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The Inhibition of Mammalian 15-Lipoxygenases by the Anti-Inflammatory Drug Ebselen: Dual-Type Mechanism Involving Covalent Linkage and Alteration of the Iron Ligand Sphere

MATTHIAS WALTHER, HERMANN-GEORG HOLZHÜTTER, RALF JÜRGEN KUBAN, RAINER WIESNER, JÖRG RATHMANN, and HARTMUT KÜHN

Institute of Biochemistry, University Clinics Charité, Humboldt University, Berlin, Germany

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ABSTRACT

Mammalian lipoxygenases have been implicated in inflammation and atherosclerosis and, thus, lipoxygenase inhibitors may be of pharmacological interest. In cells, lipoxygenases occur in a catalytically silent ground state that requires activation to become active. We found that the seleno-organic drug ebselen [2-phenyl-1,2-benzisoselenazol-3(2*H*)-one], which exhibits anti-inflammatory properties, irreversibly inhibited pure rabbit 15-lipoxygenase, with an IC_{50} in the nM range when preincubated with the enzyme in the absence of fatty acid substrates. Subsequent dialysis, gel filtration, or substrate addition did not restore the enzyme activity, and experiments with [^{14}C]ebselen indicated a covalent linkage of the drug. The presence of sulfhydryl compounds in the incubation mixture prevented both enzyme labeling and inactivation, but we did not see any reactivation when sulfhydryl compounds were added afterward. X-ray absorption studies indicated that ebselen did alter the geometry of the iron ligand sphere, and the data are consistent with an iron complexation by the drug. When fatty acid substrate was present during lipoxygenase-ebselen interaction, the inhibitory potency was strongly reduced and a competitive mode of action was observed. These data suggest that ebselen inactivated the catalytically silent ground-state lipoxygenase irreversibly by covalent linkage and alteration of the iron ligand sphere. In contrast, it functions as a competitive inhibitor of the catalytically active enzyme species. The pharmacological relevance of ebselen as a potential in vivo lipoxygenase inhibitor will be discussed.

Lipoxygenases (LOXs) are nonheme, iron-containing enzymes that catalyze the oxygenation of polyenoic fatty acids to their corresponding hydroperoxy derivatives (Funk, 1996). With respect to their positional specificity of arachidonic acid oxygenation, they may be subclassified as 5-, 8-, 11-, 12-, and 15-LOXs (Funk, 1996). 5-LOXs are involved in the biosynthesis of mediators of inflammatory and anaphylactic disease (Lewis et al., 1990), whereas 15-LOXs have been implicated in cell differentiation (Rapoport et al., 1990) and atherogenesis (Kühn and Chan, 1997). A 15-LOX inhibitor that does not lower plasma cholesterol levels appeared to protect cholesterol-fed rabbits from the development of atherosclerotic lesions (Sendobry et al., 1997). Thus, the 15-LOX may constitute a pharmacological target for the development of antiatherosclerotic drugs. Unfortunately, a rational drug design was impossible for a long time because of limited structural information. However, in recent years, the crystal structures of two plant LOX isoforms (Boyington et al., 1993; Minor et al., 1996; Skrzypczak-Jankun et al., 1997) and of a rabbit 15-LOX/inhibitor complex (Gillmor et al., 1997) were published. In addition, a variety of spectroscopic measurements have been carried out, aimed at defining the geometric and the electronic structure of the nonheme iron environment (Pavlosky et al., 1995; Solomon et al., 1997; Kuban et al., 1998).

Ebselen [2-phenyl-1,2-benzisoselenazol-3(2*H*)-one] is a seleno-organic compound (Fig. 1) of low toxicity that exhibits a unique pharmacological profile (Schewe, 1995). Originally, ebselen was developed as an anti-inflammatory drug (Cotgreave et al., 1988, 1989; Gao and Issekutz, 1993), but other interesting pharmacological activities, including immunomodulation (Wendel et al., 1997), protection from reperfusion injury (Ozaki et al., 1997), prevention of radiation-induced apoptosis (Ramakrishnan et al., 1996), and cytoprotection of somatic cells against various noxious agents (Li et al., 1994;

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ABBREVIATIONS: LOX, lipoxygenase; SH, sulfhydryl; EXAFS, extended X-ray absorption fine structure; EMBL, European Molecular Biology Laboratory.

Lin and Girotti, 1994), have since been reported. More recently, the neuroprotective activities of ebselen (Takasago et al., 1997; Yamaguchi et al., 1998) and its interaction with nitric oxide and/or peroxynitril (Sies and Masumoto, 1997; Sies et al., 1997) have been studied in detail. Many of these effects have been related to the glutathione peroxidase-mimetic effect of the drug (Sies, 1994). However, ebselen is also capable of inhibiting several enzymes involved in the inflammatory reaction, such as NADPH-oxidase (Cotgreave et al., 1989), nitric oxide synthases (Hattori et al., 1994), and 5- and 15-LOXs (Schewe et al., 1994). However, for most of these enzymes, the mechanism of inhibition has not been investigated. For the inhibition of cellular activity of 5- and 15-LOXs, a dual mode of activity has been suggested (Schewe et al., 1994): 1) indirect inhibition caused by the attenuation of the cellular hydroperoxide tone via the glutathione peroxidase-mimetic activity of the drug; and 2) direct inhibition via the formation of a catalytically inactive LOX-ebselen complex. The former mechanism, which was suggested to prevail in the presence of glutathione, is based on the fact that ebselen reacts with sulfhydryl (SH) compounds, forming a selenosulfide or selenol that subsequently reduces the cellular hydroperoxides to the corresponding alcohols (Morgenstern et al., 1992), removing them as potential enzyme activators. Intracellularly, LOXs are synthesized as inactive ground-state enzymes that require activation to become catalytically active. The mechanism of activation is not yet entirely clear, but it appears to involve peroxide-induced oxidation of the ferrous LOX to a ferric form (Holman et al., 1998). However, for the time being, the chemical nature of the active enzyme species remains a matter of discussion.

In this study, we investigated the direct interaction of a pure mammalian 15-LOX with ebselen and found that the catalytically inactive ground-state LOX is irreversibly inactivated, whereas the catalytically active enzyme species is competitively inhibited.

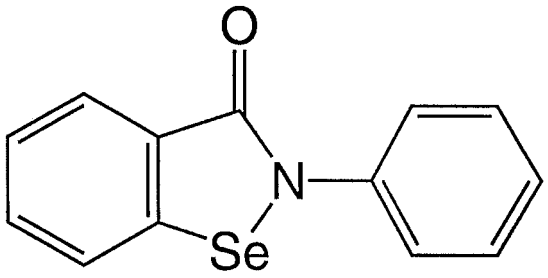

Fig. 1. Chemical structure of ebselen.

Materials and Methods

Chemicals. The chemicals used were obtained from the following sources. Methoxyethanol, linoleic acid, reduced glutathione, and sodium cholate were obtained from Serva (Heidelberg, Germany). Ebselen was kindly provided by Daiichi Pharmaceuticals Co., Ltd. (Tokyo, Japan), and [^{14}C]ebselen (uniformly labeled; specific radioactivity of 8 Ci/mol) was a kind gift from Dr. A. Wendel (University Konstanz, Germany). All solvents used were of HPLC grade and were purchased from Baker (Deventer, the Netherlands).

Preparations, Assay Systems, and Analytics. The rabbit reticulocyte LOX was prepared to apparent homogeneity from the lysate of a reticulocyte-rich blood cell suspension by fractionated ammonium sulfate precipitation and by consecutive hydrophobic interaction and anion exchange chromatography (Belkner et al., 1993). The final enzyme preparation was electrophoretically homogenous and exhibited a turnover rate of linoleic acid of 31 s^{-1}. An iron content of 0.95 mol of iron/mol of enzyme was determined by atomic absorption spectroscopy. These data indicate the high quality of the enzyme preparation used. For the inhibition studies of ebselen on the native ground-state LOX, the enzyme was preincubated with ebselen (0-8 μM final concentration) at room temperature in 1 ml of 0.1 M phosphate buffer (pH 7.4), and then the reaction was started by the addition of linoleic acid containing sodium cholate (final concentration 0.2%). The ebselen was added as highly concentrated methoxyethanolic stock solution so that the solvent concentration always was kept below 2%. At these concentrations, methoxyethanol did not influence the LOX activity. The LOX activity was assayed spectrophotometrically, measuring the increase in absorbance at 235 nm over a time interval of 1 to 2 min. To examine the effect of glutathione, a 300-fold molar excess (versus ebselen) was added

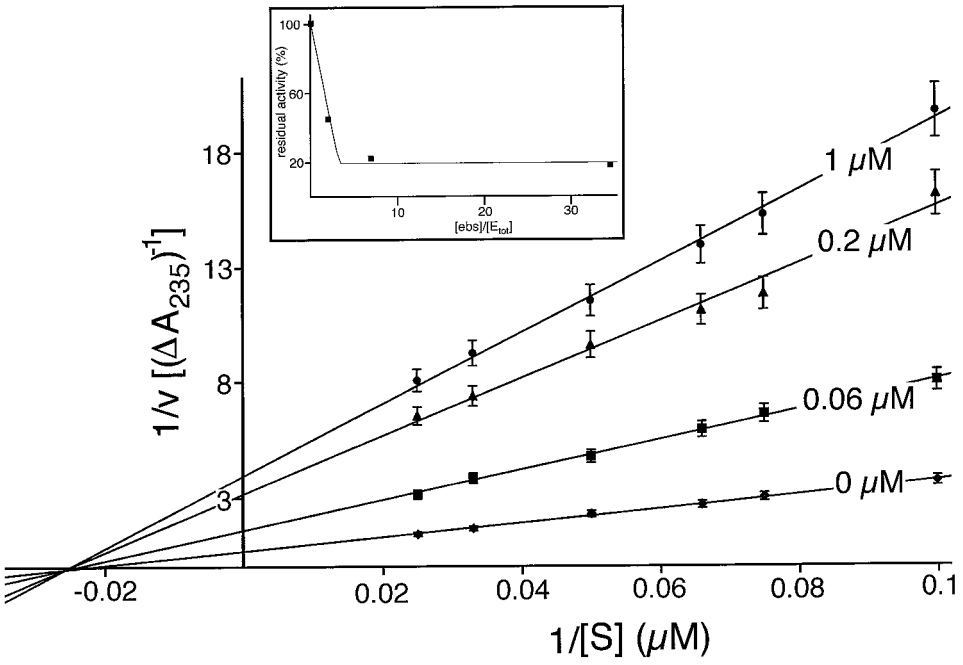

Fig. 2. Noncompetitive inactivation of the catalytically silent ground-state 15-LOX. The pure rabbit reticulocyte 15-LOX (29 nM final concentration) was preincubated with various concentrations of ebselen in 1 ml of 0.1 M phosphate buffer (pH 7.4) for 30 s at room temperature. The LOX reaction was started by the addition of various concentrations of linoleic acid and the increase in absorbance at 235 nm was monitored. Each data point represents the mean ± S. D. of three independent measurements. Inset, residual activities (V_{max}) were plotted against the ratio [ebselen]/[enzyme]. ■, measured values. The trace was calculated from equation 2. The fair agreement of the experimental data with the predicted curve suggests the validity of our kinetic model.

before or after the preincubation period. To investigate the impact of ebselen on the catalytically active enzyme species, either the drug (0–10 μM) was preincubated with linoleic acid for a short time (15 s) and then the enzyme was added or the enzyme was preincubated shortly with linoleic acid and then the ebselen was added.

SDS-electrophoresis was carried out with a 10% polyacrylamide gel (in both the absence and the presence of mercaptoethanol). The 15-LOX (0.32 nmol) was incubated for 5 min with [^{14}C]ebselen (0.5 or 2 nmol) in 1 ml of 10 mM ammonium bicarbonate buffer (pH 7.0). Afterward, the samples were dried down in a SpeedVac centrifuge (model SVC 100; Savant Instruments, Inc., Farmingdale, NY), the residue was reconstituted in electrophoresis loading buffer (no mercaptoethanol), and aliquots were applied to electrophoresis.

Reversed-phase HPLC analysis was carried out on a Shimadzu HPLC system (Shimadzu Worldwide, Kyoto, Japan) coupled with a Hewlet Packard diode array detector1040 A (Hewlet Packard Co., Palo Alto, CA). Ebselen and its metabolites were separated on a Nucleosil C-18 column (KS-system; 250 × 4 mm, 5-μm particle size; Macherey-Nagel GmbH, Düren, Germany) with a solvent system of methanol/water/acetic acid (55:45:0.1; by volume) and a flow rate of 1 ml/min. The absorbance at 325 nm was monitored.

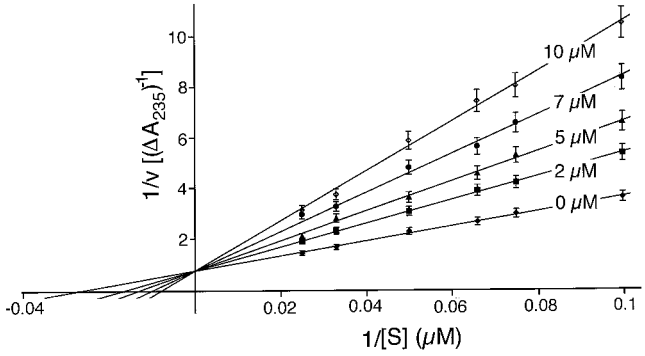

Fig. 3. Competitive inhibition of the catalytically active rabbit 15-LOX by ebselen. Various concentrations of ebselen were preincubated with different concentrations of linoleic acid for 15 s in 1 ml of 0.1 M phosphate buffer (pH 7.4). The LOX reaction was started by the addition of 29 nM purified rabbit 15-LOX and the increase in absorbance at 235 nm was monitored. Each data point represents the mean ± S. D. of three independent measurements. HPLC analysis indicated that no ebselen was lost during the preincubation period.

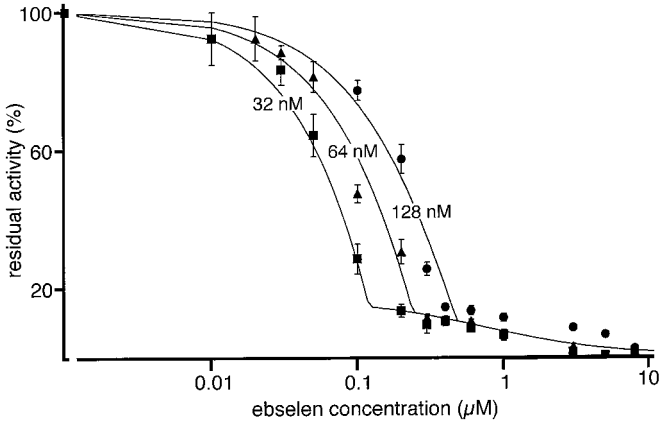

Fig. 4. Titration curves of 15-LOX inhibition by ebselen. The pure rabbit 15-LOX (various concentrations as indicated in the figure) was preincubated with different ebselen concentrations in 1 ml of 0.1 M phosphate buffer (pH 7.4) for 15 s and then the LOX reaction was started by the addition of 260 μM linoleic acid. The increase in absorbance at 235 nm was assayed spectrophotometrically. Each data point represents the mean of three independent measurements ± S. D.

X-ray Absorption Studies and Data Evaluation. For X-ray absorption studies, the pure 15-LOX (4 ml of a 40 μM solution) was incubated with a 10-fold molar excess of ebselen at room temperature for 20 min, resulting in a 90% inactivation. The excess of ebselen was removed by gel filtration (Econopak PD-10 column; Pharmacia, Inc., Uppsala, Sweden) and the buffer was exchanged to a volatile ammonium bicarbonate buffer (pH 7.0). Afterward, the enzyme was lyophilized and the remaining powder was transferred to a homemade plastic cell (1 mm thick), the measuring windows of which were covered with a Kapton membrane. The samples were stored at −80°C until measurement. X-ray absorption studies were carried out with the European Molecular Biology Laboratory (EMBL) X-ray absorption spectrometer (Hermes et al., 1984; Pettifer and Hermes, 1985) at HASYLAB (c/o Deutsches Elektronen Synchrotron, Hamburg, Germany). A Si(III) double crystal monochromator with an energy resolution of 1.9 eV at 7250 eV, resulting in a DE/E = 2.6 × 10^{-4}, was used. The second monochromator crystal was detuned to 50% to reject harmonics of higher order. The monochromator angle was converted to an absolute energy scale by applying a calibration technique (Pettifer and Hermes, 1985), resulting in an accuracy better than 0.2 eV. The 15-LOX X-ray absorption spectra were recorded and the X-ray fluorescence of the samples was monitored with a 13-element germanium solid-state detector. Series of 32 (ebselen-treated sample) and 25 (15-LOX control) spectra were taken for each sample at 20°K. The spectra were recorded in the range of 6900 to 7900 eV, with steps of 0.3 eV in the near-edge region. No evident damage of the protein sample occurred during the exposure to the X-ray beam as far as can be judged from the identity of the first and the last spectra collected.

The fluorescence raw data collected were reduced by using computer programs developed at the EMBL outstation (EXPROG:

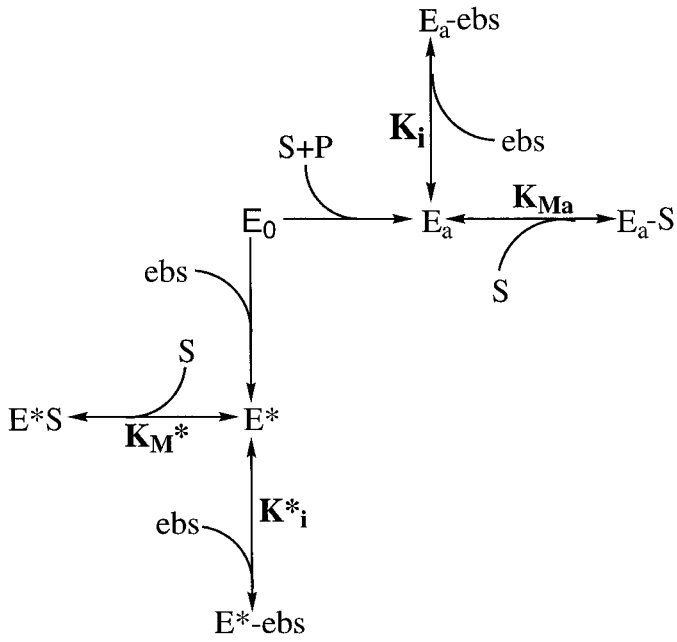

Fig. 5. Kinetic scheme of 15-LOX-ebselen interaction. The symbols represent the following enzyme species and kinetic constants: E_0, catalytically silent ground-state enzyme; E_a, catalytically active LOX species; E*, ebselen-modified LOX species, which may exhibit a residual catalytic activity; S, fatty acid substrate (linoleic acid); P, LOX product; E_a-ebs, catalytically inactive LOX-ebselen complex; E_a-S, catalytically active LOX-substrate complex; E*-ebs, ebselen-modified LOX, which has bound an additional ebselen molecule at the active site; E*-S, ebselen-modified LOX-substrate complex; K_i, affinity constant of the catalytically active enzyme species for ebselen; K^*_i, affinity constant of the ebselen-modified enzyme species for ebselen; K_{Ma}, substrate affinity of the catalytically active enzyme species; K_M*, substrate affinity of the ebselen-modified enzyme species.

EMBL extended X-ray absorption fine structure (EXAFS) data analysis and evaluation program package for PC/AT; H. F. Nolting, C. Hermes; 1992). Evaluation of the EXAFS spectra was carried out as a two-step process (Kuban et al., 1998) with the Excurve Program (version 92; Council for the Central Laboratory of the Research Councils, Daresbury Lab., Warrington, UK). In the first step, a theoretical EXAFS spectrum for the ferrous LOX was constructed that was based on the coordinates of the iron ligands (Gillmor et al., 1997). In the second step, the theoretical spectrum was fit stepwise to the measured EXAFS spectra, varying the coordinates of the atoms surrounding the central iron. A similar procedure was used for the LOX-ebselen complex. First, the sixth iron ligand in the native LOX (water oxygen) was exchanged to the ebselen selenium and a theoretical EXAFS spectrum was constructed, keeping the ligand distances constant. Then the theoretical spectrum was fit to the experimental data, changing the distances of the atoms surrounding the nonheme iron. The quality of fitting represented by the R-value was calculated from 500 data points over the entire measuring range. The R-factor is defined by the following equation:

$$R = \sum_{i}^{N} (1/\sigma_i)(|\chi^{exp}(k_i) - \chi^{the}(k_i)|)100\%$$

in which $\chi^{exp}(k_i)$ represents the experimental EXAFS data and $\chi^{the}(k_i)$ the calculated data. The $(1/\sigma_i)$ weighting term is defined by $1/\sigma_i = k_i^n \Big/ \sum_j^N k_j^n[\chi_j^{exp}(k_j)]$, in which N represents the number of data points and n a number chosen so that the term $k_j^n[\chi_j^{exp}(k_j)]$ has an approximately constant amplitude over the entire data range. For biological molecules, a R-factor of 15% indicates a good agreement of experimentally obtained traces and the calculated data. In contrast, a R-value of 40% or higher indicates poor fitting.

Modeling of the Inhibition Kinetics. Our studies on the inhibition kinetics suggested a dual mode of action: 1) ebselen acts as an irreversible inhibitor (Fig. 2) of the catalytically silent ground-state LOX (E_0), forming the inactivated enzyme species E*; and 2) it acts as a competitive inhibitor (Fig. 3) for the catalytically active enzyme species (E_a). In addition, the biphasic shape of the titration curves (Fig. 4) suggested that E*, which exhibits a residual LOX activity (about 15%), may be inhibited competitively by ebselen. These data suggested a biphasic kinetic scheme (Fig. 5). In this scheme, ebselen competes with LOX substrate for binding at the active site of the catalytically active LOX (E_a) and of the ebselen-modified enzyme species (E*) but also reacts with the catalytically silent LOX (E_0) to form E*.

$$\text{activity}(\%) = \frac{K_{Ma} + [S]}{K_{Ma}\left(1 + \frac{[ebs]}{K_i}\right) + [S]} \quad (1)$$

$$\text{activity}(\%) = \left[100 + (A_0 - 100)\frac{[ebs]}{n[E_0]}\right]\frac{K_M^* + [S]}{K_M^*\left(1 + \frac{[ebs]}{K_i^*}\right) + [S]} \quad (2)$$

The kinetic scheme can be described by equations 1 and 2. Equation 1 quantifies the competitive inhibition of the catalytically active LOX species, whereas equation 2 characterizes the two-phase inhibition consisting of the irreversible inactivation of the ground-state enzyme (absence of substrate) and of the competitive inhibition of the ebselen-modified LOX species. In equation 2, n represents the number of ebselen molecules necessary for maximal irreversible inactivation.

Results

Irreversible Inactivation of the Catalytically Silent Ground-State LOX. In mammalian cells, LOXs occur in two activity states: 1) as a ferrous, catalytically silent, ground-state enzyme, and/or 2) as a ferric, catalytically active LOX. When the ferrous ground-state LOX was prepared from rabbit reticulocytes and preincubated for 30 s at room temperature with ebselen, an IC_{50} of 65 nM (enzyme concentration of 32 nM) was observed. The inhibitory potency of ebselen was higher than that observed for other 15-LOX inhibitors that are frequently used in the literature (Table 1). 15-LOX inhibition occurred instantaneously (data not shown) and the extent of inhibition did not depend on the duration of the preincubation period (smallest preincubation interval of 5 s). Moreover, the inhibition was irreversible because gel filtration, dialysis, and the excessive addition of LOX substrate failed to restore the enzymatic activity (data not shown), and detailed studies of the inhibition kinetics confirmed a noncompetitive mode of action (Fig. 2). For the untreated enzyme, an apparent K_M for linoleic acid of 38.7 μM and a V_{max} of 31 s^{-1} (turnover rate under V_{max} conditions) were calculated from the Lineweaver-Burk plot. In the presence of 0.06 μM, 0.2 μM, and 1 μM ebselen, the following V_{max} values were obtained: 13.3 s^{-1}, 6.8 s^{-1}, and 5.5 s^{-1}, respectively. The K_M for linoleic acid determined in this study was somewhat higher than that reported previously (Ludwig et al., 1987), and this difference may be attributable to the different ways that the substrate solutions were prepared. In agreement with a previous report (Schewe at al., 1994), we found that the presence of SH compounds (glutathione or mercaptoethanol) in the preincubation mixture protected the enzyme from inactivation (data not shown).

LOX Inactivation of the Ground-State Enzyme is Paralleled by Covalent Linkage of the Drug and by

TABLE 1

Inhibitory potency of various 15-LOX inhibitors for the pure rabbit 15-LOX

The 15-LOX (29 nM) was pre-incubated for 30 s in 1 ml of 0.1 M phosphate buffer (pH 7.4) with different concentrations of the inhibitors. Then linoleic acid (260 μM) was added and the LOX activity was assayed, measuring the increase in absorbance at 235 nm. The numbers represent the means of at least duplicate measurements. It should be stressed that the IC_{50} values retrieved from the literature cannot be compared directly with the data obtained in this study because different experimental conditions (different LOX preparations, different enzyme concentrations, duration of the preincubation period) were used.

Inhibitor	Inhibition (This Study)		IC_{50}	Reference
	50 nM	500 nM		
	%		μM	
Ebselen	16	90	0.06	This study
PD 146 176	12	59	0.38	This study
Eicosatetraenoic acid	7	73	0.12	Schewe et al., 1986
Nordihydroguaiaretic acid	9	56	0.5	Schewe et al., 1986
4-Nitrocatechol	0	54	4.6	Schewe et al., 1986
tert-Butylhydroxyanisol	4	2	160	Schewe et al., 1986
Salicylhydroxymic acid	6	6	57	Schewe et al., 1986

Alterations of the Iron Ligand Sphere. Ebselen is known to react with SH groups (Ullrich et al., 1996), and the rabbit 15-LOX contains 15 free cysteins (Rapoport et al., 1979). To determine whether ebselen covalently modifies the enzyme during inactivation, the 15-LOX was incubated with [U-^{14}C]ebselen and the protein was analyzed by SDS-electrophoresis. When the electrophoresis was carried out in the absence of mercaptoethanol, one major radioactive protein band comigrating with an authentic standard of the native 15-LOX was detected, and the intensity of protein labeling did depend on the ebselen concentration (Fig. 6). However, when electrophoresis was performed in the presence of mercaptoethanol, the labeling disappeared (data not shown), suggesting that the SH compounds are capable of reversing the covalent linkage.

To investigate the stoichiometry of 15-LOX-ebselen interaction, the enzyme was inactivated by [^{14}C]ebselen and the excess of the drug was removed during long-term dialysis (28 h). At different time periods, aliquots were taken from inside the dialysis tube and the radioactivity was quantified by liquid scintillation counting. From Fig. 7 it can be seen that the radioactivity decreased gradually during the first 2 h but then remained constant over a long period of time. Quantification of the radioactivity revealed that about 10 to 12 nmol of ebselen must have been bound to 1 nmol of 15-LOX. Because the enzyme contains 15 cysteins, one may conclude that the majority of free SH groups appeared to be accessible for ebselen. If dialysis was carried out against 1 mM glutathione, much more radioactivity was removed. After 8 h of dialysis only about 2.7 nmol of ebselen remained attached to 1 nmol of enzyme (Fig. 7). After 30 h this ratio decreased to 1.6:1. These results confirm the electrophoresis data indicating that the majority of the enzyme bound drug can be removed by a molar excess of SH compounds.

Unfortunately, these experiments did not answer the question of whether removal of the covalently linked ebselen can restore the enzymatic activity because the native 15-LOX undergoes inactivation during long-term dialysis. To solve this problem, we used another experimental approach. A concentrated enzyme solution was inactivated with ebselen, and subsequently, different concentrations of glutathione were added. After removal of the low-molecular-weight compounds (free ebselen, free glutathione, and ebselen-glutathione complex) by gel filtration, the catalytic activity of the enzyme was assayed. Here, again, we did not observe any restoration of the activity when the ebselen was removed from the enzyme (data not shown). In contrast, when a control incubation (no ebselen treatment) was taken through the same experimental protocol, about 80% of the initial enzymatic activity was recovered.

Chemical modification of free SH groups might not be the only reason for enzyme inactivation. It has been reported previously that selenium compounds are capable of complexing enzyme-bound iron (Conradson et al., 1994) and that LOXs contain catalytically active nonheme iron. Thus, iron complexation may contribute to enzyme inactivation. In that case, one should be able to detect alterations in the geometry of the iron ligand sphere that can be picked up by X-ray absorption spectroscopy. Comparison of the weighted fine structure k (Fig. 8, left) and of the phase-corrected Fourier transforms (Fig. 8, right) of the X-ray absorption spectra revealed clear structural differences between the native (A) and the ebselen-treated 15-LOX (B). In both cases, a good agreement between computer-simulated model spectra (red traces) and our experimental data (yellow traces) was observed. Earlier X-ray absorption studies (Pavlosky et al., 1995; Kuban et al., 1998) and crystallographic data on the rabbit 15-LOX (Gillmor et al., 1997) suggested a six-coordinate ferrous nonheme iron, and our data on the native LOX are in line with these findings. Six nitrogen and/or oxygen atoms were identified as direct iron ligands and the following binding distances were determined: 0.190 ± 0.001 nm, 0.218 ± 0.001 nm, 0.236 ± 0.001 nm, 0.236 ± 0.001 nm, 0.239 ± 0.001 nm, and 0.240 ± 0.001 nm. The X-ray absorption spectra of the ebselen-treated 15-LOX are compatible with five nitrogen and/or oxygen atoms and one selenium atom as immediate iron ligands surrounding the central heavy metal with the following binding distances: 0.234 ± 0.001 nm, 0.235 ± 0.001 nm, 0.235 ± 0.001 nm, 0.235 ± 0.001 nm, 0.247 ± 0.001 nm (oxygen/nitrogen), and 0.217 ± 0.001 nm (selenium).

Competitive Inhibition of the Catalytically Active LOX. When ebselen was preincubated with linoleic acid

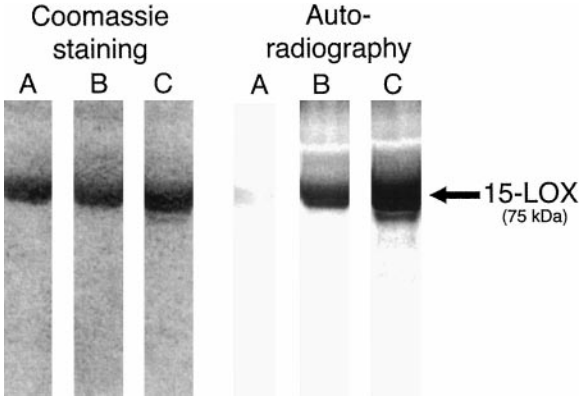

Fig. 6. Covalent linkage of ebselen to the rabbit 15-LOX during the preincubation period. The 15-LOX (0.32 nmol) was incubated for 5 min with 0 nM (A) or 0.5 (B) or 2 μM (C) [^{14}C]ebselen in 1 ml of 10 mM ammonium bicarbonate buffer (pH 7.0). Afterward, the samples were dried down in a SpeedVac centrifuge, the residue was reconstituted in electrophoresis loading buffer (no mercaptoethanol), and aliquots were applied to electrophoresis. After Coomassie staining, the gel was dried and exposed to an X-ray film.

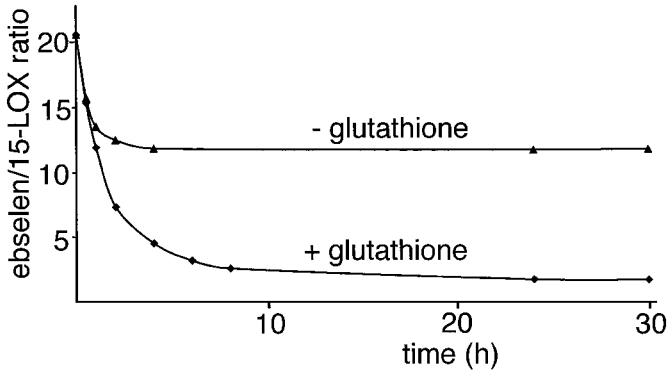

Fig. 7. Reversibility of 15-LOX labeling by [^{14}C]ebselen during dialysis. The purified 15-LOX (380 nM) was incubated with [^{14}C]ebselen (8 μM; specific radioactivity of 8 Ci/mol) in 1 ml of 0.1 M phosphate buffer (pH 7.4) for 2 min, and the incubation mixture was dialyzed against 2 liters of the same buffer. At different time points, aliquots were taken from the incubation mixture and the radioactivity was quantified by liquid scintillation counting. Glutathione concentration, 1 mM.

(LOX substrate) and the reaction was started by the addition of the enzyme, we observed a strong decrease in the inhibitory potency of the drug. Under identical conditions, the IC_{50} was shifted from 65 nM (preincubation of the enzyme with the drug) to 7 μM (preincubation of 40 μM linoleic acid with the drug). This difference was not due to a chemical modification of the drug during the preincubation period, as indicated by HPLC analysis (data not shown). Measurements of the inhibition kinetics indicated that also under these conditions, the inhibition occurred instantaneously and did not increase with time. In contrast to the inhibition of the ferrous enzyme (absence of substrate), the catalytically active enzyme species was reversibly inhibited and the drug competed with linoleic acid for binding at the active site (Fig. 3). The affinity of the enzyme for ebselen (K_i of 4 μM) was almost one order of magnitude higher than that for linoleic acid. However, it should be stressed that the K_M value for linoleic acid constitutes an apparent K_M because of the limited water solubility of the fatty acid. In fact, when the substrate solution was prepared in a different way, a lower K_M (4 μM) was observed (Ludwig et al., 1987).

Kinetic Modeling of LOX Inhibition by Ebselen. As indicated in Fig. 2, the ground-state LOX is irreversibly inactivated at low ebselen concentrations, suggesting sigmoid dose-response curves. However, titration of the enzyme with various ebselen concentrations and subsequent measurements of the residual LOX activity revealed biphasic titration curves (Fig. 4). At ebselen concentrations higher than 0.2 μM, the curves deviated significantly from the usual sigmoid shape (Hill model) in the semilogarithmic plot. To explain these deviations, one may assume an overlapping of two or more mechanisms of inhibition. When we assayed the residual LOX activity of the ebselen-treated LOX at different substrate concentrations, a K_M* of 10.7 was determined. Thus, the affinity of the ebselen-modified LOX species E* for linoleic acid was somewhat higher than that of the active enzyme species E_a. In Table 2, the kinetic constants obtained from the Lineweaver-Burk plots (Figs. 2 and 3) and from the titration curves (Fig. 4) are summarized. The affinity constant of the catalytically active LOX (E_a) for ebselen binding at the active site (K_i) was 4.1 μM. However, the corresponding constant of the ebselen-modified LOX (E*) was two orders of magnitude lower (K_i* of 0.03 μM), indicating a higher ebselen affinity of this enzyme species. During its interaction with ebselen in the absence of LOX substrate, the catalytically silent ground-state LOX is maximally inactivated to

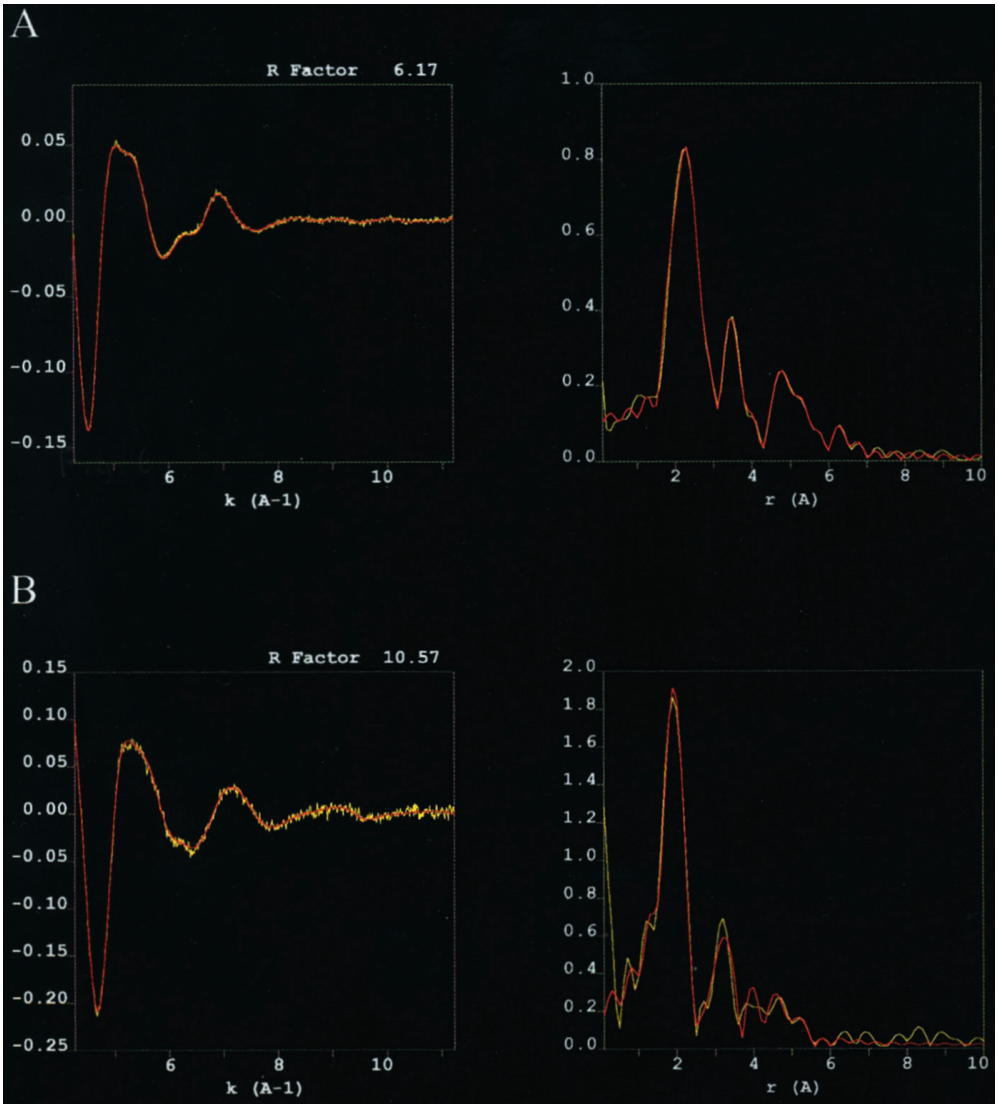

Fig. 8. X-ray absorption spectra of the rabbit 15-LOX and the 15-LOX-ebselen complex. The weighted fine structure k (left side) and the phase-corrected Fourier transforms (right side) of 15-LOX species are shown. Theoretical spectra (red lines) are superimposed by the experimental data (yellow lines). For the calculation of the model spectra, the atoms surrounding the central iron in a distance of less than 0.6 nm were considered. A, native rabbit 15-LOX. B, rabbit 15-LOX-ebselen complex.

about 85% by chemical modification (SH modification and/or iron complexing). For this inactivation, 3 to 4 mol of ebselen must have been attached to 1 mol of LOX.

Discussion

The seleno-organic compound ebselen, which exhibits anti-inflammatory properties, strongly inhibits mammalian 15-LOXs. When the ferrous ground-state enzyme was incubated with ebselen in submicromolar concentrations, an almost complete inhibition was observed. In contrast, all of the frequently used standard inhibitors (Table 1), as well as the antiatherogenic 15-LOX inhibitor PD 146 176 (Sendobry et al., 1997), were less effective when tested under identical experimental conditions. However, in the presence of SH compounds, the inhibitory potency was strongly impaired.

By studying the inhibition mechanism, we found that the native ground-state enzyme is irreversibly inactivated by the drug and that all attempts failed to restore a major part of the enzymatic activity. The catalytically active LOX was less susceptible to inhibition, and we observed a competitive mode of action. In both cases (the presence and the absence of a substrate), the inhibition occurred instantaneously, and we did not observe any time dependence of the inhibitory effect. However, it should be stressed that we did not carry out stop-flow experiments to investigate a possible time dependence in the ms range. Preincubation of the ground-state enzyme with ebselen was of dual consequence. The first was covalent linkage of ebselen to the enzyme molecule. This linkage was reversed when thiol compounds such as glutathione or mercaptoethanol were added subsequently. However, the reversal was incomplete and did not restore LOX activity. The second was alterations of the iron ligand sphere, which may be explained by a complexation of the catalytically active nonheme iron. It might be possible that the drug displaces a water molecule from the sixth iron ligand position. From our data, it was not possible to find out which portion either process (covalent linkage or alteration of the iron ligand sphere) contributed to enzyme inactivation at a given ebselen concentration.

To influence the iron ligand sphere, the drug should be capable of penetrating into the substrate binding pocket. Because ebselen is a more rigid and space-filling molecule than polyenoic fatty acids, we had to answer the question whether ebselen may fit into the substrate binding pocket without major alterations of the three-dimensional enzyme structure. We carried out molecular modeling of the enzyme-inhibitor complex based on the crystallographic coordinates (data not shown) and found that there may not be major steric constraints preventing ebselen from binding in the vicinity of the nonheme iron.

The data presented indicate that ebselen is an effective in vitro 15-LOX inhibitor, but it remains to be investigated whether it may also act in vivo. According to our findings, the catalytically silent ground-state LOX was irreversibly inactivated at nM ebselen concentrations, but the catalytically active enzyme form was only competitively inhibited in the lower micromolar range. Thus, the susceptibility of LOXs in vivo may depend on their activity state. For the human 5-LOX, it has been reported that it is silent in resting cells but becomes catalytically active after cell activation (Murakami et al., 1995). Pharmacokinetic studies in rats that received ebselen orally at a dose of 30 mg/kg body weight revealed an increase in the plasma selenium concentration of 4 μM (Takasago et al., 1997). These data suggest that ebselen plasma levels may be reached that are in the range of K_i for the catalytically active enzyme and are well above the concentrations required for irreversible inhibition of the catalytically silent ground-state enzyme.

To judge the in vivo effectiveness of ebselen as a LOX inhibitor, another point has to be considered (Schewe et al., 1994). Mammalian cells usually contain high concentrations of reduced glutathione, and SH compounds have been shown to prevent effective LOX inhibition. In the presence of 1 mM glutathione, the IC_{50} for the rabbit 15-LOX drops from 200 nM to about 300 μM (Schewe et al., 1994). In contrast, in the extracellular space, glutathione is virtually absent and, thus, it may not interfere with LOX inhibition. Under normal conditions, LOXs are intracellular enzymes, but at the site of inflammation or in growing atherosclerotic lesions where cell disruption and cell death may occur, the enzyme may be released into the extracellular space to initiate extracellular lipid peroxidation, which may contribute to the progression of disease. Selective inhibition of LOX initiated extracellular lipid peroxidation without affecting the intracellular LOX activity may be a beneficial effect for the above-mentioned pathophysiological disorders.

TABLE 2

Kinetic parameters of 15-LOX inhibition by ebselen

Equations 1 and 2 were fitted to the experimental data shown in Fig. 4 using the SIMFIT software package.

Parameter	Meaning	Estimated Value
K_i	Competitive inhibition of the catalytically active enzyme	4.1 μM
K_i*	Competitive inhibition of the ebselen-modified LOX by ebselen	0.03 μM
E*	Residual activity of the ebselen-modified enzyme	17%
n	Number of ebselen molecules involved in enzyme inactivation	3.3
K_{Ma}	Substrate affinity of the catalytically active enzyme	38.7 μM
K_M*	Substrate affinity of the ebselen-treated enzyme (data not shown)	10.7 μM

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Send reprint requests to: Dr. Hartmut Kühn, Institute of Biochemistry, University Clinics Charité, Humboldt University, Hessische Str. 3-4, D-10115 Berlin, Germany. E-mail: hartmut.kuehn@rz.hu-berlin.de