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STUDIES ON THE ANTI-INFLAMMATORY ACTIVITY OF EBSELEN

EBSELEN INTERFERES WITH GRANULOCYTE OXIDATIVE BURST BY DUAL INHIBITION OF NADPH OXIDASE AND PROTEIN KINASE C?

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Abstract—Ebselen (PZ51, 2-phenyl-1,2-benzoisoselenazol-3-(2H)-one) was shown to be an inhibitor of human granulocyte oxidative burst stimulated by phorbol myristate acetate (IC_{50} 25 μM). Estimation of the primary oxygen metabolites of the burst was complicated by the redox chemistry of Ebselen. Ebselen inhibited NADPH-stimulated superoxide generation by a partially purified NADPH oxidase preparation with an IC_{50} of 0.5–1.0 μM. Ebselen was also shown to inhibit the activity of partially purified Ca^{2+}- and phospholipid-dependent protein kinase C (IC_{50} *ca.* 0.5 μM). Phorbol ester-stimulated phosphorylation of protein in intact cells was inhibited by Ebselen (IC_{50} *ca.* 50 μM). These pharmacodynamic properties of Ebselen are discussed in terms of its anti-inflammatory activity *in vivo*. The findings are also discussed in terms of Ebselen's known ability to interact with sulfhydryl components of cells, particularly critical thiol components of the enzymes studied.

As understanding of the complex events occurring during inflammation increases, so does the possibility of designing novel anti-inflammatory agents as alternatives to the conventional steroidal, non-steroidal and "disease modifying" compounds. One current area of interest concerns the multiple roles of oxygen, reactive oxygen metabolites (ROMs) and peroxygenated lipid metabolites in the simulation, propagation and maintenance of both acute and chronic physiological and pathophysiological inflammatory processes (for reviews see Refs 1 and 2). Thus, ROMs such as $O_2^{\cdot-}$ and H_2O_2, which are produced by monocytes and granulocytes through the activity of the NADPH oxidase-dependent oxidative burst (OB), may be important both as a "triggering" signal upon initial tissue infection or injury [3] and in the development of irreversible damage to tissue (cytotoxicity, fibrosis, hyperplasia formation) in chronic inflammations [1, 4]. Concurrently ROMs stimulate the peroxidation of membrane lipids, via free radical intermediates, to hydroperoxide and aldehydic species which may possess both physiological and toxicological activities. Membrane peroxidation and other more specific stimuli also cause the release of membrane-bound arachidonic acid which, amongst other things, may be rapidly metabolised to prostaglandins (PGs) and leukotrienes (LTs) through the activity of the prostaglandin synthetase (PGS) and lipoxygenase (LO), respectively. These lipid metabolites play important roles in chemotaxis of granulocytes and the control of vascular endothelial cell function (for a review see Ref. 5).

Ebselen (PZ51, 2-phenyl-1,2-benzoisoselenazol-3-(2H)-one) [6] is a selenium-containing heterocycle which has been indicated in [7], and demonstrated efficacy on, several model inflammatory conditions in animals. These models include cobra venom paw edema [8], monoarthritis [9] and alveolitis and broncheolitis [10]; Ebselen has proven less effective in ameliorating carrageenan paw oedema and adjuvent arthritis [8]. The mechanism of Ebselen-dependent anti-inflammatory activity in these models is a matter of current debate, as the compound has been shown to have several pharmacodynamic activities in *in vitro* systems. Ebselen is a potent antioxidant inhibiting lipid peroxidation in both microsomes and isolated cells [11–13]. Ebselen also possesses a unique glutathione peroxidase-like (GSH-px-like) activity [11, 12], reducing H_2O_2 and other hydroperoxides using thiol cosubstrates such as GSH and *N*-acetylcysteine (NAC). The GSH-px-like activity of Ebselen has been shown to catalyse the conversion of LTB_4 to an inactive isomer [14], and it may also catalyse the reduction of endoperoxide products of lipoxygenases and the reduction of PGG_2 to PGH_2 [5]. Ebselen has also been shown to protect cells undergoing "redox cycling" oxidative stress [12]. In addition, Ebselen has been shown to directly inhibit mammalian leukocyte 5′ lipoxygenase [15, 16] and cyclo-oxygenase [16]. Ebselen has also been shown to inhibit the secretion of both PGE_2 and glucuronidase from macrophages [17].

Presently, it is uncertain which of Ebselen's numerous activities is responsible for the compound's efficacy. For example, Ebselen's inhibition of granulocyte infiltration and induction of interstitial oedema within the lungs of rats treated with Sephadex intratracheally was not enhanced by coadministration of thiols [10]. This indicates that the GSH-px-like activity may not be responsible for the efficacy of Ebselen in the lung edema model and

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some other specific property of Ebselen may be critical.

Previously, Ebselen was shown to inhibit oxidative burst-dependent chemiluminescence in opsonised zymosan treated macrophages, although the mechanism of this was not investigated and was thought to occur through the antioxidant potential of Ebselen [17]. Ebselen has also recently been shown to inhibit a partially purified NADPH oxidase fraction from guinea-pig peritoneal granulocytes [18]. Here we report a further pharmacodynamic property of Ebselen which may have some bearing on study of the mechanism of its anti-inflammatory activity. We have studied the effect of Ebselen on the expression of human granulocyte oxidative burst at various levels of the process. Ebselen may inhibit the phorbol ester-stimulated oxidative burst in normal human peripheral granulocytes by being able to inhibit both the activity of the terminal NADPH oxidase and the activity of protein kinase C(PKc).

MATERIALS AND METHODS

Carrier-free [^{32}P]orthophosphoric acid (in 20 mM HCl; sp. act. 9000 Ci/mmol) was purchased from New England Nuclear (Dreieich, F.R.G.). Ebselen was the kind gift of A. Nattermann and Cie GmbH, (Köln, F.R.G.). Phorbol myristate acetate (PMA), phosphatidyl-L-serine (PS), 1,2-dioleoyl-sn-glycerol (DAG), histone type III S, phenyl-methylsulfonyl-fluoride (PMSF), leupeptin and superoxide dismutase (SOD) were all obtained from Sigma Chemical Company (St Louis, MO, U.S.A.), and DEAE cellulose (DE-52) was from Whatman, U.K. All other chemicals and materials were as referenced and obtained from local suppliers.

Granulocyte preparation. Peripheral blood granulocytes (95% neutrophils by Giemsa stain) were isolated from fresh bank blood using standard Ficol-Pak separation (Pharmacia, Uppsala, Sweden). Typically, 400–500 × 10^6 cells (>95% viability by trypan blue exclusion) were obtained from 500 ml blood. Cells were suspended and incubated at 37° in, unless otherwise stated, Krebs–Henseleit buffer pH 7.4 containing 12.5 mM Hepes.

Oxygen uptake determinations. Cells (5 × 10^6) were incubated in a volume of 3 ml at 37° and oxygen consumption assessed using a Clarke electrode with a YSI model 150 oxygen monitor. Cells were stimulated by the addition of PMA (100 nM final concentration) in 10 μl DMSO, and Ebselen (from a 10 mM stock in DMSO) was administered either before PMA or at the point of maximal stimulated oxygen uptake. Under these conditions, Ebselen did not cause acute cytotoxicity as assessed by trypan blue uptake. The electrode was calibrated with dithionite.

Superoxide anion radical production. The measurement of O_2^- production from PMA-stimulated cells and NADPH-stimulated cell particulates was achieved by quantitating the reduction of acetylated cytochrome C (AcCyt C) as described previously [19]. O_2^--dependent AcCyt C reduction was assessed as the SOD-inhibitable portion of this. Ebselen was added either before the initiation of O_2^--generation or at the point of maximal rate of O_2^--generation. Controls were performed in order to assess if Ebselen reacts directly with AcCyt C. Controls were also performed in order to assess if Ebselen inhibits the activity of SOD.

Hydrogen peroxide accumulation. In some experiments H_2O_2 accumulation in incubations of PMA-stimulated cells (2 × 10^6/ml) for 15 min was assessed by formation of the ferrithiocyanate complex [20]. In order to detect the peroxide it was necessary to inhibit endogenous catalase and myeloperoxidase using azide (10 mM), which was not cytotoxic to the cells. Control experiments were performed for a similar time period to determine if Ebselen and H_2O_2 react chemically.

Partial purification of NADPH oxidase. NADPH oxidase activity was partially purified from human granulocytes essentially according to the method of Green *et al.* [21]. Briefly, cells (200 × 10^6 in 5 ml buffer) were incubated at 37° for 5 min in the presence of PMA (10 μg/ml). Cells were then pelleted and resuspended in ice-cold 10 mM Tris–HCl buffer pH 7.0 containing EGTA (2 mM), PMSF (0.1 mM), and leupeptin (0.1 mg/ml) and sonicated (4 × 15 sec pulses at 4 μm amplitude) using a Soniprep 150 probe sonicator (MSE Instruments). The lysate was then centrifuged (27,000 *g*, 30 min at 4°) and the pellet resuspended in 1 mM Tris–HCl buffer pH 8.6 containing 15% glycerol. Protein was determined by the method of Peterson [22]. Assays of O_2^- generating capacity of the NADPH-oxidase-enriched fraction were performed directly in the quartz cuvette and contained particulate protein (0.1 mg) and Triton X-100 (0.2 mM). Reactions were initiated with NADPH (2 mM) [21]. Reactions were run for 1 min and were linear over this period. Assessment of slope was made at the same time point (10 sec) for all samples.

Partial purification and assay of protein kinase C. A subcellular fraction containing Ca^{2+}- and phospholipid-dependent PKc activity was prepared from human granulocytes as follows: cells (300 × 10^6) were washed once and resuspended in 4 ml ice-cold 20 mM Tris–HCl buffer pH 7.5 containing sucrose (250 mM), EGTA (10 mM), EDTA (2 mM), PMSF (0.1 mM), and leupeptin (0.1 mg/ml), and sonicated on ice (4 × 10 sec pulses at 4 μ). Sonicates were then centrifuged (100,000 *g*, 60 min at 4°) and the supernatant (*ca.* 4 ml) was applied to a DE-52 cellulose column (6.5 ml bed volume), pre-equilibrated (at 6°) with 20 mM Tris–HCl buffer pH 7.5 containing EGTA (1 mM) and EDTA (2 mM). The column was first washed for 20 min with equilibration buffer (1 ml/min) and the PKc containing fraction was subsequently eluted with the same buffer containing NaCl (0.117 M).

Calcium- and phospholipid-dependent PKc activity was assessed by a modification of the method of Kikkawa *et al.* [23]. Ebselen was preincubated with PKc enzyme (0.1–1 μg protein) for 30 sec prior to initiation of the reaction by addition of the following assay mixture: 20 mM Tris–HCl buffer, pH 7.5 at 30°, [γ^{32}P]ATP (10 μM; *ca.* 0.1 μCi), histone III-S (200 μg/ml), $MgCl_2$ (5 mM), PS (20 μg/ml), $CaCl_2$ (5 mM), and DAG (1 μg/ml). The final volume of the complete reaction mixture was 150 μl. The amount of partially-pure PKc protein added was

adjusted so that linearity of histone phosphorylation was maintained over the entire assay period (5 min). Calcium- and lipid-independent kinase activity was measured by replacing calcium and the lipids with EGTA (5 mM) and Tris–HCl (20 mM), respectively. The final concentration of DMSO in all assays was 1%; DMSO had no effect on control PKc activity. Incubations were terminated by quenching with 0.5 ml of 50% (w/v) ice-cold TCA. The samples were stored at 4° for at least 1 hr prior to collection of the acid-precipitable radioactive material on nitrocellulose membrane filters (Sartorius SM 11300, 0.45 μm pore size). The filters were washed (4 × 0.74 ml, 10% w/v TCA) and radioactivity was then quantitated by liquid scintillation counting (Instagel scintillation cocktail, Packard) using a Beckman LS 1801 counter.

Protein phosphorylation in intact cells. In some experiments the PKc activity of intact granulocytes was determined as PMA-stimulated incorporation of ^{32}P into total cellular protein. Cells (200×10^6) were incubated for 60 min in phosphate-free Krebs-HEPES buffer pH 7.4 (5 ml) supplemented with 1% glucose and ^{32}P (500 μCi). Cells were then washed with buffer (4 × 1 ml) to remove unincorporated label and used immediately in stimulation studies. Samples containing 5×10^6 cells/ml (1 ml) were preincubated with Ebselen or DMSO for 5 min at 37° and treated with DMSO vehicle or PMA (100 nM) for 10 min at 37°. The total DMSO concentration did not exceed 1% and did not effect the response. Reactions were terminated by the addition of TCA (50% w/v, 1 ml) and the precipitated protein washed extensively with TCA (10% w/v, 4 × 1 ml) and acetone (1 ml). The protein was then dissolved in 30 mM Tris–HCl buffer pH 6.9 containing SDS (1% w/v), and glycerol (15% w/v) and aliquots taken for protein determination. Radioactivity was determined as above.

RESULTS

Cellular oxygen uptake experiments

Under the conditions employed, oxygen consumption of human granulocytes rose from <0.2 nmol/min/10^6 cells to 2.1 ± 0.2 nmol/min/10^6 cells (N = 3) upon addition of PMA (100 nM). When Ebselen was preincubated with the cells prior to stimulation with PMA there was a concentration dependent inhibition of PMA-stimulated oxygen uptake (Fig. 1), with 50% inhibition (IC_{50}) at *ca.* 22 μM. Similar effects were seen when Ebselen was added following initiation of oxygen uptake (data not shown), with an IC_{50} of *ca.* 30 μM. The inhibition of stimulated O_2 consumption was not reversed when cells were washed (×2) following addition of Ebselen.

Cellular superoxide anion radical generation

Stimulation of cells with PMA resulted in reduction of AcCyt C at a rate which indicated the production of 1.7 ± 0.1 nmol O_2^-/min/10^6 cells (N = 3) (Fig. 2A). When Ebselen (25 μM) was preincubated with the cells prior to stimulation there was total inhibition of the detection of superoxide (Fig. 2B). However, 10 μM Ebselen had no effect

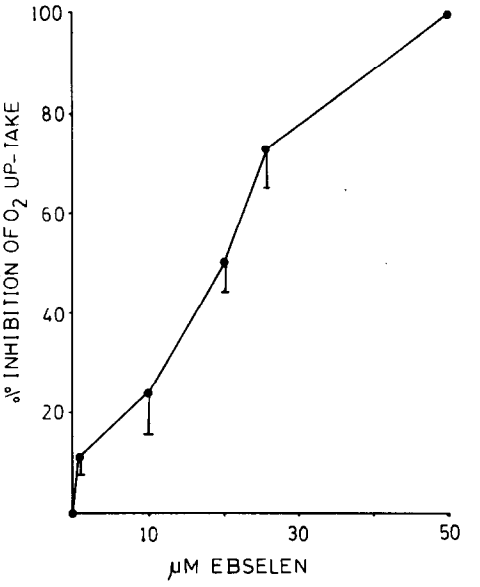

Fig. 1. The effect of Ebselen on PMA-stimulated O_2 uptake in human granulocytes. Cells (5×10^6 in 3 ml buffer) were stimulated at 37° with PMA (100 nM) in the absence and presence of Ebselen. PMA-stimulated control uptake was 2.1 ± 0.2 nmol O_2/min/10^6 cells (N = 3). Data are expressed as mean ± SEM (N = 3).

on PMA-stimulated reduction of AcCyt C (Fig. 2C). Interestingly, in the presence of 10 μM Ebselen the reduction of AcCyt C was not inhibitable by SOD; subsequent addition of more Ebselen to give a final concentration in excess of 25 μM did inhibit further AcCyt C reduction (Fig. 2C). When Ebselen was preincubated with cells at 1 μM, AcCyt C reduction was fully inhibited by SOD (Fig. 2E). Ebselen did not react directly with AcCyt C (Fig. 2D). Ebselen-dependent inhibition of superoxide production could not be washed out of cells treated with >25 μM Ebselen, nor could the apparent superoxide independent reduction of AcCyt C seen with 10 μM Ebselen. Ebselen (up to 200 μM) did not inhibit SODs inhibition of AcCyt C reduction induced by xanthine oxidase-catalysed metabolism of xanthine (data not shown).

Cellular hydrogen peroxide accumulation

When control cells were stimulated with PMA H_2O_2 could not be detected in the incubation. The inclusion of 10 mM azide, however, gave a PMA-stimulated accumulation of H_2O_2 of 4.5 ± 1.2 nmol/10^6 cells (N = 3) after 15 min (= 0.3 nmol/min/10^6 cells). Figure 3A shows a concentration-dependent inhibition of this accumulation in cells preincubated with Ebselen (IC_{50} = 20 μM).

In control experiments the chemical reaction between Ebselen and H_2O_2 was assessed. Figure 3B shows that when Ebselen and the peroxide were incubated together under the conditions of the cell experiments, i.e. for 15 min, considerable reduction of H_2O_2 resulted.

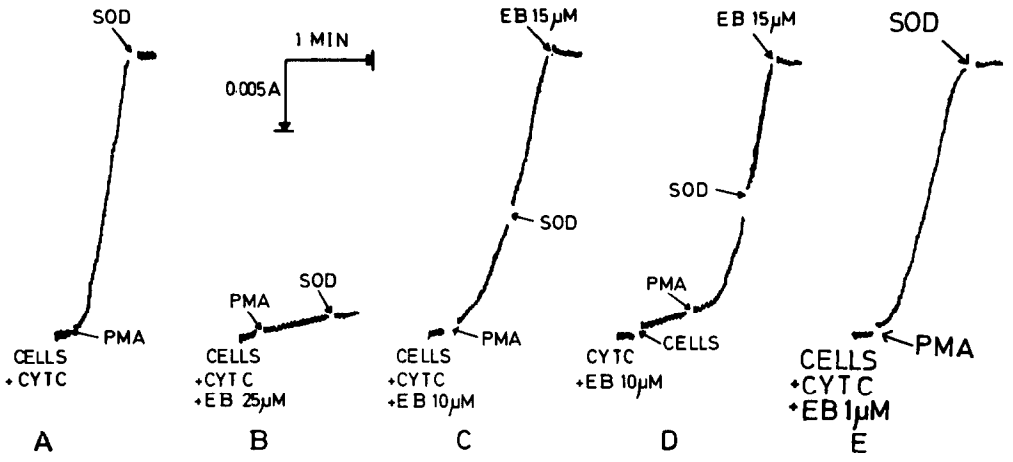

Fig. 2. The inhibitory effect of Ebselen on PMA-stimulated acetylated cytochrome C reduction by human granulocytes. Cells (10^6 in 1 ml) were incubated with the detection system described in the methods. Trace A shows the SOD-inhibitable portion of control reduction (1.7 ± 0.1 nmol O_2^-/min/10^6 cells). Trace B depicts control cells preincubated with Ebselen (25 μM). Trace C shows the effect of preincubation with Ebselen (10 μM) on PMA-stimulated cytochrome C reduction. Trace D was obtained from the incubation of cytochrome C and Ebselen (10 μM) before addition of cells and PMA. Trace E shows the effect of Ebselen (1 μM) on SOD-inhibitable cytochrome C reduction.

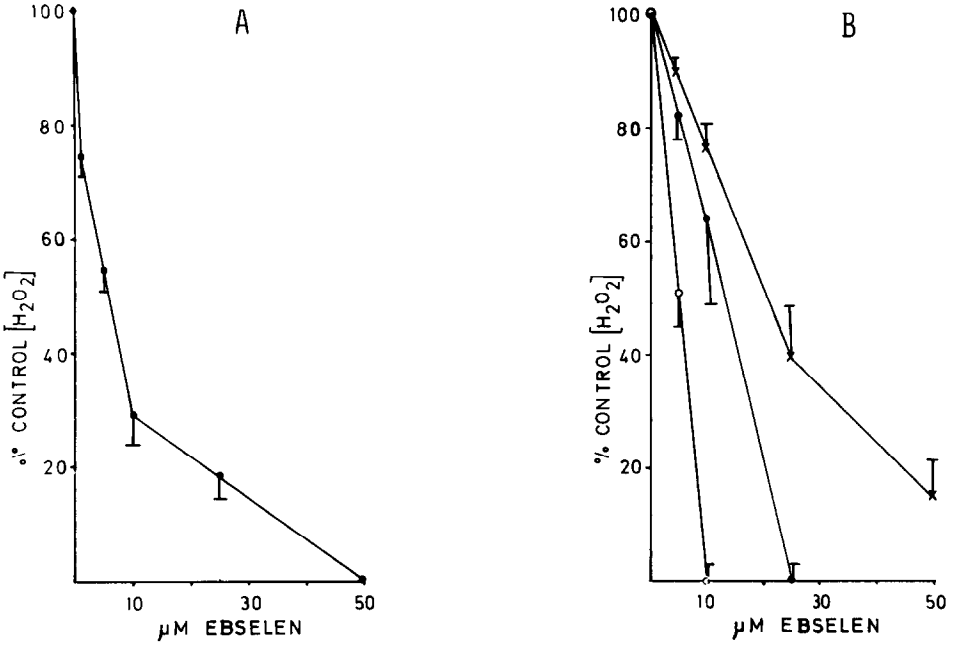

Fig. 3. Inhibition of PMA-stimulated H_2O_2 accumulation in human granulocytes by Ebselen. Azide-treated (10 mM) cells (2×10^6 in 2 ml) were incubated at 37° in the absence and presence of Ebselen for 15 min. Control accumulation was 4.3 ± 1.2 nmol H_2O_2/15 min/10^6 cells (*ca.* 0.3 nmol/min/10^6 cells) (N = 3). A shows the effect of preincubated Ebselen on control accumulation. B shows the chemical reduction of H_2O_2 (○—○ 5 μM; ●—● 10 μM; ×—× 25 μM) by Ebselen in control incubations at 37° for 15 min without cells. Data expressed as mean ± SEM (N = 3).

Superoxide production by partially purified NADPH oxidase

The particulate fraction of PMA-stimulated granulocytes was shown to produce 8.5 ± 0.1 nmol O_2^-/min/mg protein upon addition of NADPH. Figure 4 shows that Ebselen caused a concentration-dependent inhibition of O_2^- production when either preincubated with the protein or added after stimulation (IC_{50}s = 0.5 μM and 1.0 μM Ebselen, respectively). In contrast to intact granulocytes,

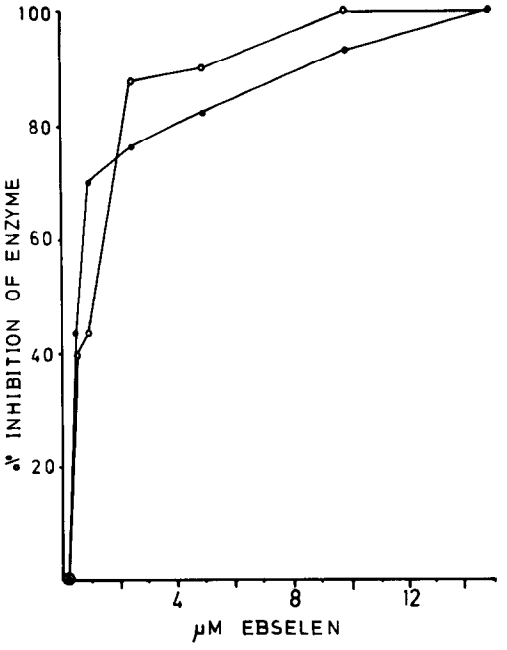

Fig. 4. The inhibition of human granulocyte NADPH oxidase by Ebselen. NADPH oxidase was partially purified and O_2^- generation assessed as described in Materials and Methods. Control generation was 8.5 ± 0.1 nmol O_2^-/min/mg protein (N = 3). ●—● = preincubation with Ebselen; ○—○ = addition of Ebselen after NADPH.

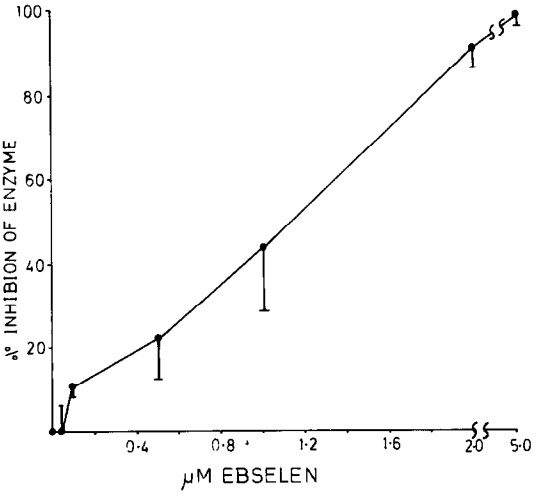

Fig. 5. Ebselens inhibition of a partially purified PKc activity from human granulocytes. Ca^{2+}/PS/OAG-dependent PKc was partially purified and assayed as described in Materials and Methods. Control incorporation of radioactivity into histone III-S was 5.0 ± 0.4 nmol ^{32}P/min/mg protein (N = 4). Data expressed as mean ± SEM (N = 4).

SOD completely inhibited AcCyt C reduction at all concentrations of Ebselen tested.

Partially purified PKc activity

The partially purified granulocyte cytosolic PKc preparation catalysed the incorporation of 5.0 ± 0.4 nmol ^{32}Pi/min/mg histone protein (N = 4) when stimulated with Ca^{2+}, PS and DAG. When Ebselen was preincubated with the preparation there was a concentration-dependent inhibition of this reaction (Fig. 5) with an IC_{50} of 1 μM. Ca^{2+}- and lipid-independent kinase activity constituted no more than 3% of the total kinase activity.

PKc-dependent protein phosphorylation in intact cells

The data shown in Table 1 indicates that there was considerable background ^{32}P-labeling of cellular proteins during the prestimulation loading period with carrier-free ^{32}Pi (*ca.* 10^5 dpm/mg protein). Stimulation of the cells with PMA (100 nM) resulted in a 35% increase in protein labeling (to 1.36×10^5 dpm/mg protein). Addition of Ebselen at increasing concentrations to loaded cells without stimulation had no effect on background labeling. However, Ebselen did produce a concentration-dependent inhibition of PMA-stimulated incorporation of label (IC_{50} *ca.* 50 μM). When Ebselen was included during the loading period, some inhibition of background incorporation of label was seen which amounted to *ca.* 40% inhibition at concentrations above 100 μM Ebselen (data not shown).

DISCUSSION

We have shown that Ebselen inhibits the oxidative burst of human granulocytes by interaction with at least two critical components of the burst mechanism, PKc and NADPH oxidase. Ebselen inhibited PMA-stimulated O_2-uptake in intact cells with an IC_{50} of 25 μM. Inhibition was not reversed by extensively washing the cells, possibly indicating irreversible binding of Ebselen to the inhibitory site(s). Ebselen inhibited the rate of the burst but not the induction time, a property reported for other agents, such as acrolein, which are known to interact with membrane and intracellular sulfhydryl groups and inhibit OB with similar IC_{50}s to Ebselen [24]. Measurement of the effects of Ebselen on production of the primary ROMs of the burst were complicated in a number of ways.

At higher concentrations (>25 μM) Ebselen totally prevented reduction of AcCyt C without reacting with AcCyt C itself. However, below 15 μM significant SOD-insensitive reduction was seen (Fig. 2C). The reason for this is unclear, but in intact cells may involve rapid reduction of Ebselen by O_2^- to yield the selenium free radical which may then reduce AcCyt C. Selenium is a good electron donor and therefore it is able to interact rapidly with free radical species, a property which is probably responsible for Ebselen's antioxidant properties [11, 12]. Another explanation for this may lie in an interaction of Ebselen with cellular GSH and subsequent reaction of this species with O_2^- generating species which are themselves capable of directly reducing cytochrome C. These await further investigation.

Dismutation of O_2^- yields H_2O_2 which is rapidly reduced by the action of catalase and a variety of peroxidases including myeloperoxidase. If H_2O_2 is to be determined in incubations containing activated granulocytes, these enzymes must be inhibited by

Table 1. Ebselen's inhibition of PMA-stimulated incorporation of ^{32}P into total protein of human granulocytes

Apparent concentration of Ebselen (μM)	10^{-5} × Dpm ^{32}P/mg protein		
	− TPA	+ TPA	Δ Dpm × 10^{-5}
0	1.00 ± 0.05	1.36 ± 0.04	+0.36
10	0.95 ± 0.05	1.25 ± 0.10	+0.30
50	1.03 ± 0.07	1.18 ± 0.15	+0.15
100	1.00 ± 0.13	1.15 ± 0.08	+0.15
200	1.03 ± 0.06	0.94 ± 0.04	−0.07
300	1.08 ± 0.03	1.10 ± 0.09	+0.02

Cells were loaded with $^{32}PO_4^{2+}$ as described in Materials and Methods. Cells were pretreated with Ebselen for 5 min at 37° and then incubated in the presence and absence of PMA (100 nM, 37°, 10 min) and irreversibly bound ^{32}P was assessed by scintillation counting. Data expressed as mean ± SEM (N = 3).

azide prior to initiation of the H_2O_2 assay. In the presence of azide, however, optimal measurement of total H_2O_2 accumulation is not possible as the peroxide may be reduced by other cellular components (e.g. the GSH/GSH-px couple). Ebselen reacts directly with H_2O_2 to form the selenium oxide, a reaction probably central to the GSH-px-like activity of the compound [12, 13]. Indeed, the control experiments clearly demonstrate that the direct reduction of H_2O_2 by Ebselen may totally account for the observed effect of Ebselen on H_2O_2 accumulation in incubations of PMA-stimulated granulocytes (Fig. 3) over the time period tested. This property should be borne in mind when performing similar experiments as the kinetics of reaction appear rapid and dependent on the concentration of both Ebselen and H_2O_2.

In accordance with previous observations [21, 25] a membrane-enriched fraction from PMA-treated granulocytes produced O_2^- upon incubation with NADPH. In contrast to intact cells, Ebselen inhibited O_2^- generation in the membrane fraction with an IC_{50} well below 1 μM, without demonstrating any of the SOD-insensitive reduction of AcCyt C shown in intact cells at 15 μM Ebselen. This latter effect may be due to the higher rate of generation O_2^- by this subcellular fraction relative to intact cells, coupled to the lower concentrations of Ebselen used. Additionally, in terms of the discrepancy between intact cells and the oxidase fraction, it will be noted that GSH was totally absent from the partially purified fraction. Also, it may be that Ebselen is sequestered by specific/nonspecific binding sites in intact cells (e.g. lipids, nucleophiles such as GSH, protein thiols, etc.) thus preventing reaction with O_2^- in solution. This may be supported by the extreme differences in inhibition constants in intact cells and lysed cell fractions. In related experiments, the generation of LTB_4 by intact human granulocytes was inhibited by Ebselen with an IC_{50} of *ca.* 25 μM, whereas the activity of 5′ LO in a cytosolic fraction of the cells was inhibited maximally at 1–2 μM (authors' unpublished observations). Indeed, similar activity was evident for the PKc activities determined in this work. Ebselen has demonstrated similar potency of inhibition of the production of O_2^- from a particulate fraction of guinea pig peritoneal granulocytes [18].

The mechanism of Ebselen-dependent inhibition of NADPH oxidase may involve reaction with critical thiol/disulfide groups in the enzyme. The chemistry of the selenium atom in Ebselen allows it to react directly with free SH-groups and/or exchange with disulfides under physiological conditions. Nonspecific alkylating agents have been shown to inhibit granulocyte OB [24]. Recently, a group of potential anti-inflammatory iodonium compounds has been described possessing some specificity for NADPH oxidase; inhibition of NADPH oxidase by these compounds is probably by sulfhydryl group alkylation [25].

The cyanide-insensitive burst of oxygen consumption by granulocytes, such as neutrophils, results from activation of the otherwise dormant NADPH oxidase in the plasma membrane and other cellular organelles. NADPH oxidase activation results in the rapid production of ROMs via O_2^- [21, 26]. The mechanism of activation of this enzyme is at present under debate. The burst may be triggered by diverse stimuli including the phagocytosis of bacteria and other particulates [27], the binding of chemotactic factors and complement components [28], and the activity of tumor promotor phorbol esters and diacylglycerol analogues [29]. Although all of these stimuli possess differences in their mechanisms of signal transduction, several common events may be involved in generating OB. These include the involvement calcium and calmodulin and the activity of PKc [30]. Protein kinase C is known to be the cellular receptor for the phorbol esters and DAG analogues [31] and may phosphorylate NADPH oxidase during final induction of the OB [32].

Under the conditions employed, incubation of granulocytes with ^{32}Pi resulted in labeling of cellular ATP pools and the irreversible binding of ^{32}Pi to cellular protein to a similar extent as in previous reports [30]. Ebselen inhibited both the PMA-stimulated incorporation ^{32}Pi into the protein of intact cells and the PMA-stimulated phosphorylation of histone III-S by a partially purified Ca^{2+}- and phospholipid-dependent "PKc" preparation. These data strongly suggest that Ebselen may interfere with granulocyte PKc. Again there was great discrepancy between the IC_{50}s obtained with the purified enzyme

preparation and in intact cells, with the former being inhibited at much lower concentrations. In view of the somewhat higher IC_{50} for inhibition of PMA-stimulated protein phosphorylation in intact cells than that seen for inhibition of O_2-uptake, it may be that this inhibitor capacity does not play a major role in the inhibitory effect on the physiological process. As granulocyte PKc has a number of substrates [30] it remains to be seen if even a partial inhibition of the kinase can have a selective inhibition on phosphorylation of granulocyte proteins. In addition to inhibiting PMA-stimulated protein phosphorylation in intact cells, inclusion of Ebselen during the loading period also inhibited the background labeling of protein. This may be due to inhibition of ATP biosynthesis and/or other protein kinases. The mechanism of the apparent inhibition of PKc may again lie in disturbance of protein thiol redox balance. In a similar partially purified PKc preparation to the one described here, PKc activity was readily inhibited by thiol-reactive agents such as *N*-ethylmaleimide and *p*-chloromercuribenzoate (SKD and GK, manuscript in preparation). Indeed, these results with "thiol-specific" reagents strongly indicate that granulocyte PKc is sensitive to changes in thiol redox balance.

Whether these inhibitions of PKc and NADPH oxidase have any relevance to the anti-inflammatory activity of Ebselen *in vivo* remains to be seen. Compounds able to inhibit the OB of granulocytes are being actively investigated as anti-inflammatory agents [25], and it is clear that such activity would greatly affect both acute and chronic inflammation processes. As Ebselen possesses a wide variety of other pharmacodynamic properties relevant to inflammation, experiments must be performed in order to determine if granulocyte OB is inhibited by Ebselen *in vivo* in treated animals undergoing inflammation.

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