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EFFECTS OF EBSELEN ON ARACHIDONATE METABOLISM BY OCULAR AND NON-OCULAR TISSUES

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Abstract—The formation of cyclooxygenase products in rabbit and rat ocular and non-ocular tissues *in vitro*, detected by radio-thin-layer chromatography, was inhibited in a concentration-dependent manner by ebselen (PZ 51), an anti-inflammatory seleno-organic compound which has glutathione peroxidase and anti-oxidant activities. The exception was prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) formation in the rabbit iris-ciliary body which was stimulated by ebselen in the concentration range 2–10 μM. These observations were confirmed by gas chromatography–mass spectrometry. The concentration that inhibited 50% of prostaglandin biosynthesis (IC_{50}) in the rabbit iris-ciliary body was 9.3 μM. Ebselen also inhibited the formation of 12-hydroxyeicosatetraenoic acid (12-HETE) in rabbit and rat ocular tissues and rabbit platelets. The IC_{50} in the rabbit cornea was 4 μM, whereas higher concentrations were generally required to achieve similar inhibition in other tissues. The formation of 12-HETE by rabbit spleen, however, was not decreased by ebselen at concentrations that were inhibitory in other tissues.

Ebselen [PZ 51: 2-phenyl-1,2-benzisoselenazol-3-(2*H*)-one] is a novel anti-inflammatory seleno-organic compound with low toxicity [1–3]. The reported anti-oxidant and glutathione peroxidase-like activities may contribute to its anti-inflammatory property [4–6] since hydrogen peroxide and lipid peroxides are phlogistic and toxic [7]. Peroxides have also been reported to be elevated in inflamed tissues [8] and are implicated in the pathophysiology of cancer and atherosclerosis [8, 9]. Recently, ebselen has been shown to inhibit the formation of cyclooxygenase and lipoxygenase products [10–12] which are important mediators and modulators of inflammatory processes. Low concentrations of ebselen have been reported to inactivate the 5-lipoxygenase product, leukotriene B_4 (LTB_4), a potent leukotactic agent, by isomerization to biologically inert compounds [11]. In this study, we have investigated the effects of ebselen on [^{14}C]arachidonate metabolism by the iris-ciliary body and cornea from rabbit and rat *in vitro*. For comparison, we have also examined the effect of ebselen on arachidonate metabolism by the rabbit spleen and platelet.

MATERIALS AND METHODS

Male New Zealand albino rabbits (1.5–2.5 kg) and Wistar rats (200–250 g) were killed with an overdose of sodium pentobarbital (Butler, Columbus, OH). All animal experiments conformed to the NIH policy statement, Publication No. NIH-80-23. The anterior segments of the eyes were removed rapidly, and the iris-ciliary bodies and corneas were excised, rinsed in chilled isotonic saline, blotted and weighed. Pooled tissues were homogenized 1:17 (w/v) in chilled 50 mM phosphate buffer, pH 7.5, by a Polytron homogenizer (Brinkmann, Westbury, NY). Spleens were rapidly removed and homogenized in chilled phosphate buffer as above. Platelets were prepared as previously reported [13]. The platelet pellet was suspended in a volume of phosphate buffer that was approximately one-tenth that of the original blood volume and homogenized by Polytron.

Unlabeled arachidonic acid (approximately 99% pure) and chemicals of the highest purity available were obtained from the Sigma Chemical Co. (St Louis, MO). Organic solvents (liquid chromatography grade), and silica gel 60 thin-layer chromatography (TLC) plates, without fluorescent indicator, were purchased from EM Science (Cherry Hill, NJ). Prostaglandin standards were obtained from Seragen (Boston, MA). [^{14}C]Arachidonic acid (sp. act. 58.3 mCi/mmol) was obtained from the Amersham Corp. (Arlington Heights, IL). Ebselen was a gift from the Ciba-Geigy Corp. (Ardsley, NY).

Tissue incubations and extraction. Duplicate aliquots of fresh tissue homogenates (0.4 ml, approximately 2 mg protein/ml [14]) were incubated aerobically with [^{14}C]arachidonate (0.1 μCi, 3.43 μM) and either 10 μl dimethyl sulfoxide (DMSO), controls, or the same volume of ebselen dissolved in DMSO, in a total incubation volume of 0.5 ml at 37° for 30 min in a shaking water bath. The reactions were terminated by acidification to pH 4, with the addition of 0.4 M HCl, and the eicosanoids were extracted with 6 vol. of ethyl acetate. The solvent was evaporated under nitrogen. The recovery for the arachidonate metabolites by the ethyl acetate extraction was approximately 80%. Each extract was reconstituted with 30 μl ethyl acetate and applied to two TLC plates.

Each extract was subsequently developed in two different solvent systems. One plate was developed

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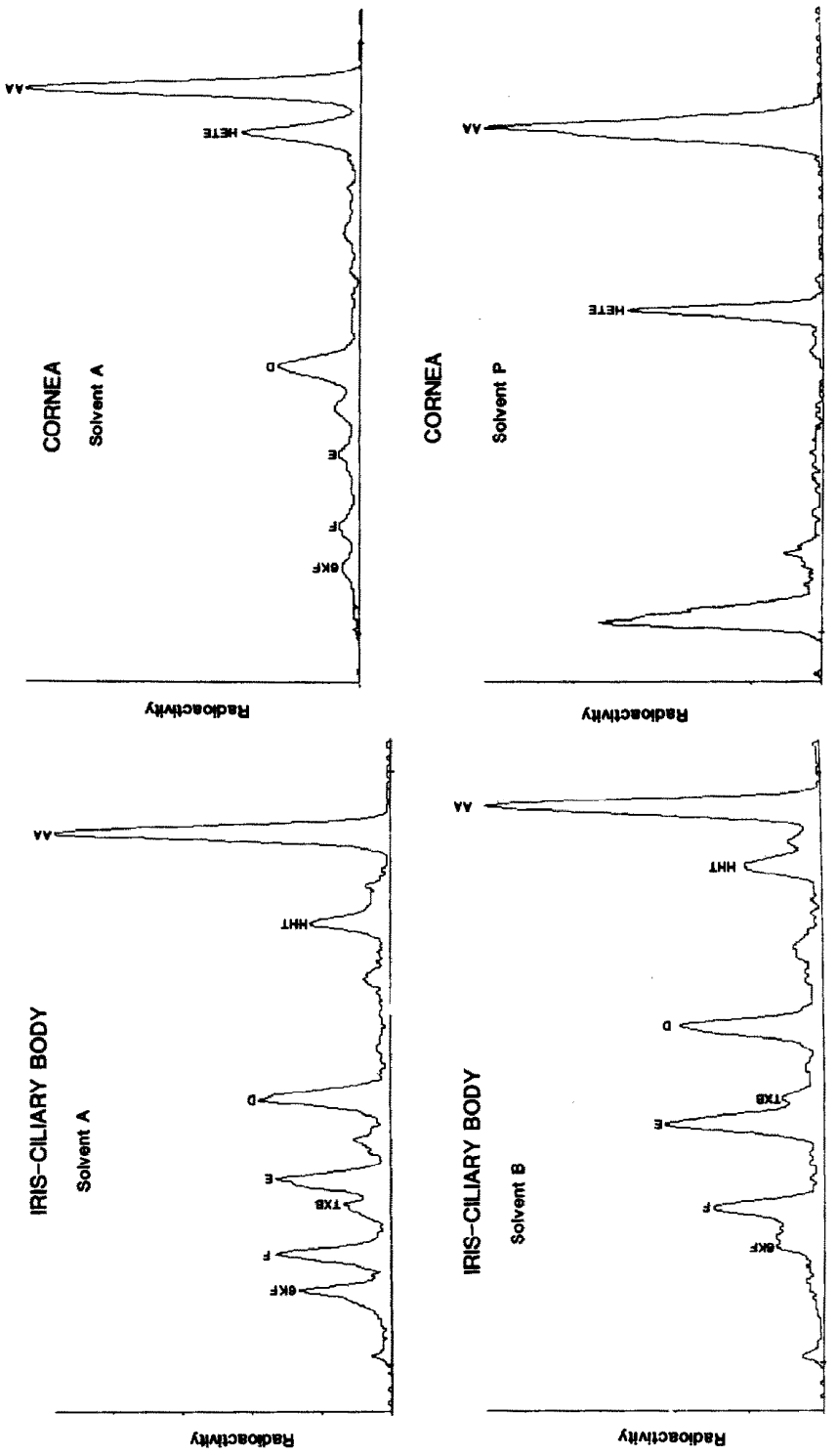

Fig. 1. Typical radio-thin-layer chromatography (TLC) scans of tissue extracts obtained from rabbit iris-ciliary body (left panel) and cornea (right panel). Tissue homogenates were incubated in the presence of [^{14}C]arachidonate, extracted, and applied to TLC plates that were developed in the solvent systems (A), (B) and (P), as described in the text. Abbreviations: 6KF, 6-keto-$PGF_{1\alpha}$; TXB, thromboxane B_2; HHT, hydroxyheptadecatrianoic acid; AA, arachidonic acid; and F, E and D, prostaglandins $F_{2\alpha}$, E_2 and D_2 respectively.

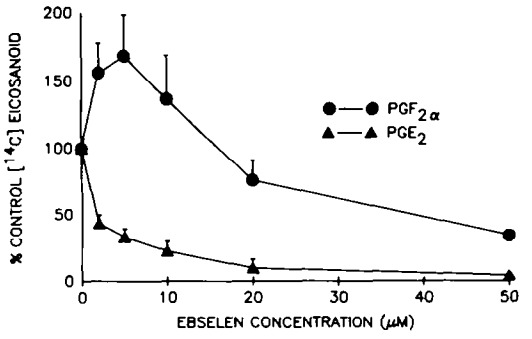

Fig. 2. Effect of ebselen on the metabolism of [^{14}C]arachidonate (0.1 μCi, 3.43 μM) by rabbit iris-ciliary body, using PGE_2 and $PGF_{2\alpha}$ as markers. Data are from radio-TLC scans of homogenate extracts applied to TLC plates developed in solvent system (A). The values correspond to the means ± SD of six different experiments (except for 20 and 50 μM ebselen where N = 4 and 2 respectively). The percentage conversion for $PGF_{2\alpha}$ from arachidonate in the absence of ebselen (control) was 10.3 ± 1.8 and 11.2 ± 1.5, respectively.

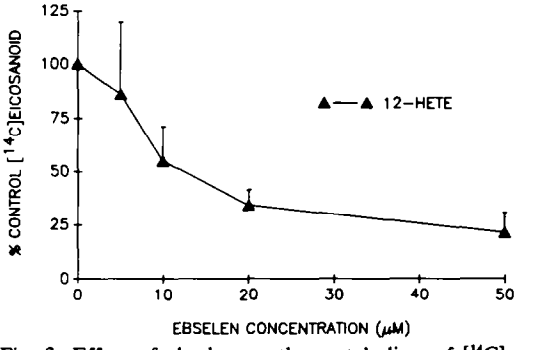

Fig. 3. Effect of ebselen on the metabolism of [^{14}C]arachidonate (0.1 μCi, 3.43 μM) by rat iris-ciliary body, using 12-HETE as a marker. Data are from radio-TLC scans of homogenate extracts applied to TLC plates developed in solvent system (A). The values correspond to the means ±SD of five different experiments. The percentage conversion for 12-HETE from arachidonate in the absence of ebselen (control) was 22.5 ± 6.6.

in solvent system (A): trimethyl pentane/ethyl acetate/water/acetic acid (25:55:50:10, by vol.), for the separation of polar eicosanoids including prostaglandins. The other plate was developed in either solvent system (B): benzene/dioxane/acetic acid (30:20:1, by vol.), an alternative system for the separation of prostanoids, or solvent system (P): diethyl ether/petroleum-ether/acetic acid (60:40:1, by vol.), which more clearly separates the less polar arachidonate metabolites such as hydroxyheptadecatrianoic acid (HHT) and the monohydroxyeicosatetraenoic acids and arachidonate. Thin-layer chromatograms were scanned by an Isomess IM-3016 radio-TLC analyzer (Raytest U.S.A. Inc., McMurray, PA). The radioactive peaks that co-migrated with unlabelled standard arachidonate metabolites were analyzed by the software package accompanying the radio-TLC analyzer. The percentage conversion for each eicosanoid generated from [^{14}C]arachidonate in the absence of ebselen (control) was designated as 100%. Radioactive counts for each arachidonate metabolite, in the presence of ebselen, were expressed as a percentage of the control value for respective metabolites.

Gas–liquid chromatography and mass spectrometry (GLC–MS). Iris-ciliary body homogenates were incubated with unlabeled sodium arachidonate (3.43 μM) in the presence or absence of ebselen (5 μM). The eicosanoids were extracted with ethyl acetate as described above. The extracts were derivatized into *tert*-butyl bis-(dimethylsilyl), (BDMS), ethers before GLC–MS analysis by a process that converted prostaglandins, in a sequence of reactions, to methyl esters, methoximes and BDMS ethers. The GLC–MS analysis was performed on an HP-580 gas chromatograph with a mass selective detector (Hewlett–Packard, Palo Alto, CA). The GLC was fitted with an HP-1, methyl silicone column (15 m × 0.18 mm, Hewlett–Packard). Helium was used as the carrier gas, and the electron energy was 70 eV.

RESULTS

Representative thin-layer radio-chromatograms demonstrating separation of arachidonate metabolites from cornea and iris-ciliary body, using the solvent systems described above, are shown in Fig. 1. In general, the principal data described are those obtained with the solvent system (A), although the other solvent systems were valuable from a confirmatory standpoint.

Effect of ebselen on arachidonate metabolism by ocular tissues. The biosynthesis of cyclooxygenase products by the rabbit iris-ciliary body, with the exception of material co-chromatographing with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in both TLC solvent systems (A) and (B), was inhibited strongly by ebselen in a concentration-dependent manner. Figure 2 shows the pattern of cyclooxygenase inhibition by ebselen using PGE_2 as a marker of cyclooxygenase metabolism; the stimulation of $PGF_{2\alpha}$ synthesis is also depicted. The formation of $PGF_{2\alpha}$ by the iris-ciliary body was stimulated by ebselen at low concentrations, but inhibited at higher concentrations. The identification of $PGF_{2\alpha}$ was confirmed by GLC–MS analysis. The IC_{50} values for ebselen inhibition in iris-ciliary body were 2 μM for PGE_2 alone and 9.3 μM for the sum of all cyclooxygenase products, including $PGF_{2\alpha}$. Indomethacin at a 0.4 μM concentration inhibited cyclooxygenase activity in iris-ciliary body to the same extent as 9.3 μM ebselen. The formation of $PGF_{2\alpha}$ by iris-ciliary body in the presence of 5 μM ebselen had increased by 69% (Fig. 2); data obtained from chromatograms developed in solvent system (B) indicated an 80% stimulation of the biosynthesis of $PGF_{2\alpha}$. Analysis by GLC–MS of derivatized extracts from homogenates incubated with unlabeled arachidonate, in the presence of 5 μM ebselen, also showed an increase in the production of $PGF_{2\alpha}$ and a strong inhibition of the formation of other cyclooxygenase metabolites.

The principal metabolite of arachidonic acid formed by the rat iris-ciliary body co-chroma-

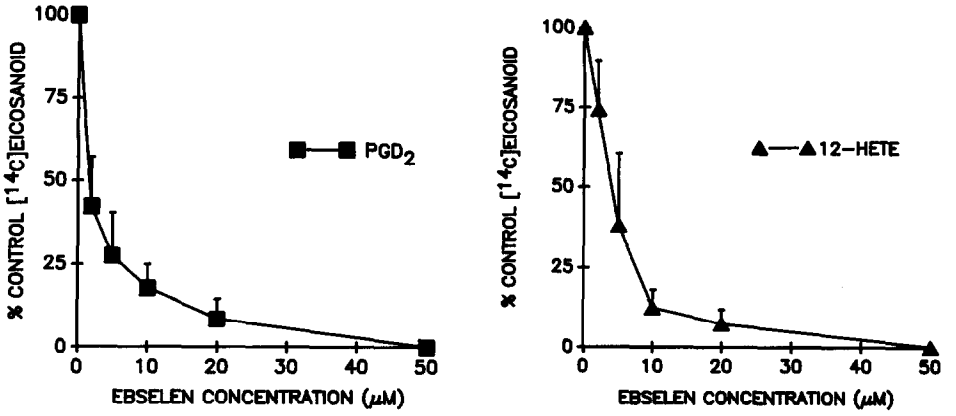

Fig. 4. Effect of ebselen on the metabolism of [^{14}C]arachidonate (0.1 μCi, 3.43 μM) by rabbit cornea, using PGD_2 and 12-HETE as markers. Data are from radio-TLC scans of homogenate extracts applied to TLC plates developed in solvent system (A). The values correspond to the means ± SD of five different experiments (except for 50 μM ebselen where N = 2). The percentage conversion for PGD_2 and 12-HETE from arachidonate in the absence of ebselen was 11.6 ± 2.5 and 15.2 ± 3.6, respectively.

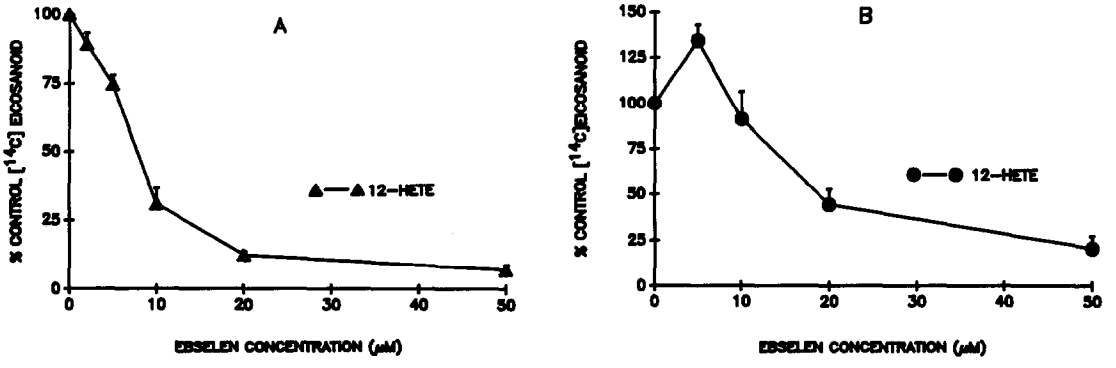

Fig. 5. Effect of ebselen on the metabolism of [^{14}C]arachidonate (0.1 μCi, 3.43 μM) by rat cornea, using 12-HETE as a marker. Data are from radio-TLC scans of homogenate extracts applied to TLC plates developed in solvent system (A). With the addition of glutathione (1 mM), 12-HETE production was inhibited by all concentrations of ebselen (panel A). In the absence of GSH (panel B), 12-HETE production was stimulated by ebselen at low concentrations. Values are the means ± SD of five different experiments. The percentage conversion for 12-HETE from arachidonate in the absence of glutathione and ebselen was 30.8 ± 5.1; in the absence of ebselen, but with added glutathione, the control conversion was 58.8 ± 7.31.

tographed with 12-hydroxyeicosatetraenoic acid (12-HETE) in both TLC solvent systems (A) and (P). This was in marked contrast to arachidonate metabolism by the rabbit iris-ciliary body which produced predominantly cyclooxygenase products. Ebselen at a concentration of 5 μM did not influence the formation of 12-HETE (Fig. 3), but at higher concentrations (10–50 μM) was inhibitory. The IC_{50} of ebselen for 12-HETE production by the rat iris-ciliary body was 12 μM. Similar data were generated by the TLC solvent system (P).

In the rabbit cornea the formation of all cyclooxygenase products, including $PGF_{2\alpha}$ and material co-migrating with the lipoxygenase metabolite, 12-HETE, in both TLC solvent systems (A) and (P) was decreased by ebselen in a concentration-dependent manner. Figure 4 demonstrates inhibition of prostaglandin and HETE formation, using PGD_2 and 12-HETE as markers. The IC_{50} was 3.8 μM for the collective inhibition of the biosynthesis of prostaglandins and 4 μM for inhibition of the formation of 12-HETE. Ebselen was a more potent inhibitor of 12-HETE formation than nordihydroguiaretic acid (NDGA). However, ebselen and NDGA equipotently suppressed cyclooxygenase activity. Similar data for the inhibition of 12-HETE formation, in the presence of ebselen, were obtained from TLC solvent system (P).

The major arachidonate metabolite formed by the rat cornea was material that co-migrated with 12-HETE in both TLC solvent systems (A) and (P). Data obtained from solvent system (A) are shown in Fig. 5. Ebselen at a 5 μM concentration stimulated the formation of 12-HETE (by approximately 34%) (Fig. 5B). Data from solvent system (P) indicated a similar increase in the production of 12-HETE. At higher concentrations of ebselen there was a concentration-dependent inhibition of 12-HETE for-

mation. The IC_{50} was 19 μM for inhibition of 12-HETE biosynthesis. Similar data were generated by TLC solvent system (P). In the presence of 1 mM reduced glutathione (GSH), the biosynthesis of 12-HETE increased by 81%. Similar data were also generated using TLC solvent system (P). However, there was a concentration-dependent inhibition of 12-HETE production in homogenates incubated with ebselen and GSH, and the IC_{50} was 8 μM for the inhibition of 12-HETE formation in the presence of GSH (Fig. 5A). Similar information was obtained with solvent system (P). Cyclooxygenase metabolism in the rat cornea was inconsistent; in preparations where cyclooxygenase was demonstrated, the principal metabolite formed by the rat cornea co-chromatographed with 6-keto-$PGF_{1\alpha}$ in solvent system (A). The collective IC_{50} for inhibition by ebselen of cyclooxygenase activity in this preparation was 5 μM.

Effect of ebselen on arachidonate metabolism by rabbit non-ocular tissues. In the spleen, the formation of material co-chromatographing with the cyclooxygenase metabolites thromboxane B_2 (TXB_2), PGE_2 and HHT was decreased by ebselen. Figure 6 shows the pattern of inhibition using TXB_2 as a marker. The IC_{50} was 4.1 μM ebselen for the collective inhibition of cyclooxygenase activity. The production of the material co-chromatographing with 12-HETE in both solvent systems (A) and (B) was only inhibited by ebselen at concentrations greater than 20 μM.

In the platelet, the production of material co-migrating with both HHT and TXB_2 was inhibited by ebselen in a concentration-dependent manner (Fig. 7, A and B). Ebselen also suppressed the formation of material co-migrating with 12-HETE (Fig. 7C). The IC_{50} values were 4.7 and 9 μM, respectively, for the inhibition of cyclooxygenase and lipoxygenase activities. Similar data were obtained with TLC solvent system (P).

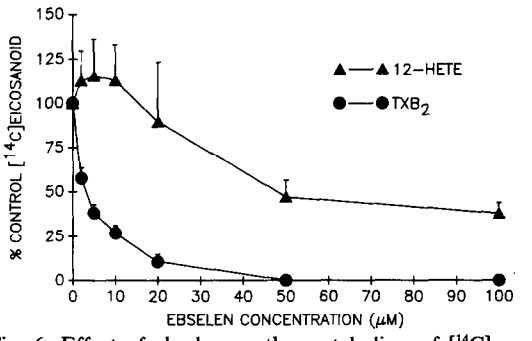

Fig. 6. Effect of ebselen on the metabolism of [^{14}C]arachidonate (0.1 μCi, 3.43 μM) by rabbit spleen, using TXB_2 and 12-HETE as markers. Data are from radio-TLC scans of homogenate extracts applied to TLC plates developed in solvent system (A). The values correspond to the means ± SD of four different experiments. The percentage conversion for 12-HETE and TXB_2 from arachidonate in the absence of ebselen (control) was 20.5 ± 5.0 and 20.6 ± 5.1, respectively.

DISCUSSION

With the exception of $PGF_{2\alpha}$ biosynthesis by the rabbit iris-ciliary body, ebselen inhibited the formation of cyclooxygenase and lipoxygenase metabolites in ocular and non-ocular tissues. Previous studies indicated that cyclooxygenase activity was more sensitive than lipoxygenase to ebselen [11]. The inhibitory mechanism of ebselen on arachidonate oxygenation could be explained by its chemical properties. Ebselen has been shown to have anti-oxidant activity and, in the presence of GSH, functions as a peroxidase [1, 4] and could modulate the "peroxide tone" that is hypothesized to regulate arachidonate metabolism by removing peroxide activators of cyclooxygenase and lipoxygenase activities [15]. Ebselen also reacts with sulfhydryl groups, and enzyme inactivation by the interaction of ebselen with amino acid residues at catalytic and/or allosteric sites has been suggested to explain inhibition of NADPH–cytochrome P-450 reductase activity [16] by ebselen.

The stimulation of $PGF_{2\alpha}$ production in rabbit iris-ciliary body by low concentration ebselen was confirmed in this study by identification of the metabolite by GLC–MS. Enhanced $PGF_{2\alpha}$ could represent a diversion of cyclooxygenase activity, inhibited by ebselen, into the $PGF_{2\alpha}$ pathway. A number of mechanisms have been proposed for the conversion of arachidonate into $PGF_{2\alpha}$. A specific reducing agent of unknown structure, that is heat-labile and contains a thiol group, has been demonstrated in tissues such as the guinea pig and bovine endometrial microsomes to directly catalyze the reduction of the end-product of the cyclooxygenase–PG endoperoxide reductase complex, PGH_2, to $PGF_{2\alpha}$ [17]. Glutathione *S*-transferases (ligandins) are also reported to transform PGH_2 to $PGF_{2\alpha}$ [18]. PGH_2 may also be non-enzymatically reduced to $PGF_{2\alpha}$ in the presence of GSH and trace transition metals [18]. Both PGE_2 and PGD_2 have been observed to be reduced to $PGF_{2\alpha}$ by reactions catalyzed by specific PG NAD-dependent 9-keto-reductases [19]. Why concentration of ebselen that inhibited the formation of $PGF_{2\alpha}$ and other cyclooxygenase metabolites by the rabbit cornea and non-ocular tissues should stimulate the biosynthesis of $PGF_{2\alpha}$ by the rabbit iris-ciliary body cannot be explained by the data.

The selective stimulation of $PGF_{2\alpha}$ formation by the iris-ciliary body, in the presence of ebselen, may be desirable since $PGF_{2\alpha}$ has been reported recently to act as an ocular hypotensive agent [20, 21] and is under consideration for glaucoma therapy. However, the anomalous stimulatory effect of ebselen on rabbit iris-ciliary body biosynthesis of $PGF_{2\alpha}$ is species and tissue related and must be evaluated further.

Another possible beneficial effect of ebselen could be attenuation of the inflammatory response because it not only inhibits prostaglandin formation but also suppresses the biosynthesis of 12-HETE by the cornea. The (*R*) isomer of 12-HETE has been identified recently in corneal epithelial microsomes [22] and has been demonstrated to be more chemotactic for polymorphonuclear leukocytes than the (*S*) isomer generated by platelet and lung lipoxygenases [23]. The enhanced inhibition of 12-HETE formation in the rat cornea by ebselen in combination with GSH, compared to ebselen alone, was presumably related

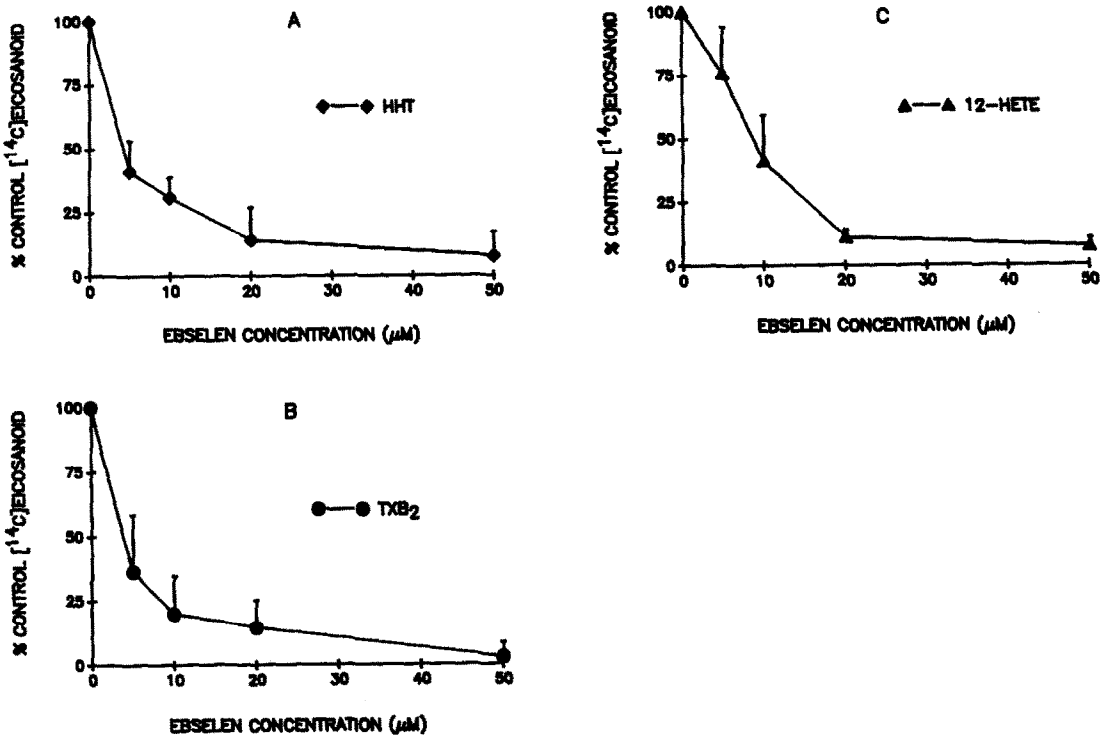

Fig. 7. Effect of ebselen on the metabolism of [^{14}C]arachidonate (0.1 μCi, 3.43 μM) by rabbit platelets, using HHT, TXB_2 and 12-HETE as markers (panels A, B, and C respectively). Data are from radio-TLC scans of homogenate extracts applied to TLC plates developed in solvent system (A). The values correspond to the means ± SD of four different experiments. The percentage conversion for HHT, 12-HETE and TXB_2 from arachidonate was 7.7 ± 3.7, 37.4 ± 6.4, and 10.6 ± 2.5, respectively.

to its peroxidase activity scavenging the required peroxide activators for enzyme activity. It is possible, therefore, that the endogenous levels of GSH in tissues could influence the inhibition of arachidonate metabolism by ebselen.

The formation of 12-HETE in the rabbit spleen was in marked contrast to its biosynthesis in ocular tissues, and rabbit platelet was not inhibited by ebselen. Spleen and platelet are of mesodermal origin, and it is most unlikely that their lipoxygenases are structurally different. Lipoxygenases, unlike the microsomal cyclooxygenase and PG isomerases, are predominantly cytosol enzymes. It is conceivable, therefore, that the lack of effect of ebselen in the spleen may be a result of metabolism in the cytosol, or perhaps the accessibility of ebselen to the spleen lipoxygenase is restricted by interaction of the selenium atom of ebselen with sulfhydryls of spleen cytosol proteins forming disulfide bridges.

Ebselen, because it inhibits both cyclooxygenase and lipoxygenase activities, is of potential clinical interest in the treatment of inflammatory conditions such as uveitis, which are mediated by arachidonate metabolites. Ebselen is presently under evaluation as an anti-inflammatory agent in animal models of ocular inflammation in our laboratory.

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