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# Differential Effects of Ebselen on Neutrophil Recruitment, Chemokine, and Inflammatory Mediator Expression in a Rat Model of Lipopolysaccharide-Induced Pulmonary Inflammation

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**We postulated that the seleno-organic compound ebselen would attenuate neutrophil recruitment and activation after aerosolized challenge with endotoxin (LPS) through its effect as an antioxidant and inhibitor of gene activation. Rats were given ebselen (1–100 mg/kg i.p.) followed by aerosolized LPS exposure (0.3 mg/ml for 30 min). Airway inflammatory indices were measured 4 h postchallenge. Bronchoalveolar lavage (BAL) fluid cellularity and myeloperoxidase activity were used as a measure of neutrophil recruitment and activation. RT-PCR analysis was performed in lung tissue to assess gene expression of TNF-$\alpha$, cytokine-induced neutrophil chemoattractant-1 (CINC-1), macrophage-inflammatory protein-2 (MIP-2), ICAM-1, IL-10, and inducible NO synthase. Protein levels in lung and BAL were also determined by ELISA. Ebselen pretreatment inhibited neutrophil influx and activation as assessed by BAL fluid cellularity and myeloperoxidase activity in cell-free BAL and BAL cell homogenates. This protective effect was accompanied by a significant reduction in lung and BAL fluid TNF-$\alpha$ and IL-1$\beta$ protein and/or mRNA levels. Ebselen pretreatment also prevented lung ICAM-1 mRNA up-regulation in response to airway challenge with LPS. This was not a global effect of ebselen on LPS-induced gene expression, because the rise in lung and BAL CINC-1 and MIP-2 protein levels were unaffected as were lung mRNA expressions for CINC-1, MIP-2, IL-10, and inducible NO synthase. These data suggest that the anti-inflammatory properties of ebselen are achieved through an inhibition of lung ICAM-1 expression possibly through an inhibition of TNF-$\alpha$ and IL-1$\beta$, which are potent neutrophil recruiting mediators and effective inducers of ICAM-1 expression.** ***The Journal of Immunology*****, 2002, 169: 974–982.**

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one) is a heterocyclic seleno-organic compound of low toxicity that exhibits a unique pharmacological profile (1). Originally, ebselen was developed as an anti-inflammatory drug (2, 3), but other interesting pharmacological activities, including immunomodulation (4), prevention of inositol trisphosphate binding to its receptors (5), protection from reperfusion injury (6), DNA protection from single-strand break formation caused by peroxynitrite (7), and prevention of radiation-induced apoptosis of mouse thymocytes (8), have since been reported. Furthermore, ebselen acts as a scavenger of NO and/or peroxynitrite (9). Ebselen is also capable of inhibiting several enzymes involved in the inflammatory responses, such as NADPH oxidase and protein kinase C, nitric oxide synthases, and 5- and 15- lipoxygenases (1, 9).

It has also been reported that ebselen inhibited human polymorphonuclear leukocyte (PMN)[2] adhesion to, and migration through, cytokine-activated human umbilical vein endothelium in vitro (10). In an in vivo experimental setting in rat immunized with adjuvant (*Mycobacterium butyricum*), it was further shown that ebselen treatment (100 mg/kg/day for 3 days) after the development of arthritis inhibited by 72–79% the PMN migration into arthritic joints (11). Ebselen also inhibited by 50–60% the PMN migration into dermal inflammatory reactions induced with zymosan-activated rat serum (C5adesArg), LPS, or IL-1$\alpha$. The effect of ebselen was apparent within 3 h of initial administration (11). Because the effects of ebselen include a large variety of different molecular actions, it is therefore difficult to decide which properties of the plethora of mechanisms account for the anti-inflammatory effect of ebselen in vivo.

The mechanism of pulmonary PMN migration in rats given intratracheal LPS has been partially characterized and involves production by airway cells of inflammatory cytokines such as TNF-$\alpha$ and IL-1$\beta$ as well as neutrophil-specific chemoattractants known as CXC chemokines, which in rats include the family of cytokine-induced neutrophil chemoattractants (CINCs) and macrophage-inflammatory protein-2 (MIP-2) also known as CINC-3 (12–19). CD18 integrins have also been shown to play a role for full polymorphonuclear cell (PMN) migration. Expression of CD18 adhesion molecules on the PMN surface can be induced by exposure to LPS or TNF-$\alpha$ and to chemokines such as CINC-1 and MIP-2 (20–23). In rats, treatment with Abs to CD11a/CD18 or CD11b/CD18, as well as to their ICAM-1 endothelial cell ligand, attenuated the migration of PMNs to intrapulmonary LPS (24, 25).

The aim of the present study was to investigate the anti-inflammatory effect of ebselen in an inhalational LPS model of airway inflammation and to dissect out the underlying mechanisms. Particularly, we

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[2] Abbreviations used in this paper: PMN, polymorphonuclear cell; CINC, cytokine-induced neutrophil chemoattractant; MIP-2, macrophage-inflammatory protein-2; BAL, bronchoalveolar lavage; iNOS, inducible NO synthase; MPO, myeloperoxidase; LIX, LPS-induced CXC chemokine.



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evaluated the effect of ebselen on LPS-induced neutrophil recruitment and activation, lung and bronchoalveolar lavage (BAL) fluid CXC chemokine, pro- and anti-inflammatory cytokine expression, and ICAM-1 expression in Wistar rats and compared this with the effect of the synthetic steroid dexamethasone. We provide evidence that ebselen inhibits neutrophil recruitment and activation through an inhibitory effect on airway TNF-$\alpha$, IL-1$\beta$, and ICAM-1 expression. These effects were not a global effect of ebselen on LPS-induced gene expression, because the rise in lung and BAL fluid levels for several candidate mediators including CINC-1, MIP-2, inducible NO synthase (iNOS), and IL-10 did not differ between untreated and ebselen-treated animals at the protein or the mRNA level.

## Materials and Methods

### *Induction of acute lung inflammation and drug treatment*

Male Wistar rats (150–180 g) were purchased from Harlan-Olac (Bicester, U.K.) and allowed to acclimatize for at least 5 days before use. Food and water were supplied ad libitum. U.K. Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act 1986 were strictly observed. In preliminary experiments, concentration- and time-dependent relationships for cell influx and cytokine release into the airway lumen were determined in response to aerosolized LPS. In subsequent experiments rats were challenged with either saline or LPS (0.3 mg/ml for 30 min) aerosol using an Ultra-Neb 99 (Sunrise Medical, Wollaston, U.K.). Rats were dosed (1 ml/kg) with ebselen (1–100 mg/kg i.p.), dexamethasone (1 mg/kg p.o.), ebselen vehicle (25% DMSO, 75% polyethylene glycol i.p.), or dexamethasone vehicle (0.5% methylcellulose/0.2% Tween 80 p.o.) 2 h before and 1 h after exposure to aerosolized saline or LPS (0.3 mg/ml (w/v), for 30 min). Animals were sacrificed (sodium pentobarbitone, 200 mg/kg i.p.) 4 h after the end of challenge. Each treatment group consisted of eight animals.

For the therapeutic dosing regimen, Wistar rats (Charles River, Kingston, NY) were dosed with either vehicle or ebselen (30 mg/kg, 1 ml/kg i.p.) at 0.5, 1, and 2 h before LPS challenge. One additional group of rats was received ebselen 1 h before and 2 h after LPS aerosol challenge. Animals were sacrificed 4 h after saline or LPS challenge. BAL fluid and lung tissue samples were processed as described below.

### *Quantification of BAL cellularity*

Four hours after saline or LPS challenge, animals were euthanized with sodium pentobarbitone (200 mg/kg i.p.), and the trachea was cannulated. BAL cells were recovered from the airway lumen by flushing the airways with 10 ml/kg RPMI 1640 delivered through the tracheal cannula and removed after a 30-s interval. This procedure was repeated and samples were then pooled for each animal. The samples were then refrigerated for later use. Total whole cell counts were obtained in BAL samples by using an automated cell counter (Cobas Argos; Roche ABX Hematologie, Montpellier, France). Cytospins of these samples were prepared by centrifugation of 100-$\mu$l aliquots in a cytospin (Shandon, Runcorn, U.K.) at 700 rpm for 5 min with low acceleration at room temperature. Slides were fixed and stained on a Hema-tek 2000 (Ames, Elkhart, IN) with modified Wright's-Giemsa stain. Four-part differential counts on 200 cells/slide were performed following standard morphological criteria, and the percentage of eosinophils, macrophages/monocytes, lymphocytes, and neutrophils were determined.

### *Lung tissue sampling and processing*

After BAL was performed, the thoracic cavity was exposed, the heart and lungs were removed en bloc. The two major lung lobes were dissected out, and the pulmonary vasculature of the lobes was perfused with ice-cold sterile PBS using a Microperspex peristaltic pump to remove the blood pool of cells. The lobes were cut into 5-mm pieces using a tissue chopper, flash frozen in liquid nitrogen, and stored at −80°C for RT-PCR analysis of cytokine protein and gene expression. For the determination of cytokine protein levels, ~200 mg of lung tissue were homogenized in PBS using an Ultraturrax T25 homogenizer (G. Heinemann, Schwäbisch Gmünd, Germany). The samples were then spun in a benchtop microcentrifuge (13,000 × *g*, 20 min, 4°C). The resulting supernatants were used for cytokine protein quantification.

### *Lung and BAL fluid cytokine protein expression*

Cytokine levels in lung homogenates and BAL fluids were determined by ELISA using commercially available kits according to the manufacturer's instructions. TNF-$\alpha$ was determined using a rat-specific sandwich immunoassay kit obtained from R&D Systems (Minneapolis, MN). IL-1$\beta$ and MIP-2 (CINC-3) concentrations were determined using a solid phase sandwich ELISA kit, which used peroxidase and tetramethylbenzidine as a detection method (BioSource International, Camarillo, CA). The detection limit of these assays was determined to be in the range of 1–5 pg/ml. There was no detectable cross-reactivity with other rat and mouse cytokines and chemokines. Because of the high degree of similarity maintained in chemokines across species, kits that used polyclonal Abs for the detection of mouse chemokines could also be used to detect the rat cognate provided that a standard curve was obtained using known concentrations of rat chemokine. Thus, rat CINC levels were assayed using a kit to detect mouse KC (R&D Systems). ODs were read at 450 nm on a microplate reader (Biotek Instruments, Winooski, VT). For lung tissue, cytokine levels were further corrected for protein content using the assay of Lowry et al. (26).

### *Neutrophil myeloperoxidase (MPO) assay*

BAL samples were first processed into cell-free BAL fluid and BAL cell homogenates. Cell-free BAL fluid was prepared by sedimenting the cells by centrifugation (400 × *g*) for 10 min at 4°C, and then the supernatant was collected and stored. The cell pellets were resuspended in 1 ml of lysis buffer (0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer, pH 6.0), and this fraction was designated as BAL cell homogenate. MPO content was determined in BAL fluid fractions using a spectrophotometric reaction with *o*-dianisidine hydrochloride as a substrate as previously described (27). Briefly, 280 $\mu$l of substrate solution (50 mM phosphate buffer containing 0.167 mg/ml of *o*-dianisidine hydrochloride), 10 $\mu$l of $H_2O_2$ (0.0005%), and 10 $\mu$l of sample were added to each cuvet. The addition of sample starts the reaction. $OD_{460}$ was monitored after 0.5 s and then every 10 s for 1 min, and the initial rate was calculated using the kinetics module in the spectrophotometer (Cobas Bio., Roche, France). The OD change per minute was used as a measure of MPO activity. The peroxidase activity was calculated using a molar extinction coefficient (for the oxidized product of *o*-dianisidine hydrochloride) of 0.0113 mol/cm. Data were expressed as units per milliliter (or micromols per minute per milliliter).

### *Lung cytokine gene expression (RT-PCR)*

***RNA extraction.*** Total cellular RNA from rat lung recovered 4 h post-challenge was isolated by guanidium thiocyanate-phenol-chloroform extraction according to the method described by Chomczynski and Sacchi (28). The purity and integrity of the RNA samples were assessed by $OD_{260}/OD_{280}$ spectrophotometric measurements and by agarose gel (1% agarose-formaldehyde gel containing 20 mM morpholinosulfonic acid, 5 mM sodium acetate, and 1 mM EDTA, pH 7.0) electrophoresis.

***Reverse transcription.*** A 1-$\mu$g portion of total RNA was subjected to first-strand cDNA synthesis in a 25-$\mu$l reaction mixture containing avian myeloblastosis virus reverse transcriptase (10 U), dNTP mixture (2 mM concentrations of each dNTP), oligo$(dT)_{12-18}$ primers (10 $\mu$M), and reaction buffer as supplied with the enzyme (50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, and 10 mM DDT). The samples were incubated in a PerkinElmer 480 thermal cycler (PerkinElmer, Wellesley, MA) at 42°C for 60 min followed by enzyme denaturation step at 94°C for 2 min. The reverse transcription mixture was diluted with 25 $\mu$l of RNase-free water and stored at −80°C for use in PCR. All reagents were obtained from Promega (Southampton, U.K.).

***PCR.*** PCR was performed on 4 $\mu$l of reverse transcriptase product using Ready-To-Go PCR beads (Amersham Pharmacia Biotech, Albans, U.K.), containing *Taq* DNA polymerase, dNTP, buffer, and 0.5 $\mu$M concentrations of each gene-specific forward and reverse primers (obtained from Life Technologies, Paisley, U.K.) in a total volume of 25 $\mu$l. Gene-specific oligonucleotide primers, listed in Table I, were designed from published rat sequences. The PCR was conducted in PerkinElmer GeneAmp PCR system 9700. After an initial denaturation at 95°C for 5 min, amplification was conducted through 25–35 cycles of denaturation at 94°C for 30s, annealing at 55°C (GAPDH) or 60°C (for all other transcripts) for 30 s and extension at 72°C for 45 s. Final extension was at 72°C for 7 min followed by a final hold at 4°C. Negative controls (PCR mixture without cDNA) and positive controls (PCR mixture with a standard cDNA sample) were included in preliminary PCR runs. Initial experiments were conducted to determine the optimal annealing temperature for each set of gene-specific primers and also the linear phase of the product amplification curve (data not shown).

The PCR products were separated by electrophoresis using 2% agarose gels stained with ethidium bromide to visualize cDNA products. Bands of each target transcript were visualized by ultraviolet transillumination and captured using a digital camera. ODs for each band were quantified by

Table I. *Rat gene-specific oligonucleotide primer sequences used in PCR*

| Gene | | Primer Sequence (5′→3′) | Product Size (bp) |
|---|---|---|---|
| *TNF-α* | Sense | TACTGAACTTCGGGGTGATTGGTCC | 295 |
| | Antisense | CAGCCTTGTCCCTTGAAGAGAACC | |
| *CINC-1* | Sense | TGAGCTGCGCAGTCAGTGCCTGCA | 205 |
| | Antisense | ACACCCTTTAGCATCTTTTGGACA | |
| *MIP-2* | Sense | GGCACAATCGGTACGATCCAG | 287 |
| | Antisense | ACCCTGCCAAGGGTTGACTTC | |
| *IL-10* | Sense | TGACAATAACTGCACCCACTT | 402 |
| | Antisense | TCATTCATGGCCTTGTAGACA | |
| *ICAM-1* | Sense | AGACACAAGCAAGAGAAGAAAAGG | 425 |
| | Antisense | TTGGGAACAAAGGTAGGAATGTAT | |
| *GAPDH* | Sense | TGAAGGTCGGTGTCAACGGATTTGGC | 983 |
| | Antisense | CATGTAGGCCATGAGGTCCACCAC | |

image analysis software (Imagemaster; Amersham Pharmacia Biotech, Piscataway, NJ). The level of gene expression of each transcript was normalized to that of the housekeeping gene *GAPDH*.

### *Materials*

Ebselen and dexamethasone were synthesized at Aventis Pharma (Dagenham Research Center, Dagenham, Essex, U.K.). LPS from *Escherichia coli* serotype 0111:B4 and all other materials were purchased from Sigma (Poole, U.K.) except for RPMI 1640 (Life Technologies) and sodium pentobarbitone (euthatal; Rhône Mérieux, Harlow, U.K.).

### *Data analysis*

Values are expressed as mean ± SEM of *n* independent observations. Statistical comparisons were made using the Kruskal-Wallis test followed by Dunnett's post-test. All treatments were compared with vehicle-treated groups.

## Results

### *Effect of ebselen on cell recruitment*

Initial LPS dose-response and kinetics studies were performed to provide a reference point for studies described subsequently in which the effect of ebselen on neutrophil recruitment and cytokine and chemokine expression were investigated. Dose-response studies showed that LPS concentrations above 0.1 mg/ml (aerosolized over 30 min) produced significant BAL neutrophilia which was evident at 60 min, reached a plateau at 2–6 h, and returned toward basal levels by 24–48 h postchallenge (data not shown). The LPS dose of 0.3 mg/ml aerosolized for 30 min was selected in subsequent studies with inflammatory indices measured 4 h postchallenge. LPS inhalation induces a significant increase in total cell counts recovered in BAL fluid. The cell population contributing to this increase is mainly neutrophils (Fig. 1).

Injection of ebselen (1–100 mg/kg i.p.) 2 h before and 1 h after exposure to aerosolized LPS challenge significantly attenuated the number neutrophils in BAL fluid as compared with that of vehicle-treated animals receiving LPS (Fig. 1). Ebselen afforded similar inhibition to that seen with the synthetic glucocorticoid dexamethasone (1 mg/kg p.o.) used as an internal standard. Ebselen on its own has no effect on neutrophil influx when compared with saline-vehicle-treated animals.

It has previously been demonstrated that MPO activity is directly related to neutrophil number and can be used as a neutrophil marker (27, 29). Therefore, we have used the MPO assay to assess the extent of neutrophil recruitment into the airway lumen as well

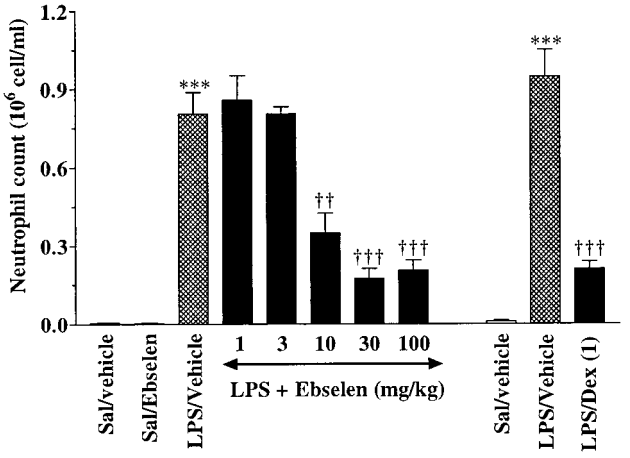


**FIGURE 1.** Effect of ebselen and dexamethasone (Dex) on LPS-induced neutrophil accumulation in BAL fluid. Rats were pretreated with ebselen (1–100 mg/kg i.p.), dexamethasone (1 mg/kg p.o.), or compound vehicles 2 h before and 1 h after exposure to aerosolized saline (Sal) or LPS (0.3 mg/ml w/v) for 30 min as described in *Materials and Methods.* The lungs were then lavaged 4 h after exposure, and neutrophil number was determined in BAL fluid by differential cell counting. Results are expressed as mean ± SEM of eight animals. Statistical significance was assessed using the Kruskal-Wallis test with Dunnett's post-test. ***, $p < 0.001$, compared with saline-vehicle-treated groups; ††, $p < 0.01$; and †††, $p < 0.001$, compared with LPS-vehicle-treated groups.

as to assess the activation state of neutrophils after LPS challenge and the effect of ebselen and dexamethasone. LPS inhalation induces a marked increase in MPO content in BAL cell homogenates, which was significantly attenuated by ebselen and dexamethasone pretreatment, consistent with the BAL neutrophil differential cell count. Ebselen alone did not modify the MPO content in BAL cell homogenates (Fig. 2*A*). We have also quantified MPO activity in cell-free BAL as an index of neutrophil degranulation and activation. LPS-induced MPO activity in cell-free BAL was significantly inhibited by ebselen at 10, 30, and 100 mg/kg and dexamethasone (Fig. 2*B*).

### *Effect of ebselen on airway CXC chemokine expression*

Within the CXC chemokine subfamily, the presence of a glutamate-leucine-arginine (ELR) motif before the first conserved cysteine residue confers selectivity in recruiting neutrophils (30), and the ELR-containing CXC chemokines, CINC, and MIP-2 are potent inducers of neutrophil activation and their directional migration (17, 18, 31–33). We have therefore investigated whether the effect of ebselen on neutrophil recruitment might be achieved through inhibition of the macrophage CXC chemokines CINC-1 and MIP-2. Using RT-PCR, CINC-1 and MIP-2 signals were barely detectable in lung from saline-vehicles or saline-ebselen groups. After LPS inhalation, the mRNA expression for these transcripts was substantially induced in the lungs (Fig. 3). Surprisingly, ebselen pretreatment did not alter the levels of CINC-1 and MIP-2 mRNA expression (Figs. 3 and 4). LPS inhalation releases more MIP-2 than CINC-1 proteins measured in either BAL fluid or lung homogenates (Fig. 5). The LPS-induced increases in BAL and lung protein levels for CINC-1 and MIP-2 were also unaffected by ebselen pretreatment, which is consistent with the gene expression data (Fig. 5). Unlike ebselen, dexamethasone slightly but significantly inhibited lung protein and mRNA levels for CINC-1 and MIP-2 and marginally inhibited BAL protein expression for these chemokines (Fig. 5).

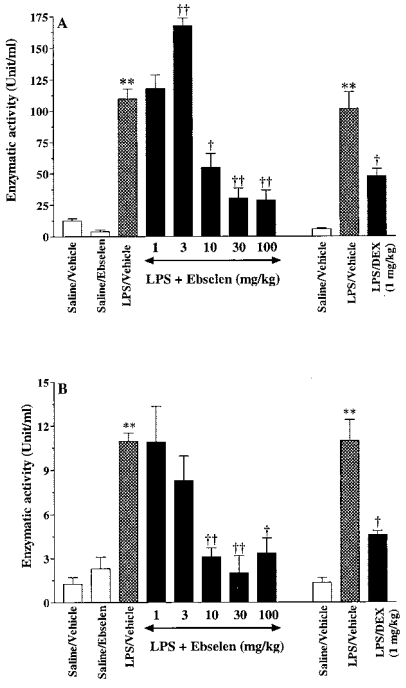


**FIGURE 2.** Effect of ebselen and dexamethasone (DEX) on MPO content in BAL fluid fractions after airway challenge with saline or LPS. Rats were pretreated with ebselen (i.p.), dexamethasone (1 mg/kg, p.o.), or compound vehicles 2 h before and 1 h aftr exposure to aerosolized saline or LPS (0.3 mg/ml w/v) for 30 min. MPO activity was measured in BAL cell homogenates (*A*) and cell-free BAL (*B*) as described in *Materials and Methods*. Results are expressed as mean ± SEM of eight animals. Statistical significance was assessed using the Kruskal-Wallis test with Dunnett's post-test. **, $p < 0.01$ compared with saline-vehicle-treated groups; †, $p < 0.05$ and ††, $p < 0.01$ compared with LPS-vehicle-treated groups.

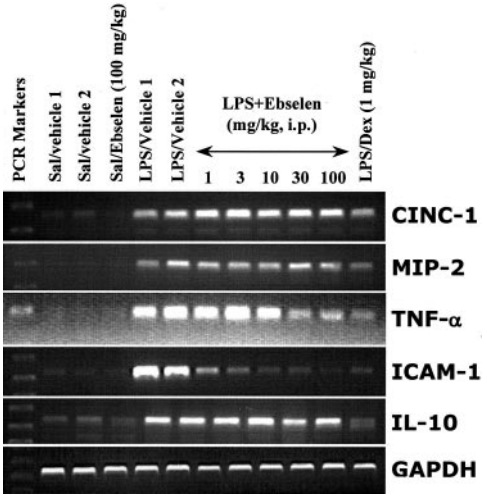


**FIGURE 3.** Effect of ebselen and dexamethasone (Dex) on LPS-induced pro- and anti-inflammatory mediator gene expression in rat lung. A representative RT-PCR analysis showing lung CINC-1, MIP-2, TNF-$\alpha$, ICAM-1, and IL-10 mRNA expression together with the mRNA expression for the housekeeping gene *GAPDH* used as an internal standard. Two hours before and 1 h after airway challenge with saline (Sal) or LPS (0.3 mg/ml for 30 min), rats were treated with ebselen (1–100 mg/kg i.p.), dexamethasone (1 mg/kg p.o.), ebselen vehicle (25% DMSO, 75% polyethylene glycol i.p. (vehicle 1)) or dexamethasone vehicle (0.5% methylcellulose, 0.2% Tween 80 p.o. (vehicle 2)). Lung RNAs extracted 4 h after saline or LPS exposures were reverse-transcribed into cDNA and transcripts were amplified using gene-specific primers as described in *Materials and Methods*. Each band represents mRNA expression obtained from one animal in each experimental group.

### *Effect of ebselen on airway cytokine expression*

TNF-$\alpha$ has been implicated as a mediator of LPS-induced airway inflammation (12, 13, 15, 19). TNF-$\alpha$ was shown to initiate and amplify pulmonary inflammatory responses by stimulating the release of chemotactic factors by up-regulating the expression of leukocyte and endothelial adhesion molecules (16, 23). To determine whether ebselen mediated its protective effect against LPS-induced neutrophil recruitment and activation through this pathway, we evaluated activation of the TNF-$\alpha$ gene in vivo by assessing whole lung TNF-$\alpha$ mRNA expression and accumulation of TNF-$\alpha$ protein in lung and BAL fluid. As demonstrated in Figs. 3 and 4, there was a marked increase in lung TNF-$\alpha$ mRNA in response to LPS challenge. Furthermore, prior treatment with ebselen prevented the induction of TNF-$\alpha$ mRNA expression which was significant at 30 and 100 mg/kg. Consistent with the gene expression data, the up-regulated lung and BALF protein levels for TNF-$\alpha$ were also inhibited after ebselen pretreatment (Fig. 6). Dexamethasone was also able to significantly inhibit protein and mRNA expression for TNF-$\alpha$ and was more potent than ebselen (Figs. 4 and 6).

IL-1$\beta$ shares many of the same biological properties as TNF-$\alpha$, including stimulation of chemokine release and up-regulation of adhesion molecules (34). In this study, LPS inhalation also induced IL-1$\beta$ protein expression in BAL fluid and lung homogenates, which was significantly and dose-dependently inhibited by ebselen pretreatment. The levels of inhibition achieved by ebselen were comparable with those obtained with dexamethasone (Fig. 6).

Beside the multifunctional cytokines TNF-$\alpha$ and IL-1$\beta$, we also evaluated the potential contribution of the anti-inflammatory cytokine IL-10 to the protective effect of ebselen. IL-10 mRNA was detected constitutively in rat lungs and was up-regulated after LPS inhalation. Ebselen appears to have had no effect on the increased expression, whereas dexamethasone treatment appears to have reduced the up-regulated IL-10 mRNA expression (Figs. 3 and 4). Similar data were obtained when analyzing lung iNOS mRNA expression (data not shown).

### *Effect of ebselen on lung ICAM-1 expression*

IL-1$\beta$ together with TNF-$\alpha$ are potent inducers of ICAM-1 expression which has previously been shown to be a requirement for neutrophil recruitment after airway instillation of LPS (24, 25, 35). Therefore, we postulated that the beneficial effect of ebselen might occur through the ability of this agent to prevent lung ICAM-1 expression. There is a constitutive expression of ICAM-1 mRNA which was significantly up-regulated after LPS challenge (Figs. 3 and 7). Furthermore, ebselen appears to modulate the up-regulated ICAM-1 gene, because there was a significant difference in lung ICAM-1 mRNA expression after induction of lung injury with and without pretreatment with this agent. This inhibition occurred in a dose-dependent manner, with residual efficacy at doses as low as 3 mg/kg (Fig. 7).

### *Effect of therapeutic administration of ebselen on cellular recruitment and cytokine expression*

We have also examined the effect of ebselen administered in a therapeutic dosing regimen on airway inflammatory indices in Wistar rats. The compound (30 mg/kg i.p.) was given at various time points after LPS challenge, and as a positive control ebselen was also given before and after challenge. The results depicted in

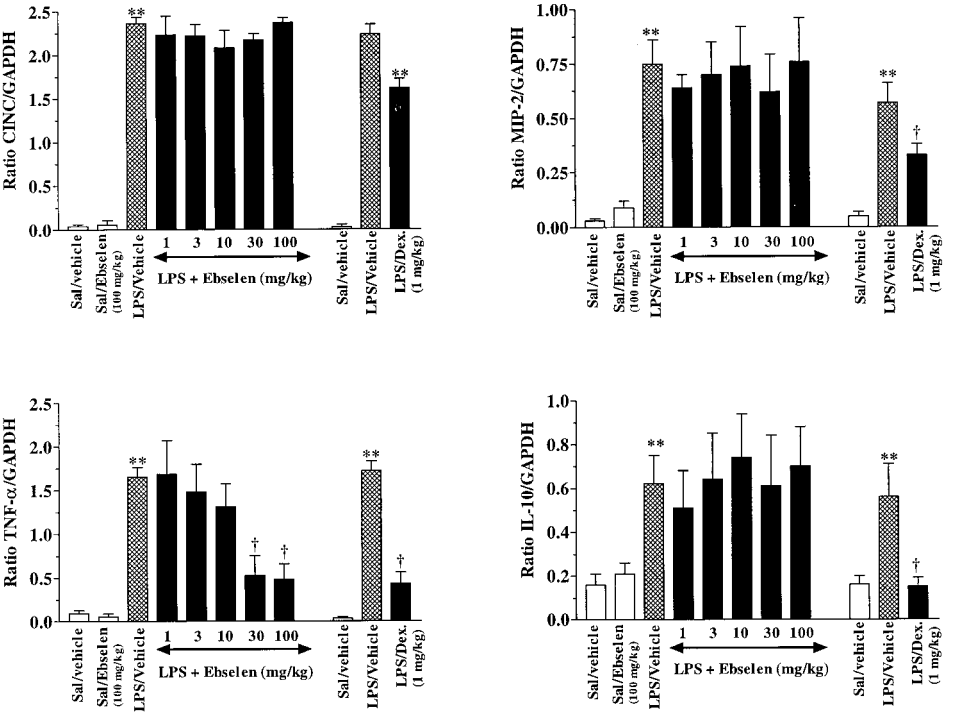


**FIGURE 4.** Effect of ebselen and dexamethasone (Dex.) on pro- and anti-inflammatory mediator gene expression in rat lung after airway challenge with saline (Sal) or LPS. The graphs show TNF-$\alpha$, CINC-1, MIP-2, and IL-10 (see ordinates) mRNA signals expressed as a ratio to GAPDH mRNA as measured by densitometric scanning of the autoradiograms (representative gels are shown in Fig. 3) obtained in eight animals in each experimental group. Data are expressed as mean ± SEM. Statistical significance was assessed using the Kruskal-Wallis test with Dunnett's post-test. **, $p < 0.01$ compared with saline-vehicle-treated groups; †, $p < 0.05$ compared with LPS-vehicle-treated groups.

Fig. 8 showed that ebselen given even after LPS challenge was still effective in inhibiting neutrophil recruitment in BAL fluid collected 4 h after challenge. The same results were also obtained when assessing the effect of therapeutically administered ebselen on BAL fluid and lung tissue TNF-$\alpha$ and IL-1$\beta$ protein levels (Fig. 9). Vehicle treatment at various time points before or after LPS challenge had no effect on neutrophil recruitment or cytokine production in BAL fluid and lung tissue samples.

## Discussion

The selenium-containing compound ebselen possesses antioxidant and anti-inflammatory properties (1, 9). In this study, we investigated its effects on pulmonary inflammation indices in an LPS inhalational challenge model in the rat. We show that the inhibition of LPS-induced neutrophilic inflammation by ebselen is not achieved through modulation of the gene and/or protein expression of the neutrophil chemotactic factors CINC-1 and MIP-2 (CINC-3) or the anti-inflammatory inflammatory cytokine IL-10. Our data rather suggest that the beneficial effect of ebselen is achieved through an inhibition of lung ICAM-1 expression possibly through an inhibition of TNF-$\alpha$ and IL-1$\beta$ expression which are potent neutrophil-recruiting mediators and effective inducers of ICAM-1 expression. Finally, our data further suggest that macrophage CXC chemokines may be necessary but

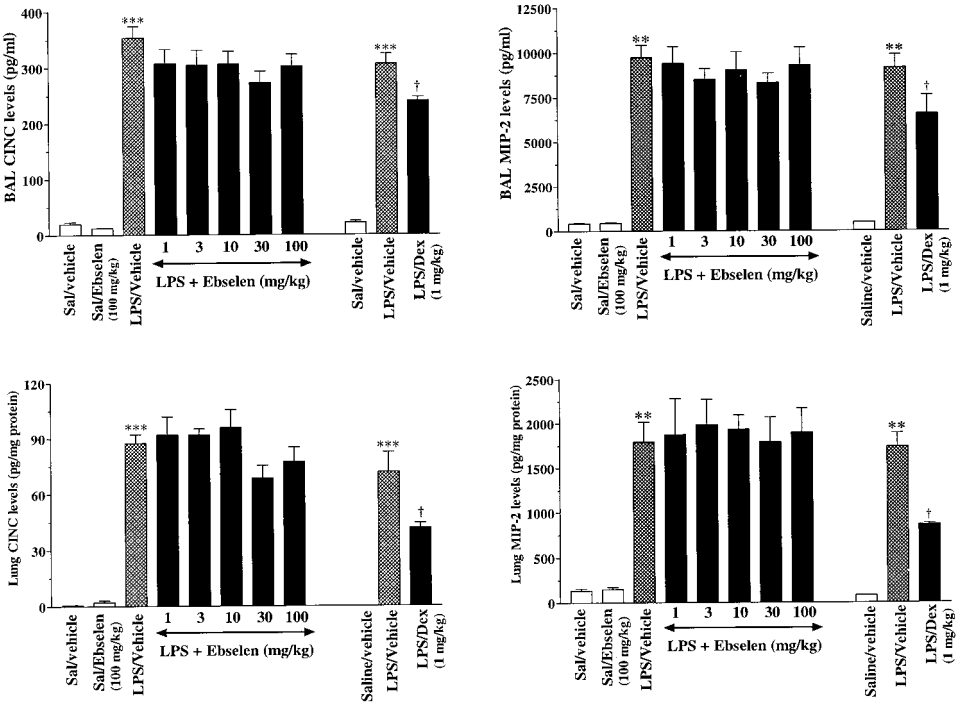


**FIGURE 5.** Effect of ebselen and dexamethasone (Dex) on LPS-induced CINC-1 and MIP-2 protein expression in rat lung and BAL fluid. Rats were pretreated with ebselen (1–100 mg/kg i.p.), dexamethasone (1 mg/kg p.o.), or compound vehicles 2 h prior and 1 h post exposure to aerosolized saline (Sal) or LPS (0.3 mg/ml w/v) for 30 min. BAL and lung samples were harvested 4 h postchallenge. Chemokine protein levels in BAL fluids (*top*) and lung homogenates (*bottom*) were measured by ELISA, as described in *Materials and Methods*. Data are expressed as mean ± SEM of eight animals. Statistical significance was assessed using the Kruskal-Wallis test with Dunnett's post-test. **, $p < 0.01$ and ***, $p < 0.001$ compared with saline-vehicle-treated groups; †, $p < 0.05$ compared with LPS-vehicle-treated groups.

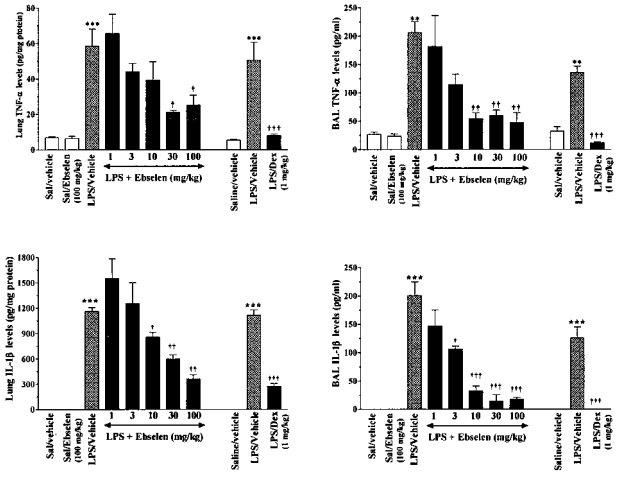


**FIGURE 6.** Effect of ebselen and dexamethasone (Dex) on LPS-induced TNF-$\alpha$ and IL-$\beta$ protein expression in rat lung and BAL fluid. Rats were pretreated with ebselen (1–100 mg/kg i.p.), dexamethasone (1 mg/kg p.o.), or compound vehicles 2 h before and 1 h after exposure to aerosolized saline (Sal) or LPS (0.3 mg/ml w/v) for 30 min. BAL and lung samples were harvested 4 h postchallenge. Protein levels in BAL fluid and lung homogenates were measured by specific rat TNF-$\alpha$ and IL-1$\beta$ ELISA kit according to the manufacturer's instructions. Data are expressed as mean ± SEM of eight animals. Statistical significance was assessed using the Kruskal-Wallis test with Dunnett's post-test. **, $p < 0.01$ and ***, $p < 0.001$ compared with saline-vehicle-treated groups; †, $p < 0.05$, ††, $p < 0.01$ and †††, $p < 0.001$, compared with LPS-vehicle-treated groups.

not sufficient to cause neutrophilia and that additional mechanisms may be responsible for neutrophil influx in this rat model of airway inflammation.

LPS inhalation induces a marked neutrophilic inflammation as assessed by BAL cellular profile and MPO content in BAL cell homogenates recovered 4 h postinhalation. This increase in neutrophil count and MPO activity was abrogated by ebselen pretreatment. Ebselen produced a comparable degree of inhibition to that produced by the synthetic glucocorticosteroid dexamethasone in agreement with published data (36). MPO activity in cell-free

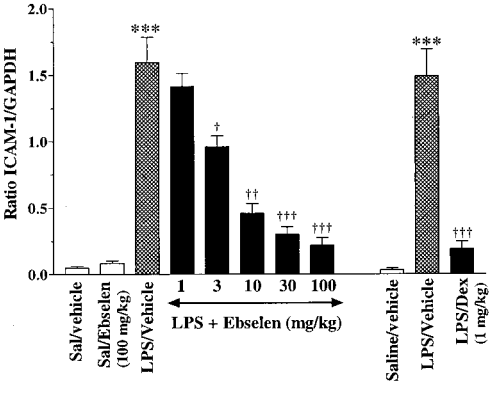


**FIGURE 7.** Effect of ebselen and dexamethasone (Dex) on ICAM-1 mRNA expression in rat lung after airway challenge with saline (Sal) or LPS. The graph shows ICAM-1 mRNA signals expressed as a ratio to GAPDH mRNA as measured by densitometric scanning of the autoradiograms (representative gels are shown in Fig. 3) obtained in 8 animals in each experimental group, as described in *Materials and Methods*. Data are expressed as mean ± SEM. Statistical significance was assessed using the Kruskal-Wallis test with Dunnett's post-test. ***, $p < 0.001$ compared with saline-vehicle-treated groups; †, $p < 0.05$, ††, $p < 0.01$ and †††, $p < 0.001$, compared with LPS-vehicle-treated groups.

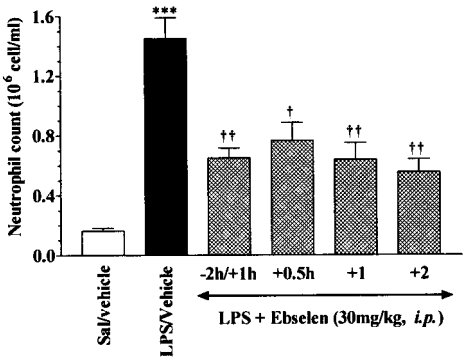


**FIGURE 8.** Effect of ebselen and dexamethasone on LPS-induced neutrophil accumulation in BAL fluid. Rats were pretreated with ebselen (30 mg/kg i.p.), or vehicle 0.5, 1, and 2 h before exposure to aerosolized saline (Sal) or LPS (0.3 mg/ml w/v) for 30 min as described in *Materials and Methods*. As a positive control, an additional group was treated with ebselen 2 h before and 1 h after challenge. The lungs were then lavaged 4 h after exposure, and neutrophil number was determined in BAL fluid by differential cell counting. Results are expressed as mean ± SEM of six to eight animals. Statistical significance was assessed using the Kruskal-Wallis test with Dunnett's post-test. ***, $p < 0.001$, compared with saline-vehicle-treated groups; †, $p < 0.05$ and ††, $p < 0.01$, compared with LPS-vehicle-treated groups.

BAL, used as an index of neutrophil degranulation, was significantly inhibited by ebselen and the standard anti-inflammatory drug dexamethasone. These data show that ebselen provided a significant protection against LPS-induced neutrophil recruitment and activation.

To explore the underlying mechanisms by which ebselen achieves its beneficial effects, we investigated its effect on macrophage CXC chemokine mRNA and protein expression. In rats, the predominant $\alpha$-chemokines appear to be the homologs of the three human growth-related oncogene proteins, CINC-1, CINC2, and CINC3/MIP2 (37, 38). It has previously been shown that among these chemokines, CINC-1 and MIP-2 (CINC-3) play an important role in the recruitment of neutrophils to the lung after intratracheal instillation of cytokines or cytokine-inducing stimuli such as LPS (14, 39–41). In our model, CINC-1 and MIP-2 both exhibited early (0.5- to 2-h) peak levels of mRNA expression after LPS challenge, consistent with their role in the recruitment of neutrophils. Surprisingly, the marked induction of CINC-1 and MIP-2 protein and/or mRNA expression in lung and BAL fluid was not inhibited by ebselen pretreatment, despite the inhibitory effect of ebselen on neutrophil influx as assessed by both BAL fluid cellularity and MPO content. This result suggests that the inhibitory effect of ebselen is independent of the macrophage CXC chemokine expression and that additional mechanisms are involved in neutrophil recruitment.

Dexamethasone, unlike ebselen, modestly inhibited lung protein and mRNA levels for CINC-1 and MIP-2 and had only a marginal effect on BAL fluid protein expression. In rats, modest glucocorticoid effects on LPS-induced lung expression of CINC and MIP-2 have been reported (42, 43). The observed effect of dexamethasone on CXC chemokine BAL fluid protein level expression is largely in agreement with published data in which airway CINC-1 and/or MIP-2 expression were unaffected by glucocorticoids (36, 44, 45). Stimulus-specific differences in glucocorticoid sensitivity may account for the more potent effect of dexamethasone on lung CINC and MIP-2 expression observed in ozone-exposed brown Norway rats (31, 32).

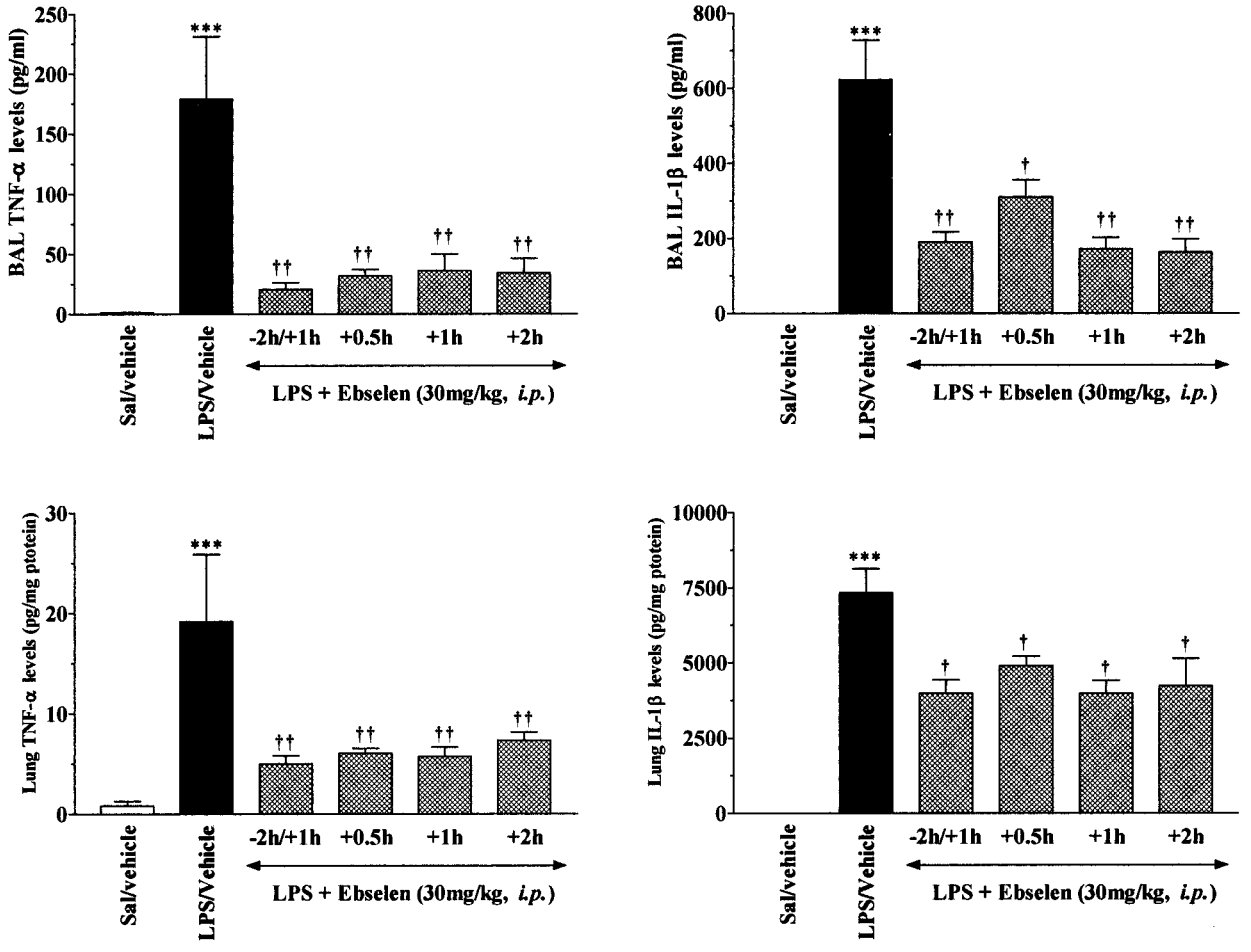


**FIGURE 9.** Effect of ebselen and dexamethasone on LPS-induced TNF-$\alpha$ and IL-$\beta$ protein expression in rat lung and BAL fluid. Rats were pretreated with ebselen (30 mg/kg i.p.), or vehicle 0.5, 1, and 2 h before exposure to aerosolized saline (Sal) or LPS (0.3 mg/ml w/v) for 30 min as described in *Materials and Methods*. As a positive control, an additional group was treated with ebselen 2 h before and 1 h after challenge. BAL and lung samples were harvested 4 h postchallenge. Cytokine protein levels in BAL fluid and lung homogenates were measured by specific rat TNF-$\alpha$ and IL-1$\beta$ ELISA kit according to the manufacturer's instructions. Data are expressed as mean ± SEM of six to eight animals. Statistical significance was assessed using the Kruskal-Wallis test with Dunnett's post-test. ***, $p < 0.001$ compared with saline-vehicle-treated groups; †, $p < 0.05$, and ††, $p < 0.01$, compared with LPS-vehicle-treated groups.

Our data with ebselen and dexamethasone suggest that additional mechanisms may be responsible for neutrophil chemoattraction. In this respect, LPS-induced CXC chemokine (LIX) has recently been cloned as a glucocorticoid-attenuated response gene and is a potent chemotactic factor for neutrophils (46, 47). LIX message is induced by LPS in fibroblasts, but not in macrophages (46). Beside fibroblast and macrophage chemokines, other CXC chemokines such as epithelial neutrophil-activating peptide-78 and lungkine were recently shown to play a role in neutrophil influx in airway inflammation (48, 49). Whether these chemokines play a role in LPS-induced airway inflammation and their sensitivity to ebselen and glucocorticoids remains to be determined.

IL-10 is among the most potent anti-inflammatory agents induced in response to LPS (50). Several studies have demonstrated the ability of IL-10 to down-regulate LPS-inducible mRNA expression of proinflammatory cytokines and chemokines partly through inhibition of NF-$\kappa$B (51, 52). It was also recently shown that mice pretreated orally with ebselen were dose-dependently protected from Con A-induced liver injury through up-regulation of IL-10 (53). We have therefore examined the potential contribution of IL-10 and its sensitivity to modulation by ebselen. In our model, the up-regulated expression of lung IL-10 was not affected by ebselen pretreatment, ruling out the contribution of this pathway to the protective effect of ebselen. Dexamethasone, however significantly reduced lung IL-10 mRNA expression.

Another mechanism by which LPS promotes acute lung inflammation is by up-regulating the expression of adhesion molecules on endothelial cells and circulating leukocytes (16, 35, 54, 55). Studies have shown protective effects of both anti-CD11/CD18 and anti-ICAM-1 mAbs in models of acute lung injury, which was attributed to inhibition of PMN sequestration in the lungs (24, 56–58). ICAM-1 has previously been shown to be a requirement for neutrophil recruitment after airway instillation of LPS with peak levels of expression associated with maximum leukocyte adherence (35). In agreement with published data, we found that lung ICAM-1 expression was up-regulated by LPS exposure (24, 25). Ebselen dose-dependently abrogated ICAM-1 mRNA expression. Dexamethasone also diminished ICAM-1 expression to a level similar to that produced by ebselen. Therefore, it is likely that the diminished ICAM-1 expression by ebselen and dexamethasone may have contributed to the impaired neutrophil recruitment. This finding suggests that ebselen exerts its actions directly on endothelial cells in vivo. However, we cannot preclude any additional effects that ebselen may have on PMN-endothelial cell interactions apart from the effect on ICAM-1 expression demonstrated in the present studies. Indeed, ebselen may have additional effects on

other endothelial cell adhesion molecules involved in lung leukocyte sequestration, and these possibilities require further investigations. Alternatively, ebselen might inhibit other cells in vivo from releasing cytokines such as TNF-$\alpha$ and IL-1$\beta$ that induce endothelial cell adhesion molecules (see below).

TNF-$\alpha$ plays an important role in promoting and amplifying lung inflammation in response to inhaled LPS through the release of chemotactic factors and by up-regulating the expression of leukocyte and endothelial cell adhesion molecules (12–16, 19, 23, 25, 44). TNF-$\alpha$ stimulates chemokine release by alveolar macrophages and by cells that do not respond directly to LPS in vitro, such as epithelial cells and fibroblasts (16). Exposure to aerosolized or endotracheally administered rTNF-$\alpha$ has been associated with chemokine release in bronchoalveolar lining fluid (59), up-regulation of ICAM-1 on pulmonary vascular endothelium (23), margination of leukocytes in the pulmonary vasculature (60), and neutrophilic infiltration of the interstitium or alveolar septae (60). In agreement with published data, we found that LPS challenge induces TNF-$\alpha$ protein and/or mRNA expression in lung tissue and BAL fluid, suggesting an important role of this pleiotropic cytokine in this model (12, 44, 61). Furthermore, ebselen and dexamethasone were able to inhibit protein and mRNA expression for TNF-$\alpha$. These results are in agreement with data showing that ebselen inhibited, in a dose-related manner, BAL TNF-$\alpha$ induced by Sephadex particle instillation in the rat and also provided a significant protection against Sephadex-induced lung edema (62). Our data suggest that the inhibitory effect of ebselen on neutrophil influx and lung ICAM-1 expression may be achieved, at least in part, through inhibition of lung and BAL fluid TNF-$\alpha$ protein and/or mRNA expression. Many of the ebselen effects have been related to the glutathione peroxidase-mimetic effect of the drug (1, 9). Therefore, the antioxidant properties of ebselen are likely to account for the observed effects of ebselen on inflammatory indices induced by LPS. In support of this hypothesis, it has been shown that the glutathione-depleting agent diethylmaleate prevented the intratracheal LPS-induced increase in rat lung PMN influx by inhibiting up-regulation of lung ICAM-1 mRNA expression independent of any effect on CINC-1 (63, 64). However, unlike ebselen, the effect of diethylmaleate was not achieved through modulation of BAL fluid TNF-$\alpha$ levels (63). The effects of ebselen and diethylmaleate on neutrophil influx and CINC-1 and ICAM-1 expression are not a general feature of antioxidants because *N*-acetylcysteine was able to prevent PMN influx, partly through an inhibitory effect on CINC-1 induction (65).

Our data suggest that the protective effect of ebselen involve, at least in part, an inhibitory effect on ICAM-1 expression and TNF-$\alpha$ production. However, it is likely that other inflammatory mediators with overlapping functions can also partially fulfil the proinflammatory activities of TNF-$\alpha$. In this respect, IL-1$\beta$ shares many of the same effects as TNF-$\alpha$, including neutrophil recruitment, stimulation of chemokine release, and up-regulation of adhesion molecules (12, 15, 34). In this study, LPS inhalation was associated with a significant increase in lung and BAL IL-1$\beta$ levels, which was dose-dependently abrogated by ebselen. These data suggest that the inhibitory effect of ebselen on LPS-induced neutrophil influx and ICAM-1 expression is partially achieved through an inhibitory effect on IL-1$\beta$. In agreement with an important role of IL-1$\beta$ in this model are the findings showing that an anti-IL-1 Ab reduced LPS-induced lung ICAM-1 mRNA expression, which is necessary for neutrophil recruitment (25).

Finally, we have also examined the effect of therapeutically administered ebselen on airway inflammatory indices. The compound given up to 2 h after LPS challenge was still effective in inhibiting BAL neutrophilia as well as BAL fluid and lung tissue TNF-$\alpha$ and IL-1$\beta$ levels. These data are in agreement with published work showing ebselen to be efficacious in a therapeutic regimen. Indeed, in rat immunized with adjuvant (*M. butyricum*), treatment with ebselen (100 mg/kg/day for 3 days) when arthritis was fully developed (13 days after immunization) inhibited by 72–79% the neutrophil migration into arthritic joints and tail (spondylitis) and by 50–60% that into dermal inflammatory reactions induced with zymosan-activated rat serum, endotoxin (LPS), or IL-1$\alpha$ (11).

Collectively, these data show that ebselen possesses anti-inflammatory properties in this model of airway inflammation. Compounds with glutathione peroxidase activity similar to those of ebselen or ebselen derivatives have been shown to be potent inhibitors of adhesion molecule (ICAM-1 and VCAM-1) expression, leukocyte recruitment, and TNF-$\alpha$ expression in vitro and in vivo (66–69).

In summary, we have shown that the inhibition of neutrophilic inflammation by ebselen is independent of any effect on the macrophage CXC chemokine expression. We have also described a novel and specific mechanism by which ebselen achieved its beneficial effects. We have shown that ebselen modulates ICAM-1 expression possibly through inhibition of both TNF-$\alpha$ and IL-1$\beta$ which are a potent neutrophil-recruiting mediators and potent inducers of ICAM-1 expression. These effects do not seem to be a global effect of ebselen on LPS-induced gene expression given that the rise in airway levels for several candidate mediators including CINC-1, MIP-2, IL-10, and iNOS were unaffected by this agent. In conclusion, ebselen may be a useful therapy in lung pathologies in which neutrophilic inflammation is a feature and where TNF-$\alpha$ and IL-1$\beta$ productions are the predominant mediators.

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