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A NOVEL BIOLOGICALLY ACTIVE SELENO-ORGANIC COMPOUND—V

INHIBITION BY EBSELEN (PZ 51) OF RAT PERITONEAL NEUTROPHIL LIPOXYGENASE

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Abstract—Suspensions of rat peritoneal PMNLs elicited with glycogen were stimulated by calcium and an ionophore to produce leukotrienes from endogenous arachidonic acid. We investigated the effect of the non-toxic, anti-inflammatory seleno-organic compound, ebselen (PZ 51). When ethanolic extracts of the medium of stimulated cells were analysed by HPLC, a dose-dependent inhibition by ebselen of LTB_4 formation with a concomitant decrease of 5-HETE production was found. Half-maximum inhibition was observed at 20 μmoles/l ebselen. Similar findings were obtained after analysis of chloroform extracts of both cells and medium using a different HPLC system. Under these conditions, enhanced 5-HETE formation was associated with reduced production of LTB_4 and other di-HETE isomers, when purified glutathione peroxidase + GSH were present. We conclude that the reported GSH peroxidase-like activity of ebselen, catalysing the reduction of 5-HPETE to 5-HETE, can not account for our findings. Therefore, the lipoxygenase reaction itself apparently represents the site of inhibition of LTB_4 formation by ebselen.

Ebselen (PZ 51, 2-phenyl-1,2-benzoisoselenazol-3(2H)on)† is a selenium compound with very low toxicity which exhibits anti-inflammatory activity in various animal models [1]. *In vitro*, the compound catalysed a glutathione peroxidase-like reaction, i.e. the reduction of hydroperoxides by GSH [2, 3]. In isolated liver microsomes, the signs of lipid peroxidation were markedly reduced in the presence of μmolar quantities of ebselen [2]. Similar results were obtained with isolated hepatocytes [4]. *In vivo*, however, the selenium moiety of the drug was not available for selenoprotein synthesis in mouse liver [3]. The observation that prostaglandin E_2 production by peritoneal macrophages was only partially inhibited by ebselen over a wide concentration range [5] prompted us to study its effect on another class of inflammatory mediators, i.e. leukotrienes.

It has been shown that the chemokinetic and aggregating activities, towards PMNL and eosinophils, released from rat and human PMNL exposed to the calcium ionophore A 23187 [6, 7] are due to the release of leukotriene B_4, which is active over a concentration range from 10 pg/ml to 5 ng/ml [8]. We chose this system in order to find a further rationale for the anti-inflammatory properties of ebselen.

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† Abbreviations used: PZ 51, 2-phenyl-1,2-benzoisoselenazol-3(2H)on; PMNL, polymorphonuclear leukocytes; 5-HETE, 5-hydroxy-eicosa-tetraenoic acid; 5-HPETE, 5-hydroperoxy-eicosa-tetraenoic acid; di-HETE, di-hydroxy-eicosa-tetraenoic acid; PBS, phosphate-buffered saline; LT, leukotriene; GSH, glutathione; PG, prostaglandin.

MATERIALS AND METHODS

Materials

PGB_2 and calcium ionophore A 23187 were supplied by Sigma (F.R.G.). Amberlite XAD-8 was obtained from Serva (F.R.G.). The four standard di-HETE isomers as well as 5-HETE were a kind gift of Dr. G. Loschen, and Dr. W. Martin (Grünenthal GmbH, Aachen). Their identity was confirmed by GC/MS as well as u.v.-spectroscopy. PZ 51 and related compounds were a gift from Nattermann (Köln). The prepacked reverse phase column (250 × 4 m, LiChrosorb C 18, 5 μm) was obtained from Merck (F.R.G.). Glutathione peroxidase was isolated to homogeneity from bovine red blood cells as described [9].

Cell suspensions

Peritoneal exudate leukocytes were collected from Sprague–Dawley rats 4 hr after the injection of a glycogen solution in Dulbecco's phosphate-buffered saline [10] into the peritoneal cavity (1 g glycogen per kg body weight). The rats were anaesthetized, killed and the PMNL were collected from the opened peritoneal cavities. The cells were washed twice with PBS at 4° and suspended in the incubation buffer (137 mM NaCl, 2.7 mM KCl, 2 mM KH_2PO_4, 5 mM Na_2PO_4, 5 mM glucose) pH 7.2. Cells were typically 90 ± 5% PMNL, the remainder being mononuclear cells, as determined by Wright's stained smears [11]. The cells exhibited greater than 95% viability, as measured by the trypan blue exclusion test [12].

Cell incubations

Samples of 10^7 cells (5×10^6 cells/ml incubation buffer without and with 0.1 mM glutathione added,

pH 7.2) were prewarmed for 5 min with A 23187 (1 μg/ml) at 37°. Having added the calcium solution to the final calcium concentration of 1.75 mM, the mixture was shaken for a further 10 min at 37°.

For experiments on the inhibition of leukotriene synthesis, PZ 51 dissolved in DMSO was added to the cells prior to A 23187 addition. All incubations including controls were carried out in 0.35% DMSO.

Incubations of cell homogenates. 6×10^7 packed cells were lysed by adding 1.2 ml distilled water. Samples of 200 μl containing the lysate of 10^7 cells were incubated at 37° for 10 min in the presence of 0.1 mmole/l GSH, 83 μmole/l arachidonic acid, 1.75 mmole/l $CaCl_2$, with or without 4.5 units GSH-peroxidase. The incubate was then extracted with chloroform/methanol and evaporated as described later on. The analysis of these experiments were run on system III as described.

Extraction procedures

(a) *Ethanolic extraction of the incubation medium.* The reaction was stopped by cooling to 0° and by centrifugation at 700 *g* for 7 min. 1.5 ml of the supernatant was made up to 80% ethanol with 6 ml absolute ethanol containing 600 ng PGB_2 as an internal standard. A 200 μl aliquot of the supernatant was stored separately at 4° for the lactate dehydrogenase determination [12].

The ethanolic extract was filtered and rotatory evaporated to dryness at 35°. The residue was dissolved in 1.5 ml bidistilled water and applied to an XAD-8 column (0.8 × 5 cm). The column was washed with 1 vol. bidistilled water and the LTs eluted with 1.5 vol. 80% ethanol. The eluent was evaporated to dryness at 35° and stored at −20° for HPLC-analysis. Prior to analysis, it was dissolved in 300 μl methanol and filtered through a 0.2 μm diameter cellulose filter.

(b) *Total extraction of the incubation medium and the cells.* After addition of 600 ng PGB_2 as internal standard, the cell suspensions was acidified to pH 3 with formic acid and extracted twice with an equal volume of chloroform/methanol (1:1). The extract was evaporated to dryness and stored for HPLC-analysis.

Reversed-phase high performance liquid chromatography

Twenty microlitres of a methanolic solution were injected via a Rheodyne 7125 device into an SP 8 700 HPLC from Spectra Physics using a detection wavelength of 280 nm. After elution of the di-HETE isomers, the wavelength was switched to 235 nm in order to allow detection of 5-HETE.

System I: isocratic elution with methanol:water: acetic acid = 67:33:0.02, pH = 4.7, flow = 1 ml per min.

System II: isocratic elution with methanol:water: acetic acid = 72:28:0.2, pH = 4.8, flow = 1 ml per min.

System III: isocratic elution with methanol: water:acetic acid = 83.2:16.7:0.1, pH = 4.8, flow = 1 ml per min.

RESULTS

Figure 1A shows an HPLC elution diagram of an extract from the medium of cells which were stimulated to release eicosanoids from endogenous arachidonic acid. By comparison with the standards shown in Fig. 1C, it is concluded that the system separates four di-HETE isomers. Neglecting PGB_2 which was added as internal standard, the cells released primarily LTB_4. The chromatogram in Fig. 1B demonstrates that in the presence of 30 μmoles/l ebselen the medium contained less di-HETEs. Among them, the amount as well as the distribution of the all-*trans* isomers of leukotriene B_4 (I, II), were affected. However, the most pronounced effect was observed on the release of LTB_4 and its primary metabolite, according to its retention time, most probably 20-carboxy-LTB_4 (M). For these reasons

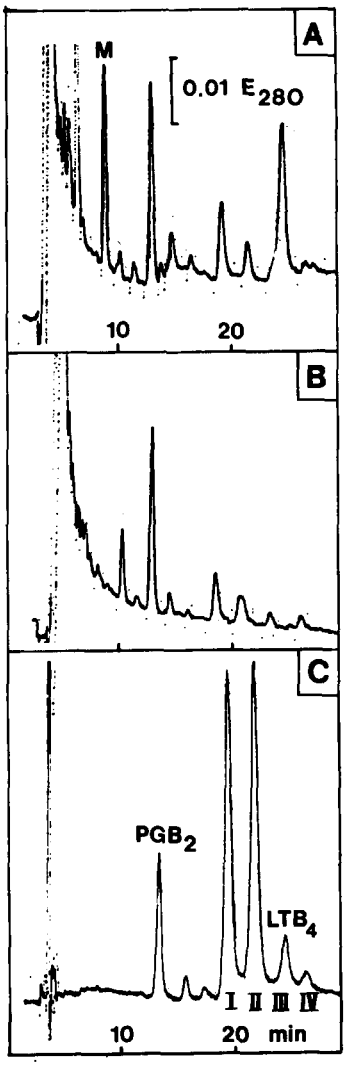

Fig. 1. HPLC elution profiles (system I) of ethanol extracts from medium which had been incubated with ionophore-stimulated rat peritoneal polymorphonuclear leukocytes (A), in the presence of 30 μmole/l ebselen (B). Standards (C): PGB_2 = 20 ng prostaglandin B_2. I = 65 ng 5(*S*),12(*R*)-all *trans*-LTB_4; II = 75 ng 5(*S*),12(*S*)-all *trans*-LTB_4; III = 10 ng 5(*S*),12(*R*)-dihydroxy-6-*cis*,8,10-*trans*-14-*cis*-eicosatetraenoic acid (LTB_4); IV = 3 ng 5(*S*),12(*S*)-dihydroxy-6-*trans*-8-*cis*-10-*trans*-14-*cis*-eicosatetraenoic acid; M = metabolite:20 carboxy- or 20-hydroxy-LTB_4.

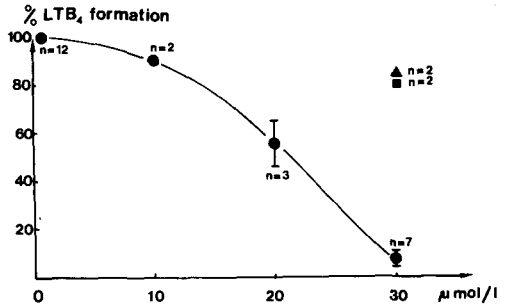

Fig. 2. Concentration dependence of ebselen-induced (●) inhibition of leukotriene B_4 formation by inophore-stimulated rat peritoneal polymorphonuclear leukocytes. Controls: glutathione adduct of ebselen 30 μmoles/l (▲); sodium selenite, 30 μmoles/l (■). Data in means ± S.D.; 100% = 900 ng LTB_4/5 × 10^6 cells (N = 12).

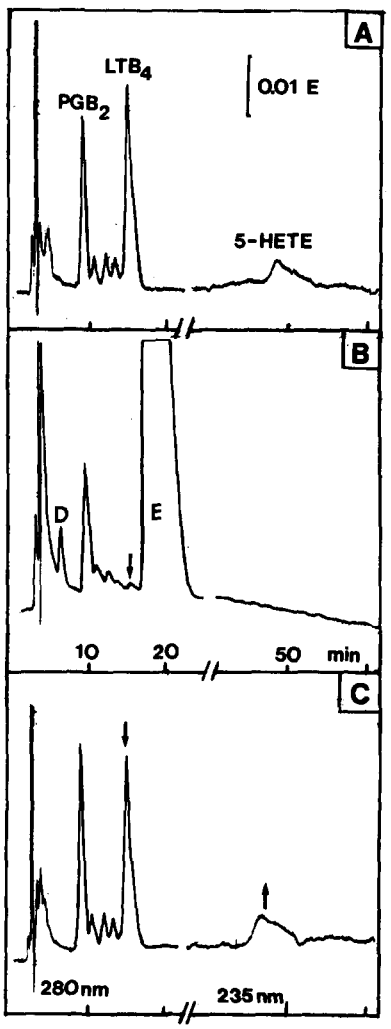

Fig. 3. HPLC elution profiles (system II) of chloroform extracts of ionophore-stimulated PMNL's *plus* medium in the presence of: A, 0.1 mmole/l GSH; B, 0.1 mmole/l GSH; 30 μmole/l ebselen; C, 0.1 mmole/l GSH; 4.5 U/ml GSH peroxidase (500 U/mg). Peak D = ebselen, peak E = ebselen-metabolites (retention time of ebselen = 5 min). The figure shows one out of four experiments.

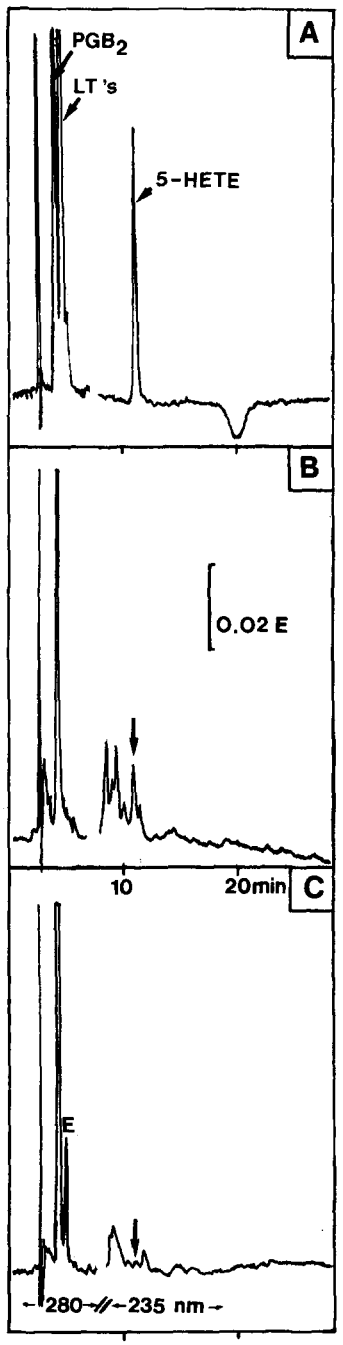

Fig. 4. HPLC elution profiles (system III) of standards (A) (cf Fig. 1C) or of chloroform extracts of PMNL lysates containing 83 μmoles/l exogenous arachidonic acid in presence of 4.5 U GSH-peroxidase (B) or of 30 μmoles/l ebselen (C). Peak E = ebselen metabolites (not LTs).

and with respect to its prominent biological relevance we focused our interest on the influence of ebselen on LTB_4 formation. The results in Fig. 2 illustrate that the ebselen-induced inhibition of LTB_4 release was concentration-dependent. Fifty per cent inhibition was obtained at about 20 μmole/l ebselen. At a concentration of 30 μmole/l, Na_2SeO_3 or the GSH adduct of ebselen (*S*-(2-benzylcarbamoyl-phenylselenyl)-glutathione) showed no significant effect on

LTB_4 concentrations in the medium. In none of the experiments described in this section were we able to detect 5-HETE in the medium of stimulated cells (after ethanolic extraction of medium without cells).

In order to distinguish inhibition of LT synthesis from impaired release, in the following experiments cells plus medium were extracted with chloroform and analysed in a second HPLC system. The chromatograms in Fig. 3 show that, compared to an incubation in the presence of GSH (A), an incubation of cells in the presence of GSH *plus* ebselen (B) led to a decrease in both LTB_4 and 5-HETE formation. In order to circumvent permeability problems and to improve the chromatographic resolution of 5-HETE, we repeated the control experiment using GSH-peroxidase in rat neutrophil homogenates followed by a different chromatographic procedure. Figure 4B shows neutrophil lysate converts exogeneously added arachidonic acids to 5-HETE, while no 5-HETE (and no LTB_4, cf. legend) is detectable in the presence of ebselen (Fig. 4C). Thus a potentially ebselen-catalyzed reduction of 5-HPETE to 5-HETE was not apparent under these conditions. The experiment shown in Fig. 3C demonstrates that in the presence of high activities of extracellularly added GSH peroxidase and its substrate GSH, a 30% increase in 5-HETE accompanied by a 30% decrease in LTB_4 formation is observed.

Inhibition experiments were repeated in another laboratory using 20 μmole/l exogenous arachidonic acid and thin layer chromatography analysis with essentially the same results with respect to the parallel inhibition by ebselen of LTB_4 and 5-HETE formation (G. Loschen, W. Martin and L. Flohé, 1984, personal communication).

In our experiments, we also monitored the viability of the cells by determination of extracellular lactate dehydrogenase activity at the end of the experiments, i.e. after 10 min incubation. A consistently better viability was observed with the cells incubated in the presence of ebselen. For example, leukocytes incubated at 30 μmole/l ebselen released only 62 ± 15 ($N = 3$) mU per ml of the lactate dehydrogenase compared to controls incubated with ionophore and solvent alone (209 ± 25, $N = 8$). Thus, in agreement with the observation of a delayed Fe/ADP-induced lipid peroxidation in hepatocytes [4], the compound seems to exhibit a cytoprotective effect on stimulated neutrophils.

DISCUSSION

Leukotriene B_4 is thought to be a potent mediator of chemotaxis and aggregation of neutrophils. Therefore, inhibition of its production might be a key process in diminishing the inflammatory response.

In the presence of ebselen the formation of both 5-HETE and LTB_4 was decreased suggesting that less hydroperoxide was formed. This interpretation is corroborated by using two mutually exclusive analytical systems, one of which primarily resolves the leukotriene isomers (system II, Fig. 3), and one that analyses 5-HETE (system III, Fig. 4). The absence of detectable amounts of 5-HETE in the presence of ebselen focuses the site of inhibition of this compound to the first step, i.e. the lipoxygenase reaction. Since the inhibitory effect of the selenium compound was also observed when exogenous arachidonic acid was the substrate, an inhibition of phospholipase seems unlikely.

This study offers an alternative explanation to the glutathione peroxidase-like property of ebselen for its pharmacological effects. Firstly, the extracellular concentration of GSH is very low, e.g. in man less than 1 μmole/l [15]. Secondly, the putative reaction product, 5-HETE, is not found—in contrast to a control experiment with GSH-peroxidase. The alternative rationale for the anti-inflammatory action of ebselen as proposed by the findings reported here is further supported by a quantitative consideration: oral administration of 50 mg/kg ebselen to rats leads to serum selenium contents of 3–4 μg Se per ml after several hours (J. P. Löhr, 1984, personal communication). This means that effective extracellular ebselen and its metabolites concentrations of roughly 50 μmoles/l might be reached—a level at which the inhibition phenomena described here could be relevant.

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