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STRONG INHIBITION OF MAMMALIAN LIPOXYGENASES BY THE ANTIINFLAMMATORY SELENO-ORGANIC COMPOUND EBSELEN IN THE ABSENCE OF GLUTATHIONE

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Abstract—Both human recombinant 5-lipoxygenase (EC 1.13.11.34) and 15-lipoxygenase (EC 1.13.11.33, mammalian enzyme) purified from rabbit reticulocytes were inhibited in the absence of glutathione (GSH) by submicromolar concentrations of the seleno-organic compound ebselen. These concentrations were comparable to those of the enzymes. Soybean lipoxygenase-1 (EC 1.13.11.33, plant enzyme) was not inhibited, whereas prostaglandin endoperoxide synthase-1 (EC 1.14.99.1) was inhibited only at much higher concentrations of ebselen ($IC_{50} = 37.7 \pm 4.3$ μM). The action of ebselen on reticulocyte 15-lipoxygenase ($IC_{50} = 0.17 \pm 0.01$ μM) was studied in detail. Inhibition occurred instantaneously and appeared to be reversible and was largely abolished by a 20-fold molar excess of GSH over ebselen. In the presence of 1 mM GSH 50% inhibition was observed only at ebselen concentrations as high as 234 ± 27 μM. 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid, the lipoxygenase product formed from linoleic acid, augmented the inhibitory effect at low concentrations and caused a partial reversal at high concentrations. A variety of derivatives or structural analogues of ebselen were also tested and proved to be either inactive or weaker inhibitors of 15-lipoxygenase. We have concluded that the potent inhibition of 15-lipoxygenase by ebselen is due neither to GSH peroxidase-like activity nor to lowering of the hydroperoxide tone. The pharmacological implications of these unique characteristics of the action of ebselen on lipoxygenases are then discussed.

Key words: selenium; ebselen; lipoxygenase; cyclooxygenase; glutathione; hydroperoxy fatty acids

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one) is an anti-inflammatory drug with low toxicity and a unique pharmacological profile [1, 2 and Refs. cited therein]. The mode of action of this seleno-organic compound has been studied extensively during the last decade. It has proven to be an effective reductant of organic hydroperoxides such as free polyenoic fatty acids, phospholipids and cholesterol [3, 4]. In the presence of GSH‡ or other thiols, it has been suggested that several catalytic cycles, depending on the actual concentrations of peroxides and GSH, may result [2–6]: a mono-oxygenated derivative of ebselen; a mixed selenodisulphide; or diselenide, selenol and selenenic acid derivatives of ebselen [2, 3]. Ebselen thus exhibits glutathione peroxidase-like activities and resembles the selenium-containing enzyme phospholipid hydroperoxide glutathione peroxidase with respect to its catalytic action [7]. The molecular turnover rate of ebselen, however, is several orders of magnitude lower than that of the enzyme [6]. The reductive and catalytic action of ebselen affords effective protection against the cytotoxic action of hydroperoxy-polyenoic fatty acids [8]. Moreover, non-enzymatic lipid peroxidation is inhibited [9, 10] by the removal of the peroxides needed for the initiation and propagation of radical chain reactions. Hence, ebselen is an antioxidant with a mode of action different from that of radical scavengers [4].

Further biochemical action has been observed which may also be relevant to the anti-inflammatory activities of ebselen: (i) inhibition of the 5-lipoxygenase pathway of arachidonic acid metabolism in leukocytes [11], which appears to occur also *in vivo* as shown in a specific animal model of experimental hepatitis [12–14]; (ii) isomerisation of leukotriene B_4 to its biologically inactive *trans*-isomer [15]; and (iii) inhibition of the microsomal electron transfer system at the level of the flavin enzymes [16, 17]. As far as the inhibition of the 5-lipoxygenase pathway is concerned, earlier work [11, 14] has revealed that ebselen primarily affects the first step (formation of 5-HpETE) rather than the following one via accelerated reductive removal of 5-HpETE, the common precursor of all biologically active leukotrienes. However, no clear evidence appeared as to whether ebselen causes inhibition indirectly by lowering the hydroperoxide tone [18]

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‡ Abbreviations: GSH, glutathione; 5-HpETE, 5*S*-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; 13-H(p)ODE, 13*S*-hydro(pero)xy-9*Z*,11*E*-octadecadienoic acid; PGHS-1, prostaglandin endoperoxide synthase-1; LDL, low-density lipoproteins.

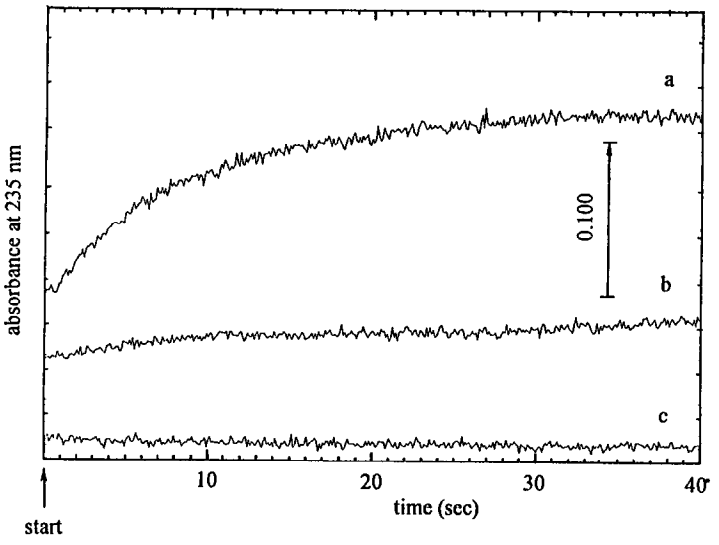


Fig. 1. Progress curves of human recombinant 5-lipoxygenase activity in the absence (a) and in the presence of 0.1 μM (b) or 1.0 μM (c) ebselen, respectively. This activity was measured spectrophotometrically at 235 nm in 1.0 mL of Hanks' solution, pH 7.4, containing phosphatidylcholine (12 μg/mL), ATP (0.1 mM), EDTA (0.1 mM) and human recombinant 5-lipoxygenase (3.4 μg, specific activity 10.2 sec^{-1}), T = 14.5°. The reaction was started by addition of arachidonic acid (final concentration: 20 μM). The "noise" of the traces was due to the turbidity of the reaction samples. The formation of 5-HpETE in samples a and b was verified by HPLC analysis (not documented).

Table 1. Action of ebselen on pure mammalian lipoxygenases

Ebselen (μM)	Activity* 5-Lipoxygenase †(0.044 μM) nkat (mg protein)$^{-1}$	%	15-Lipoxygenase ‡(0.080 μM) nkat (mg protein)$^{-1}$	%
0	132	100	240	100
0.1	26.5	20	125	52
1.0	0	0	26.4	11

* The values refer to the linear part of the progress curves either † measured spectrophotometrically; conditions as in Fig. 1; [28]; or ‡ measured oxygraphically; conditions as in Fig. 2.

(shown to regulate the 5-lipoxygenase pathway in neutrophils [19, 20]) or whether it interacts directly with the enzyme. This accounts for our studying the action of ebselen on various pure lipoxygenases. A preliminary short report of some of these results has been presented elsewhere [21].

MATERIALS AND METHODS

Enzymes. 15-Lipoxygenase (EC 1.13.11.33, mammalian enzyme) from rabbit reticulocytes was prepared as described in Ref. 22. Human recombinant 5-lipoxygenase (EC 1.13.11.34) [23] was a gift from Dr Denis Riendeau (Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Québec, Canada). Soybean lipoxygenase-1 (EC 1.13.11.33, plant enzyme) was prepared as described previously [24]. Particulate PGHS-1 (EC 1.14.99.1) was prepared from sheep vesicular glands as published elsewhere [25].

Chemicals. Ebselen and its congeners were obtained from Rhône-Poulenc-Rorer Nattermann International (Cologne, Germany) and dissolved in freshly distilled 2-methoxyethanol. A solution of ebselen oxide was prepared by treatment of a 1 mM suspension of ebselen in 0.1 M phosphate buffer, pH 7.4, with a 3-fold molar excess of hydrogen peroxide at 37° under shaking which caused solubilization of the reaction product; the excess of hydrogen peroxide was removed by addition of 1 mg catalase (19 U) per mL and after 2 min the solution was rapidly heated to 60° in order to denature the catalase.

Linoleic acid (9*Z*,12*Z*-octadecadienoic acid), arachidonic acid (5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid) and sodium cholate were purchased from Serva

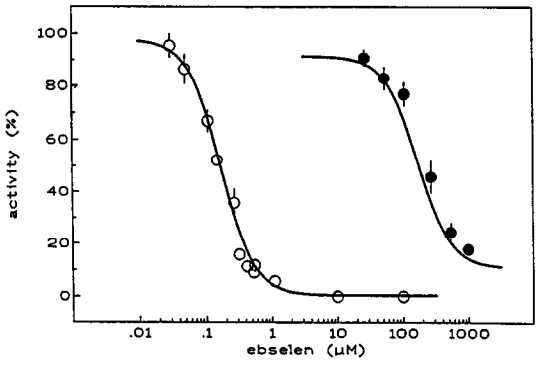


Fig. 2. Dose–response curve of the inhibition of reticulocyte 15-lipoxygenase by ebselen in the absence (○) and in the presence (●) of 1 mM GSH. 10.53 μg of pure 15-lipoxygenase from rabbit reticulocytes (specific activity: 18.1 sec^{-1}) were added to 1.75 mL air-equilibrated 0.1 M phosphate buffer, pH 7.4. Varying amounts of ebselen dissolved in peroxide-free 2-methoxyethanol were added and the samples preincubated for 1 min at 25°. The amount of solvent did not exceed 20 μL and did not affect activity. The reaction was started by potassium linoleate (final concentration: 0.26 mM) dispersed in sodium cholate (final concentration: 0.2%) and followed oxygraphically. The linear part of the progress curves reached after the short lag period had ceased was evaluated. The curves were fitted with ALLFITT [42]. The constraint of a common slope for the two plots did not significantly alter the fit for the two curves ($P = 0.69$). The IC_{50} values were calculated as 0.17 ± 0.01 μM and 234 ± 27 μM in the absence and presence of GSH, respectively (mean values ± estimate of the error according to Ref. 42. The latter value proved to be 1348 ± 179-fold higher than the first one.

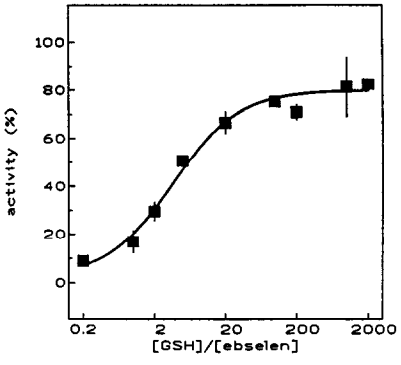


Fig. 3. Effect of GSH concentration on reticulocyte 15-lipoxygenase inhibition by ebselen. Ebselen (final concentration 0.55 μM) and varying concentrations of GSH were mixed with the assay buffer and the 15-lipoxygenase added. The samples were preincubated for 1 min before the reaction was started by linoleate. Other conditions are as in Fig. 2. The ED_{50} value for the GSH/ebselen ratio was calculated according to Ref. 42 as 3.7 ± 1.3.

(Heidelberg, Germany). 13-HpODE and 13-HODE were prepared from linoleic acid with commercial soybean lipoxygenase (Serva) [26] and purified by HPLC. All other chemicals were of analytical grade, while the solvents used (Serva) were of HPLC grade.

Enzyme assays. 15-Lipoxygenase activity was measured oxygraphically with 0.26 mM linoleic acid as substrate in 0.1 M phosphate buffer pH 7.4 containing 0.2% (w/v) sodium cholate [27]. 5-Lipoxygenase activity was measured spectrophotometrically with 20 μM arachidonic acid as substrate in Hanks' solution supplemented with ATP, EDTA and phosphatidylcholine [28]. Soybean lipoxygenase activity was assayed oxygraphically with linoleic acid as substrate in 0.05 M sodium borate buffer pH 9.0.

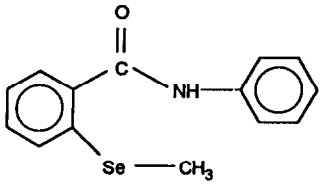
PGHS-1 activity was measured oxygraphically with 30 μM arachidonic acid as substrate in 0.05 M Tris–HCl buffer pH 8.0 containing 0.5 mM phenol [25].

Analytics. Separation of 13-HpODE and 13-HODE was performed by straight-phase HPLC on a Zorbax-SIL column (250 × 4.6 mm, 5 μm particle size) with the solvent system *n*-hexane/isopropanol/acetic acid 98.5:1.5:0.5 (by vol.) and a flow rate of 1 mL/min. The retention times of 13-HODE and 13-HpODE were determined in separate runs using authentic standards.

RESULTS

We studied the effect of ebselen on two pure mammalian lipoxygenases in the absence of GSH or any other thiol. The photometrically measured progress curves of the reaction of human recombinant 5-lipoxygenase with arachidonic acid as substrate [28] are shown in Fig. 1. Ebselen concentrations as low as 0.1 μM produced an inhibition of 80%, with inhibition completed at 1 μM (Table 1). Thus, ebselen proved to be a much more potent inhibitor of pure 5-lipoxygenase than of cellular 5-lipoxygenase activity (reported to be inhibited by 50% at 20 μM ebselen [11]). Ebselen was also a comparatively potent inhibitor of pure 15-lipoxygenase from rabbit reticulocytes (Table 1).

Due to the limited amount of 5-lipoxygenase available we performed further experiments with the reticulocyte 15-lipoxygenase. As shown in Fig. 2, a dose-dependent inhibition of this lipoxygenase was observed with an IC_{50} value of 0.17 ± 0.01 μM. This concentration of ebselen is of the same order of magnitude as that of the enzyme under the conditions of our assay. Such high inhibitory potency with the mammalian 15-lipoxygenase was only observed towards the suicide inactivators eicosatetranoic acid [29] and hexanalphenylhydrazone (unpublished results) while a variety of other known lipoxygenase inhibitors were much less potent. Thus ebselen represents one of the most powerful known *in vitro* inhibitors of mammalian 15-lipoxygenase. However, its inhibitory potency was drastically reduced in the presence of 1 mM GSH (Fig. 2, right curve); under these conditions an IC_{50} value of about 234 ± 27 μM was obtained. The dependence of ebselen inhibition on GSH concentration is presented in Fig. 3. A molar excess of GSH approximately 20-fold that of ebselen was sufficient to largely abolish the inhibitory effect. This might be due to the ability of thiol compounds to react with ebselen via opening the



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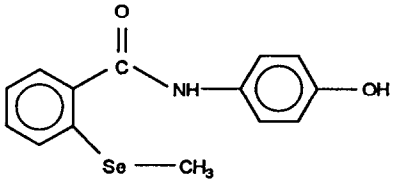

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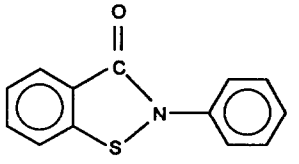

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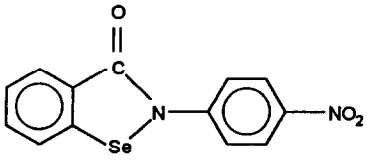

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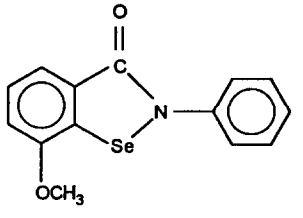

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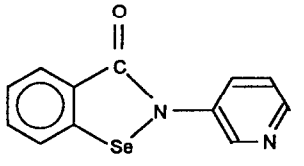

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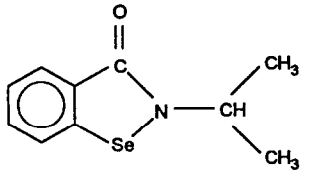

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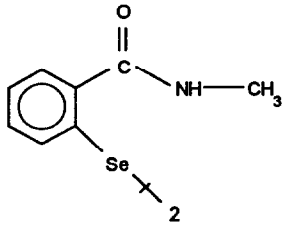

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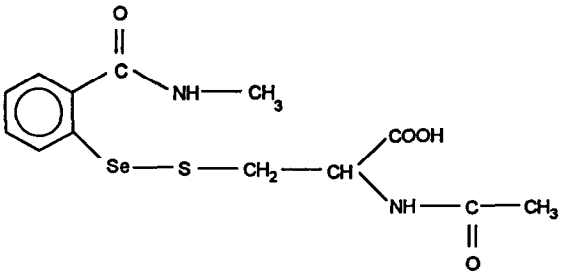

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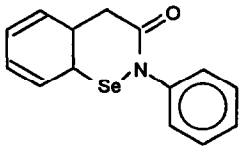

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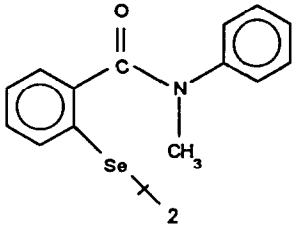

XI

Scheme 1. Structures of compounds tested in Table 2.

isoselenazol ring, thereby forming a selenenyl-sulphide adduct [3] which affects the 15-lipoxygenase only at high concentrations. When the authentic GSH adduct of ebselen was tested, it did indeed prove to inhibit 15-lipoxygenase with an IC_{50} value of 125 μM (Table 2).

It seemed conceivable that ebselen-induced lipoxygenase inhibition might be caused by a reduction in hydroperoxy fatty acids, usually present as traces in the lipoxygenase assay or formed by autoxidation and which are necessary to initiate the enzyme reaction [30]. Therefore we analysed the

Table 2. Comparison of the inhibitory potencies of some ebselen congeners on reticulocyte 15-lipoxygenase

Compound	Inhibition at 1 μM (%)	IC_{50} (μM)	Remarks
Ebselen	85	0.14	
Ebselen oxide	0		not inhibitory at 100 μM
Ebselen-GSH-adduct	0	125	
I	0	100	
II	0	100–200	
III	30 . . . 65	0.3 . . . 3*,†	
IV	0		not inhibitory at 100 μM
V	36	2	
VI	72	0.3	
VII	45	1 . . . 10*	
VIII	34	5*	
IX	40	5*	
X	71	0.35‡	
XI	0		not inhibitory at 100 μM

* Very flat dose–response curve so that complete inhibition was not achieved at 100 μM.
† Large variations on repeated measurement.
‡ Complete inhibition was not achieved at 100 μM.
The structures of compounds I–XI are shown in Scheme 1.

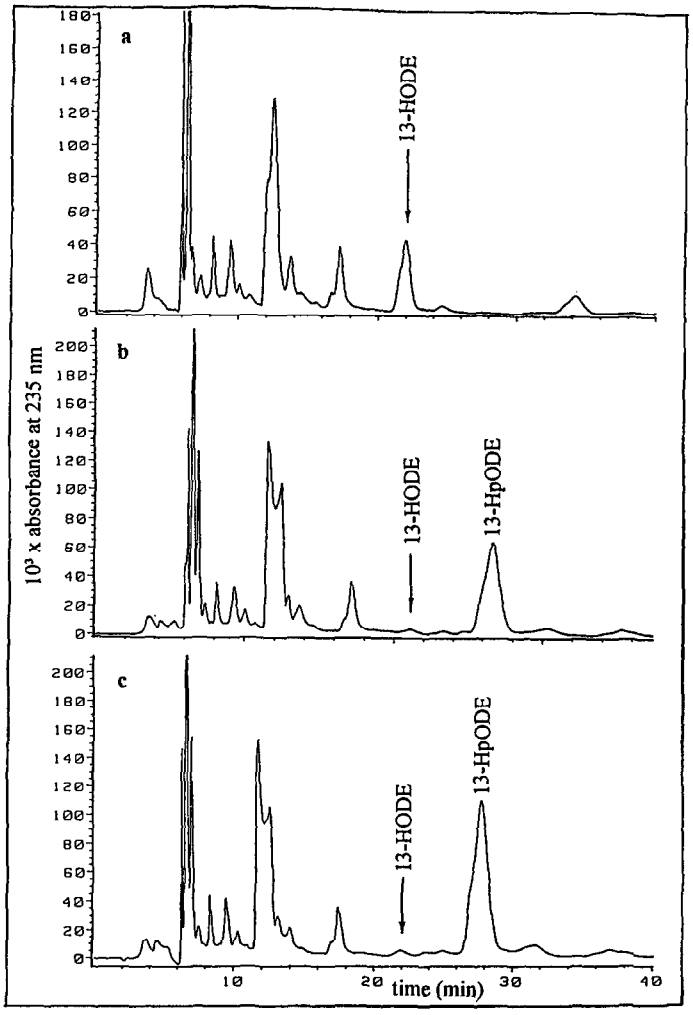

Fig. 4. HPLC separation of the reaction products of 15-lipoxygenase from linoleic acid under conditions of partial ebselen inhibition in the absence and presence of 1 mM GSH. The samples were allowed to react for 2 min as described in Fig. 2. The reaction was stopped by the addition of 0.18 mL 1 M citric acid and the lipids were extracted with 4 mL ethylacetate for 2 min with vigorous shaking. After centrifugation the organic extract was evaporated to dryness under argon and dissolved in 500 μL *n*-hexane. An aliquot of 380 μL of this solution was subjected to straight-phase HPLC (see Materials and Methods): (a) 270 μM ebselen + 1 mM GSH; (b) 0.16 μM ebselen; (c) control (without or with GSH). The reaction rates of samples a and b amounted to 51 and 64%, respectively, that of control sample c. A further sample with 0.27 μM ebselen but double reaction time yielded a chromatogram similar to sample b (not shown).

reaction products under the conditions of half-inhibition by ebselen both in the presence and absence of 1 mM GSH. It can be seen in Fig. 4 that in the absence of GSH, linoleic acid was virtually exclusively converted to its regular 15-lipoxygenase product 13-HpODE. It may therefore be concluded that in the absence of thiols, ebselen-induced inhibition is not due to the lowering of the "hydroperoxide tone", but to direct interaction with the enzyme. However, in the presence of GSH and the correspondingly high ebselen concentration thereby required to produce sizable inhibition, only the reduction product, 13-HODE, was found. This would suggest that under these conditions an ebselen-catalysed GSH peroxidase-like reaction [2–6] takes place. The absence of 13-HpODE, within the limits of detection of our analytic method, corroborates the assumption that, in the presence of GSH, ebselen or its selenenylsulphide adduct intermediately formed inhibits 15-lipoxygenase by lowering the hydroperoxide tone.

We observed varying effects of the lipoxygenase product 13-HpODE on ebselen inhibition. Pre-treatment of 15-lipoxygenase with 1 μM 13-HpODE augmented the inhibitory action (Fig. 5). If present in a concentration stoichiometric to the enzyme, 13-HpODE is known to convert the rest of the ferrous form of the lipoxygenase to the active ferric form [31]. The effect of low concentrations of 13-HpODE may therefore be interpreted as indicating that

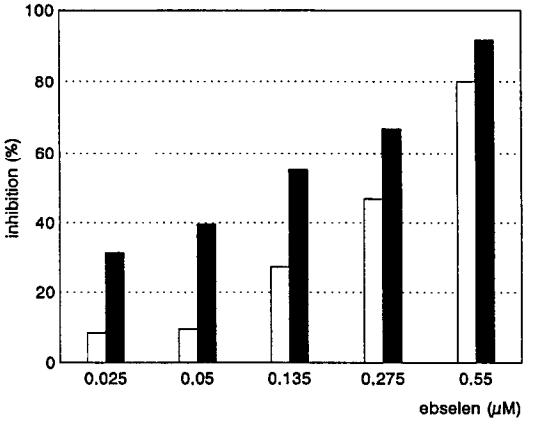

Fig. 5. Augmentation of ebselen inhibition of 15-lipoxygenase upon pretreatment of the enzyme with 13-HpODE. The lipoxygenase and 1 μM 13-HpODE were mixed with the assay buffer and varying concentrations of ebselen were then added (filled bars). The open bars correspond to control samples lacking 13-HpODE. Other conditions as in Fig. 2.

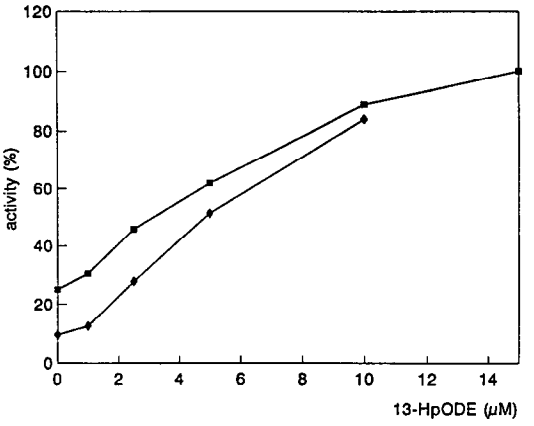

Fig. 6. Reversal of the inhibitory action of ebselen on 15-lipoxygenase by pretreatment of ebselen with 13-HpODE. 0.275 μM (■) or 0.55 μM (◆) ebselen (final concentration) and varying concentrations of 13-HpODE were mixed with the assay buffer and the 15-lipoxygenase subsequently added. After a preincubation period of 1 min the reaction was started by addition of linoleate. The values refer to the control sample without ebselen. Other conditions as in Fig. 2. The correlation coefficient amounts to 0.979 for (■) and 0.995 for (◆).

ebselen reacts preferentially with the ferric form of the enzyme. In contrast, pretreatment of ebselen with higher concentrations of 13-HpODE markedly diminished the inhibition of the 15-lipoxygenase (Fig. 6). This may be due to the oxidation of ebselen to its monooxygenated derivative [3]. This assumption is strongly supported by the observation that ebselen oxide did not inhibit the 15-lipoxygenase even at concentrations as high as 100 μM. The hypothesis by which the inhibitory action of ebselen is due to its conversion to ebselen oxide may thus be excluded.

The inhibition of mammalian 15-lipoxygenase by ebselen occurred instantaneously. Preincubation of the samples containing enzyme and ebselen for 5 min under aerobic conditions lowered rather than enhanced the inhibitory effect (not shown). The latter observation may indicate a partial destruction of the ebselen complex by oxygen. Preincubation of the samples containing enzyme and ebselen with either GSH or 13-HpODE caused a marked partial reversal of the inhibition (Table 3). This observation is consistent with the results presented in Figs 2 and 6 and indicates the reversibility of the lipoxygenase–ebselen complex. We also tested a variety of compounds structurally analogous to ebselen, none of which exhibited inhibitory potency comparable to ebselen (Table 2). As expected, the inhibitory effects did not correlate with the GSH peroxidase-like activities observed (not documented).

In contrast to mammalian lipoxygenases, soybean lipoxygenase-1 was resistant to ebselen; even at concentrations as high as 1 mM ebselen, no more than 28% inhibition was observed. Since soybean lipoxygenase-1 is also a 15-lipoxygenase with respect to its reaction with arachidonic acid [31], ebselen's ability to inhibit lipoxygenases is unlikely to be

Table 3. Partial reversal of the inhibitory effect of ebselen on 15-lipoxygenase by subsequent addition of GSH or 13-HpODE

Ebselen (μM)	Subsequent addition	Relative activity (%)
0	None	100
0	GSH	100
0	13-HpODE	97
0.27	None	35
0.27	GSH	62.5
0.55	None	14.5
0.55	GSH	56
0.55	13-HpODE	35

Reticulocyte lipoxygenase was preincubated for 1 min with ebselen (conditions as in Fig. 2). GSH (1 mM) or 10 μM 13-HpODE was added and the sample was incubated for another minute before the reaction was started by the addition of linoleate. The values represent at least duplicate measurements.

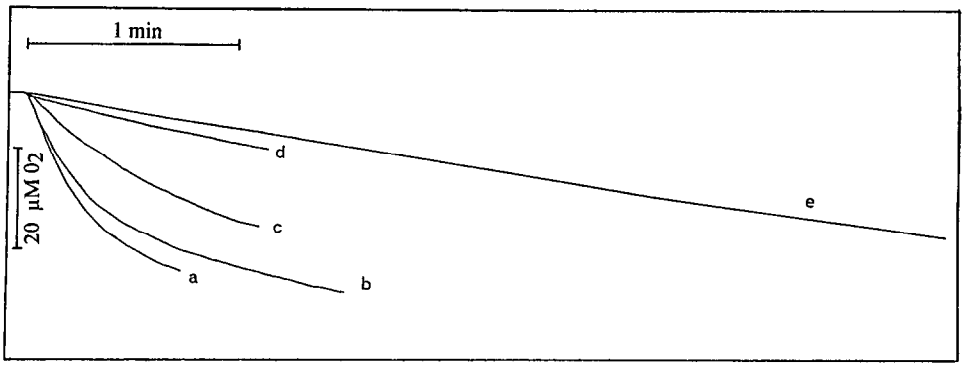

Fig. 7. Action of ebselen on ovine vesicular gland particulate PGHS-1. A sample of 0.8 mg protein of ovine vesicular gland microsomes was added to 1.75 mL air-equilibrated Tris–HCl buffer pH 8.0 containing 0.1 mM EDTA, varying concentrations of ebselen dissolved in 20 μL 2-methoxyethanol and 0.5 mM phenol at 25°. After a pre-incubation period of 1 min the reaction was started by 30 μM arachidonic acid: (a) control; (b) 11 μM; (c) 50 μM; (d) 100 μM; and (e) 250 μM ebselen. The dose–response curve for inhibition using the initial rates of these and other progress curves revealed an IC_{50} of 37.7 ± 4.3 μM (not shown).

related to their positional specificity towards fatty acids.

A particulate preparation of PGHS-1 shown to be highly sensitive to non-steroidal antiinflammatory drugs such as indomethacin, sodium diclofenac, piroxicam etc. [25], was only moderately inhibited (Fig. 7), with an IC_{50} of 37.7 ± 4.3 μM (compare indomethacin = 0.085 μM under identical assay conditions). At concentrations partially inhibiting PGHS-1, ebselen exhibited a second remarkable effect: it protected the enzyme from the well-known suicide inactivation [32] (see in particular trace e in Fig. 7). The self-inactivation of PGHS-1 is ascribed to a tyrosyl radical, possibly formed via a side reaction of the catalytic cycle of the enzyme and perhaps involved in the hydroperoxidase activity of the enzyme [33]. It is conceivable that ebselen scavenges this or another free radical responsible for self-inactivation. Such an assumption is in line with the radical-scavenging activity of ebselen observed by other authors [34]. Ebselen thus exerts a dual action on PGHS-1: (i) inhibition of the initial activity and (ii) protection from suicide inactivation and thus prolongation of product formation. This dual action is consistent with the earlier observation that ebselen inhibits prostaglandin E_2 production by peritoneal macrophages only partially over a wide concentration range [35]. Ebselen's capacity to offer protection from suicide inactivation was also observed for the partially inhibited mammalian 15-lipoxygenase (data not shown).

DISCUSSION

The data clearly show that ebselen is a direct-acting, potent inhibitor of mammalian 5- and 15-lipoxygenases provided thiols are not present. The elucidation of the chemical structure of the lipoxygenase–ebselen complex requires further study. It is conceivable that ebselen reacts with a defined SH group in mammalian lipoxygenases in forming an ebselen-protein-selenodisulphide as appears to be the case with the reaction on serum albumin [2, 36]. Although lipoxygenases are not believed to contain essential SH groups, binding to a non-essential cysteine residue located in the vicinity of the active site may block the hydrophobic substrate binding site by the aromatic ring(s) of the ebselen molecule. Such an assumption would be in accordance with the observation that soybean lipoxygenase-1 is quite insensitive towards ebselen. The capacity of GSH, HpODE or oxygen to reverse ebselen inhibition does not contradict this mechanism inasmuch as all these agents should be able to cleave the putative ebselen-selenodisulphide bond [2].

Since intact eukaryotic cells contain 2–8 mM GSH [37], it seems unlikely that the inhibition of mammalian lipoxygenases by stoichiometric concentrations of ebselen could occur under these conditions. However, a further pharmacodynamic quality should be considered based on the following considerations: the possible role of 15-lipoxygenase in the pathogenesis of atherosclerosis has been suggested [38], given the fact that this enzyme is able to oxygenate LDL [39]. The oxidized LDL are taken up by monocytes/macrophages via scavenger receptors. These processes are believed to lead to the formation of foam cells and fatty streaks, the early stage of an atherosclerotic lesion. The concept of a 15-lipoxygenase mediated oxidative modification of LDL is strongly supported by the observation that 15-lipoxygenase and its mRNA are found in atherosclerotic lesions together with oxidatively modified LDL [40]. It has, however, not yet been demonstrated whether the LDL are taken up and subsequently oxygenated or whether the 15-lipoxygenase is released from cells upon damage and acts extracellularly. In the latter case, ebselen would be the drug of choice to prevent this 15-lipoxygenase reaction, since extracellular GSH concentration in man is less than 1 μM [41], whereas ebselen concentrations in plasma up to 50 μM might be achieved [11]. Thus, ebselen may selectively suppress the unfavourable extracellular action of 15-

lipoxygenases without affecting their intracellular functions. The mechanism suggests a new element in the therapeutic applications of ebselen.

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