RESEARCH COMMUNICATION

Attenuation of oxidation and nitration reactions of peroxynitrite by selenomethionine, selenocystine and ebselen

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The effect of the selenium-containing compounds selenomethionine, selenocystine and ebselen [2-phenyl-1,2-benzisoselenazol-3(2H)-one] on the oxidation of dihydrorhodamine 123 caused by peroxynitrite and on the nitration of 4-hydroxyphenylacetate by peroxynitrite was studied in comparison with their sulphur analogues methionine, cystine and ebsulfur [2-phenyl-1,2-benzisothiazol-3(2H)-one]. The selenocompounds protected dihydrorhodamine 123 from oxidation and 4-hydroxyphenylacetate from nitration more effectively than their sulphur analogues. Sodium selenite exhibited no effect. These observations are corollaries to the recent finding [Roussyn, Briviba, Masumoto and Sies (1996) Arch. Biochem. Biophys. **330**, 216–218] that selenium-containing compounds are efficient in protecting against peroxynitrite-induced DNA single-strand breaks.

INTRODUCTION

Peroxynitrite/peroxynitrous acid ($ONOO^-$/ONOOH) is an oxidant of biological interest [1] that can be produced by endothelial cells [2], Kupffer cells [3], neutrophils [4] and macrophages [5]. $ONOO^-$ is a relatively stable species, but its protonated form decays with a rate constant of 1.3 s^{-1} at 25 °C [1]. This oxidant is thought to be a mediator of toxicity in inflammatory states, with strong oxidizing properties towards biological molecules, including thiol groups [6], ascorbate [7], lipids [8], methionine [9], tryptophan [10] and DNA [11], and it can cause strand breaks in DNA [12]. A wide range of free or protein-associated tyrosine residues [13] and other phenolics [14] can be nitrated by peroxynitrite.

The selenium-containing compound, ebselen [2-phenyl-1,2-benzisoselenazol-3(2H)-one], reacts with peroxynitrite very efficiently [15]. Furthermore, ebselen, selenocystine and selenomethionine protect the DNA from single-strand-break formation caused by peroxynitrite more effectively than their sulphur-containing analogues [16]. Ebselen is known as a GSH peroxidase mimic, while no activity of the sulphur-containing analogue of ebselen, ebsulfur [2-phenyl-1,2-benzisothiazol-3(2H)-one], was observed for hydroperoxide reduction (see [17] for a review).

Fluorescent probes, such as rhodamine 123, have been used for detecting the formation of cellular reactive oxygen species [18]. Peroxynitrite-mediated oxidation of dihydrorhodamine 123 to rhodamine 123 was used as a probe of peroxynitrite production [19]. Regarding nitration reactions, the Fe(III)–EDTA-catalysed nitration of 4-hydroxyphenylacetate (4-HPA) by peroxynitrite has been established as a model system [14]. Here we studied the effects of selenium-containing compounds in comparison with their sulphur-containing analogues on these oxidation or nitration reactions of peroxynitrite.

MATERIALS AND METHODS

Reagents

Selenomethionine, selenocystine, methionine, cystine, diethylenetriaminepenta-acetic acid (DTPA) and 4-HPA were from Sigma (Deisenhofen, Germany). MnO_2 was from Fluka (Buchs, Switzerland). Ebselen and ebsulfur were kindly given by Rhône–Poulenc–Rorer (Cologne, Germany). Dihydrorhodamine 123 was from Molecular Probes (Eugene, OR, U.S.A.), and rhodamine 123 was from ICN Biomedicals (Aurora, OH, U.S.A.). Other chemicals and solvents were from Merck (Darmstadt, Germany).

Peroxynitrite was synthesized from sodium nitrite and H_2O_2 using a quenched-flow reactor as described in [20] with minor modifications [15]. H_2O_2 was eliminated by passage of the peroxynitrite solution over MnO_2 powder. Peroxynitrite was concentrated by freeze fractionation, and the concentration was determined spectrophotometrically at 302 nm (ϵ 1670 $M^{-1} \cdot cm^{-1}$).

Assay of peroxynitrite-mediated oxidation of dihydrorhodamine 123

The peroxynitrite-mediated oxidation of dihydrorhodamine 123 was monitored as described in [19] with minor modifications. Fluorescence intensity was measured on a fluorescence spectrophotometer LS-5 (Perkin–Elmer Co., Norwalk, CT, U.S.A.) 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 $\pm$ S.D. for the final fluorescence intensity minus background fluorescence.

Fe(III)–EDTA-dependent nitration of HPA by peroxynitrite

The iron complex, Fe(III)–EDTA, was prepared by mixing equimolar solutions of $FeCl_3$ and sodium EDTA. Peroxynitrite (final concentration 50 μM) was added to 4-HPA (1.0 mM) in 0.1 M sodium phosphate buffer, pH 7.3, containing Fe(III)–EDTA (0.5 mM) and various concentrations of the tested compounds while vortex-mixing. Samples were incubated for 30 min at room temperature. Alternatively, as controls, 4-HPA was

Abbreviations used: DTPA, diethylenetriaminepenta-acetic acid; 4-HPA, 4-hydroxyphenylacetate; NO_2-HPA, 4-hydroxy-3-nitrophenylacetate.

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added 5 min after peroxynitrite and to buffer alone. The pH was measured after the reaction to account for the slight alkaline shift caused by the alkali from the stock solution of peroxynitrite. The pH was adjusted to 10.0 with 3 M NaOH before absorbance measurement at 430 nm. The yield of 4-hydroxy-3-nitrophenylacetate (NO_2-HPA) was calculated from an ϵ value of 4400 $M^{-1} \cdot cm^{-1}$ [14]. Results were reported as the yield of NO_2-HPA. The yield of NO_2-HPA from reaction of 4-HPA with peroxynitrite alone was set equal to 100%. Data are given as means ± S.D.

RESULTS

Dihydrorhodamine 123 oxidation

The inhibition of the oxidation of dihydrorhodamine 123 to rhodamine 123 by peroxynitrite is shown for the three couples of selenium- and sulphur-containing compounds in Figure 1. Without additions, 10 nM rhodamine 123 was formed, and the selenium-containing compounds (closed symbols) were capable of decreasing the formation of rhodamine 123 in concentrations between 0 and 1 μM in a statistically significantly manner ($P < 0.01$), whereas the sulphur-containing analogues showed practically no effect over this concentration range. The most efficient protection against the peroxynitrite-mediated oxidation in this system was observed for ebselen and selenomethionine, with half-maximal inhibitory concentrations of 0.2 and 0.3 μM respectively (Table 1). The corresponding value for selenocystine was about 10 times higher. The half-maximal values for the sulphur analogues, obtained from the corresponding concentration dependence up to 1 mM (results not shown), were roughly two orders of magnitude higher than for the seleno-organic compounds. Cystine values were more than 1 mM; higher concentrations were not suitable for assaying because of the low solubility of cystine. Sodium selenite, as an inorganic selenocompound, was ineffective at 1 mM concentration, and almost no effect was observed even at 10 mM (Table 1).

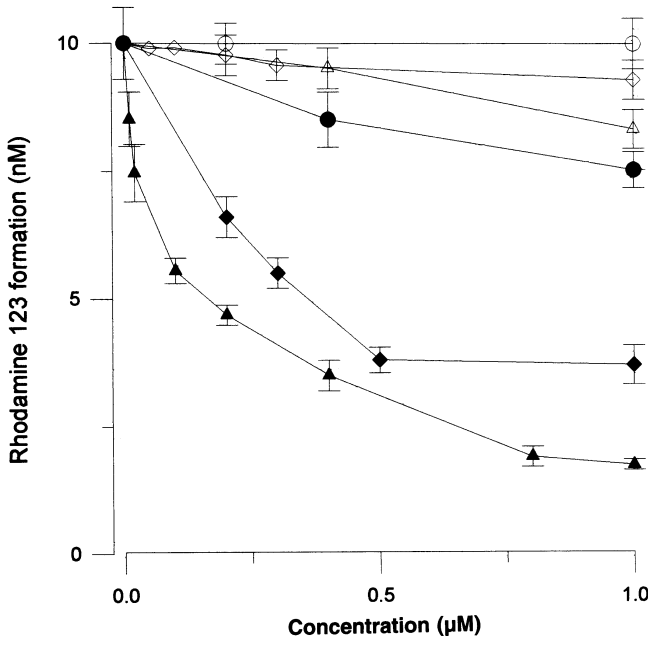

Figure 1 Effect of selenium- and sulphur-containing compounds on dihydrorhodamine 123 oxidation caused by peroxynitrite

Peroxynitrite (100 nM) was added to dihydrorhodamine 123 (0.5 μM) and selenomethionine (◆), methionine (◇), selenocystine (●), cystine (○), ebselen (▲) or ebsulfur (△) in 0.1 M phosphate buffer, pH 7.3, containing DTPA (0.1 mM), under intensive stirring at room temperature. Results are given as means ± S.D. ($n = 3$–5).

Table 1 Half-maximal inhibitory concentrations of the peroxynitrite-mediated oxidation of dihydrorhodamine 123 (DHR 123) and of the peroxynitrite-mediated nitration of 4-HPA for some seleno-organic compounds and their sulphur analogues

Data were obtained from experiments such as shown in Figures 1 and 2, carried out in 0.1 M sodium phosphate buffer, pH 7.3, at 25 °C, in the appropriate concentration ranges of the compounds studied.

Compound	Half-maximal inhibitory concentration (μM)	
	DHR 123 oxidation*	4-HPA nitration†
Ebselen	0.2	60
Selenomethionine	0.3	50
Selenocystine	2.5	30
Ebsulfur	15	300
Methionine	20	750
Cystine	$> 10^3$	$> 10^3$
Sodium selenite	$> 10^4$	$> 10^4$

* DHR 123 (0.5 μM), DTPA (0.1 mM) and peroxynitrite (0.1 μM).
† 4-HPA (1 mM), Fe(III)–EDTA (0.5 mM) and peroxynitrite (50 μM).

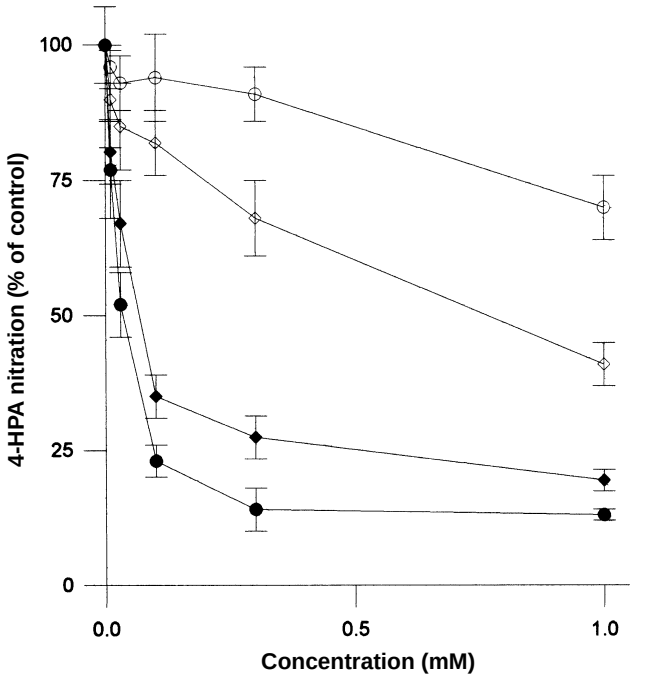

Figure 2 Effect of selenomethionine, methionine, selenocystine and cystine on the nitration of 4-HPA caused by peroxynitrite

Peroxynitrite (final concentration 50 μM) was added to 4-HPA (1.0 mM) in 0.1 M sodium phosphate buffer, pH 7.3, containing Fe(III)–EDTA (0.5 mM) and various concentrations of selenomethionine (◆), methionine (◇), selenocystine (●) or cystine (○). Results are given as means ± S.D. ($n = 3$–5).

Nitration of 4-HPA

The inhibition of the nitration of 4-HPA by peroxynitrite was also studied (Figure 2; Table 1). As for the effects on the oxidation of dihydrorhodamine 123, the selenium-containing compounds were more effective inhibitors of the nitration reaction than the sulphur-containing analogues. The half-maximal inhibitory concentrations obtained in the corresponding concentration range are higher than in the oxidation assay (Table 1), due to the higher concentration of peroxynitrite employed in the nitration assay (50 μM versus 0.1 μM). The inhibition of nitration was most pronounced for selenocystine, and again selenite was ineffective.

DISCUSSION

The data reported here demonstrate that reactions caused by peroxynitrite can be protected against by seleno-organic compounds, and that the protection is more efficient than that exerted by the corresponding sulphur analogues. The reactions examined here, an oxidation reaction to generate a fluorescent product and a nitration reaction of a phenolic compound, have been used previously as model reactions for peroxynitrite effects that might occur in biological systems. Differences in the ranking order for inhibition by seleno-organic compounds (Table 1) apparently reflect further parameters such as hydrophobic interactions etc.

The present results are in line with our recent observation of the protection against peroxynitrite-induced single-strand breaks in plasmid DNA [16]. It is possible that selenomethionine and selenocysteine residues in proteins may carry out similar functions, namely that selenoproteins or selenopeptides might have a biological function as a defence line against peroxynitrite [17]. A number of different selenopeptides and selenoproteins, many of them with a so far unknown function, have been described *in vivo* [21]. The presence of one of them, selenoprotein P, has been associated recently with the protection against liver damage in two oxidant-injury models [22].

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