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Research Paper

Diphenyl diselenide in its selenol form has dehydroascorbate reductase and glutathione S-transferase-like activity dependent on the glutathione content

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Abstract

Objectives The antioxidant action of diphenyl diselenide ($(PhSe)_2$) is attributed to the mechanism by which $(PhSe)_2$ has pharmacological activity. Although $(PhSe)_2$ has glutathione peroxidase mimetic activity, the exact mechanism involved in its antioxidant effect has not yet been completely elucidated. In the present study, mechanisms involved in the antioxidant property of $(PhSe)_2$ (1–50 μM) were investigated.

Methods Dehydroascorbate (DHA) reductase- and glutathione S-transferase (GST)-like activity, 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical-scavenging activity and the protection against the oxidation of Fe^{2+} were evaluated.

Key findings $(PhSe)_2$ at concentrations equal to, or greater than, 5 μM showed DHA reductase- and GST-like activity. $(PhSe)_2$ was not a scavenger of DPPH or ABTS radicals and did not protect against the oxidation of Fe^{2+}.

Conclusions These results clearly indicated that DHA reductase- and GST-like activity are the mechanisms involved in the antioxidant effect of $(PhSe)_2$.

Keywords antioxidant; dehydroascorbate reductase-like; diphenyl diselenide; glutathione s-transferase-like; selenium; organoselenium

Introduction

The concept that selenium-containing molecules may be better nucleophiles (and therefore antioxidants) than classical antioxidants has led to the design of synthetic organoselenium compounds.[1] In-vitro studies have suggested that organoselenium compounds can be considered potential antioxidant compounds.[2,3]

Diphenyl diselenide ($(PhSe)_2$), an organoselenium compound, has been shown to reduce lipid peroxidation induced by a variety of oxidants.[4,5] In addition, $(PhSe)_2$ has many pharmacological properties, such as anti-ulcer [6], anti-inflammatory and antinociceptive[7] as well as anti-hyperglycaemic[8] activity. Moreover, the antioxidant property of $(PhSe)_2$ has been shown in several animal models of oxidative stress.[3,9–17] The antioxidant action of $(PhSe)_2$ is suggested as being the mechanism by which $(PhSe)_2$ exerts its pharmacological activity.

However, the exact mechanism involved in the antioxidant property of $(PhSe)_2$ has not been completely elucidated. Of particular importance, the antioxidant activity of various organoselenium compounds, such as ebselen and $(PhSe)_2$ seems to be related, at least in part, to their glutathione peroxidase (GPx) mimetic effect.[5,18] Evidence in the literature indicates that $(PhSe)_2$ has thiol peroxidase activity[3,19] and acts in different antioxidant lines of defence.[8,12–17] In addition, Jung *et al*.[20] demonstrated that besides the GPx-like activity of ebselen, the thioltransferase and dehydroascorbate (DHA) reductase-like actions seem to be related to the antioxidant effect of this compound.

Therefore, the novelty of this study was to investigate the mechanisms involved in the antioxidant property of $(PhSe)_2$ to better elucidate the well-documented efficacy of this compound as a potent agent with different pharmacological properties.

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Materials and Methods

Chemicals

Reduced glutathione (GSH), 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma (St Louis, USA). 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Aldrich Chemical Co (St Louis, USA). $(PhSe)_2$ was prepared in our laboratory according to the method described in the literature.[21] Dehydroascorbate (DHA) was prepared by oxidation of ascorbic acid with bromine as described previously.[22]

Analysis of the ^1H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra showed that $(PhSe)_2$ obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of the compound (99.9%) was determined by gas chromatography/high-performance liquid chromatography (GC/HPLC). This drug was dissolved in dimethyl sulfoxide (DMSO), which was obtained from a standard commercial supplier. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Dehydroascorbate reductase-like assay

DHA reductase activity of $(PhSe)_2$ was assayed as described previously[22,23] with minor modifications. In brief, $(PhSe)_2$ (1–50 μM) was incubated (1–2 min) in 100 mM sodium phosphate buffer, pH 6.9 at 25°C in the presence of GSH (1–3 mM). DHA reductase activity was initiated by adding DHA (0.5 mM) to a final volume of 1.0 ml. Ascorbic acid regeneration was recorded at 265 nm. A blank without $(PhSe)_2$ was run, and the difference gave the $(PhSe)_2$ DHA reductase activity in nmol/min using the molar extinction coefficient of ascorbic acid of 14 700/cm/M. Ebselen (1–50 μM) was used as a positive control.

At the end of the DHA reductase-like assay, $(PhSe)_2$ was determined in the samples. The samples (1.0 ml, final volume of DHA reductase-like assay) were mixed with ethyl acetate in a ratio of 2 : 1 (v/v) in the tube. The extraction was performed by vortex-mixing the tubes for 3 min. After, the samples were centrifuged at 3000 rev/min for 5 min. The supernatants were transferred to a clean test tube and a 1-μl volume was injected into the chromatographic system for analysis. Qualitative analysis of $(PhSe)_2$ in samples was conducted by gas chromatography (GC 2010 Shimadzu) in association with a flame ionized detector system (FID) and using a 5% diphenyl–95% dimethyl column, 30 m × 0.25 mm × 0.25 μm, from Restek. The limit of detection (LOD) for $(PhSe)_2$ was 0.5 μg/ml. A standard solution was used to obtain the retention time of $(PhSe)_2$.

Glutathione S-transferase-like assay

The reaction of GSH with CDNB is typically the preferred system used to measure the catalysis imparted by naturally occurring glutathione S-transferases.[24] Reaction of $(PhSe)_2$ with CDNB demonstrates GST-like activity. $(PhSe)_2$ (1–50 μM) was incubated with 2.0 mM GSH at 25°C for 3 min. The reaction was initiated by adding 1.0 mM CDNB to a final volume of 1.0 ml in 100 mM sodium phosphate buffer, pH 6.9, and recorded for 3 min at 340 nm. A blank without $(PhSe)_2$ was included and the difference was expressed as ΔA/min. Ebselen (1–50 μM) was used as a positive control.

DPPH radical scavenging effect

The DPPH stable radical was performed in accordance with the method of Choi *et al*.[25] Briefly, 50 μM DPPH was added to a medium containing $(PhSe)_2$ (10–50 μM). The medium was incubated for 30 min at 25°C in the dark. The decrease in absorbance was measured at 518 nm, which depicted the scavenging activity of $(PhSe)_2$ against DPPH radicals. Ascorbic acid (10–50 μM) was used as a positive control to determine the maximal decrease in DPPH absorbance. The results are expressed as percentage of the blank (without compound).

ABTS radical scavenging effect

Determination of the ABTS radical scavenging effect of $(PhSe)_2$ was performed according to the method of Re *et al*.[26] with some modifications. Initially, the ABTS radical was generated by reacting 7 mM ABTS solution in water with 140 mM potassium persulfate in the dark for 12–16 h. On the day of assay, the pre-formed ABTS radical solution was diluted 1 : 88 (1 ml ABTS radical + 87 ml 10 mM potassium phosphate buffer, pH 7.0). Briefly, ABTS radical was added to a medium containing $(PhSe)_2$ (10–50 μM). The medium was incubated for 30 min at 25°C. The decrease in absorbance was measured at 734 nm, which depicted the scavenging activity of $(PhSe)_2$ against ABTS radicals. Ascorbic acid (10–50 μM) was used as a positive control to determine the maximal decrease in ABTS absorbance. The results are expressed as percentage of the blank (without compound).

Autooxidation of Fe^{2+}

Interaction of $(PhSe)_2$ with iron was evaluated as described by Yoshino and Murakami,[27] with some modifications. The samples of 2 ml contained 10 mM Tris-HCl pH 7.1, 0.1 mM $FeSO_4$ and $(PhSe)_2$ (10–50 μM) in the absence and presence of 0.1 mM isocitrate. The reaction was started by the addition of $FeSO_4$. Volumes of 0.6 ml were mixed with 0.3 ml of 1 mM 1,10-phenanthroline at 40 min and the absorbance at 540 nm was measured. Ascorbate (0.1 mM) was used as a positive control. The values are expressed as the percentage of isocitrate in relation to the control values without $(PhSe)_2$.

Statistical analysis

Data are expressed as means ± SD. Statistical analysis was performed using a one-way analysis of variance followed by the Duncan's test. $P < 0.05$ was considered statistically significant.

Results

Dehydroascorbate reductase-like activity

Ebselen, a positive control, from concentration of 5 μM had DHA reductase-like activity dependent on the GSH concentration (data not shown). $(PhSe)_2$ had a GSH-dependent DHA reductase-like activity, and the rate of reduction was closely proportional to the concentration of GSH and $(PhSe)_2$.

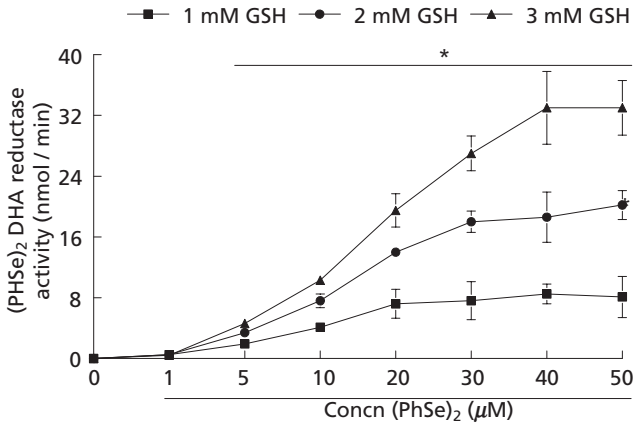

Figure 1 DHA reductase-like activity of $(PhSe)_2$. $(PhSe)_2$ (1–50 μM) was incubated in 100 mM sodium phosphate buffer, pH 6.9, at 25°C in the presence of 1, 2 and 3 mM GSH. DHA reduction was measured by absorbance increase at 265 nm. Values are the means ± SD of three or more determinations. *$P < 0.05$ compared with the blank (without $(PhSe)_2$) (one-way analysis of variance/Duncan's test).

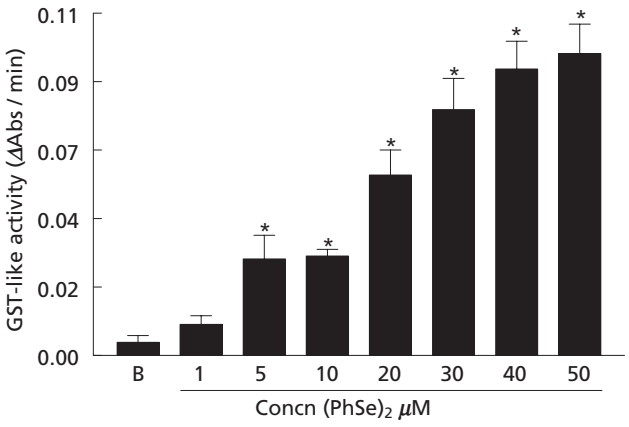

Figure 2 GST-like activity of $(PhSe)_2$. $(PhSe)_2$ (1–50 μM) was incubated at 25°C in the presence of 2 mM GSH and 1 mM CDNB in 100 mM sodium phosphate buffer, pH 6.9. The reaction was measured by absorbance increase at 340 nm. Values are the means ± SD of three or more determinations. *$P < 0.05$ compared with the blank (B, without $(PhSe)_2$) (one-way analysis of variance/Duncan's test).

Table 1 Scavenging activity of $(PhSe)_2$ and ascorbic acid on DPPH and ABTS radicals

Concn (μM)	DPPH Radical		ABTS Radical	
	$(PhSe)_2$	Ascorbic acid	$(PhSe)_2$	Ascorbic acid
0	100.0 ± 5.0	100.0 ± 5.0	100.0 ± 5.0	100.0 ± 5.0
10	103.7 ± 5.8	85.6 ± 6.5*	95.0 ± 7.0	64.0 ± 2.8*
20	105.3 ± 7.4	68.8 ± 2.5*	94.0 ± 6.1	32.0 ± 11.3*
30	106.6 ± 8.0	67.0 ± 1.0*	98.0 ± 3.6	4.9 ± 0.2*
40	104.0 ± 5.6	63.3 ± 5.1*	97.3 ± 6.0	2.2 ± 0.2*
50	100.0 ± 2.0	50.3 ± 7.8*	100.7 ± 6.4	2.4 ± 0.3*

Data are reported as mean ± SD of four independent experiments. 2,2′-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) are expressed as percentage of blank (without compound – 0). *$P < 0.05$ compared with the blank (0) (one-way analysis of variance/Duncan's test).

At concentrations equal to, and greater than, 5 μM $(PhSe)_2$ reduced DHA to ascorbic acid (Figure 1). The DHA reductase-like activity of $(PhSe)_2$ was similar to that of ebselen. At the end of the DHA reductase-like assay, $(PhSe)_2$ was detected by GC.

Glutathione S-transferase-like activity

Ebselen, a positive control, from a concentration of 10 μM had GST-like activity in the presence of GSH (data not shown). $(PhSe)_2$, at concentrations equal to, and greater than, 5 μM had GST-like activity in the presence of GSH (Figure 2). The reaction rate was essentially proportional to $(PhSe)_2$ concentration. The GST-like activity of $(PhSe)_2$ was superior to that of ebselen.

DPPH radical scavenging activity

Ascorbic acid at a concentration equal to, and greater than, 10 μM showed DPPH radical-scavenging activity. $(PhSe)_2$ at all concentrations tested did not present DPPH radical-scavenging activity (Table 1).

ABTS radical scavenging activity

Ascorbic acid at a concentration of 10 μM had ABTS radical-scavenging activity. $(PhSe)_2$ at all concentrations did not have ABTS radical-scavenging activity (Table 1).

Autooxidation of Fe^{2+}

$(PhSe)_2$ (10–50 μM) did not affect the reduced state of iron (data not shown). Isocitrate stimulated the autooxidation of Fe^{2+} to Fe^{3+} ion and ascorbate was able to maintain the iron in reduced form, inhibiting the isocitrate-mediated oxidation of Fe^{2+} ion effectively. $(PhSe)_2$ (10–50 μM) was not effective in protecting against the oxidation of Fe^{2+} (Figure 3).

Discussion

This study showed, for the first time, that $(PhSe)_2$ has DHA reductase- and GST-like activity dependent on the glutathione concentration. Therefore, the well-documented efficacy of $(PhSe)_2$ as a potent antioxidant agent could be a result of its catalytic activity (i.e. its property in operating as enzyme

mimic). The study also demonstrated that the scavenger activity of radicals and protection against autooxidation of Fe^{2+} are not mechanisms involved in the antioxidant action of $(PhSe)_2$.

DHA reductase is an enzyme that catalyses the reduction of DHA to ascorbic acid.[22,28] In this study, we demonstrated that $(PhSe)_2$ at a low concentration (5 μM) had DHA reductase-like activity. This result corroborates previous in-vivo studies in which animals treated with $(PhSe)_2$ had an increase in the levels of ascorbic acid.[12–17] Taking these results together, we suggest that the increase in ascorbic acid content in $(PhSe)_2$-treated animals is a consequence of its DHA reductase-like activity, which could increase the reduction of DHA to ascorbic acid.

Although the intermediate phenylseleno-hemiketal was not isolated and identified in this study, we propose a mechanism for DHA reductase-like activity of $(PhSe)_2$ (Figure 4, adapted from the pathway for DHA reductase-like activity of ebselen reported by Jung *et al.*[20]). In this proposed mechanism $(PhSe)_2$ is reduced by GSH to its selenol form (phenylselenol) giving the oxidized form glutathione disulfide, GSSG (reaction **1**). Phenylselenol reacts with DHA to form phenylseleno-hemiketal (reaction **2**), which reacts with another molecule of GSH to release ascorbic acid and the intermediate, phenylseleno-glutathione sulfide (reaction **3**). This compound then reacts with another GSH to regenerate $(PhSe)_2$ and GSSG (reaction **4**). Intracellular systems rely on the GSH regeneration by NADPH and glutathione reductase activity. Based on the reaction stoichiometry and the detection of $(PhSe)_2$ after DHA reductase-like assay, we suggest that this is a catalytic cycle.

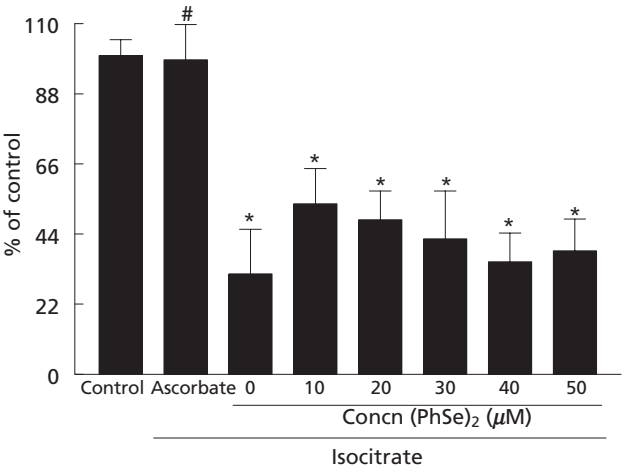

Figure 3 Effect of $(PhSe)_2$ (10–50 μM) on autooxidation of ferrous ion in the absence and presence of isocitrate. Ascorbate was used as positive control. Iron autooxidation was evaluated at 40 min. Results are expressed as percentage of control. Values are the means ± SD of three or more independent experiments. $*P < 0.05$ compared with the control; $\#P < 0.05$ compared with isocitrate.

Moreover, the scheme shown in Figure 4 clearly highlights the importance of glutathione for the $(PhSe)_2$ DHA reductase-like activity, since glutathione acts as a reducing agent of $(PhSe)_2$, generating its selenol form.

Glutathione S-transferases, also known as phase II enzymes, are widely distributed catalysing and binding proteins that promote the conjugation of GSH with a variety of reactive electrophilic compounds resulting in the formation of substances that are easily excreted from the body.[29,30] Moreover, several authors have reported that GST is an antioxidant defence and serves to protect the tissues against oxidative stress.[31–33] In this context, low-molecular-mass compounds operating as enzyme mimics have been used as antioxidants. This study demonstrated that $(PhSe)_2$ had GST-like activity, supporting the idea that the GST-like activity of $(PhSe)_2$ is involved in its antioxidant effect. Corroborating with these in-vitro findings, we previously demonstrated that GST activity is increased in animals treated with $(PhSe)_2$.[15,16]

Stable radicals DPPH and ABTS have been widely used for the determination of primary antioxidant activity of pure antioxidant compounds, plant and fruit extracts, and food materials.[34,35] However, the findings of this study clearly indicated that the antioxidant effect of $(PhSe)_2$ is not related to the scavenger activity of DPPH and ABTS radicals.

In addition, it is known that formation of ROS is closely related to the redox state of transition metals such as iron.[36]

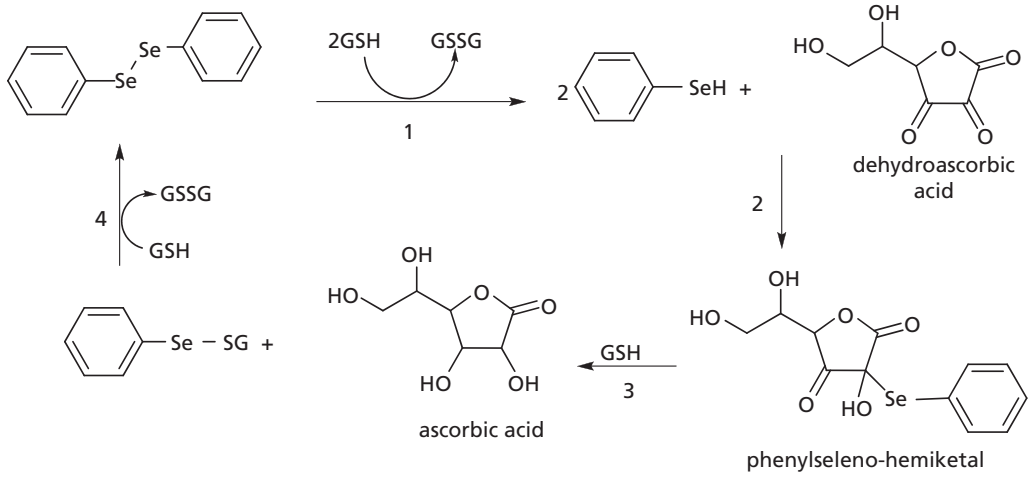

Figure 4 Mechanism of $(PhSe)_2$ DHA reductase-like activity.

The superoxide anion is readily produced through the one-electron reduction of oxygen by ferrous ion, and is largely dismuted into hydrogen peroxide by enzymatic and nonenzymatic mechanisms.[37] Hydrogen peroxide is further converted to hydroxyl radical by the Fenton reaction, which requires reduced iron.[36] Reduced iron also binds to molecular oxygen, and forms the perferryl ion (Fe^{2+}–O_2).[38] Hydroxyl radicals and perferryl ions are highly reactive, and act as the actual initiating species for cellular lipid peroxidation.[37] Effective inhibition of the isocitrate-mediated enhancement of Fe^{2+} autooxidation results in decreased formation of perferryl ions by keeping the iron in a reduced state. This study showed that $(PhSe)_2$ was not effective in protecting against the oxidation of Fe^{2+}, discarding this as one of the mechanisms involved in the $(PhSe)_2$ antioxidant action.

Ebselen is an organoselenium compound that has various pharmacological properties.[39] Moreover, the mechanisms behind the antioxidant property of this compound have been reported.[18–20,40,41] In this study, the GST-like activity of $(PhSe)_2$ was superior to that of ebselen, while the DHA reductase-like activity of $(PhSe)_2$ and ebselen were similar. Regarding the scavenging activity, ebselen has been shown to act as a scavenger of DPPH and ABTS radicals.[40,41] Different from ebselen, $(PhSe)_2$ was not a scavenger of DPPH and ABTS radicals. Therefore, if one compares $(PhSe)_2$ and ebselen, $(PhSe)_2$ has superior GST-like activity but does not act as a scavenger of DPPH and ABTS radicals. In addition, both are small-molecular-weight molecules but $(PhSe)_2$ is easier to prepare than ebselen. Considering the results presented above, we deduce that $(PhSe)_2$ would have an antioxidant effect in certain situations. In fact, $(PhSe)_2$ exerts an antioxidant effect by its mimetic activity of antioxidant enzymes but not by its radical scavenger property.

Conclusions

In conclusion, the results of this study indicate that DHA reductase- and GST-like actions are the mechanisms behind the antioxidant property of $(PhSe)_2$. The scavenging activity of DPPH and ABTS radicals and protection against autooxidation of Fe^{2+} seems not to be involved in the antioxidant property of $(PhSe)_2$.

Declarations

Conflicts of interest

The Author(s) declare that they have no conflicts of interest to disclose.

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