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Research Report

Diphenyl diselenide confers neuroprotection against hydrogen peroxide toxicity in hippocampal slices

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ABSTRACT

The present study aimed at investigating the potential *in vitro* protective effect of the organochalcogenide diphenyl diselenide – $(PhSe)_2$ – against hydrogen peroxide (H_2O_2)-induced toxicity in rat hippocampal slices. Hippocampal slices were treated for 1 h with H_2O_2 (2 mM) in the presence or absence of $(PhSe)_2$ (0.1–10 μM). H_2O_2 treatment significantly decreased cell viability (measured by MTT test) and the co-incubation with $(PhSe)_2$ (10 μM) significantly blunted such phenomenon. The non permeable thiol compounds dithiothreitol (DTT) (100 μM) or reduced glutathione (GSH) (100 μM), which did not display protective effects against H_2O_2-induced loss of cell viability *per se*, significantly improved the protective effects elicited by $(PhSe)_2$. Conversely, the permeable form of GSH (GSH monoethyl ester) was unable to alter the neuroprotection mediated by $(PhSe)_2$. The treatment of rat hippocampal slices with H_2O_2 also increased the lipid peroxidation and decreased the intracellular GSH levels. Moreover, $(PhSe)_2$ (from 0.1 μM) significantly decreased H_2O_2-induced lipid peroxidation. Interestingly, H_2O_2 decreased GSH levels and this phenomenon was partially prevented by $(PhSe)_2$. The potential effects of H_2O_2 on MAPKs phosphorylation (ERK1/2, $p38^{MAPK}$ and JNK1/2) were also evaluated. Even though H_2O_2 (2 mM) did not alter $p38^{MAPK}$ and JNK1/2 phosphorylation in hippocampal slices, it stimulated ERK1/2 phosphorylation and the co-incubation with $(PhSe)_2$ (10 μM) blocked this effect. Taken together, the present results indicate that $(PhSe)_2$ exerts protective effects against H_2O_2-induced oxidative damage in hippocampal slices and avoided the increase in ERK1/2 phosphorylation promoted by H_2O_2. The neuroprotective effect of compound seems to be related to its thiol-peroxidase-like activity and appears to occur at the extracellular milieu because a permeable form of GSH was unable to improve the protective effect of the compound as did the impermeable GSH.

1. Introduction

There is substantial evidence that the brain, which consumes large amounts of oxygen and has a high content of easily peroxidizable long-chain polyunsaturated fatty acids, is particularly vulnerable to oxidative damage (Floyd and Hensley, 2002). In this regard, reactive oxygen/nitrogen species (ROS/RNS), such as superoxide anion, nitric oxide and hydrogen peroxide (H_2O_2),

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are continuously generated within the brain and should be maintained at low levels in order to avoid oxidative damage to cellular molecules (Marnett et al., 1985; Fraga et al., 1990; Stadtman, 1992). In order to counteract the oxidative damage promoted by these reactive molecules, cells possess a complex defense system regulating ROS levels and maintaining physiological homeostasis. Such a defense system involves enzymatic antioxidants, including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) and a non-enzymatic system, including glutathione (GSH), vitamins A, C and E (Finkel and Holbrook, 2000).

Since the overproduction of ROS/RNS is considered to be involved in the pathogenesis of various neurodegenerative diseases (Coyle and Puttfarcken, 1993; Kanterewicz et al., 1998; Woltjer et al., 2005) as well as in the aging process (Tolmasoff et al., 1980; Sohal and Allen, 1990; Finkel and Holbrook, 2000), the search for new compounds with antioxidant properties and their potential utilization in antioxidant therapies have been stimulated. Of particular importance, seleno-organic compounds, which display thiol-peroxidase activity, have been experimentally studied as potential protective molecules against brain oxidative stress (Nogueira et al., 2004). Ebselen (Muller et al., 1985) and diphenyl diselenide $(PhSe)_2$ (Fig. 1A; Paulmier, 1986), whose catalytic activity involves the reduction of peroxides at the expense of thiol compounds, represent important molecules whose protective properties against experimental oxidative stress have been reported (Dawson et al., 1995; Takasago et al., 1997; Porciuncula et al., 2001; Rossato et al., 2002a; Rossato et al., 2002b; Farina et al., 2003; Moretto et al., 2005; Santos et al., 2005a; Santos et al., 2005b; Xu et al., 2006; Posser et al., 2006; Borges et al., 2006).

A cellular response to a diversity of stimuli depends on integrated networks of signaling pathways that function in a precisely coordinated manner. The control of these signaling networks is exerted, at least in part, at the level of both protein phosphorylation and dephosphorylation, catalyzed by protein kinases and phosphatases, respectively (Wong and Scott, 2004; Svenningsson et al., 2004). In this context, mitogen-activated protein kinases (MAPKs), a group of serine–threonine kinases, play a central role in transduction, amplification and integration of cell signaling from the cell surface to the nucleus (Chang and Karin, 2001; Thomas and Huganir, 2004). The MAPK family includes extracellular regulated protein kinase (ERK1/2) and the stress activated protein kinases (SAPKs), JNK1/2 and $p38^{MAPK}$ (Chang and Karin, 2001; Chen et al., 2001). These kinases are involved in the regulation of complex cellular processes such as cell proliferation, differentiation, survival and death (Chang and Karin, 2001; Chen et al., 2001). In the central nervous system it is well demonstrated that ERK1/2 may regulate a diversity of processes such as cell survival and neuroplasticity (Sweatt, 2001; Thomas and Huganir, 2004). However, it is noteworthy that ERK1/2 signaling during oxidative neuronal injury has been involved with several neuropathological conditions, such as cerebral ischemia, brain trauma and neurodegenerative diseases (Chu et al., 2004). ERK1/2 may be activated in response to oxidative stress under several *in vitro* conditions (Kanterewicz et al., 1998; Lee et al., 2005; Park et al., 2005) and the blocking of ERK1/2 activation may exert protective effects in cell lines and neurons submitted to oxidative insults by peroxinitrite (Oh-hashi et al., 1999), glutathione depletion (Stanciu and DeFranco, 2002), zinc excess (Seo et al., 2001) and amyloid beta peptide plus iron (Kuperstein and Yavin, 2002).

In spite of the well demonstrated beneficial properties of the aforementioned organoselenium compounds ebselen and $(PhSe)_2$ against pro-oxidative insults, neuroprotective properties have been reported almost exclusively for ebselen. In fact, the protective effect of ebselen has been demonstrated in neural insults such as ischemia (Dawson et al., 1995; Takasago et al., 1997; Imai et al., 2003), glutamate neurotoxicity (Porciuncula et al., 2001; Centuriao et al., 2005; Xu et al., 2006;), spinal cord injury (Kalayci et al., 2005) and methylmercury mediated neurotoxicity (Farina et al., 2003). On the other hand, under specific conditions, ebselen may present pro-oxidative effects, probably via GSH depletion (Farina et al., 2004). In this regard, $(PhSe)_2$ has been reported to present lower toxicity than ebselen (Nogueira et al., 2004). $(PhSe)_2$ caused minimal toxicity when administrated acutely to mice and rats (Nogueira et al., 2001) and after a long term exposure to rabbits (de Bem et al., 2006).

Taking into account the potential pharmacological properties of organoselenium compounds and the lower toxicity of $(PhSe)_2$ when compared to ebselen, as well as the scarcity of studies on the molecular mechanisms related to $(PhSe)_2$-induced neuroprotection, the present study aimed at investigating the potential protective effects of $(PhSe)_2$ in hippocampal slices against the acute oxidative insult with H_2O_2. The relationship between intra- and extracellular thiol-peroxidase activities and the modulation of MAPKs phosphorylation were investigated as potential events involved with $(PhSe)_2$ effects in hippocampal slices exposed to H_2O_2.

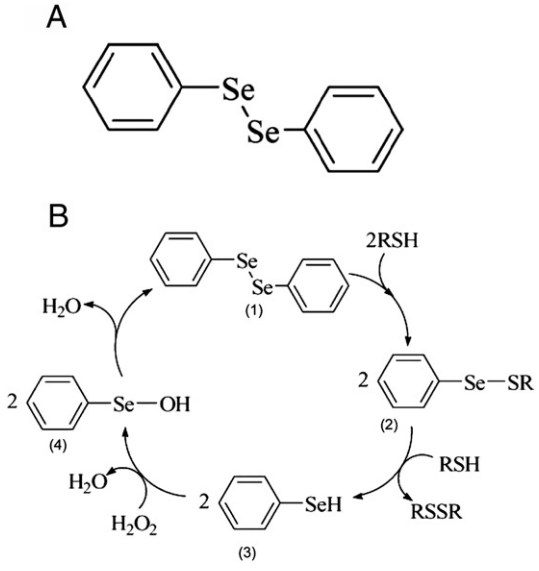

Fig. 1 – Chemical structure of $(PhSe)_2$ (A) and its derivatives (B) during the catalytic detoxification of H_2O_2.

2. Results

$(PhSe)_2$ did not affect the viability of slices when incubated for 1 or 2 h (Fig. 2A) at the concentration ranging from 0.1–100 μM. The treatment of hippocampal slices with 2 mM H_2O_2, during 1 h, decreased the cell viability by ~25% and $(PhSe)_2$ (10 μM)

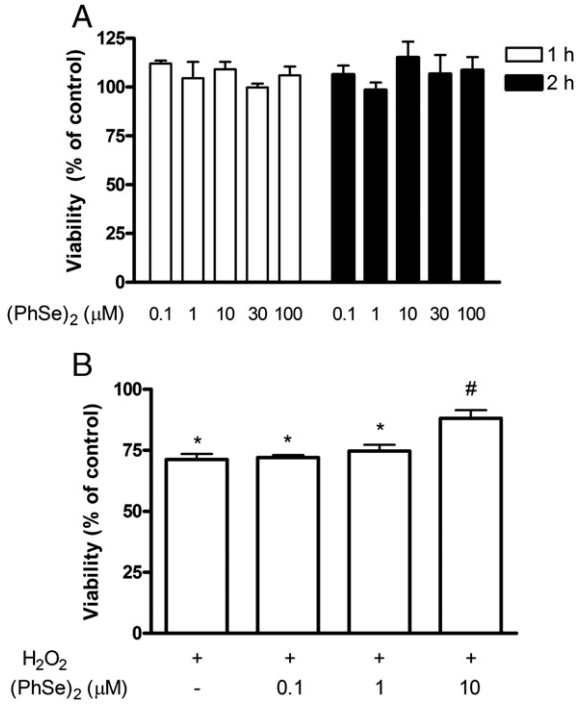

Fig. 2 – Effects of $(PhSe)_2$ on the viability of hippocampal slices. (A) Hippocampal slices were incubated in the presence or absence of $(PhSe)_2$ (0.1–100 μM) during 1 or 2 h. Values are expressed as % of control (without treatment) (means ± S.D., $n=3$, performed in triplicate). (B) Hippocampal slices were incubated during 1 h with H_2O_2 (2 mM) in the presence or absence of $(PhSe)_2$ (0.1, 1 and 10 μM). Cell viability was measured by MTT test. Values are expressed as % of control, which was defined as untreated slices (values are means ± S.D., $n=8$–12). $^{*}p<0.001$ as compared to control. $^{\#}p<0.05$ as compared to control and $p<0.01$ compared to all other groups.

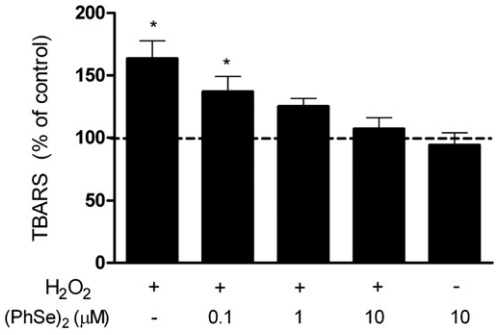

Fig. 3 – Effect of $(PhSe)_2$ on H_2O_2 (2 mM) induced lipid peroxidation (LPO) in hippocampal slices. Results are expressed as percent of control (mean ± S.D.) of eight independent experiments. The value of LPO for control group was 3.02 + 0.18 nmol TBARS/mg protein. $^{*}p<0.0001$ compared to control group.

partially protected the hippocampal slices from H_2O_2-mediated toxicity (Fig. 2B).

The GSH and protein thiols (PSH) content, as well the GPx activity were analyzed after different treatments of hippocampal slices. Table 1 shows that the acute treatment (1 h) with 2 mM H_2O_2 decreased around 23% the GSH content, but did not modify PSH levels and GPx activity. The concomitant treatment with $(PhSe)_2$ partially reversed the fall in GSH levels promoted by H_2O_2 and did not change the PSH levels and GPx activity.

Table 1 – Effects of $(PhSe)_2$ and H_2O_2 (2 mM) on GPx activity and proteic (PSH) and non-proteic thiol (total GSH) content

$(PhSe)_2$	H_2O_2	GSH	PSH	GPx
0	2 mM	77.2 ± 6.5*	100.7 ± 5.4	91.1 ± 4.5
0.1	2 mM	81.5 ± 7.2**	96.99 ± 14.4	95.2 ± 19.5
1	2 mM	86.0 ± 9.0**	102.9 ± 16.7	101.5 ± 10.6
10	2 mM	86.0 ± 9.0**	102.9 ± 16.7	101.5 ± 10.6
10	0	93.56 ± 0.05	94.52 ± 13.83	116.65 ± 15.31

Results are expressed in percent of control and represent mean ± S.D. of 5–8 independent experiments. The values of total GSH and PSH in the control group was 37.9 ± 3.8 μM/slice and 68.6 ± 6.5 μM/slice, respectively. The value for GPx activity of control was 11.06 ± 3.9 mU/mg protein. $^{*}p<0.0001$ related to control group. $^{**}p<0.0001$ related to H_2O_2 and control group.

In parallel with the neuroprotective action of $(PhSe)_2$ regarding cell viability (Fig. 2), the compound demonstrated a concentration dependent antioxidant activity (Fig. 3), preventing completely the lipid peroxidation induced by H_2O_2 at the dose of 1 and 10 μM.

It has been described that $(PhSe)_2$ possesses glutathione peroxidase mimetic action. Therefore, in this study, we investigated if the co-incubation of $(PhSe)_2$ with thiol compounds would modify the antioxidant potential of $(PhSe)_2$ against the H_2O_2 insult in hippocampal slices. Fig. 4 demonstrates that 100 μM GSH (A) or DTT (B), *per se*, did not protect hippocampal slices against H_2O_2-induced loss of cell viability (assessed by MTT assay). However, when GSH and DTT were co-incubated with $(PhSe)_2$, it was possible to observe a significant protective effect of the chalcogen, even at low concentrations (0.1 μM or 1 μM), which were not sufficient to afford neuroprotection without the presence of thiols such as GSH or DTT. Such result supports a thiol-peroxidase-like activity for $(PhSe)_2$. In another set of experiments, the hippocampal slices were pre-incubated (30 min) in the presence of glutathione monoethyl ester (GSHEE — 2 mM), a permeable form of glutathione which readily crosses the blood–brain barrier and is demonstrated to increase intracellular levels of glutathione. After pre-incubation, slices were further submitted to $(PhSe)_2$ and/or H_2O_2 treatments. Interestingly, even if the intracellular GSH levels in the GSHEE-exposed slices were increased two-fold (Fig. 4D), GSHEE pre-treatment did not alter the $(PhSe)_2$ effects toward H_2O_2-induced toxicity (Fig. 4C).

H_2O_2 may alter cell signaling pathways since it can inhibit tyrosine phosphatase activity which in turn may reinforce the tyrosine kinase activity (Finkel, 1998; 2000; Hensley and Floyd, 2002; Tonks, 2005). Furthermore, it has been well determined that H_2O_2 may activate MAPKs in cell culture models and slices (Kanterewicz et al., 1998; Park et al., 2005; Song et al., 2005). Therefore, we investigated the time dependent modulation of MAPKs by this oxidant in our model. It was verified that ERK1/2 phosphorylation was markedly stimulated by H_2O_2 in the shorter

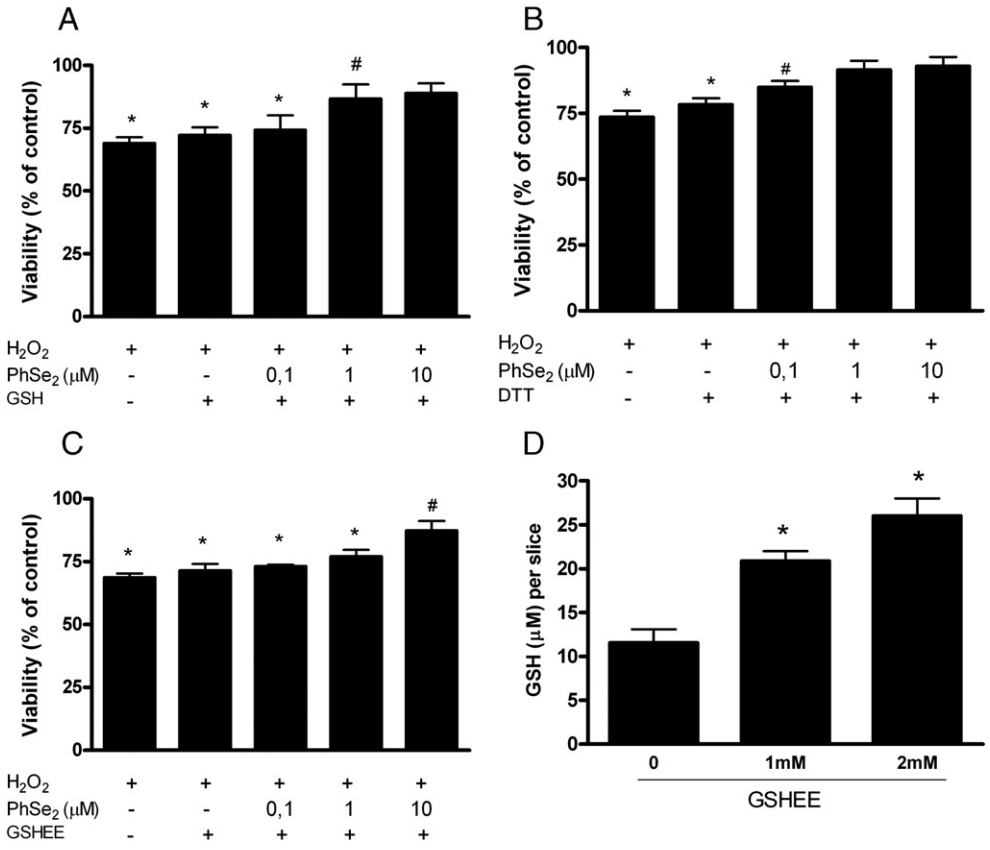

Fig. 4 – Effect of DTT, GSH and GSH monoethyl ester (GSHEE) additions in the treatment with $(PhSe)_2$ and H_2O_2 on cell viability of hippocampal slices. The slices were incubated in the presence of (A) GSH (100 μM) or (B) DTT (100 μM) during the treatment by 1 h with $(PhSe)_2$ and H_2O_2 (2 mM). (C) The slices were pre-incubated with 2 mM of GSHEE before the treatment with $(PhSe)_2$ and H_2O_2. Results are expressed as percent of control (mean ± S.D.) of 5–8 independent experiments. $^{*}p<0.0001$ compared to control group. $^{\#}p<0.0001$ compared to H_2O_2 and control group. (D) After the period of pre-treatment in the presence or absence of GSHEE diluted in incubation buffer, the slices were processed for analyses of intracellular content of total GSH. The results are expressed in μM GSH/slice (mean ± S.D.) of two independent experiments performed in duplicate. $^{*}p<0.05$ compared to control group.

period of incubation (15 min) (Fig. 5). In contrast, the $p38^{MAPK}$ and JNK1/2 phosphorylation were not altered by the treatment with H_2O_2 in the period of 15–60 min.

Concerning the alteration verified in ERK1/2 phosphorylation after 15 min of H_2O_2 treatment, the capacity of $(PhSe)_2$ to reverse this effect was evaluated. Fig. 6 shows that 10 μM $(PhSe)_2$ prevented the increase of ERK1/2 phosphorylation in response to 15 min H_2O_2 (2 mM) treatment. Additionally, we treated the slices with higher concentrations of $(PhSe)_2$ (10, 30 and 100 μM) in the absence of H_2O_2 with the purpose of analyzing a possible *per se* effect of the compound on MAPKs phosphorylation. We observed that $(PhSe)_2$ was not able to alter the MAPKs phosphorylation compared to the basal conditions (Fig. 7). This data in parallel with the data of Fig. 2A, in which $(PhSe)_2$ did not cause alteration of cell viability, reinforces the low toxicity of this compound in biological systems (Nogueira et al., 2004).

3. Discussion

The main aim of this work was to evaluate the potential neuroprotective role of the organoselenium compound diphenyl diselenide $(PhSe)_2$ in hippocampal slices submitted to oxidative insult with H_2O_2. In addition, we intended to investigate the antioxidant properties of this compound and the possible participation of MAPKs in its mechanism of action. It was showed that $(PhSe)_2$ was able to protect the slices against the acute insult with H_2O_2 in the highest concentration applied (10 μM). However, concentration as low as 0.1 μM partially decreased the lipid peroxidation induced by H_2O_2, while the highest concentration (10 μM) returned TBARS to control levels.

The antioxidant properties of $(PhSe)_2$ have been tested in many studies where the compound demonstrated a protective potential against oxidative insults in such tissues as lung (Luchese et al., 2007), blood (Posser et al., 2006) and liver (Borges et al., 2006). However, there is a scarcity of studies on the antioxidant activity of this compound within the brain, which is more susceptible to oxidative stress, when compared with other tissues, due to its high oxygen consumption, high concentration of polyunsaturated fat acids and low to moderate activity of antioxidant enzymes (Dringen, 2000; Gilgun-Sherki et al., 2002). The hippocampus is known to be critical for certain forms of learning and memory. Additionally, this brain area appears to be particularly vulnerable to a variety of conditions leading to an

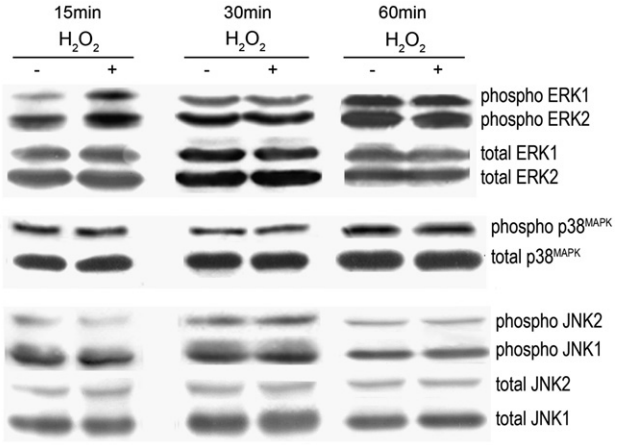

Fig. 5 – Modulation of MAPK phosphorylation by H_2O_2 (2 mM) in a time dependent manner. After treatments by 15, 30 and 60 min with H_2O_2 (2 mM), the slices were homogenized using SDS and proteins were separated by one-dimensional electrophoresis and transferred to a nitrocellulose membrane. Phosphorylation and total content of ERK1/2, $p38^{MAPK}$ and JNK1/2 were detected by specific antibodies against the phosphorylated and total forms of these proteins and the reactions were developed by ECL. Each of the blots shown above is representative of at least four experiments with similar outcomes.

overproduction of ROS such as aging, ischemia and neurodegenerative diseases (Serrano and Klann, 2004; Baloyannis, 2006). Herein, using hippocampal slices of adult rats, we demonstrated a neuroprotective and antioxidant effect of $(PhSe)_2$, in the concentration range of 1–10 μM, against an insult mediated by acute treatment with H_2O_2. From a methodological point of view, cerebral slices can be considered an appropriate model to study biochemical events *in vitro* in the brain, since they maintain the natural extracellular matrix, neuronal connectivity and neuronal–glial interactions (Rodnight et al., 1991; Cordova et al., 2004). The treatment of slices with $(PhSe)_2$ was able to recover cell viability and decrease lipoperoxidation induced by H_2O_2, preventing the cell damage against the pro-oxidative challenge.

From a molecular point of view, it has been proposed that the mechanism to explain the antioxidant effect of $(PhSe)_2$ is similar to that catalyzed by glutathione peroxidase. According to Fig. 1B, $(PhSe)_2$, reacts with the thiols (step 1) to afford a selenenyl sulphide (step 2). The selenenyl sulphide reacts with a second equivalent of GSH to yield a single product that is characterized as selenol (step 3). Finally, selenol reacts with H_2O_2 to form H_2O and a seleninic acid (step 4), which spontaneously produces another molecule of H_2O and turns into $(PhSe)_2$ (Nogueira et al., 2004; Borges et al., 2006). However, it is important to state that organoselenium compounds with thiol-peroxidase activity behave differently with different concentrations of peroxide and thiol (Mugesh and Singh, 2000). Importantly, the oxidation of diselenides by excess peroxide is known to give oxidized derivatives such as selenenic acid and seleninic acid (Mugesh et al., 2001). Despite this phenomenon, in our study, $(PhSe)_2$ significantly protected against peroxide-induced glutathione oxidation in the hippocampal slices, that could suggest a possible occurrence of a direct chemical interaction between $(PhSe)_2$ and H_2O_2, avoiding the GSH oxidation by oxidized derivatives that may occur when a high concentration of H_2O_2 is present in the medium. However, more studies are necessary to confirm this hypothesis.

Previous studies have demonstrated the neuroprotective properties of ebselen, another organoselenium compound, which acts as an efficient antioxidant in diverse animal experimental models of neurotoxicity (Ozaki et al., 1997; Porciuncula et al., 2001; Rossato et al., 2002a; Rossato et al., 2002b; Burger et al., 2005; Xu et al., 2006), as well as in ischemic stroke (Yamaguchi et al., 1998) and in aneurismal subarachnoid hemorrhage (Saito et al., 1998) in humans. In contrast with neuroprotective actions, the toxicity of ebselen has also been demonstrated in previous studies (Guerin and Gauthier, 2003; Farina et al., 2004), motivating the search for new organoselenium compounds with antioxidant activity and lower toxicity than ebselen. In this regard, $(PhSe)_2$ has been reported to display lower toxicity than ebselen when administered acutely (Meotti et al., 2003).

GSH and DTT, when co-incubated with the chalcogen, increased 10 and 100 fold the neuroprotection mediated by $(PhSe)_2$, respectively. In agreement with our data, it has been reported that, contrasting to the reaction catalyzed by GPx,

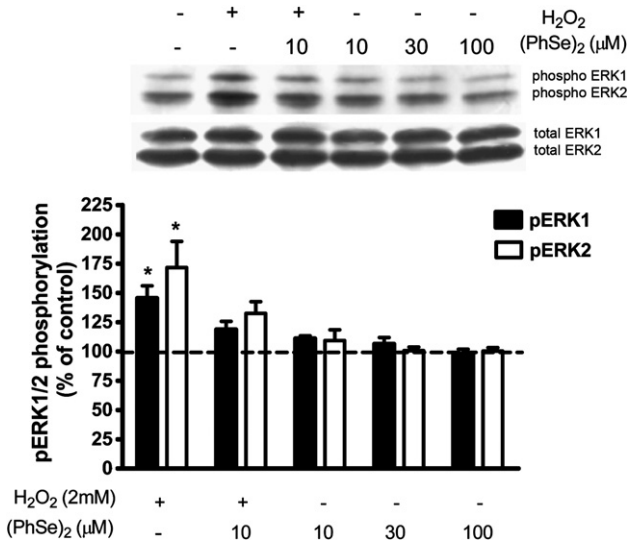

Fig. 6 – Effect of $(PhSe)_2$ on H_2O_2 induced ERK1/2 phosphorylation in hippocampal slices. After treatments by 15 min with H_2O_2 (2 mM) in the presence or absence of $(PhSe)_2$ (10 μM) or in the presence of $(PhSe)_2$ 10, 30 and 100 μM, slices were homogenized using SDS and proteins were separated by one-dimensional electrophoresis and transferred to a nitrocellulose membrane. Phosphorylation and total content of ERK1/2 was detected by a specific antibody against phosphorylated and total forms of the kinases and reactions were developed by ECL. The panel shows western blotting of phosphorylated and total forms of ERK1/2. The data represent a ratio between phospho/total forms of ERK1/2 and are expressed as percentage of the control (considered as 100%). The values are mean ± S.D. of four independent experiments. *$p < 0.01$ compared to control.

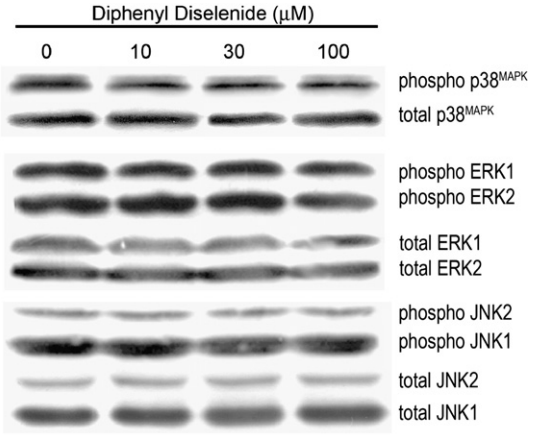

Fig. 7 – Analyses of MAPKs phosphorylation after incubation of hippocampal slices with different concentration of $(PhSe)_2$. After treatments by 1 h with $(PhSe)_2$ (10 μM–100 μM) slices were homogenized using SDS and proteins were separated by one-dimensional electrophoresis and transferred to a nitrocellulose membrane. Phosphorylation of $p38^{MAPK}$, JNK1/2 and ERK1/2 was detected by specific antibody against the bi-phosphorylated form of kinases and reactions were developed by ECL. The panel shows western blotting of phosphorylated and total forms of MAPKs, each picture is representative of at least four experiments realized independently.

organoselenium compounds can use thiol compounds other than GSH as substrate for reducing peroxide to their respective nontoxic alcohols (Muller et al., 1985; Cotgreave et al., 1987; Nogueira et al., 2004). Thus, as observed here, the combination of $(PhSe)_2$ and the thiol compounds GSH and DTT may provide a more effective protective effect, reinforcing the idea that its beneficial effects against H_2O_2 is related to its thiol-peroxidase activity.

In order to investigate a possible intracellular effect of $(PhSe)_2$, another set of experiments with a permeable form of glutathione, the GSH monoethyl ester (GSHEE), was performed. This form of GSH, in which the carboxyl group of the glycine residue is esterified, in contrast to glutathione, can be effectively transported into various cells types and intracellularly converted into glutathione (Levine et al., 1993; Ceccon et al., 2000; Gabryel and Malecki, 2006; Zeevalk et al., 2007). Herein, GSHEE (2 mM) was able to increase significantly the intracellular levels of GSH as verified in Fig. 4D. However, this treatment did not cause alterations on the protective effect of $(PhSe)_2$. Taking this data into account, it is possible to attribute an extracellular effect to $(PhSe)_2$, since an increase in intracellular GSH content was not able to affect its neuroprotective action against H_2O_2. Although previous studies suggest that $(PhSe)_2$, a very lipophilic compound, can cross the blood–brain barrier, leading to increasing selenium levels into the brain after acute and chronic treatments (Jacques-Silva et al., 2001; Maciel et al., 2003), the unaltered activity of intracellular GPx, observed in this study, reinforce the hypothesis that $(PhSe)_2$ is acting extracellularly in our experimental model. Previous studies of our research group have demonstrated that the presence $(PhSe)_2$ in the incubation medium significantly increased the activity of GPx in human platelets and this activity was related to the intrinsic GPx-like activity of the chalcogen (Posser et al., 2006). Thus, the unaltered GPx activity in the slices, even after the acute treatment with $(PhSe)_2$, may point to a very small or even the absence of the compound in the intracellular medium, showing that the period of treatment was not able to cause a considerable entrance of $(PhSe)_2$ throughout the plasmatic membrane.

Transient activation of ERK1/2 by oxidative insult with H_2O_2 under *in vitro* conditions has been widely demonstrated (Guyton et al., 1996; Lee et al., 2003; Ruffels et al., 2004; Park et al., 2005). $(PhSe)_2$ *per se* was not able to alter the MAPKs phosphorylation, however, it decreased significantly the activation of ERK1/2 stimulated by H_2O_2. Since an increase in ERK1/2 phosphorylation has been correlated to oxidative stress in patients with Alzheimer's and Parkinson's diseases (Zhu et al., 2002a; Zhu et al., 2002b; Chu et al., 2004), the present data render $(PhSe)_2$ a promising molecule for pharmacological studies on neurological disorders related to oxidative stress.

The members of MAPK family, JNK and p38 are preferentially activated by environmental stresses including oxidative stress as well pro-inflammatory cytokines (Mielke and Herdegen, 2000). It was previously demonstrated that JNK1/2 and p38 phosphorylation increase in response to oxidative stress in different cell lineages (Ali et al., 2004; Park et al., 2005), however in the present work, the incubation of hippocampal slices with H_2O_2 was not able to modulate the phosphorylation state of these kinases. Our results are in agreement with Guyton et al., (1996), who have demonstrated a modest increase in JNK1 and p38 activation compared with ERK1/2 activation in response to H_2O_2 in several cell types. Additionally in neuroblastoma cell lineages, H_2O_2 treatment did not modify p38 phosphorylation (Ruffels et al., 2004).

In conclusion, our data shows, for the first time, the neuroprotective effects of $(PhSe)_2$ in hippocampal slices submitted to an H_2O_2 insult. This neuroprotection, observed as a recovery of cell viability, may be a consequence of a decrease in lipid peroxidation and/or inhibition of H_2O_2-induced ERK1/2 activation. We propose that the GPx-like activity of the chalcogen acting in an extracellular manner, responds by the observed neuroprotective effects, which appear to be related to the modulation of peroxide availability in the extracellular milieu.

4. Experimental procedures

4.1. *Materials*

4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 2-amino-2-hydroxymethyl-1,3-propanediol (Tris), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), secondary antibodies (anti-rabbit or -mouse IgG)-horse radish peroxidase (HRP) conjugated and an Enhanced Chemiluminescence kit (ECL) were purchased from Amersham Pharmacia Bioscience. β-nicotinamide adenine dinucleotide phosphate sodium salt — reduced form (NADPH), 5-5'-dithio-bis (2-nitrobenzoic) acid (DTNB), glutathione reductase (GR) from baker's yeast (*S. cerevisiae*), dithiothreitol (DTT), glutathione reduced form (GSH), and primary antibodies anti-ERK1/2 and anti-phospho-ERK1/2 were obtained from Sigma

(St. Louis, MO, USA). Anti-phospho-JNK1/2 and anti-phospho-p38^{MAPK} antibodies were purchased from Cell Signaling (Beverly, MA, USA). Anti JNK1/2 and GSH monoethyl ester were purchased from Calbiochen (U.S., Canada). All other chemicals were of the highest grade available commercially.

4.2. *Animals*

Two months old male Wistar rats weighting approximately 130 g were used. Rats were maintained in a temperature controlled room (22–25 °C) on a 12-h light/dark cycle with food and water available *ad libitum*. They were manipulated and sacrificed according to the "Principles of Laboratory Animal Care" (NIH publication no. 80-23, revised 1996) and approved by the local Ethical Committee for Animal Research (CEUA).

4.3. *$(PhSe)_2$ synthesis*

$(PhSe)_2$ (Fig. 1) was synthesized according to literature methods (Paulmier, 1986) and was dissolved in ethanol for further incubation with tissue samples. Analysis of the ^{1}H-NMR and ^{13}C-NMR spectra showed that the obtained compound presented analytical and spectroscopic data in full agreement with its assigned structure.

4.4. *In vitro treatment and hippocampal slices preparation*

Rats were killed by decapitation, brains were removed and hippocampi dissected (4 °C) into "cutting solution" (110 mM saccharose, 60 mM NaCl, 3 mM KCl, 1.25 mM KH_2PO_4, 0.5 mM $CaCl_2$, 7 mM $MgSO_4$, 5 mM glucose; 25 mM HEPES pH 7.4). Hippocampal slices of 400-μM thickness were prepared using a McIlwain Tissue Chopper (Cordova et al., 2004). The slices were individually pre-incubated for 30 min in HEPES-saline buffer (124 mM NaCl, 4 mM KCl, 1.2 mM MgSO4, 12 mM glucose, 1 mM $CaCl_2$ and 25 mM HEPES pH 7.4) previously oxygenated for 30 min (300 μl/slice). The medium was replaced by fresh buffer in the absence (control) or presence of H_2O_2 (2 mM) and diphenyl diselenide (0–10 μM) and slices were then incubated for 1 h (35 °C). In some assays, glutathione (GSH; 100 μM) or dithiothreitol (DTT; 100 μM) were co-incubated with diphenyl diselenide and H_2O_2 during the period of treatment. In another series of experiments, the incubation with GSH monoethyl ester (GSHEE) (2 mM) was done during the period of pre-incubation. After the medium was removed the slices were washed before the addition of the respective treatments. For the treatments, $(PhSe)_2$ was solubilized in ethanol; the concentration of ethanol in the medium was 0.5%, this concentration of ethanol was not able to alter the parameters analyzed in this study. All the other reagents were diluted in HEPES-saline buffer before the treatments.

4.5. *Slice viability*

Cellular viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Slices were normalized by size, and immediately after incubation with treatments, slices were assayed for an MTT test (0.05% in HEPES-saline) for 30 min (35 °C) (Cordova et al., 2004). MTT is converted into a purple formazan after cleavage of the tetrazolium ring by mitochondrial dehydrogenases. Formazan was dissolved by the addition of DMSO, resulting in a colored compound whose optical density was measured in an ELISA reader (λ = 550 nm) (Liu et al., 1997).

4.6. *Western blot*

Western blot analysis was performed as previously described (Leal et al., 2002; Cordova et al., 2004; Posser et al., 2007). Briefly, the slices were solubilized with SDS-stopping solution (4% SDS, 2 mM EDTA, 8% β-mercaptoethanol, and 50 mM Tris, pH 6.8). Samples (100 μg of total protein/track) were separated by SDS-PAGE using 10% gels. The proteins were transferred to a nitrocellulose membrane using a semidry electroblotter (Amersham Biosciences) at 1.2 mA/cm^2, for 1.5 h according to Bjerrum and Heegaard (1998). The membranes were blocked with 5% skim milk (1 h) in tris buffer saline (TBS; 10 mM Tris, 150 mM, NaCl, pH 7.5). All steps were followed by three times washing with TBS-T (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5). ERK1/2, JNK1/2 and p38^{MAPK} total and phosphorylated forms, were detected using specific antibodies diluted in TBS-T containing 2.5% BSA in the dilutions: 1:1000 (anti-phospho-p38^{MAPK} and anti-phospho-JNK1/2), 1:10,000 (anti-phospho-ERK1/2 and anti-total-p38^{MAPK}) and 1:40,000 (anti-total-ERK1/2 and anti-total-JNK1/2). The reactions were developed by ECL. The optical density (OD) of the bands was quantified using Scion Image software®. The phosphorylation level of each MAPK was determined as a ratio of OD of phosphorylated band/OD of total band (Calloni et al., 2005; Posser et al., 2007).

4.7. *Lipid peroxidation assay*

The end products of the lipid peroxidation were determined in tissue samples by the method of Ohkawa et al. (1979) as thiobarbituric acid reactive substance (TBARS) with minor modifications. For *in vitro* lipid peroxidation the slices were incubated at 35 °C for 60 min with different concentrations of $(PhSe)_2$ (0–10 μM) in the presence or absence of H_2O_2 (2 mM). Subsequently, four slices per treatment group were homogenized in 20 mM HEPES buffer (pH 7.0) and incubated with 0.45 M acetic acid/HCl buffer pH 3.4, 0.28% thiobarbituric acid, 1.2% SDS, and thereafter at 95 °C for 60 min to promote color reaction, measured at 532 nm. Malondialdehyde (ranged from zero to 3 nmol) was used as a standard. The results represent a mean of 5 independent experiments done in duplicates. The TBARS values were normalized by protein concentration.

4.8. *Thiol status*

Glutathione (GSH) was measured as nonprotein thiols based on Ellman (1959) with minor modifications (Franco et al., 2006). Briefly, two slices per experimental group were homogenized in 0.5 M perchloric acid and centrifuged at 15,000 *g* for 2 min (4 °C). An aliquot of the supernatant was removed and incubated for 20 min at room temperature in the presence of a solution containing 0.15 mM DTNB, and 0.5 M Tris–HCl, pH 8.0 for measurements of GSH. The pellets were washed with 0.5 M perchloric acid and resuspended in 0.5 M Tris–HCl/0.1% SDS pH 8.0. An aliquot was incubated for 20 min at room

temperature in 0.15 mM DTNB, 0.5 M Tris–HCl, pH 8.0 in order to determine protein thiols (PSH). After colorimetric reaction, absorbance was measured at 412 nm in a UV/visible Varian® Cary 50 spectrophotometer. GSH and PSH were estimated using the molar extinction coefficient of 13,600 M^{-1} cm^{-1}. A sample blank without Ellman's reagent was run simultaneously.

4.9. *GPx activity*

For measurements of GPx activity, six slices per experimental group were homogenized in 20 mM HEPES, pH 7.0. GPx activity was determined based on Wendel (1981). The procedure is based on the indirect consumption of NADPH resulting in a decrease in absorbance at 340 nm, following the degradation of *tert*-butylhydroperoxide at the expense of GSH.

4.10. *Protein quantification*

Protein concentration in slices was estimated by the method of Bradford (1976) using bovine serum albumin as the standard.

4.11. *Statistical analysis*

The data are expressed as percentage of the control (considered as 100%) and the values are presented as mean ± S.D. Statistical significance was assessed for all measurements by one-way ANOVA. Post hoc analysis was performed by Duncan's test when appropriate. A value of $p<0.05$ was considered significant.

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