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Glutathione Peroxidase Protects against Peroxynitrite-mediated Oxidations

A NEW FUNCTION FOR SELENOPROTEINS AS PEROXYNITRITE REDUCTASE*

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There is a requirement for cellular defense against excessive peroxynitrite generation to protect against DNA strand breaks and mutations and against interference with protein tyrosine-based signaling and other protein functions due to formation of 3-nitrotyrosine. Here, we demonstrate a role of selenium-containing enzymes catalyzing peroxynitrite reduction using glutathione peroxidase (GPx) as an example. GPx protected against the oxidation of dihydrorhodamine 123 by peroxynitrite more effectively than ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one), a selenoorganic compound exhibiting a high second-order rate constant for the reaction with peroxynitrite, 2×10^6 M^{-1} s^{-1}. Carboxymethylation of selenocysteine in GPx by iodoacetate led to the loss of "classical" glutathione peroxidase activity but maintained protection against peroxynitrite-mediated oxidation. The maintenance of protection by GPx against peroxynitrite requires GSH as reductant.

When peroxynitrite was infused to maintain a 0.2 μM steady-state concentration, GPx in the presence of GSH, but neither GPx nor GSH alone, effectively inhibited the hydroxylation of benzoate by peroxynitrite. Under these steady-state conditions peroxynitrite did not cause the loss of classical GPx activity. GPx, like selenomethionine, protected against protein 3-nitrotyrosine formation in human fibroblast lysates, shown in Western blots. The formation of nitrite rather than nitrate from peroxynitrite was enhanced by GPx or by selenomethionine. The results demonstrate a novel function of GPx and potentially of other selenoproteins containing selenocysteine or selenomethionine, in the GSH-dependent maintenance of a defense line against peroxynitrite-mediated oxidations, as a peroxynitrite reductase.

Peroxynitrite is a potent biological oxidant (1) generated, *e.g.* by endothelial cells, Kupffer cells, neutrophils, and macrophages (see Beckman (2) for review). Peroxynitrite ($ONOO^-$) is a relatively stable species compared with free radicals, but peroxynitrous acid (ONOOH) decays with a rate constant of 1.3 s^{-1}. Peroxynitrite is a mediator of toxicity in inflammatory processes with strong oxidizing properties toward biological molecules, including sulfhydryls, ascorbate, lipids, amino acids, and nucleotides, and it can cause strand breaks in DNA. Free or protein-bound tyrosine residues and other phenolics can be nitrated by peroxynitrite (see Beckman (2) for review). Protein tyrosine nitration may interfere with phosphorylation/dephosphorylation signaling (3), and the *in vivo* occurrence of protein nitration in the human has been demonstrated in patients chronically rejecting renal allografts (4). 3-Nitrotyrosine plasma levels of up to 0.12 mM were observed in chronic renal failure patients with septic shock (5).

The selenium-containing compound, ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one) (6) and its main metabolite *in vivo*, 2-(methylseleno)benzanilide (7), react with peroxynitrite very efficiently. Ebselen, selenocysteine, and selenomethionine protected DNA from single-strand break formation caused by peroxynitrite more effectively than their sulfur-containing analogs (8). Furthermore, these selenocompounds were protective in model oxidation and nitration reactions mediated by peroxynitrite (9).

Ebselen is known as a mimic of the GSH peroxidase (GPx)[1] reaction (10). We hypothesized (11) that its newly found reactivity with peroxynitrite (6) mimics a so far undescribed peroxynitrite reductase activity of selenoproteins. The present work provides evidence for a protective function of GPx against peroxynitrite.

MATERIALS AND METHODS

Reagents—Glutathione peroxidase from bovine erythrocytes was from Calbiochem. Diethylenetriamine pentaacetic acid (DTPA), glutathione, glutathione disulfide reductase, and sodium iodoacetate were from Sigma (Deisenhofen, Germany). MnO_2 was from Fluka (Buchs, Switzerland). NADPH was from Boehringer (Mannheim, Germany). Sephadex G-25 was from Pharmacia (Uppsala, Sweden). Ebselen and its derivatives, 2-(methylseleno)benzanilide, and ebsulfur, 2-phenyl-1,2-benzisothiazol-3(2*H*)-one, were kindly provided by Rhône-Poulenc-Rorer (Cologne, Germany). Dihydrorhodamine 123 was from Molecular Probes (Eugene, OR), and rhodamine 123 was from ICN Biomedicals (Aurora, OH). Other chemicals and solvents were from Merck (Darmstadt, Germany).

Peroxynitrite was synthesized from potassium superoxide and nitric oxide as described in Koppenol *et al.* (12), and H_2O_2 was eliminated by passage of the peroxynitrite solution over MnO_2 powder. Peroxynitrite concentration was determined spectrophotometrically at 302 nm (ϵ = 1670 M^{-1} cm^{-1}).

GSH Peroxidase Assay—GPx activity was followed spectrophotometrically at 340 nm as described in Roveri *et al.* (13) with minor modifications. The test mixture contained GSH (1 mM), DTPA (1 mM), glutathione disulfide reductase (0.6 unit/ml), and NADPH (0.1 mM) in 0.1 M sodium phosphate, pH 7.3. GPx samples were added to the test mixture at room temperature, and the NADPH oxidation rate was recorded for 1 min. The reaction was started by the addition of *tert*-butyl hydroperoxide (1.2 mM). Activity was calculated from the rate of NADPH oxidation. Carboxymethylation of the selenol in selenocysteine

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[1] The abbreviations used are: GPx, glutathione peroxidase; DTPA, diethylenetriamine pentaacetic acid; BSA, bovine serum albumin.

of GPx was carried out according to Roveri *et al.* (13), 5 μM GPx was incubated with 2 mM iodoacetate and 3 mM GSH for 10 min at 37 °C and then dialyzed.

Assay of Peroxynitrite-mediated Oxidation of Dihydrorhodamine 123—The peroxynitrite-mediated oxidation of dihydrorhodamine 123 was followed as described previously (9, 14) using a fluorescence spectrophotometer LS-5 (Perkin-Elmer Co.) with excitation and emission wavelengths of 500 nm and 536 nm, respectively, at room temperature. Fluorescence intensity was linearly related to rhodamine 123 concentration between 0 and 400 nM. Results are reported as means ± S.D. (n = 3–6) for the final fluorescence intensity minus background fluorescence.

Hydroxylation of Benzoate Caused by Steady-state Infusion of Peroxynitrite—Peroxynitrite-mediated hydroxylation of benzoate was measured as described elsewhere (15). Peroxynitrite was infused with a micropump at a rate of 175 μl/min from a stock solution of 50 μM under constant mixing with a magnetic stirrer at room temperature into a mixture (1.5 ml) containing benzoate (10 mM) and DTPA (0.1 mM) in 0.5 M potassium phosphate buffer (pH 7.4). Peroxynitrite infusion was for 3 min to give a cumulated concentration of 13 μM. The final volume was 2025 μl. The pH in the mixture did not change detectably following the addition of peroxynitrite. The steady-state input concentration of peroxynitrite was calculated by using the infusion rate of peroxynitrite (72 nM/s) and its decay rate in phosphate buffer at 25 °C and at pH 7.4 (0.41 s^{-1}) (16). GSH, GPx alone, or GPx in the presence of GSH were added before peroxynitrite infusion. In control experiments, the peroxynitrite solution was incubated with phosphate buffer at pH 7.4 for 10 min at room temperature to decompose the peroxynitrite before infusion into the reaction mixture. The benzoate hydroxylation data were corrected for the dilution by the infused volume.

Albumin-Ebselen Complex—Ebselen, dissolved in dimethylformamide at a concentration of 4 mM, was mixed with an aqueous solution of bovine serum albumin (580 μM) at a ratio of 1:1 (v/v) and incubated for 15 min at 37 °C (17). Unbound ebselen was removed by passing the mixture through Sephadex G-25 column (10 × 250 mm). The absorption spectrum of the complex was used to determine the bound ebselen concentration, ϵ_{330} = 7700 M^{-1} cm^{-1}.

Western Blot Analysis—After lysis of human skin fibroblasts grown to near confluency and separation of proteins by SDS-polyacrylamide gel electrophoresis, Western blots using a mouse monoclonal anti-nitrotyrosine antibody (kindly provided by J. S. Beckman, Birmingham, AL) were performed essentially as described in MacMillan-Crow *et al.* (4). The exposure to peroxynitrite (200 μM) was by injection into cell lysate (1 mg of protein/ml) under vortexing. GPx, selenomethionine, or ebselen, when included, were present from the beginning. After usual processing and incubation with a secondary goat anti-mouse antibody coupled to alkaline phosphatase and appropriate washings, nitrated proteins were detected using a chemiluminescent substrate (Starlight, ICN, Costa Mesa, CA). The reaction was abolished in the presence of 10 mM 3-nitrotyrosine as ascertained in dot blots.

Nitrate and Nitrite Formation from Peroxynitrite—Nitrate and nitrite concentrations were measured according to Verdon *et al.* (18).

RESULTS

Protection by GPx against Dihydrorhodamine 123 Oxidation by Peroxynitrite—The peroxynitrite-mediated oxidation of dihydrorhodamine 123 to fluorescent rhodamine 123 is an efficient and selective probe of peroxynitrite production in model systems (14). When peroxynitrite (100 nM) was added to 500 nM dihydrorhodamine 123, about 10 nM rhodamine 123 was formed. In the experiments shown in Fig. 1, this value is set to 100%. As shown in Fig. 1*A*, addition of a GPx preparation from bovine erythrocytes up to 200 nM had no effect on rhodamine 123 formation (*open circles*). However, in the presence of the low concentration of 1 μM GSH, GPx exhibited a pronounced inhibition of rhodamine 123 formation (*solid circles*). It can be noted at the y axis in Fig. 1*A* that the addition of 1 μM GSH alone, without GPx, led to a 15% loss of rhodamine 123 production. The half-maximal inhibitory concentration of GPx is 150 nM. Fig. 1*B* shows that, in this concentration range, neither bovine serum albumin (BSA) had any effect (*open stars*) nor did 1 μM GSH potentiate rhodamine 123 formation in the presence of BSA (*solid stars*).

To test whether the reduced form of GPx can inhibit peroxynitrite-induced oxidation, we incubated the enzyme (5 μM GPx) with 5 mM 2-mercaptoethanol and then dialyzed this against 1000 volumes of phosphate buffer. As shown in Fig. 1*B*, reduced GPx diminished rhodamine 123 formation effectively (*open triangles*), while in the presence of 1 μM GSH (*solid triangles*) the effect was simply additive as in the case of BSA. Furthermore, reoxidation of reduced GPx by incubation with *tert*-butyl hydroperoxide for 10 min at 37 °C caused a loss of the ability of GPx of protecting against peroxynitrite-mediated dihydrorhodamine 123 oxidation (data not shown).

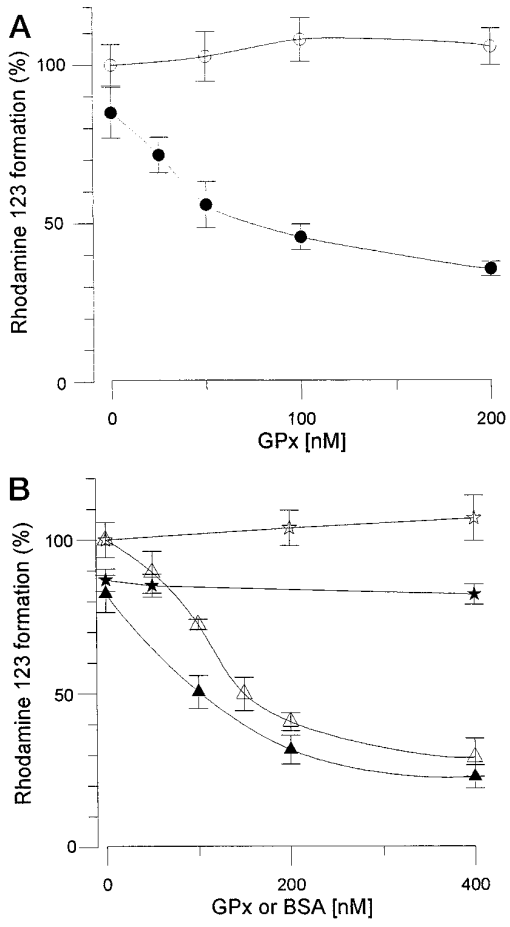


FIG. 1. **Protection by GPx against dihydrorhodamine 123 oxidation caused by peroxynitrite.** Peroxynitrite (100 nM) was added to 0.5 μM dihydrorhodamine 123 and different concentrations of GPx without (*open circles*) and with 1 μM GSH (*solid circles*) in 0.1 M phosphate buffer, 0.1 mM DTPA, pH 7.3, under intense stirring at room temperature. *A*, GPx preparation without further treatment; *B*, GPx (5 μM) reduced with 5 mM 2-mercaptoethanol and dialyzed against 1000 volumes of phosphate buffer before the experiment. The effect of reduced GPx was studied in the absence (*open triangles*) and in the presence of 1 μM GSH (*solid triangles*). In *B*, BSA was also assayed without (*open stars*) or with 1 μM GSH (*solid stars*).

When GPx was carboxymethylated by iodoacetate, the glutathione peroxidase activity was lost (Fig. 2*A*; *solid diamonds versus open triangles*), whereas its protective activity against peroxynitrite-mediated oxidation of dihydrorhodamine 123 was retained or even slightly enhanced (Fig. 2*B*; *solid diamonds versus open triangles*).

Protection by GPx against Hydroxylation of Benzoate under Steady-state Infusion of Peroxynitrite—A suitable detector system for examining steady-state conditions is given by the hydroxylation of benzoate (15). In the experiments shown in Fig. 3, peroxynitrite was infused with a micropump to give a steady-state concentration of 0.2 μM over 3 min. The cumulative peroxynitrite concentration was 13 μM (see "Materials and Meth-

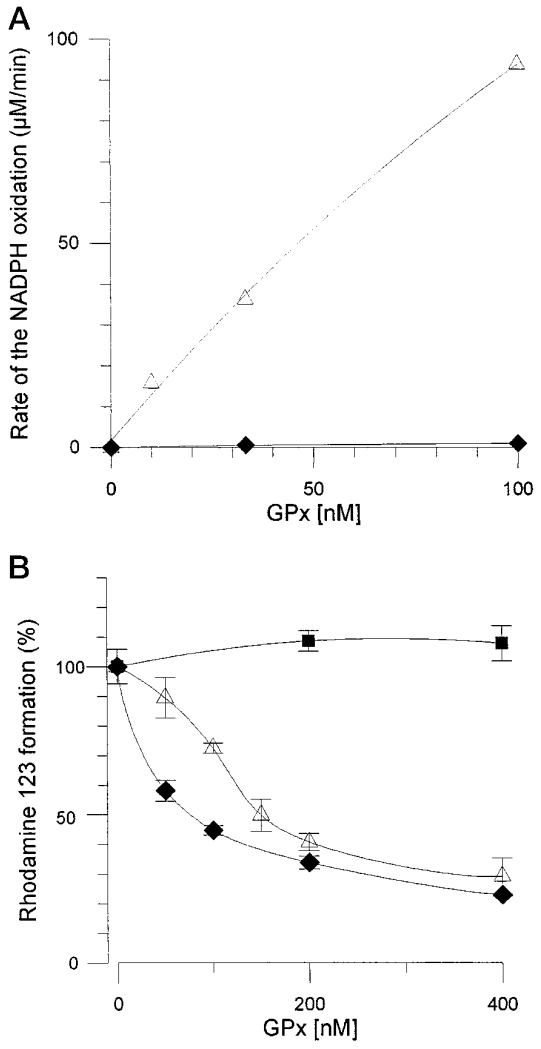


FIG. 2. **GSH peroxidase activity (*A*) and peroxynitrite-mediated rhodamine 123 formation (*B*) of GPx without (*open triangles*) or with (*solid diamonds*) carboxymethylation of the selenocysteine in GPx by iodoacetate.** Oxidation of dihydrorhodamine 123 was as in Fig. 1. In *B*, the effect of dialysis buffer after GPx dialysis (*solid squares*) is presented as a control.

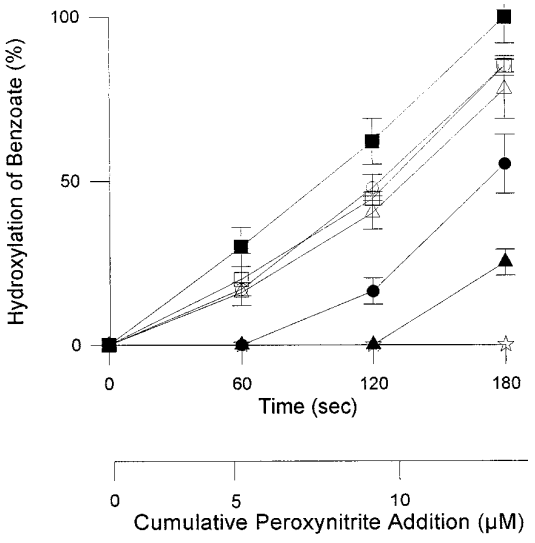


FIG. 3. **Protection by GSH peroxidase against hydroxylation of benzoate caused by a steady-state infusion of peroxynitrite.** Peroxynitrite (cumulative concentration 13 μM) was infused at a rate of 175 μl/min over 3 min from a stock solution of 50 μM to yield a 0.2 μM steady-state concentration. The reaction mixture (1.5 ml) contained 10 mM benzoate and 0.1 mM DTPA in 0.5 M potassium phosphate buffer (pH 7.4) (*solid squares*). The final volume at 3 min was 2025 μl. The pH in the mixture was not changed following the addition of peroxynitrite. 10 μM GSH (*open squares*), 20 μM GSH (*open triangles*), 330 nM GPx alone (*open circles*), 330 nM GPx in the presence of 10 μM GSH (*solid circles*) or 330 nM GPx in the presence of 20 μM GSH (*closed triangles*) were added before peroxynitrite infusion. Infusion of peroxynitrite after decomposition at pH 7.4 did not cause hydroxylation of benzoate (*open stars*).

ods"). The hydroxylation of benzoate (control) is shown as *solid squares* in Fig. 3. 330 nM GPx (*open circles*), 10 μM GSH (*open squares*), or 20 μM GSH (*open triangles*) alone had only a small protective effect. However, GPx in the presence of 10 μM GSH (*solid circles*) completely suppressed benzoate hydroxylation until 5 μM peroxynitrite had been infused, *i.e.* within the first min of infusion in Fig. 3. Likewise, in the presence of 20 μM GSH, the effect of 10 μM peroxynitrite infused for 2 min was abolished by the same amount of GPx (*solid triangles*), and again the GSH/peroxynitrite ratio necessary for the inactivation of peroxynitrite in the presence of GPx was 2/1. These data establish that GPx inactivates peroxynitrite in a catalytic reaction at the stoichiometry known for that of hydroperoxide reduction, *i.e.* the classical GPx reaction.

It has been shown that peroxynitrite can inactivate a number of enzymes such as Mn-superoxide dismutase (19) or alcohol dehydrogenase (20). Even the precursor of peroxynitrite, nitric oxide is capable of inactivating GPx (21). To test whether the activity of GPx is lowered under conditions used in this work we estimated the GPx activity using *tert*-butyl hydroperoxide as a substrate. Table I shows that 330 nM GPx treated with peroxynitrite under steady-state conditions used in the experiments of Fig. 3 (cumulative concentration, 13 μM) maintained the capability to reduce *tert*-butyl hydroperoxide, *i.e.* that the classical GPx activity was retained upon the exposure to a steady-state concentration of peroxynitrite. Further, exposure of 150 nM GPx in phosphate buffer at pH 7.3 to a bolus addition of peroxynitrite up to 30 μM did not detectably change the capability of GPx to reduce *tert*-butyl hydroperoxide.

The increase in the formation of nitrite from peroxynitrite by GPx and GSH in the steady-state experiments shown in Fig. 3 is presented in the right-hand column of Table I; when the nitrite measurement was carried out without benzoate, results were similar. Correspondingly, the levels of nitrate were lowered (data not shown).

The protection against hydroxylation of benzoate (Fig. 3) was half-maximal at 0.1 μM GPx. The pH dependence is shown in Fig. 4, indicating a more efficient protection at more alkaline pH. This is inverse to the pH dependence observed in similar experiments carried out with selenomethionine, also shown in Fig. 4.

Ebselen and Derivatives—Ebselen inhibits peroxynitrite-mediated dihydrorhodamine 123 oxidation with a half-maximal inhibitory concentration of 0.2 μM (9). Since the catalytic mechanism of ebselen and GPx for GSH peroxidase activity is the same (for review, see Sies (22)), we used ebselen as a model compound to examine effects of selenium modification regarding the mechanism of selenoenzyme action. In the whole organism, extracellular ebselen is present as an albumin complex, presumably as an inactive (with respect to its GSH peroxidase-like action) selenodisulfide, linked to the reactive cysteine residue on albumin (17, 23). Fig. 5 shows that ebselen bound to BSA (molar ratio of 0.3 mol of ebselen/1 mol of BSA) had no additional effect on the peroxynitrite-mediated dihydrorhodamine 123 oxidation as compared with BSA alone (*solid* and *open circles* in Fig. 5, respectively). However, in the presence of 2 μM dithiothreitol the ebselen-BSA complex (*solid squares*) attained activity against peroxynitrite-mediated dihydrorho-

TABLE I

GPx activity and formation of nitrite during steady-state exposure to peroxynitrite

The activity of GSH peroxidase was assayed before and after infusion of peroxynitrite to give a steady-state concentration of ~200 nM; at 180 s the cumulative concentration of peroxynitrite was 13 μM. The reaction mixture contained benzoate (10 mM) and DTPA (0.1 mM) in phosphate buffer (0.5 M) at pH 7.4 (see Fig. 3). The activity of GPx was assayed as described under "Materials and Methods." GSH and GPx were present as indicated. The rate of spontaneous NADPH oxidation and spontaneous formation of nitrite were subtracted. Data are expressed as means ± S.D. (n = 3–6).

Addition	Rate of the NADPH oxidation		Formation of nitrite
	Before exposure to $ONOO^-$ (zero time in Fig. 3)	After exposure to $ONOO^-$ (180 s in Fig. 3)	After exposure to $ONOO^-$ (180 s in Fig. 3)
	$\mu M/min$		μM
None	0	0	0
GSH (20 μM)	0	0	0.3 ± 0.3
GPx (330 nM)	393 ± 12	386 ± 10	ND[a]
Plus GSH (20 μM)	390 ± 17	389 ± 16	4.0 ± 0.9
Carboxymethylated GPx (330 nM)	4 ± 2	5 ± 2	ND
Plus GSH (20 μM)	6 ± 1	5 ± 1	ND

[a] ND, not determined.

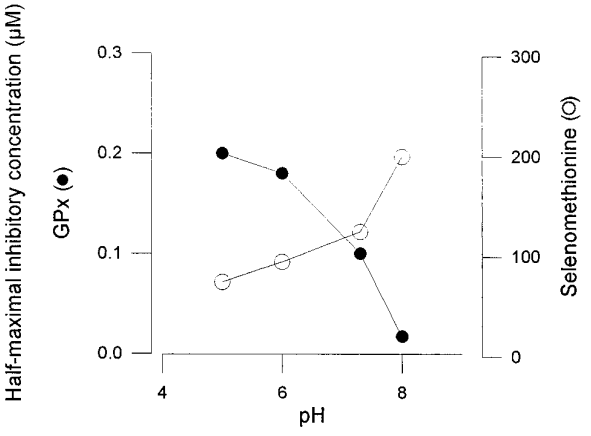


FIG. 4. **pH Dependence of half-maximal inhibitory concentrations of GPx or selenomethionine for peroxynitrite-mediated hydroxylation of benzoate.** Experiments were carried out as in Fig. 3 in phosphate buffer at the pH indicated. Data are given for GPx in the presence of 60 μM GSH (*solid circles*) or selenomethionine (*open circles*).

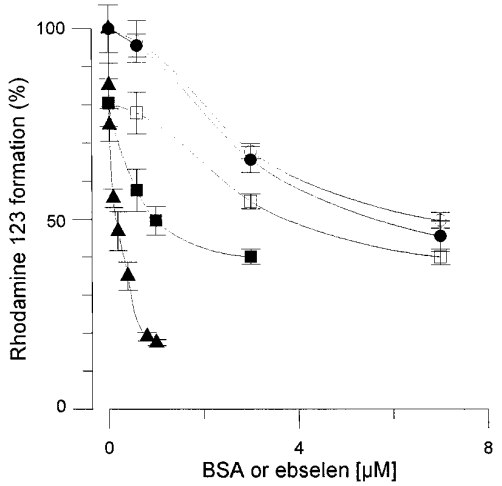


FIG. 5. **Effect of ebselen, BSA, and BSA-bound ebselen on peroxynitrite-mediated dihydrorhodamine 123 oxidation.** Peroxynitrite was added to dihydrorhodamine 123 solution as described under "Materials and Methods" in the presence of ebselen (*solid triangles*), BSA (*open circles*), BSA-bound ebselen (*solid circles*); and in the presence of 2 μM dithiothreitol plus BSA (*open squares*) and BSA-bound ebselen (*solid squares*).

damine 123 oxidation similar to that of free ebselen at low concentration (*triangles* in Fig. 5). Thus, selenium present as selenodisulfide is not effective against dihydrorhodamine 123 oxidation by peroxynitrite. Data on BSA alone in the presence of 2 μM dithiothreitol are also given (*open squares*).

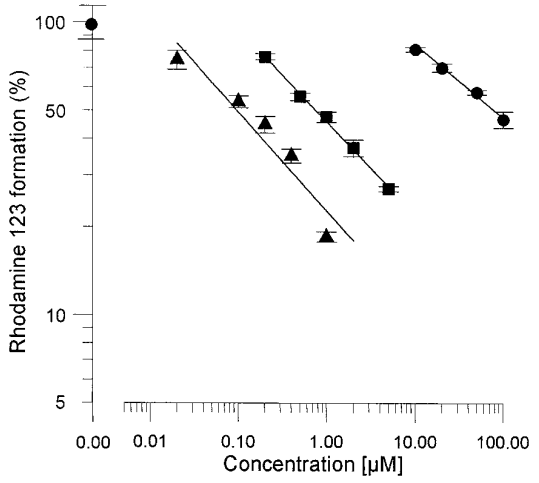


FIG. 6. **Effect of ebselen, ebselen selenoxide, and 2-(methylseleno)benzanilide on peroxynitrite-mediated dihydrorhodamine 123 oxidation.** Assay was performed as described in Fig. 1 in the presence of ebselen (*triangles*), 2-(methylseleno)benzanilide (*squares*), and ebselen selenoxide (*circles*).

Ebselen selenoxide (*solid circles* in Fig. 6) was about three orders of magnitude less effective than ebselen (*triangles*), 100 *versus* 0.2 μM for half-maximal inhibition, respectively. Methylation of the selenium in ebselen (–Se–CH_3), forming 2-(methylseleno)benzanilide (*squares* in Fig. 6), a structure analogous to selenomethionine, led to a 4-fold increase in half-inhibitory concentration (0.8 μM) over that of ebselen. Dithiothreitol as a reductant of the ebselen selenoxide almost completely recovered the protective activity of ebselen against dihydrorhodamine 123 oxidation by peroxynitrite (Table II). Data on the half-maximal inhibitory concentrations for several proteins and selenium-containing compounds are collected in Table III.

Protein Nitration in Cells—Fig. 7 presents Western blots from human fibroblast lysates exposed to peroxynitrite using a monoclonal anti-3-nitrotyrosine antibody. There are several bands of nitrated protein, the bands observed at 25 and 41 kDa being assigned to Mn-superoxide dismutase and actin, respectively (4).[2] Reduced GPx, but not oxidized (untreated) GPx (Fig. 7*A*), and selenomethionine (Fig. 7*B*) were protective against tyrosine nitration by peroxynitrite. Ebselen also was protective (data not shown).

Nitrite Formation from Peroxynitrite—As the spontaneous decay of peroxynitrite generates nitrate, the increase in the yield of nitrite in the presence of selenocompounds is a measure of peroxynitrite reduction. We found 100 μM nitrate and about

[2] J. S. Beckman, personal communication.

TABLE II
The effect of dithiothreitol (DTT) on the activity of ebselen selenoxide against peroxynitrite-mediated dihydrorhodamine 123 oxidation

Conditions	Rhodamine 123 formation
	%
Control[a]	100 ± 7
Plus ebselen (0.2 μM)	48 ± 5
Plus ebselen selenoxide (0.2 μM)	94 ± 5
Plus DTT (2 μM)	73 ± 3
Plus ebselen selenoxide (0.2 μM) + DTT (2 μM)	46 ± 2

[a] Dihydrorhodamine 123 (0.5 μM), DTPA (0.1 mM), in 0.1 M sodium phosphate buffer, pH 7.3, at 25°C; plus peroxynitrite (0.1 μM). Data are expressed as means ± S.D. (n = 6).

TABLE III
Half-maximal inhibitory concentrations of selenoproteins and some selenocompounds in peroxynitrite-mediated oxidation of dihydrorhodamine 123

Compound	Half-maximal inhibitory concentration[a]
	μM
PHGPx[b]	0.05
Carboxymethylated GPx	0.1
GPx	0.15
Ebselen[c]	0.2
2-(Methylseleno)benzanilide	0.8
Ebselen selenoxide	100
Ebselen-BSA complex	7
Ebselen-BSA complex + dithiothreitol	0.3
BSA	7
GSH	12
Se-methionine[c]	0.3
Se-cystine[c]	2.5
Sodium selenite[c]	$>10^4$

[a] Concentration of the compound obtaining half-maximal inhibition.
[b] Phospholipid hydroperoxide GPx (jointly with F. Ursini and M. Maiorino, unpublished results).
[c] Data from Briviba *et al.* (9).

50 μM nitrite after spontaneous decay of 100 μM peroxynitrite; *i.e.* the initial nitrite concentration was approximately 50 μM resulting from the synthesis of peroxynitrite. As shown in Fig. 8, selenomethionine generated a pronounced increase (up to 70% at 0.5 mM) in nitrite formation when 100 μM peroxynitrite was employed. This indicates successful competition with the spontaneous decay to nitrate. The increase in the formation of nitrite is commensurate with the decrease in the generation of nitrate, 70 μM at 0.5 mM selenomethionine (Fig. 8). Potentially, nitrogen-containing species different from nitrite also may be formed during scavenging of peroxynitrite by selenomethionine; this was not analyzed further.

DISCUSSION

Peroxynitrite Reductase—Selenoproteins, and selenocysteine in particular, carry out a variety of catalytic functions, many of which are redox reactions (see Refs. 24 and 25 for reviews). We here report a novel function for selenoproteins, the reduction of peroxynitrite. The study was prompted by the observation of a very efficient reduction of peroxynitrite by ebselen (6), exhibiting the highest second-order rate constant for a low molecular weight compound with peroxynitrite known so far, 2.0×10^6 M^{-1} s^{-1} (26). In analogy to the reaction cycle for ebselen (6, 24), Scheme 1 presents the proposed sequence. In the first step, the selenocysteine, probably as the selenolate, reacts with peroxynitrite to be oxidized to the corresponding seleninic acid, yielding nitrite (Table I; Fig. 3). The data in Fig. 4 show a higher efficiency of GPx at more alkaline pH, in agreement with peroxynitrite being the reacting species. However, peroxynitrous acid may also react to yield nitrous acid. A more detailed analysis as to whether peroxynitrite or peroxynitrous

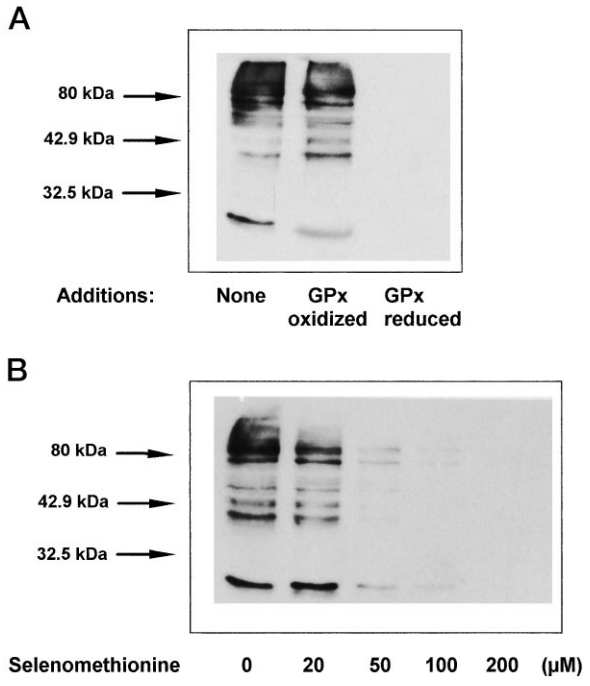


FIG. 7. **Suppression of protein tyrosine nitration mediated by peroxynitrite in human skin fibroblast lysates by GPx (*A*) or selenomethionine (*B*).** Cell lysates (1 mg of protein/ml) were exposed to peroxynitrite (200 μM). Protein nitration was examined by Western blotting using a monoclonal anti-3-nitrotyrosine antibody. In *A*, the addition of GPx (30 μM) showed protection with the reduced, but not with the oxidized (untreated), enzyme. See "Materials and Methods."

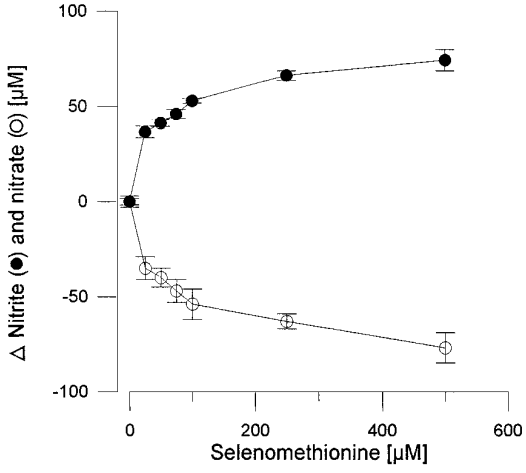
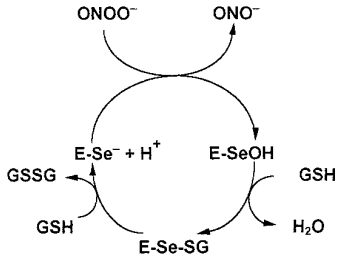


FIG. 8. **Changes in nitrite and nitrate concentrations in the presence of selenomethionine.** Peroxynitrite (100 μM) was added to phosphate buffer (0.1 M, pH 7.3) in the presence of increasing amounts of selenomethionine. Nitrite (*solid circles*) and nitrate (*open circles*) were assayed as described under "Materials and Methods."

acid or both react with GPx under a given condition would require the use of stopped-flow methods. The subsequent two steps in the reaction cycle are facile regeneration reactions at the expense of reducing equivalents provided by GSH in cells, as known from the extensive work on GPx (24), and as studied with ebselen selenoxide (27–30). Regarding the chemical mechanism, it might be concluded that the selenolate form of the selenocysteine residue is required. However, a selenol moiety is not strictly necessary for peroxynitrite reductase activity, in contrast to the GSH peroxidase action, since the carboxymethylated selenium derivative maintained activity (Fig. 2). This is in accord with the high rate constant obtained for 2-(methylseleno)benzanilide (7) and for selenomethionine (31). Data con-



SCHEME 1. **Proposed catalytic mechanism of selenoperoxidases in the reduction of peroxynitrite to nitrite (or peroxynitrous acid to nitrous acid).** The mechanism is based on that established for GSH peroxidases and the mimic, ebselen (22, 24, 32), which use ROOH and ROH as substrate and product, respectively. See text.

cerning the activity of other selenocompounds (Table III) are also in support of this suggestion. The high activity of ebselen and its selenium-methylated derivative, as well as of selenomethionine, points to the fact that the existence of C–Se–C, N–Se–C, or C–Se–Se–C bonds in selenocompounds does not strongly affect selenium reactivity with peroxynitrite. On the other hand, oxidation to the selenoxide or to the selenodisulfide as in the case of the albumin-ebselen complex, *i.e.* formation of one or more covalent bonds of selenium with other chalcogenides, oxygen or sulfur, diminishes the protective action, apparently due to decreasing the nucleophilicity of the selenium atom.

The only product of the reaction of ebselen and its methyl-seleno-derivative with peroxynitrite is the corresponding selenoxide, >Se=O (6, 7). Ebselen selenoxide can be reduced to the catalytically active form in the presence of reducing thiol equivalents (Table II). Redox shuttling of the selenium can be maintained with GSH, similar to the GSH peroxidase reaction. It is of interest to note that substitution of selenium by sulfur in PHGPx by site-directed mutagenesis lowered the k_{+1} rate constant by about three orders of magnitude (32), an effect which is comparable to the lower rate constants for sulfur compared with selenium compounds in the reaction with peroxynitrite (9, 31) (Table III).

The lack of peroxynitrite reductase activity with the GPx preparation without prior reduction with 2-mercaptoethanol (Fig. 1*A*) may have a simple explanation; a decrease in GPx activity upon storage in aerated solutions is a routinely observed feature of all types of glutathione peroxidases (24). This effect is reversible upon incubation with thiols and is commonly interpreted as resulting from oxidation of the active site selenium to a seleninic acid derivative (Fig. 1*B*).

Physiological Significance—The present results are in line with our recent observation of the protection against peroxynitrite-induced single-strand breaks in plasmid DNA by selenoorganic compounds, selenomethionine and selenocysteine (8). It is possible that selenomethionine and selenocysteine residues in proteins in general may carry out similar functions, *i.e.* that selenoproteins or selenopeptides might have a biological function as a defense line against peroxynitrite (9, 11). A number of different selenopeptides and selenoproteins, many of them with still unknown function, have been described *in vivo* (33). The presence of one of them, selenoprotein P, has been associated recently with the protection against liver damage in two oxidant injury models (34).

While the 100–1000-fold higher second-order reaction rate constants of the selenium-containing compounds as compared with sulfur analogs make for a kinetic advantage, it should be considered that there are multiple other defense mechanisms against peroxynitrite in the organism. For example, there is prevention of the formation of peroxynitrite by control of nitric oxide synthase and by control of the level of nitric oxide by oxyhemoglobin and other binding sites, as well as control of superoxide levels by superoxide dismutase. Second, there are reactions of peroxynitrite, once formed, with other compounds such as ascorbate (35), cysteine (36), GSH (37), or CO_2 (16, 38–40), all of which will share in the modulation of potentially deleterious reactions caused by peroxynitrite. A special feature of the peroxynitrite reductase activity of selenoproteins may reside in the catalytic nature and in the high efficiency of the reaction.

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Glutathione Peroxidase Protects against Peroxynitrite-mediated Oxidations: A NEW FUNCTION FOR SELENOPROTEINS AS PEROXYNITRITE REDUCTASE

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