Biochemical Pharmacology, Vol. 43, No. 4, pp. 793–802, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

α-(PHENYLSELENENYL)ACETOPHENONE DERIVATIVES WITH GLUTATHIONE PEROXIDASE-LIKE ACTIVITY

A COMPARISON WITH EBSELEN

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(*Received* 7 *June* 1991; *accepted* 21 *October* 1991)

Abstract—Here we describe a new class of organoselenium compounds possessing glutathione peroxidase-like activity. The parent compound, α-(phenylselenenyl)acetophenone (PSAP), increased the rate of reaction of glutathione with H_2O_2, *tert*-butylhydroperoxide, cumene hydroperoxide, linoleic acid hydroperoxide and dilinoleyl lecithin hydroperoxide by 7.0, 25.1, 34.1, 19.1 and 8.4-fold, respectively, as assessed by the oxidized glutathione (GSSG) reductase enzyme assay. Direct assay of the removal of hydrogen peroxide and glutathione from reaction mixtures confirmed the peroxidase-like activities of these selenoorganic compounds, but indicate that the conventional coupled GSSG reductase assay may be unsuitable for the assessment of the catalytic capacity of PSAP and Ebselen. One possible mechanism of catalysis by PSAP involves an initial oxidation at selenium. Thiol may then react with the selenoxide to yield a selenium (II) compound, H_2O and a disulfide. Compounds derived from PSAP may provide potential selenium-based anti-inflammatory agents.

It has been postulated that granulocyte-derived reactive oxygen metabolites (ROM¶) such as $O_2^{\cdot-}$, H_2O_2, $\dot{O}H$ and HOCl may be centrally involved in the processes of inflammation [1–3]. Their localized accumulation in the extracellular milieu may be associated with events ranging from acute cytotoxicity [2, 3] to cell-specific alterations in the metabolism of lipids by lipid peroxidation. In turn, effects on lipid turnover may lead to the release of bioactive mediators such as arachidonate metabolites (leukotrienes, prostaglandins and thromboxanes), which help to propagate the inflammation response [5, 6]. It is of interest to note that the key enzyme in leukotriene biosynthesis, 5^1-lipoxygenase, is stimulated by lipid hydroperoxides [7]. There is also some evidence that fatty acid hydroperoxides themselves have biological activity in these respects [8], as do further break-down products of the peroxidative process such as long-chain aldehydes [9]. Thus, the existence of hydroperoxides at the site of inflammation may have an important amplificatory function in the propagation of the acute inflammatory stimulus.

Thus, it is of therapeutic interest to manipulate the production and accumulation of these kinds of species during the inflammation response, particularly during chronic episodes. One logical approach is to attempt to modulate components of the endogenous antioxidant defence 'network' [10] present both within cells and in the extracellular milieu. During excessive exposure to oxidant species, cellular antioxidant defences may become overwhelmed, leading to alterations to cellular biochemistry and often cytotoxicity. One key component in this defence network are the glutathione peroxidases (GSH-pxs) with specificity for reduction and inactivation of organic hydroperoxides, lipid hydroperoxides and H_2O_2.

Several investigations have established the anti-inflammatory activity of Ebselen (PZ51, 2-phenyl-1,2-benzisoselenazol-3(2*H*)-one) (**2**), [11] in a variety of model inflammations [12–14]. Studies conducted *in vitro* have shown Ebselen to possess various pharmacodynamic properties associated with the redox activity of the selenium atom. These include GSH-px-like activity [15–17] and potent antioxidant activity [15–18]. It is still unclear, though, which structural elements are required for the respective activities, which molecular mechanisms they rely on and, importantly, which activity is responsible for the observed anti-inflammatory profile of Ebselen *in vivo*.

In the rational search for the structural elements required for the catalytic activity of Ebselen it was shown that PSAP (**1**, Fig. 1) possessed GSH-px-like activity. Here we report this finding together with studies on some structural analogues performed in

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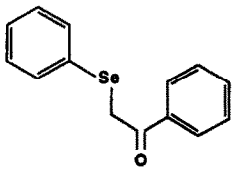
¶ Abbreviations: GSSG, oxidized glutathione; GSH, reduced glutathione; GSH-px, glutathione peroxidase; PSAP, α-(phenylselenenyl)acetophenone; t-BOOH, *tert*-butylhydroperoxide; CuOOH, cumene hydroperoxide; LinOOH, linoleic acid hydroperoxide; PL(LinOOH)$_2$, dilinoleyl lecithin hydroperoxide; ROM, reactive oxygen metabolite; DMSO, dimethyl sulphoxide.



1; PSAP

Fig. 1. The chemical structures of PSAP.

an effort to understand the mechanism of catalysis of PSAP.

MATERIALS AND METHODS

Compounds

Ebselen, (**2**) was the kind gift of A. Nattermann GmbH (Köln, F.R.G.). Other organoselenium compounds used in this study were prepared as described below or by literature methods.

Typical procedure. PSAP (**1**) [19]: $PhSeCl_3$ (3.0 g, 11.4 mmol) and acetophenone (3.0 g, 25.0 mmol) were stirred in dry ethyl ether (15 mL) for 13 hr.

Filtration afforded 2.89 g (73%) of 1-phenyl-(2-phenyl-selenyl)ethanone-Se-Se-dichloride, m.p. 107–109° dec., ^{1}H-NMR ($CDCl_3$): 5.88 (s, 2H) 7.50–8.10 (several peaks, 10H).

1-Phenyl-(2-phenylselenenyl)ethanone-Se-Se-dichloride (1.0 g, 2.9 mmol) was added to a stirred solution of thiourea (0.50 g, 6.6 mmol) in acetone (30 mL). After 30 min at ambient temperature, CH_2Cl_2 (50 mL) was added and the reaction mixture poured into water (100 mL). The organic phase was separated, washed with water, dried and evaporated. Flash chromatography (SiO_2; CH_2Cl_2) afforded 0.78 g (98%) of compound **1**, m.p. 43–44°. The ^{1}H-NMR spectrum of the material showed excellent agreement with literature data [20].

The following compounds were similarly prepared: 1-(4-Nitrophenyl)-2-(phenylselenenyl)ethanone (**3**): m.p. 83–84°. ^{1}H-NMR ($CDCl_3$): 4.15 (s, 2H), 7.26–7.32 (several peaks, 3H), 7.47–7.52 (several peaks, 2H), 7.98 (d, 2H), 8.24 (d, 2H). Anal. Calcd for $C_{14}H_{11}NO_3Se$: C, 52.51; H, 3.46. Found: C, 52.36; H, 3.46.

2-(Phenylselenenyl)-1-(2-thienyl)ethanone (**4**): m.p. 44–45° (lit. 45–46° [21]. ^{1}H-NMR ($CDCl_3$): 4.06 (s, 2H), 7.06 (m, 1H), 7.26–7.30 (several peaks, 3H), 7.52–7.65 (several peaks, 4H).

1-(2-Naphthyl)-2-(phenylselenenyl)ethanone (**5**): m.p. 70–72° ^{1}H-NMR ($CDCl_3$): 4.28 (s, 2H), 7.25–7.30 (several peaks, 3H), 7.50–7.60 (several peaks, 4H), 7.81–7.77 (several peaks, 3H), 7.97 (dd, 1H), 8.28 (s, 1H). Anal. Calcd for $C_{18}H_{14}OSe$: C, 66.47; H, 4.34. Found: C, 66.20; H, 4.37.

3,3-Dimethyl-1-(phenylselenenyl)-2-butanone (**8**): m.p. 28° ^{1}H-NMR ($CDCl_3$): 1.18 (s, 9H), 3.88 (s, 2H), 7.26–7.30 (several peaks, 3H), 7.53–7.58 (several peaks, 2H). Anal. Calcd for $C_{12}H_{16}OSe$: C, 56.47; H, 6.32. Found: C, 56.22; H, 6.25.

1-(Phenylselenenyl)-2-propanone (**9**): Oil. ^{1}H-NMR data were in good agreement with literature data [20].

1-(4-Methoxyphenyl)-2-(phenylselenenyl)ethanone (**7**): was synthesized in analogy with a literature procedure [22]. ^{1}H-NMR ($CDCl_3$): 3.86 (s, 3H), 4.15 (s, 2H), 6.90 (d, 2H), 7.25–7.30 (several peaks, 3H), 7.54 (m, 2H), 7.86 (d, 2H). Anal. Calcd for $C_{15}H_{14}O_2Se$: C, 59.0; H, 4.6. Found: C, 59.0; H, 4.6.

2-(Phenylselenenyl)-2-cyclohexenone (**6**) [23], 2-(phenylselenenyl)cyclohexanone (**10**) [24], 2-acetoxy-2-phenylethyl phenyl selenide (**11**) [25] and 1-phenyl-2-(phenylselenenyl)ethanol (**12**) [26] were prepared according to literature methods.

LinOOH and $PL(LinOOH)_2$ were the gift of Dr R. Morgenstern. H_2O_2 (30% v/v), t-BOOH (70% v/v), CuOOH (80% v/v), GSSG (>99.8%), glutathione reductase (grade 1, from yeast) and NADPH were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Stock hydroperoxide concentrations were determined using literature absorbance coefficients. All other reagents and chemicals were obtained from local suppliers in the highest grades available.

Assessment of GSH-px-like activity

The GSH-px-like catalytic activity of the compounds under study was assessed as their ability to catalyse the reaction between the chosen peroxide and glutathione in an aqueous buffer at physiological pH using a variety of approaches.

Indirect enzymatic assay. The oxidation of GSH to GSSG was measured indirectly by spectrophotometrically assessing the stimulated oxidation of NADPH in the presence of glutathione reductase [27]. The incubations were mainly constructed in the following manner: incubations in quartz curvettes were with 50 mM potassium phosphate buffer pH 7.4 (1 mL). In experiments where organic hydroperoxides were used as substrates the buffer was supplemented with 0.2% (w/v) Triton X-100. This did not affect the basal rate of oxidation. Additions were made in the order (all final concentrations): NADPH (250 μM), GSH (1 mM), test substance (50 μM) or vehicle (DMSO), check for precipitation and record baseline, GSSG reductase (1 unit), record, peroxide (1 mM), record. Incubations were conducted at room temperature in an Aminco Bowman Model 940 Scanning Double Beam Spectrophotometer recording at 340 nm with air as a reference. All substances were dissolved in DMSO and the vehicle concentration in incubations usually did not exceed 0.5% (v/v). Control and substance-stimulated oxidation of GSH to GSSG was quantified according to Racker [27]. In some cases, 20% DMSO was included in the incubations due to precipitation of certain test substances. DMSO did not affect the activity of reductase and under these conditions and other controls indicated that Ebselen/PSAP alone or in combination with H_2O_2 in the absence of GSSG reductase did not directly oxidize NADPH. This assay was also used to test the catalytic nature of PSAP's effects. Assays were constructed as above and the oxidation of NADPH followed to completion. Repeated addition of NADPH (250 μM) were made until no more stimulation was recorded. At this point, GSH (1 mM) and H_2O_2 (1 mM) were reintroduced into the incubation and the above procedures repeated.

Direct assay of peroxide removal. In other experiments, the catalytic potential of Ebselen and PSAP on the reaction between GSH and H_2O_2 was assessed directly by determining the disappearance of the peroxide from the incubations. Incubations were constructed essentially as above to test the reduction of the peroxide (250 μM) in the presence of GSH (1 mM) only, GSH and GSSG reductase (1 unit)/NADPH (250 μM) and these agents in the presence of either Ebselen or PSAP (both at 50 μM). Aliquots of the incubation (0.5 mL) were mixed with 6.5% trichloroacetic acid (0.5 mL) and the peroxide content determined by its oxidation of ferrous ammonium sulfate and subsequent reaction of the ferric ions with potassium thiocyanate to yield the coloured iron–thiocyanate complex [28]. Controls demonstrated that the addition of catalase (10 units) to the incubations prior to the assay of peroxide completely quenched the subsequent reaction with ferrous ions. This indicates a lack of interference from peroxide-catalyst intermediates in the assay of the peroxide. Similarly, no reaction was seen for incubations lacking peroxide but containing Ebselen/PSAP and GSH only.

Direct assay of glutathione removal. A similar series of incubations to the ones above were constructed containing H_2O_2 (1 mM), Ebselen or PSAP (50 μM), and GSSG reductase (1 unit)/NADPH (250 μM) in various combinations. The reactions were initiated by the addition of GSH (1 mM) and the free GSH content of the incubations determined at intervals by derivatization of aliquots (100 μL) with monobromobimane and subsequent HPLC separation and fluorescence quantitation of the monobromobimane–GSH adducts [29]. Controls were performed for the possible reaction of Ebselen/PSAP with GSH in the absence of peroxide. Briefly, Ebselen or PSAP (50 μM) were reacted with GSH (50 μM) for 1 min in 50 mM phosphate buffer, pH 7.4. The free GSH content was then determined as above. The samples were then treated with GSSG reductase (1 unit) or NADPH (250 μM), or a combination of these for a further minute and the free GSH content redetermined.

RESULTS

The indirect GSSG reductase assay indicated that the control oxidation of GSH by hydrogen peroxide amounted to 13 ± 3 nmol NADPH/min (N = 6) in control incubations and 35 ± 4 nmol NADPH/min (N = 6) in incubations containing DMSO (20%) under the conditions of incubation. This basal rate was accelerated *ca.* 5–6-fold by Ebselen (50 μM) in accordance with previous observations [15–17], yielding a rate of 70 ± 5 nmol NADPH/min (N = 4). On the other hand, PSAP had a larger stimulatory effect amounting to a *ca.* 8-fold increase over baseline and a total rate of oxidation of 103 ± 4 nmol NADPH/min (N = 4). These data are shown in Table 1. Figure 2 shows that both Ebselen and PSAP produced a concentration-dependent catalysis of the reaction between GSH and H_2O_2, each demonstrating log linearity over the concentration range 0.5 μM to 100.0 μM.

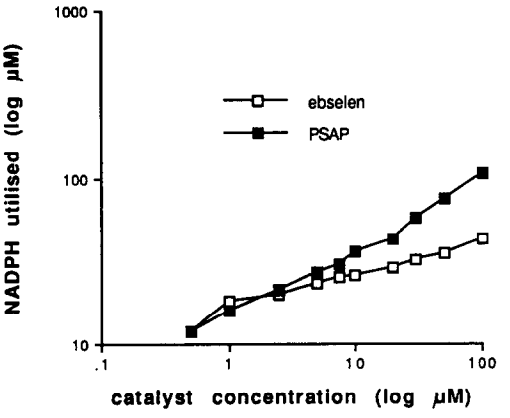


Fig. 2. The concentration dependency of the Ebselen- and PSAP-dependent catalysis of the reduction of H_2O_2 by GSH. Catalytic rates were determined by the enzymatic assay. Incubations contained GSH and H_2O_2 (1 mM each), GSSG reductase (1 unit) and NADPH (250 μM). Compounds were added in DMSO (<0.5% final concentration).

The catalytic natures of PSAP's effect on the reaction of H_2O_2 and GSH is shown in Fig. 3. The 250 μM NADPH included in the assay was quickly exhausted from the incubation [Fig. 3(1)]. Repeated administration of 250 μM aliquots of NADPH re-stimulated the reaction until no further effect was seen after a total concentration of 1 mM had been included [Fig. 3(2–4)]. At this point, re-administration of substrates and NADPH to the reaction re-stimulated the oxidation of NADPH through a new cycle [Fig. 3(5–9)].

Various other compounds containing the intact phenylselenenyl-moiety but substituted in other parts of the molecule were tested for their stimulatory effects on the reaction of GSH and H_2O_2. It can be seen *p*-nitro-substitution of the phenyl ring of the acetophenone (**3**) resulted in slight potentiation of the catalytic effect, whereas substitution of this position by a methoxy-group (**7**) diminished the catalytic potency. Removal of the aromatic function totally abolished catalytic activity, as did other manipulations of the structure designed to hamper enolizations (Table 1).

Results of the assay of hydrogen peroxide during basal and Ebselen/PSAP-catalysed reaction with GSH are shown in Fig. 4. Keeping the peroxide concentration at 250 μM, for the convenience of the assay, GSH caused a linear-dependent removal of H_2O_2 from the incubation at an average rate of 2.1 μM/min over the 60 min of incubation. The inclusion of GSSG reductase and NADPH in the incubation slightly increased this basal rate to 2.7 μM/min. The inclusion of Ebselen or PSAP increased the basal reaction rates to 5.0 and 10.0 μM/min, respectively, over the initial 5 min of reaction. Additionally, inclusion of GSSG reductase and NADPH in the incubations containing either Ebselen or PSAP further accelerated the reaction of H_2O_2 and GSH to 24 and 34 μM/min, respectively, over the initial 5 min of incubation.

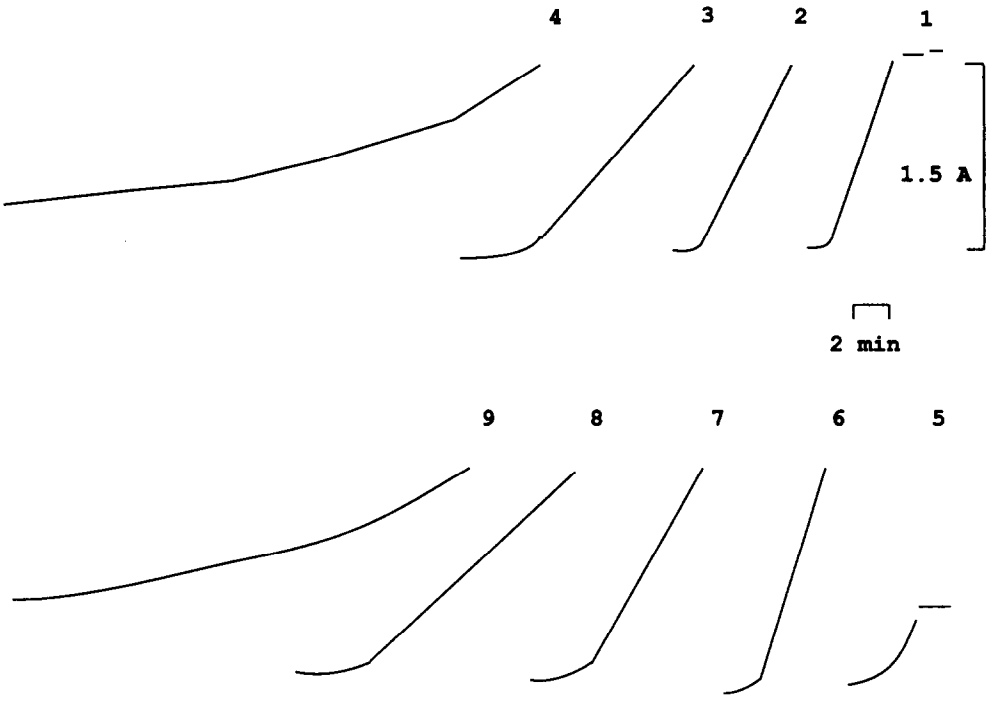


Fig. 3. The effect of repeated additions of NADPH and substrates on the catalysis of reduction of H_2O_2 by GSH with PSAP. (1) Incubation constructed as in Fig. 2. (2–4) Incubation serially resupplemented with three aliquots of NADPH (250 μM). (5) Incubation resupplied with H_2O_2 (1 mM) and GSH (1 mM). (6–9) Incubation resupplemented with four aliquots of NADPH (250 μM) at completion of the reaction.

Table 1. The glutathione peroxidase-like catalytic activity of Ebselen and a variety of PSAP analogues with hydrogen peroxide as co-substrate assessed by the enzymatic assay of GSSG

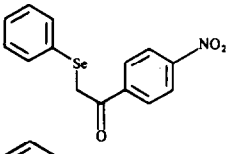
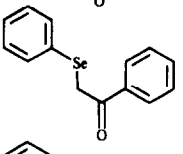
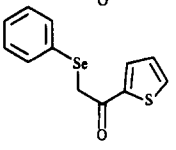
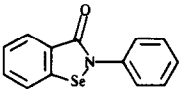
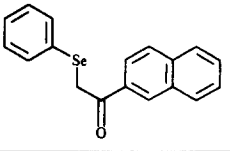
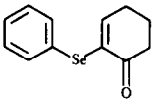
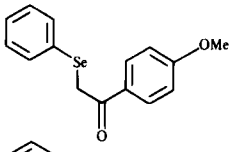
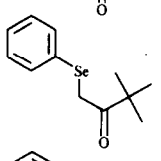
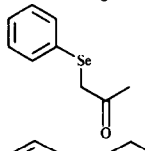
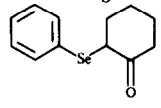
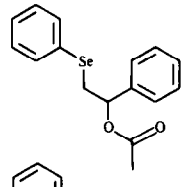
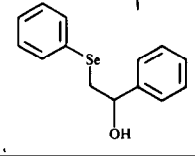
Compound	Substance	nmol NADPH/min*	% GSH†
3		157 ± 5	1207
1		103 ± 6	790
4		75 ± 3	576
2		70 ± 5	538
5		94 ± 4‡	268‡

Table 1 *continued.*

Compound	Substance	nmol NADPH/min*	% GSH†
6		30 ± 2	230
7		25 ± 2	192
8		20 ± 1	153
9		20 ± 2	153
10		14 ± 1	107
11		35 ± 3‡	100‡
12		13 ± 1	100

* Recorded at points of optimal and sustained NADPH oxidation. Measurements were averaged for a 20 sec recording period. Incubations contained GSH and H_2O_2 (1 mM each), compound (50 μM) in DMSO (<0.5% final concentration), GSSG reductase (1 unit) and NADPH (250 μM). Results expressed as mean ± SEM, N = 4, performed on the same day.

† The compound's percentage increase of the basal reaction rate between GSH and H_2O_2 calculated as:

$$\frac{\text{rate of NADPH oxidation + substance}}{\text{rate of NADPH oxidation + vehicle}} \times 100\%.$$

Vehicle controls performed on-the-day (N = 6).

‡ DMSO added to 20% (v/v). Comparative rates were corrected for DMSO's effect on basal reduction of H_2O_2 by GSH.

Figure 5 shows the results of the assay of GSH in incubations similar to those above. Control removal of GSH (1 mM) was linear over the initial 10 min of incubation, amounting to nearly 14 μM/min over this period. The inclusion of Ebselen (50 μM) or PSAP (50 μM) increased the rate of removal of GSH to 50 and 70 μM/min, respectively, over the initial 10 min of incubation. The inclusion of GSSG reductase (1 unit) and NADPH (250 μM) in control incubations retarded the removal of GSH over the initial 10 min. However, when either selenoorganic compound was introduced into this reaction, a stimulated rate of removal of the thiol was initiated over the initial 10 min, with rates amounting to 70 and 90 μM/min for Ebselen and PSAP, respectively.

Figure 6 shows that GSH (50 μM) is rapidly removed from incubations containing Ebselen (50 μM) in the absence of peroxide. Over 60% of the free GSH could subsequently be recovered by introduction of GSSG reductase and NADPH into

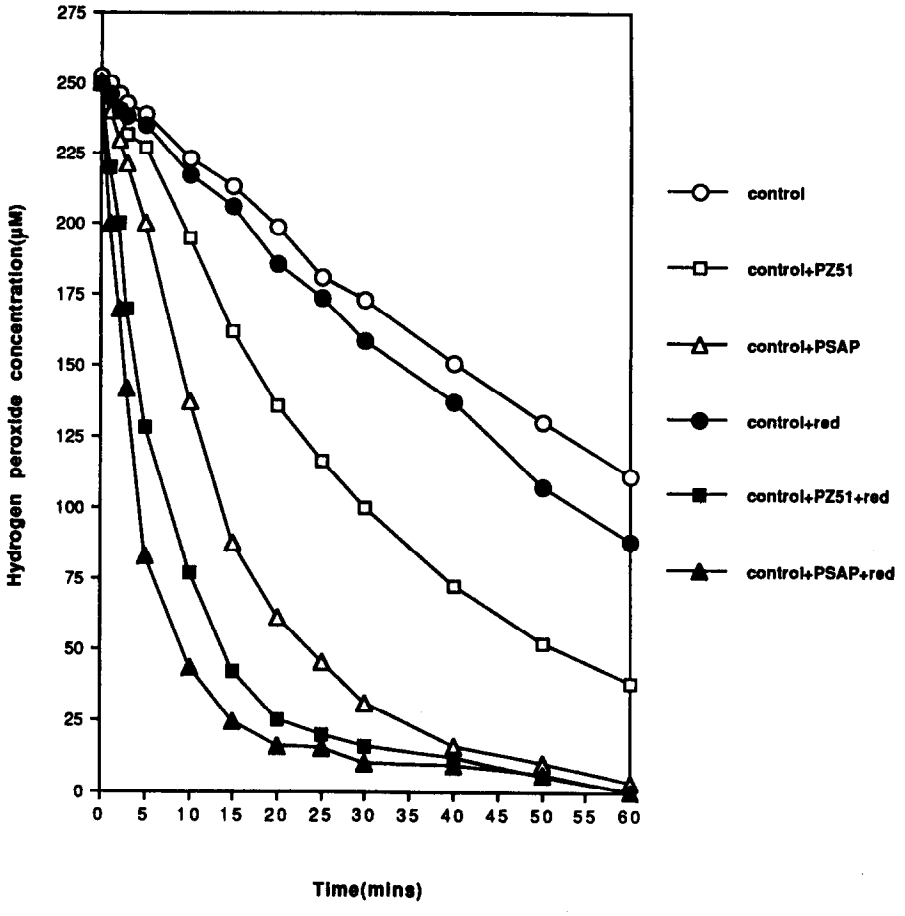


Fig. 4. The glutathione peroxidase-like activities of Ebselen and PSAP determined by the assay of hydrogen peroxide utilization. Incubations were constructed in Fig. 2 except that the H_2O_2 concentration was 250 μM throughout and Ebselen or PSAP (both 50 μM) were added in DMSO (<0.5% final concentration). Red = complete reducing system comprising GSSG reductase (1 unit) and NADPH (250 μM). Samples were removed at intervals for the assay of H_2O_2 as described in Materials and Methods. Data are mean of three observations (SEM <6% on all points), and the error bars omitted for clarity.

the incubation. This behaviour was not seen with PSAP.

Both Ebselen and PSAP catalysed the reduction of other hydroperoxides by GSH, as assessed by the enzymatic assay (Table 2). The basal reaction rates of GSH with t-BOOH, CuOOH, LinOOH or PL(LinOOH)$_2$, were much lower than with H_2O_2. However, Ebselen increased these rates of reaction proportionally more than with H_2O_2, i.e. by 6.9-, 29.2-, 48.9- and 23.3-fold, respectively. Similarly, PSAP catalysed the reaction of GSH with these substrates to the varying extents of 25.1-, 34.1-, 19.1- and 8.4-fold, respectively.

DISCUSSION

In this communication, we demonstrate the capacity of PSAP and some selected derivatives to catalyse the glutathione-dependent reduction of H_2O_2 and several organic hydroperoxides. Early work proposed that Ebselen exerts its GSH-px-like catalysis via a GSH-induced ring opening followed by oxidation of the selenium atom, eventually furnishing GSSG, water and Ebselen [30]. Alternatively, it was argued that an oxidation at selenium in the intact isoselenazolone ring occurs first, followed by rapid reduction and GSSG formation [30]. More recently, Mairoino *et al.* [31] and Haenen *et al.* [32] have proposed that Ebselen's catalytic mechanism involves initial formation of the reactive selenol of Ebselen, from Ebselen–glutathione selenosulfide, followed by formation of Ebselen diselenide which then reacts with H_2O_2 to yield a selenic acid anhydride which dehydrates to yield Ebselen again [32]. One of the possible mechanisms of the catalytic activity of PSAP derivatives may result from initial oxidation of the selenium atom and subsequent reaction by GSH (Fig. 7). The substituent effects observed (compare **1**, **3** and **7**) may be explained by assuming that the initial oxidation involves the enol tautomer. The following step, the thiol-mediated reduction of the selenoxide,

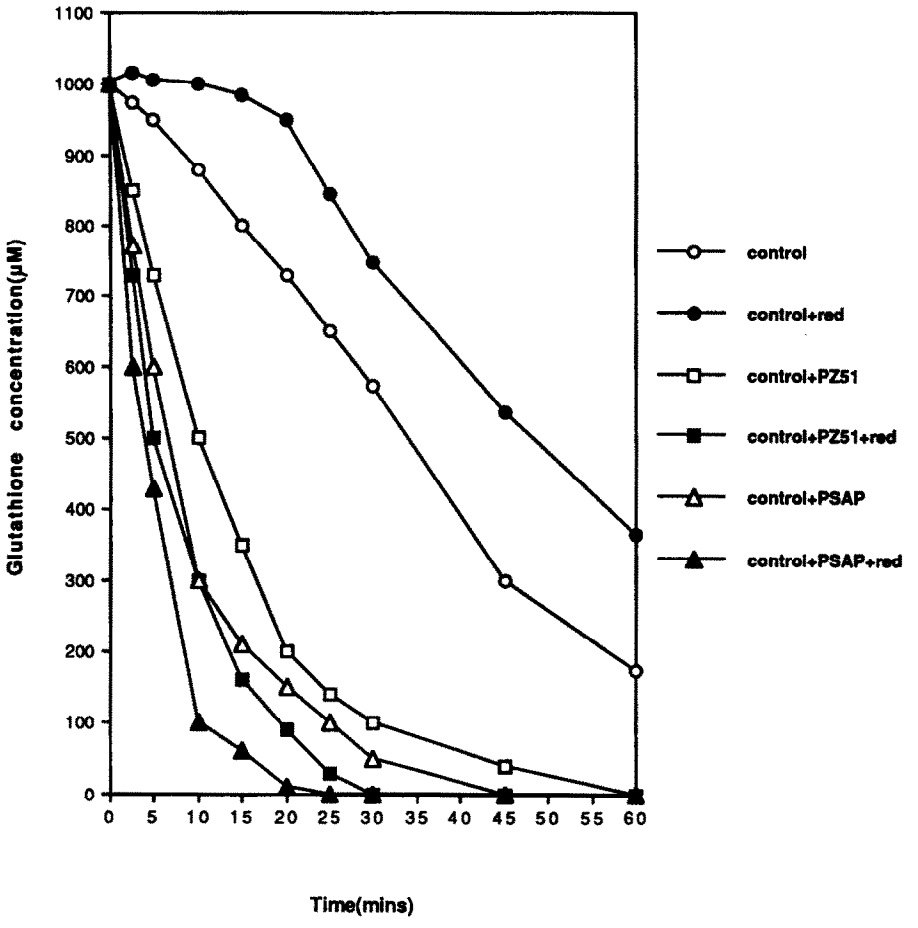


Fig. 5. The glutathione peroxidase-like activities of Ebselen and PSAP determined by the assay of glutathione utilization. Incubations were constructed as in Fig. 2. Control = GSH and H_2O_2 (1 mM each) only. Ebselen/PSAP (50 μM) were added in DMSO (<0.5% final concentration). Red = complete reducing system comprising GSSG reductase (1 unit) and NADPH (250 μM). Samples were removed at intervals for the determination of GSH as described in Materials and Methods.

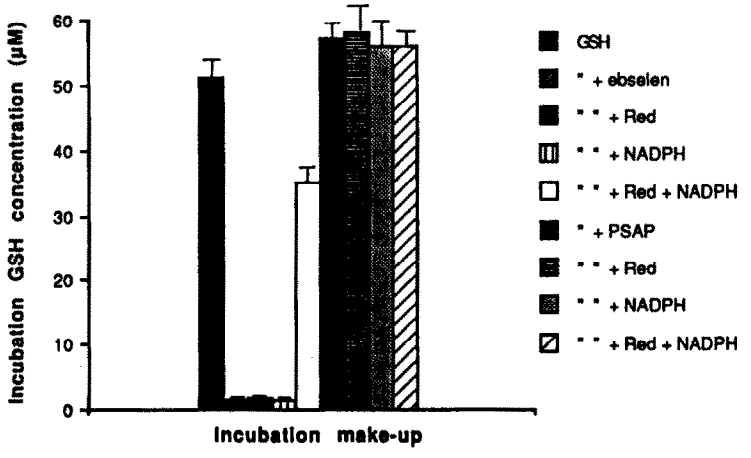


Fig. 6. The reactions of Ebselen and PSAP with glutathione and the susceptibilities of the reaction mixtures to re-reduction by glutathione peroxidase. GSH (50 μM) and Ebselen/PSAP (50 μM each) were mixed for 1 min and the GSH content determined as described in Materials and Methods. The incubations were then exposed to either GSSG reductase (1 unit) or NADPH (250 μM) or both agents for a further minute and the GSH content reassayed. The data are mean ± SEM, N = 3.

Table 2. The comparative glutathione peroxidase-like activities of Ebselen and PSAP with a variety of peroxide co-substrates as assessed by the enzymatic assay of GSSG

Catalyst	Co-substrate	nmol NADPH/min*	% Control†
—	H_2O_2	8.6 ± 0.4	—
—	t-BOOH	0.7 ± 0.1	—
—	CuOOH	1.3 ± 0.1	—
—	LinOOH	1.2 ± 0.1	—
—	$PL(LinOOH)_2$	5.2 ± 0.5	—
Ebselen			
Ebselen	H_2O_2	40.2 ± 2.3	470
Ebselen	t-BOOH	4.8 ± 0.5	685
Ebselen	CuOOH	37.9 ± 0.6	2915
Ebselen	LinOOH	58.7 ± 1.1	4891
Ebselen	$PL(LinOOH)_2$	121.2 ± 6.7	2330
PSAP	H_2O_2	60.0 ± 3.0	700
PSAP	t-BOOH	17.6 ± 0.5	2514
PSAP	CuOOH	44.3 ± 0.6	3407
PSAP	LinOOH	22.9 ± 0.4	1908
PSAP	$PL(LinOOH)_2$	43.8 ± 1.9	842

Glutathione was at 1 mM and the peroxide co-substrates introduced at 1 mM final concentration. GSSG reductase (1 unit) and NADPH (250 μM) were introduced and the reduction of GSSG monitored at 340 nm. When organic hydroperoxides were used the incubations contained 0.2% (v/v) of Triton X-100. This did not affect the activity of the GSSG reductase system.

*,† Calculated as for * and † in Table 1.

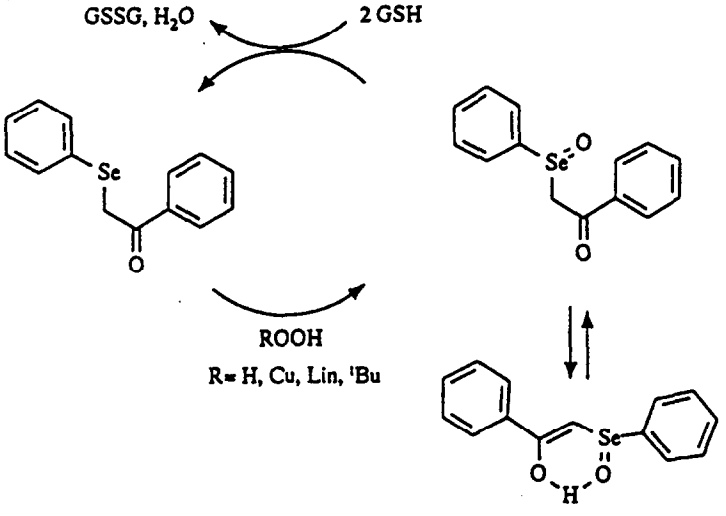


Fig. 7. One possible mechanism for glutathione peroxidase-like catalysis by PSAP (**1**).

can occur according to several different mechanisms: the selenoxide could transfer its oxygen atom to sulfur and the resulting sulfenic acid [33] could react with another thiol group to form a disulfide. Thiol could attack selenium, followed by nucleophilic attack on sulfur by a second thiol group. Finally, a Pummerer rearrangement [34] may be involved whereby sulfur is first introduced alpha to selenium and then eliminated as a disulfide by nucleophilic attack of the second thiol group. Alternatively, a mechanism involving the cleavage of the selenium–carbonyl bond and release of phenylselenol may be postulated. This cleavage would be affected in a similar manner to the resonance effects above by the substitution patterns detailed above. However, unlike the case of Ebselen [31], preliminary data indicate the inclusion of IAA (which might react with a selenol) in the reaction mixtures failed to inhibit PSAP's stimulatory effect on the reaction between H_2O_2 and GSH when the rates were corrected for IAA's effect on the basal reaction rates (Authors, unpublished). This matter awaits further investigation.

Direct assay of the removal of peroxide from incubations confirmed the applicability and sensitivity of the indirect, coupled GSSG reductase assay in

screening for the GSH-px-like catalytic activity of Ebselen, PSAP and some analogues of PSAP. In comparing both screening methods it will be noted that the rates for the catalytic effects of both Ebselen and PSAP appear three to four times greater when assessed with the enzyme assay or when GSSG reductase and NADPH were included in the assay. This indicates an artefact in the assay of GSH-px activity of both compounds by the inclusion of GSSG reductase and NADPH which causes an overestimation of the catalytic potency of both compounds in the coupled reductase assay. It will be noted, however, that the absolute rates of reaction in GSSG/NADPH-supplemented assays are lower in the peroxide assay, but H_2O_2 was only present at 250 μM as opposed to 1 mM in the coupled reductase assay.

The catalytic properties of Ebselen and PSAP were further confirmed by monitoring the removal of GSH from the reaction mixtures. Unlike the case of the peroxide measurements, the incubation for thiol determinations were constructed in exactly the same manner as for the enzymatic assays. The data again indicate that Ebselen and PSAP accelerate the reaction of GSH and H_2O_2, but by only 3.5- and 5.5-fold, respectively, over the initial 10 min of reaction. This again shows that the *actual* catalytic efficiency of the compounds may be less than the *observed* efficiencies derived from the coupled enzymatic assays. The discrepancies may again lie in the inclusion of GSSG reductase and NADPH in the system. Thus, addition of these agents into the incubations caused a 5–7-fold increase in the removal of GSH, stimulated by the catalysts, as compared to controls. This is now in agreement with the rates obtained with the enzymatic assay.

The origins of this enhancement by GSSG reductase/NADPH may lie in the enzyme keeping a high steady state of GSH available for reaction. This seems unlikely, however, as results from the assay of peroxide removal show reductase/NADPH enhancement of the catalysis, even when the GSH is in 4-fold excess over H_2O_2. Alternatively, GSSG reductase may have some reductive capacity towards intermediates of the respective catalytic cycles of Ebselen and PSAP. Indeed, preliminary results show that for Ebselen, rapid reaction with GSH may be reversed by GSSG reductase/NADPH (Fig. 6). This liberation of free GSH may either be due to reduction of the selenosulfide intermediate of Ebselen and GSH [32], or reduction of GSSG formed upon reaction of this selenosulfide with more GSH. The latter seems unlikely as care was taken to react Ebselen and GSH in a 1:1 molar ratio. Additionally, it is also difficult to envisage how reduction of the GSSG produced during catalysis should produce such a profound effect on the observed catalysis. Rereduction of the selenosulfide might, however, keep a steady state of Ebselen available for direct reaction with the peroxide [30]. These matters are being subjected to further investigation with authentic synthetic compounds.

The reasons underlying the variability of catalytic efficiency of Ebselen and PSAP with different peroxides are unclear. Both compounds were more effective with organic hydroperoxides than with H_2O_2. It remains to be determined what significance this high capacity for organic hydroperoxides may have for the protection of lipids in biological membranes.

Both Ebselen and PSAP rely on phenylselenofragments for their activity whereas the mammalian enzyme GSH-px contains a selenocysteine [35]. The proposed oxidized intermediate of GSH-pxs is a selenic acid. Recent attempts to generate GSH-px active site analogues [36, 37] have produced compounds which would seem too unstable for administration *in vivo*. Thus, as yet, synthetic compounds related to **1** and **2** represent the only stable GSH-px-like catalysts available which presently may find application *in vivo*.

Acknowledgements—We thank the National Swedish Board for Technical Development, the Swedish Natural Science and Medical Research Councils, and Carl Tryggers-Stigtelse for financial support.

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