm}^{-1}$) for 120 s.

For the succinate:ubiquinone oxidoreductase (complex II) activity assay, we carried out the experiments in two different conditions. In condition 1, mitochondrial membranes in 100 mM phosphate buffer were supplemented with succinate 5 mM as substrate, and incubated with organocompounds or 8 mM malonate, a specific inhibitor of complex II (in the presence of succinate). In condition 2, mitochondrial membranes were pre-incubated with organocompounds or 8 mM malonate 8 (in the absence of succinate). After 10 min of pre-incubation, (3–4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT 50 $\mu\text{g/mL}$; condition 1) or MTT plus succinate (5 mM; condition 2) were added to the reaction medium. The reaction was stopped 3 min after the addition of MTT with 1 mL of ethanol. The absorbance was determined at 570 nm.

For the succinate-cytochrome *c* reductase (complexes II–III) activity assay, mitochondrial membranes (0.5 mg/ml) were supplemented with succinate 5 mM as substrate and 1 mM KCN, and incubated for 10 min with organocompounds. The reaction was initiated by adding 100 μM cytochrome *c3* (oxidized cytochrome). The enzymatic activity was determined over 120 s at 550 nm ($\epsilon = 19 \text{ mM}^{-1} \text{ cm}^{-1}$).

For the cytochrome oxidase (complex IV) activity assay, mitochondrial membranes (0.5 mg/ml) were incubated for 10 min in 100 mM phosphate buffer with organochalcogens or 10 mM KCN, a specific inhibitor of complex III. The reaction was started with the addition of 100 μM cytochrome *c2* (reduced cytochrome), and monitored over 180 sec. The rate of cytochrome *c2* oxidation was calculated as the first-order reaction constant *k* per mg protein.

Synthesis of organochalcogens

(PhSe)₂ was synthesized according to a previously published method (Paulmier 1986), (PhTe)₂ according to Petragnani (Petragnani 1994) and Ebs as described by Engman (Engman 1989). Organochalcogens solutions were freshly prepared in 3% dimethylsulfoxide (DMSO).

Statistical Analysis

Results were analyzed by one-way analysis of variance (ANOVA) followed, where appropriate, by Duncan's multiple range test. The data shown in Figure 3 and the effect of the respective classical inhibitors (*vs.* control) were analyzed by Student *t* Test. A $P < 0.05$ value was considered statistically significant.

Results

Effect of organochalchogens on brain mitochondrial complex I activity

Ebs, (PhSe)₂ and (PhTe)₂ significantly ($p<0,05$) inhibited rat brain mitochondrial complex I activity in a concentration-dependent manner (Figure 1). Ebs-induced complex I inhibition was statistically significant at concentrations 5 μ M, while both (PhSe)₂ and (PhTe)₂ caused mitochondrial complex I inhibition at concentrations 10 μ M. Rotenone (100 μ M), a classical complex I inhibitor, completely inhibited mitochondrial complex I activity (Figure 1).

Effect of organochalchogens on brain mitochondrial complex I–III activity

As shown in Figure 2, Ebs at concentrations 10 μ M significantly ($p<0,05$) inhibited rat brain mitochondrial complex I–III activity, with maximal effect at 50 μ M (Figure 2). Neither (PhSe)₂ nor (PhTe)₂ (10–100 μ M) caused a change in mitochondrial complex I–III activity (Figure 2).

To better understand the inhibitory effect of organochalchogens on complexes I–III activity, we carried out experiments using two different conditions (conditions 1 and 2 as described above). As shown in Figure 3, under condition 1, Ebs (5 μ M) significantly inhibited rat brain mitochondrial complexes I–III activity, whereas (PhSe)₂ and (PhTe)₂ did not affect complexes I–III activity. However, under condition 2, both Ebs, (PhSe)₂ and (PhTe)₂ (5 μ M) significantly ($p<0,05$) inhibited (approximating complete inhibition) complexes I–III activity. Rotenone (100 μ M) caused 30% and 70% inhibition in rat brain mitochondrial complexes I–III activity in conditions 1 and 2, respectively (Figure 3).

Effect of organochalchogens on brain mitochondrial complex II activity

To examine the inhibitory effect of organochalchogens on brain complex II activity, we carried out experiments at two different conditions (conditions 1 and 2 as described above). In both conditions, Ebs and (PhTe)₂ significantly ($p<0,05$) inhibited rat brain mitochondrial complex II activity, while (PhSe)₂ did not produce a discernible change (Figure 4A and 4B). Malonate (8 mM) significantly ($p<0,05$) inhibited mitochondrial complex II activity, which varied from 40 to 70% inhibition (Figures 4A and 4B).

Effect of organochalchogens on brain mitochondrial complex II–III activity

Figure 5 shows that both Ebs and (PhTe)₂ significantly ($p<0,05$) inhibited in a concentration-dependent manner mitochondrial complexes II–III activity, whereas (PhSe)₂ failed to do so.

Effect of organochalchogens on brain mitochondrial complex IV activity

Ebs and (PhSe)₂ significantly ($p<0,05$) inhibited rat brain mitochondrial complex IV activity (Figure 6), but the effect was concentration-independent. (PhTe)₂ (10–100 μ M) did not affect mitochondrial complex IV activity, whereas KCN (10 mM) completely inhibited it.

Discussion

Our results indicate that mitochondrial complexes in rat brain are targeted by organochalcogens, corroborating our previous data (Puntel et al. 2010). We hypothesized that organochalcogens-induced mitochondrial inhibition could, at least in part, mediate organochalcogens-induced neurotoxicity (Nogueira and Rocha 2010; Nogueira et al. 2004). Here we demonstrate for the first time that organochalcogens inhibit mitochondrial electron transport chain components (mitochondrial complexes I and II; Figures 1–5), establishing a mechanism for the impairment in mitochondrial function. Specifically, we noted that complex I was the major mitochondrial complex affected by the organocompounds (Figures 1–3), followed by complex II (Figures 4–5), and to a lesser extent complexes III and IV (practically not affected by the organocompounds).

The inhibitory effect of the organochalcogens on mitochondrial function is likely modulated by their interaction with essential cysteinyl residues on complexes I and II. In fact, the mitochondrial complexes (complexes I–IV) are known to be oxidatively modified, and consequently inhibited, under different physiological and non-physiological conditions (Beltran et al. 2000; Clementi et al. 1998; Le-Quoc et al. 1981; Navarro and Boveris 2007b; Navarro et al. 2004; Navarro et al. 2005; Navarro et al. 2002; Ohnishi et al. 1998). Accordingly, organochalcogens have been reported to inhibit several sulfhydryl-containing enzymes via thiol oxidase activity (Borges et al. 2005; Folmer et al. 2005; Lugokenski et al. 2011; Nogueira et al. 2004), making us to hypothesize that they could act inhibiting the mitochondrial complexes, under our assay conditions, by this way.

As depicted in Figure 3, incubation of mitochondrial membranes with the complex I substrate, NADH, partially prevented the inhibitory effect of the organochalcogens on complex I activity. Under these conditions, NADH would be expected to decrease the access of organochalcogens to sulfhydryl groups, and by inference decrease mitochondrial complex I inhibition. Therefore, organochalcogens-induced mitochondrial complex I inhibition is likely due to their interaction with critical thiol groups in the active site of the NADH:ubiquinone oxidoreductase (complex I).

However, in contrast to the complex I findings, the organochalcogens-induced mitochondrial complex II inhibition were independent of the presence of succinate in the incubation medium (Figure 4A and 4B). Succinate dehydrogenase (SDH) possesses a single sulfhydryl group that is essential for its catalytic activity (Le-Quoc et al. 1981). We posit that the organochalcogens-induced mitochondrial complex II inhibition is likely due to their interaction with other, non-critical thiols to the enzyme's activity (located in the non-active SDH site). However, more studies will be necessary to better understand the mechanism(s) involved in organochalcogens-induced complex II inhibition.

The mitochondrial complex III and IV were practically unchanged by treatment with organocompounds. Thus, we speculate that organochalcogens do not inhibit mitochondrial complexes III and IV due to the absence of sulfhydryl groups on these complexes, which are accessible to the organochalcogens-mediated oxidation.

Our results have established Ebs as the most potent inhibitor of the mitochondrial complexes, followed by (PhTe)₂ and to a lesser extent (PhSe)₂. The “expected” order of electrophilicity (or reactivity towards nucleophile thiolate centers) should be telluride>selenides (Briviba et al. 1998). The reasons for this “hypothetical” discrepancy may be related to differences in the interaction of the non-chalcogenide moiety of Ebs, (PhSe)₂ and (PhTe)₂ with specific proteins in the mitochondrial complexes. There is sparse information on the specific targeting of thiol-containing proteins by organochalcogens. Nevertheless, our studies are consistent with observations that proteins and low-molecular weight thiols (dithiols) with vicinal thiol groups are better substrates for the catalytic oxidative effect of (PhSe)₂ and (PhTe)₂ (Nogueira and Rocha 2010; Nogueira et al. 2004). Nevertheless, in a previous study with LDH, Ebs showed a greater inhibitory effect on its activity compared to (PhSe)₂ or (PhTe)₂ (Lugokenski et al. 2011). These results suggest that Ebs may be more efficient oxidizer of high-molecular weight proteins compared to (PhSe)₂ or (PhTe)₂, and are consistent with earlier findings by our group (Puntel et al. 2010) showing that (PhSe)₂ is less potent in altering mitochondrial function compared with Ebs. Taken together, our findings indicate that mitochondrial thiol-containing proteins are more effectively targeted by Ebs than (PhTe)₂ or (PhSe)₂. However, additional studies will be needed to further clarify the molecular basis of these differences.

Damage to the mitochondrial electron transport chain is an important factor in the pathogenesis of a range of neurological diseases (dementia, cerebral ischemia, Alzheimer’s disease and Parkinson’s disease) (Fattal et al. 2006; Madrigal et al. 2001). Mitochondrial complexes inhibition is also inherent to increased reactive oxygen species (ROS) generation (Beltran et al. 2000; Clementi et al. 1998; Le-Quoc et al. 1981; Navarro and Boveris 2007b; Navarro et al. 2004; Navarro et al. 2005; Navarro et al. 2002; Navarro and Torrejon 2007; Ohnishi et al. 1998), a condition inherent to the above neurological disorders, aging as well as ischemia–reperfusion (Cadenas and Davies 2000; Caspersen et al. 2005; Hauptmann et al. 2006; Navarro and Boveris 2007a; Navarro and Boveris 2007b; Nicholls 2002; Sastre et al. 2003). Accordingly, mitochondrial complexes must be considered as putative molecular targets for organochalcogens-induced neurotoxicity.

In conclusion, our studies demonstrate that Ebs-, (PhSe)₂- and (PhTe)₂-induced mitochondrial complexes I and II inhibition. Mitochondrial complex I was the most sensitive of the complexes to organochalcogens-induced inhibition followed by complex II, whereas complex III and complex IV were almost indistinguishable from controls. Based on the structural conformation of the mitochondrial complexes, we hypothesize that the inhibitory effect of organochalcogens may be attributed to their ability to oxidize essential thiols in the enzyme complexes. Accordingly, we suggest that mitochondrial complex I and II should be considered as molecular targets for the organochalcogens. Future studies should be profitably directed at refining the understanding of the molecular mechanism(s) by which Ebs, (PhSe)₂ and (PhTe)₂ affect mitochondrial function.

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Figure 1. Effect of organochalcogens on mitochondrial complex I activity

Brain mitochondria membranes were incubated (0.5 mg/ml) for 10 min at 30°C in a medium containing 100 mM phosphate buffer, pH 7.4, in the presence of the indicated concentrations of organochalcogens (Ebs 0–50 μM; [(PhSe)₂] 0–100 μM; [(PhTe)₂] 0–100 μM). Complex I activity is expressed as % of control (absent organochalcogens). —▲— (PhSe)₂, —●— (PhTe)₂, —■— Ebs, —+— Rotenone (100 μM). The control value (in the absence of organochalcogens) was 101.78 ± 2.31. Data are expressed as means ± SEM (n=4). *p<0.05 from respective control by Duncan's multiple range test. # p<0.05 from control by t test.

Figure 2. Effect of organochalcogens on mitochondrial complex I–III activity

The mitochondria membranes were incubated (0.5mg/ml) for 10 min at 30°C in a medium containing 100 mM TFK buffer, pH 7.4, in the presence of the indicated concentrations of organochalcogens. Complex I–III activity is expressed as % of control (in the absence of organochalcogens). —▲— (PhSe)₂, —●— (PhTe)₂, —■— Ebs. The control value (in the absence of organochalcogens) was 100.94 ± 1.92. Data are expressed as means ± SEM (n=5). *p<0.05 from respective control by Duncan's multiple range test.

Figure 3. Effect of organochalcogens on mitochondrial complex I–III activity under different assay conditions

Mitochondrial membranes were incubated (0.5mg/ml) for 10 min at 30°C in a medium containing 100 mM TFK buffer, pH 7.4, in the presence of the indicated concentrations of organochalcogens in the presence (white bars) or absence (gray bars) of NADH (200μM). Complex I–III activity is expressed as % of control (a condition without organochalcogens addition). Control (in the absence of organocompounds); (PhSe)₂ (5μM); (PhTe)₂ (5μM); Ebs (5μM); Rotenone (100μM). Data are expressed as means ± SEM (n=5). *p<0.05 from respective control by t test. #p<0.05 condition 1 vs condition 2 by t test

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Figure 4. Effect of organochalcogens on mitochondrial complex II activity

Mitochondria membranes were incubated (0.5mg/ml) for 10 min at 30°C in a medium containing 100 mM TFK buffer, pH 7.4, in the presence of the indicated concentrations of organochalcogens under two different conditions: Condition 1 (with succinate 5mM); Condition 2 (without succinate). Values of complex II activity are expressed as % of control (in the absence of organochalcogens). (A) Brain mitochondrial membranes under condition 1 (Control value 101.05 ± 1.81); (B) Brain mitochondrial membranes under condition 2 (Control value 99.88 ± 2.05). —▲— (PhSe)₂, —●— (PhTe)₂, —■— Ebs, —+— Malonate (8mM). Data are expressed as means $\pm$ SEM (n=5). *p<0.05 from respective control by Duncan's multiple range test. #p<0.05 from control by t test.

Figure 5. Effect of organochalcogens on mitochondrial complex II–III activity

Mitochondria membranes were incubated (0.5mg/ml) for 10 min at 30°C in a medium containing 100 mM TFK buffer, pH 7.4, in the presence of the indicated concentrations of organochalcogens. Complex II–III activity is expressed as % of control (in the absence of organochalcogens). —▲— (PhSe)₂, —●— (PhTe)₂, —■— Ebs. The control value (in the absence of organochalcogens) was 101.91 ± 1.81. Data are expressed as means ± SEM (n=5). *p<0.05 from respective control by Duncan's multiple range test.

Figure 6. Effect of organochalcogens on mitochondrial complex IV activity

The mitochondria membranes were incubated (0.5mg/ml) for 10min at 30°C in a medium containing 100 mM TFK buffer, pH 7.4, in the presence of indicated concentrations organochalcogens. Complex IV activity is expressed as % of control (in the absence of organochalcogens). —▲— (PhSe)₂, —●— (PhTe)₂, —■— Ebs, —◆— KCN (10mM). The control value (in the absence of organochalcogens) was 102.81 ± 2.07 . Data are expressed as means $\pm$ SEM (n=5). *p<0.05 from respective control by Duncan's multiple range test. #p<0.05 from control by t test.