Glutathione peroxidase-1 protects against cigarette smoke-induced lung inflammation in mice

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Duong C, Seow HJ, Bozinovski S, Crack PJ, Anderson GP, Vlahos R. Glutathione peroxidase-1 protects against cigarette smoke-induced lung inflammation in mice. Am J Physiol Lung Cell Mol Physiol 299: L425–L433, 2010. First published May 28, 2010; doi:10.1152/ajplung.00038.2010.—Reactive oxygen species (ROS) produced from cigarette smoke cause oxidative lung damage including protein denaturation, lipid peroxidation, and DNA damage. Glutathione peroxidase-1 (gpx-1) is a detoxifying enzyme that may protect lungs from such damage. The aim of this study was to determine whether gpx-1 protects the lungs against oxidative stress-induced lung inflammation in vivo. Male wild-type (WT) or gpx-1−/− mice were exposed to cigarette smoke generated from nine cigarettes per day for 4 days to induce oxidative stress and lung inflammation. The effect of the gpx mimetic ebselen on cigarette smoke-induced lung inflammation was evaluated when given prophylactically and therapeutically, i.e., during established inflammation. Mice were killed, and the lungs were lavaged with PBS and then harvested for genomic and proteomic analysis. Gpx-1−/− mice exposed to cigarette smoke had enhanced BALF neutrophils, macrophages, proteolytic burden, whole lung IL-17A, and MIP1α mRNA compared with WT mice. The gpx mimetic ebselen (10 and 100 μM) inhibited cigarette smoke extract-induced oxidation of MH-S cells in vitro and inhibited cigarette smoke-induced increases in BALF macrophages, neutrophils, proteolytic burden, and macrophage and neutrophil chemotactic factor gene expression when administered prophylactically. In addition, ebselen inhibited established BALF inflammation when administered therapeutically. These data show that gpx-1 protects against cigarette smoke-induced lung inflammation, and agents that mimic the actions of gpx-1 may have therapeutic utility in inflammatory lung diseases where cigarette smoke plays a role.

reactive oxygen species; leukocyte; proteases; macrophage growth factors

REACTIVE OXYGEN SPECIES (ROS) are a family of highly reactive molecules that are produced by a variety of cell types in the lung in response to chemical and physical agents in the environment (33, 41). It is well known that ROS are critical in host defense as they kill invading pathogens. However, their excessive accumulation, or their impaired clearance, in the lung results in oxidative damage including DNA damage, lipid peroxidation, and protein denaturation (42). The cell has developed enzymatic defenses to combat oxidative stress, including glutathione peroxidase (gpx).

Glutathione peroxidases are a family of selenium-dependent and -independent antioxidant enzymes that catalyze the reduction of damaging hydrogen peroxide (H2O2) as well as a large variety of hydroperoxides (such as DNA peroxides and lipid peroxides) into water and alcohols, respectively, and thus protect biomembranes and cellular components against oxidative stress (6). Analysis of the selenoproteome has identified six gpxs in mammals: cytosolic gpx (cGpx, gpx-1), phospholipid hydroperoxide gpx (PHGpx, gpx-4), plasma gpx (pGpx, gpx-3), gastrointestinal gpx (GI-Gpx, gpx-2), gpx-5 (specifically expressed in mouse epididymis), and, in humans, gpx-6, which is restricted to the olfactory system (6). Although their expression is ubiquitous, the levels of each isoform vary depending on the tissue type. The cytosolic selenium-dependent gpx-1 is the predominant isoform of cellular gpx and is ubiquitously expressed throughout the body (6). Sources in the lungs include epithelium, alveolar epithelial lining fluid, and alveolar macrophages (2). We have previously shown that mice lacking the gpx-1 gene are highly susceptible to oxidative stress but do not display an overt phenotype and thus proposed gpx-1 may be an attractive target for increasing the antioxidant capacity in ischemia/reperfusion brain injury where oxidative stress is involved (12, 13, 25). However, there is now evidence to suggest that gpx-1 may have a role in regulating the inflammatory response to cigarette smoke exposure. Elevated levels of H2O2 are measured in the exhaled breath condensate of COPD patients, particularly during exacerbations (14). There is upregulation of gpx-1 gene expression in the lungs of smokers (5) and depletion of gpx activity in COPD patients and smokers (24, 47, 53). With respect to reduced gpx activity in COPD patients and smokers, erythrocyte gpx activity was significantly lower in patients with severe COPD compared with patients with moderate COPD (24). In addition, gpx activity was decreased in plasma from COPD patients (53) and total blood from smokers and ex-smokers (47). However, these studies did not identify the isofrom of gpx that was involved in reduced activity of gpx.

Given that the known biology of gpx-1 appears to be protection during oxidative stress, and evidence of a role in COPD, we propose that gpx-1 protects against cigarette smoke-induced lung inflammation. Gpx-1-deficient mice exposed to cigarette smoke had significantly elevated levels of BALF macrophages, neutrophils, proteolytic burden, and whole lung macrophage and neutrophil chemotactic factor gene expression. In addition, the gpx mimetic ebselen significantly inhibited cigarette smoke-induced lung inflammation when given prophylactically and during established inflammation. Our data provide new evidence for the role of gpx-1 in smoke-induced lung inflammation and suggest the potential therapeutic utility of targeting gpx-1 in vivo.

MATERIALS AND METHODS

Animals. Specific pathogen-free male C57BL/6 wild type (WT), glutathione peroxidase-1-deficient (gpx-1−/−, C57BL/6 genetic background), or Balb/C mice aged 7–12 wk were obtained from Monash Animal Resource Facility (Melbourne, Australia) and the Animal Resource Centre (Perth, Australia). The animals were housed at 20°C...
on a 12-h day/night cycle in sterile micro-isolators and fed a standard sterile diet of Purina mouse chow with water allowed ad libitum. The experiments described in this manuscript were approved by the Animal Experimentation Ethics Committee of The University of Melbourne and conducted in compliance with the guidelines of the National Health and Medical Research Council of Australia on animal experimentation.

**Cigarette smoke exposure.** Mice were placed in an 18-L perspex chamber in a class II biosafety cabinet and exposed to cigarette smoke generated from nine cigarettes/day for 4 days as previously described (54, 57). The mean total suspended particulate mass concentration in the chamber containing cigarette smoke was ~420 mg/m³. Sham-exposed mice were placed in an 18-L perspex chamber but did not receive cigarette smoke. On the fifth day, mice were killed by an intraperitoneal overdose of anesthetic (pentobarbital sodium, 300 mg/kg ip), and the lungs were lavaged with PBS. Commercially available filter-tipped cigarettes (manufactured by Philip Morris, Australia) of the following composition were used: 16 mg or less of tar, 1.2 mg or less of nicotine, and 15 mg or less of CO. 

**Bronchoalveolar lavage.** Lungs from each terminal anesthetized mouse were lavaged in situ with a 400-μl aliquot, followed by three 300-μl aliquots of PBS as previously described (54, 57). The total number of viable cells in the BALF was determined, cytospins were prepared using 50–200 μL of BALF, and cells were differentiated by standard morphological criteria.

**Protease expression and activity in BALF.** Zymography was used to assess protease expression in response to cigarette smoke exposure as previously described (54, 57). Briefly, BALF from animals in each treatment group was pooled, concentrated, and spun, and the pellet was washed and resuspended in 50 μl of 1 × nonreducing buffer. Twenty microliters was loaded onto 10% SDS-PAGE mini-gels containing 2 mg/ml gelatin and run at a constant voltage of 200 V for 45 min. Gels were then washed in 2.5% Triton X-100, incubated at 37°C overnight in zymography buffer, stained for 45 min with Coomassie Brilliant Blue R-250, and extensively destained. Neat BALF was also tested for net gelatinase activity using fluorescence-conjugated gelatin (Molecular Probes) as previously published (54, 57).

**RNA extraction and quantitative real-time PCR.** Whole lungs were perfused free of blood via right ventricular perfusion with 10 ml of warmed saline, rapidly excised en bloc, blotted and snap frozen in liquid nitrogen. Total RNA was extracted from 15 mg of whole lung tissue pooled from five to eight mice per treatment group using RNeasy Mini Kits (Qiagen), reverse transcription with SuperScript III (Invitrogen), and triplicate real-time PCR reactions with Applied Biosystems predeveloped assay reagents. 18S rRNA internal control was done as previously described (54, 57).

**Administration of ebselen.** In experiments designed to investigate the effects of ebselen on cigarette smoke-induced lung inflammation, Balb/C mice were treated with 10 mg/kg ebselen or vehicle (10% DMSO/90% PEG) intraperitoneally twice a day, 1 h before the first and third smoke session of the day. Balb/C mice were used for this aspect of the study because we have previously shown they are more susceptible to cigarette smoke-induced lung inflammation, and this would allow us to better observe any possible inhibitory effect of ebselen (54, 57). Mice were then killed on day 5, and the analysis described above was performed. In a therapeutic treatment regimen protocol, Balb/C mice were exposed to 4 days of cigarette smoke and then treated with ebselen or vehicle (5% CM-cellulose made up in distilled water) via oral gavage (as above) beginning on day 5. Cigarette smoke exposure and ebselen/vehicle treatment continued in the same manner until day 11. Mice were then killed on day 12, and the analyses described above were performed. To prove that ebselen was mimicking the actions of gpx-1 in the lung, we administered ebselen (10 mg/kg, 1 h before the first cigarette smoke exposure on days 1–4) or vehicle (5% CM-cellulose) via oral gavage to smoke-exposed gpx-1−/− mice. Mice were then killed on day 5, and BALF cellularity was assessed as described above.

**OxyBlot protein oxidation analysis.** A murine (Balb/C) alveolar macrophage cell line (MH-S) was used to assess the effects of ebselen on cigarette smoke extract (CSE)-induced protein oxidation. The MH-S cell line was maintained in a 75-cm² flask containing RPMI 1640 medium supplemented with 10% FBS, 10 ml sodium pyruvate, 5 ml l-glutamine, 5 ml HEPES, 5 ml sodium bicarbonate, 174 μl 2-mercaptoethanol (1:100 dilution in sterile water), and 1.25 ml gentamycin until required. Cells were plated in six-well plates at a density of 2×10⁷ cells/well. In all experiments, CSE was prepared fresh on the day of the experiment by combusting one Winfield Red cigarette with a syringe-driven apparatus and bubbled through 25 ml of RPMI 1640 medium plus 1% FBS as previously published (28). This was designated as 4% CSE, and, when required, dilutions were made with RPMI 1640 medium plus 1% FBS to the required concentration. These concentrations of CSE did not cause cytotoxicity as assessed by cell viability (double staining with acridine orange and ethidium bromide) and as previously described (28). Similar concentrations of CSE have been used with no detrimental effect on cell viability (1, 26, 27, 34, 36, 58, 62), although direct comparisons are complicated due to methods used to generate stock CSE solutions and type of cigarette used. The working solution was used within 15 min as aged CSE was less potent at suppressing inflammatory responses (28). In CSE concentration-response experiments, MH-S cells were exposed for 20 min to various concentrations of CSE (0.25, 0.5, 1, 2, and 4% made up in RPMI 1640 + 1% FBS), scraped from the wells, and centrifuged for 5 min at 3,000 rpm. The supernatant was removed via aspiration, and remaining cell pellets were snap frozen in liquid nitrogen and stored at ~80°C for OxyBlot analysis. In experiments with ebselen treatment, cells were exposed to 4% CSE and treated with 1, 10, and 100 μM ebselen (in 0.5% DMSO) for 20 min and collected and stored as above. DMSO (0.5%) was used as the vehicle-control.

**OxyBlot Protein Oxidation Detection kit (Millipore) was utilized for immunoblot detection of carbonyl groups introduced onto proteins by oxidative reactions.** Briefly, MH-S cell pellets from the above experiments were resuspended in lysis buffer and subjected to SDS-PAGE on 10% gel slabs at 200 V for 60 min, and resolved proteins were transferred onto a Hybond PVDF membrane using a Trans-Blot SD transfer cell (Bio-Rad) at 260 mA for 60 min. After transfer, PVDF membranes were incubated in blocking solution for 1 h at room temperature. The carbonylated proteins were then detected with primary antibodies and subsequently a secondary antibody as per manufacturers’ instructions. Immunoreactive bands were visualized by autoradiography with chemiluminescence (ECL Plus, Amersham). β-actin was used as a loading control.

**Statistical analyses.** As data were normally distributed, they are presented as grouped data expressed as means ± SE; n represents the number of mice. Differences in total BALF cell types and differential counts were determined by one- or two-way ANOVA followed by Bonferroni post hoc test for multiple comparisons, where appropriate. All statistical analyses were performed using GraphPad Prism for Windows (version 5.02). In all cases, probability levels less than 0.05 (P < 0.05) were taken to indicate statistical significance.

**RESULTS**

**Deletion of gpx-1 enhances cigarette smoke-induced BALF cellularity.** In WT mice exposed to cigarette smoke generated from nine cigarettes/day for 4 days, there was a significant increase in the total number of cells, neutrophils, and macrophages in BALF (Fig. 1, A–C) (P < 0.05, ANOVA followed by Bonferroni post hoc test). However, gpx-1−/− mice exposed to cigarette smoke had significantly more numbers of total cells (~50%), macrophages (~60%), and neutrophils (~46%) compared with cigarette smoke-exposed WT mice (Fig. 1, A–C) (P < 0.05, ANOVA followed by Bonferroni post hoc test).
Consistent with the zymography, there was an increase in net protease expression was identified at 90 kDa, corresponding to the molecular size of the active form of MMP-9 (Fig. 2A). A major band of gelatinase activity in the BALF from mice exposed to cigarette smoke (Fig. 2B). However, gpx-1−/− mice treated with cigarette smoke had significantly more levels of MMP-9 and net gelatinase activity than WT cigarette smoke-exposed mice (Fig. 2, A and B).

Effect of gpx-1 deletion on cigarette smoke-induced increases in blood growth factors, neutrophilic, and macrophage chemotactic factors. Wild-type mice treated with cigarette smoke had more IL-17A, MIP-1α, MCP-1, GM-CSF, and MMP-12 as measured by QPCR in whole lung compared with sham-exposed mice (Fig. 3). However, gpx-1−/− mice had markedly elevated levels of IL-17A, MIP-1α, and MMP-12, but reduced levels of MCP-1. In addition, levels of GM-CSF in smoke-exposed gpx-1−/− mice were similar to those of WT smoke-exposed mice.

Ebselen attenuates CSE-induced protein oxidation. In conjunction with examining the effects of the gpX mimetic ebselen in vivo, we wanted to determine whether ebselen could inhibit protein oxidation in vitro. Pilot concentration-response curves revealed that 4% CSE caused maximal protein oxidation in MH-S cells (data not shown). Ebselen (10 and 100 μM) decreased CSE-induced protein oxidation but was without effect at 1 μM (Fig. 4). Protein oxidation was not evident in the control (media alone), and vehicle (0.5% DMSO) did not affect CSE-induced protein oxidation. Immunodetection of β-actin was used as a loading control (data not shown).

Ebselen reduces cigarette smoke-induced BALF neutrophilia and macrophage accumulation. In Balb/C mice exposed to cigarette smoke generated from nine cigarettes/day for 4 days, there was a significant increase in the total number of cells, neutrophils, and macrophages in BALF (Fig. 5, A–C) (P < 0.05, ANOVA followed by Bonferroni post hoc test).

Total cells, macrophages, and neutrophil numbers in sham-exposed gpx-1−/− mice were similar to those in sham-exposed WT mice (Fig. 1, A–C). Administration of the gpX mimetic ebselen (10 mg/kg) to cigarette smoke-exposed gpx-1−/− mice significantly reduced cigarette smoke-induced BALF total cell number, macrophages, and neutrophils by 62 ± 6%, 68 ± 5%, and 74 ± 6%, respectively (P < 0.001, unpaired t-test, n = 6–7). In other words, the increased susceptibility to cigarette smoke-induced BALF inflammation of gpx-1−/− mice was abolished by ebselen administration. This clearly shows that there is a link between ebselen and its direct effects on gpX-1 in the smoking mice.

Smoke-induced BALF proteolytic burden is enhanced in gpx-1−/− mice. Since MMPs contribute to the movement of neutrophils/macrophages into the lung parenchyma, we measured the secretion of MMP-9 in the BALF of cigarette smoke-treated mice. There was a marked increase in protease expression in BALF from WT mice exposed to cigarette smoke as assessed by gelatin zymography (Fig. 2A). A major band of protease expression was identified at ~90 kDa, corresponding to the molecular size of the active form of MMP-9 (Fig. 2A). Consistent with the zymography, there was an increase in net protease expression in BALF from mice exposed to cigarette smoke (Fig. 2B). However, gpx-1−/− mice treated with cigarette smoke had significantly more levels of MMP-9 and net gelatinase activity than WT cigarette smoke-exposed mice (Fig. 2, A and B).

Effect of gpx-1 deletion on cigarette smoke-induced increases in blood growth factors, neutrophilic, and macrophage chemotactic factors. Wild-type mice treated with cigarette smoke had more IL-17A, MIP-1α, MCP-1, GM-CSF, and MMP-12 as measured by QPCR in whole lung compared with sham-exposed mice (Fig. 3). However, gpx-1−/− mice had markedly elevated levels of IL-17A, MIP-1α, and MMP-12, but reduced levels of MCP-1. In addition, levels of GM-CSF in smoke-exposed gpx-1−/− mice were similar to those of WT smoke-exposed mice.

Ebselen attenuates CSE-induced protein oxidation. In conjunction with examining the effects of the gpX mimetic ebselen in vivo, we wanted to determine whether ebselen could inhibit protein oxidation in vitro. Pilot concentration-response curves revealed that 4% CSE caused maximal protein oxidation in MH-S cells (data not shown). Ebselen (10 and 100 μM) decreased CSE-induced protein oxidation but was without effect at 1 μM (Fig. 4). Protein oxidation was not evident in the control (media alone), and vehicle (0.5% DMSO) did not affect CSE-induced protein oxidation. Immunodetection of β-actin was used as a loading control (data not shown).

Ebselen reduces cigarette smoke-induced BALF neutrophilia and macrophage accumulation. In Balb/C mice exposed to cigarette smoke generated from nine cigarettes/day for 4 days, there was a significant increase in the total number of cells, neutrophils, and macrophages in BALF (Fig. 5, A–C) (P < 0.05, ANOVA followed by Bonferroni post hoc test).
Mice treated with ebselen (10 mg/kg, bis in die [bid]) had significantly reduced total cells, neutrophils, and macrophages compared with vehicle (Fig. 5, A–C) (*P < 0.05, ANOVA followed by Bonferroni post hoc test). Ebselen had no effect on baseline total, macrophage, and neutrophil cell numbers in sham-exposed animals.

Ebselen inhibits cigarette smoke-induced increases in proteases, blood growth factors, and neutrophilic and macrophage chemotactic factor gene expression. Cigarette smoke-exposed Balb/C mice had markedly elevated mRNA levels of the chemokines IL-17A, MCP-1, MIP-1α, the blood growth factor GM-CSF, and the protease MMP-12 (Fig. 6). Ebselen (10 mg/kg, bid) alone had no effect on baseline levels of IL-17A, MCP-1, MIP-1α, GM-CSF, and MMP-12 mRNA but reduced smoke-induced increases in IL-17A, MCP-1, MIP-1α, GM-CSF, and MMP-12 mRNA (Fig. 6).

Ebselen reduces cigarette smoke-induced protease expression. There was a marked increase in protease expression in BALF of Balb/C mice exposed to cigarette smoke as assessed by zymography (Fig. 7). A major band of protease expression was identified at 90 kDa, corresponding to the molecular size of the active form of MMP-9 (Fig. 7). Smoke-exposed Balb/C mice treated with ebselen (10 mg/kg) had reduced levels of MMP-9 compared to smoke-exposed mice treated with vehicle (Fig. 7).

Ebselen resolves established BALF inflammation. The effect of ebselen administered in a therapeutic treatment regimen (i.e., on established lung inflammation) was examined in Balb/C mice exposed to cigarette smoke for 11 days with ebselen administration commencing on day 5 (Fig. 8). Cigarette smoke exposure significantly increased BALF total cells,

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Fig. 3. Effect of cigarette smoke exposure on whole lung gene expression in WT and gpox-1−/− mice. mRNA expression for all genes was measured simultaneously under identical conditions using quantitative real-time PCR. Responses are shown as fold increase relative to 18S. Data are shown as means ± SE of 3 replicates as previously published (54, 57). Open bars represent sham-exposed mice, and filled bars represent smoke-exposed mice.

Fig. 4. Ebselen reduces cigarette smoke extract (CSE)-induced protein oxidation of murine alveolar macrophage (MH-S) cells. OxyBlot detection of carbonylated proteins as a measurement of protein oxidation was performed using cell pellets collected in experiments where MH-S cells were exposed to 4% CSE and incubated with vehicle (0.5% DMSO in media) or ebselen (1–100 μM). The OxyBlot is representative of 4 separate experiments.
neutrophils, and macrophages (Fig. 8, A–C) \( (P < 0.05, \text{ANOVA followed by Bonferroni post hoc test}) \). However, ebselen administered at 10 mg/kg after established lung inflammation significantly lowered the total number of cells, neutrophils, and macrophages in BALF compared with vehicle-treated mice (Fig. 8, A–C) \( (P < 0.05, \text{ANOVA followed by Bonferroni post hoc test}) \).

**DISCUSSION**

The principal objective of this study was to investigate whether gpx-1 protects against cigarette smoke-induced lung inflammation and to determine whether agents that mimic the actions of gpx-1, or which restore gpx-1 activity in situations of overwhelming oxidative stress, inhibit cigarette smoke-induced lung inflammation.

Normal expression of the ubiquitous antioxidant gpx-1 is essential in protection against oxidant/antioxidant imbalance in local tissues and at a systemic level in mice \( (12, 13, 25, 29) \). Gpx activity is significantly reduced in smokers and subjects with COPD, highlighting its prominent role in lung antioxidant defense \( (5) \). It has been shown that COPD patients are deficient in selenium and that this could explain the observed reduction in gpx activity \( (47) \). Moreover, selenium is an important element in the gpx catalysis of the reaction between GSH and ROS. Consequently, gpx mimetics such as ebselen increase the efficiency of GSH as an antioxidant. There is a direct relationship between systemic gpx activity and FEV\(_1\) \( (24) \), and oxidative stress correlates with both lung function and body mass index in COPD \( (53) \). When investigating the gene expression of specific isoforms of gpx, gpx-1 has been shown to be increased in COPD \( (5) \), and gpx-2 showed a three- to fivefold upregulation in epithelial cells of smokers compared with nonsmokers \( (18, 39, 49) \). It has been shown that gpx-2 is induced in lungs of mice in response to cigarette smoke and that basal and cigarette smoke-inducible expression of gpx-2 is directly dependent on Nrf2 \( (43, 48) \). Gpx-3 showed a twofold upregulation in epithelial cells of smokers compared with...
nonsmokers (11, 39). There was little evidence of differential regulation of gpx-4, gpx-5, or gpx-7 by disease status (39).

This study showed that gpx-1−/− mice exposed to subchronic cigarette smoke have enhanced BALF inflammation, suggesting that gpx-1 is required to control cigarette smoke-induced lung inflammation. In addition, the fact that gpx-1−/− mice have normal BALF cell counts at baseline (i.e., no cigarette smoke exposure) strengthens the hypothesis that gpx-1 may be protective in cigarette smoke-induced lung inflammation where there is an enhanced oxidant burden and is entirely consistent with the known biology of gpx-1, which appears to be limited to protection during oxidative stress (12, 13, 25). It should also be noted that the similarity in cell count numbers between sham-exposed WT and gpx-1−/− mice can help explain the absence of any overt phenotype in gpx-1−/− mice. Of interest was that the approximately twofold increase in BALF cellularity (total cell number, macrophages, and neutrophils) of gpx-1−/− mice exposed to cigarette smoke was abolished by ebselen administration. This clearly shows that there is a link between ebselen and its direct effects on gpx-1 in smoke-exposed mice. Moreover, this is in accord with our previous study showing that pretreatment of gpx1−/− mice with ebselen restored microvascular perfusion, limited the induction and activation of MPP-9, and attenuated the increases in infarct size and vascular permeability (59). In the present study, we did not explore the role of gpx-1 in the development of emphysema. We and others have proposed that short-term responses to cigarette smoke exposure may be a useful predictor of the development of emphysema, and such models may be a useful screen by which to identify therapeutic targets (9, 10, 55, 57, 60). Thus, given that gpx-1 protects against cigarette smoke-induced lung inflammation in the present study, we would predict that gpx-1 also protects against cigarette smoke-induced emphysema. This prediction is in accordance with work by Foronjy and colleagues (16) showing that transgenic gpx-1 mice exposed to cigarette smoke for 12 mo were completely protected against the formation of emphysema.

Macrophages and neutrophils are increased in patients with COPD where the numbers correlate with disease severity (44). Cigarette smoke can increase cell numbers by promoting cell proliferation, recruitment from the circulation, or prolonged survival in the airways. The observed numbers of BAL macrophages increased by subchronic cigarette smoke exposure in mice have been postulated as representative of both the progeny of resident alveolar macrophages and influx of blood monocytes adopting macrophage-like morphology (“alveolar monocytes”) (57). We have previously published that smoke-exposed mice had an increased percentage of BAL macrophages in mitosis (4.8 ± 0.1% compared with sham-exposed mice 0.6 ± 0.1%) based on nuclear mitotic figures indicating active cell division (57). Macrophage proliferation occurs in smokers’ alveolar macrophages since there is increased expression of the antiapoptotic long isoform of B cell leukemia/lymphoma (Bcl)-X and increased cytoplasmic expression of the cyclin-dependent kinase inhibitor p21CIP/WAF1, an inhibitory regulator of the cell cycle (51), and may account for the increased number of macrophages in smoking and COPD. Increased expression of the proliferation marker, Ki-67, in macrophages of smoking patients also supports the proliferative state of these cells (22). In addition, enhanced expression of the transcription factor MafB by cigarette smoke exposure inhibits mouse alveolar macrophage apoptosis and prolongs their survival in the cigarette smoke-exposed lung (32). While we have not explored the role of apoptosis in our present study, the enhanced infiltration of macrophages and neutrophils in BALF of gpx-1−/− mice is in accordance with whole lung gene upregulation of the chemotactic factors MIP-1α and IL-17A, respectively. IL-17A is involved in activating neutrophil-mobilizing factors such as IL-6 and GM-CSF and hence sustaining the accumulation and activity of neutrophils locally in the lungs (31). It was interesting to note that the levels of other chemotactic factors MIP-2 and GROα (data not shown) and the blood growth factor GM-CSF (a cytokine produced readily by the respiratory epithelium which regulates the activation and survival of macrophages and potently increases neutrophil survival and activation) were similar to those observed in the WT smoke-exposed lung. Interestingly, the levels of MCP-1, a recruiter of monocytes, were reduced in gpx-1−/− mice. Mixed reports have found either no increase or increased concentrations of MCP-1 in BAL and sputum of smokers where the correlation of MCP-1 is positive with neutrophil numbers (7, 52). However, MCP-1 gene expression is increased in peripheral lung tissue of COPD patients and smokers (50).

Proteases that break down connective tissue components are found in increased amounts in people with COPD. In patients with emphysema, there is an increase in BALF concentrations and macrophage expression of MPP-1 (collagenase) and MPP-9 (gelatinase) (15). Alveolar macrophages from normal smokers express more MPP-9 than those from normal subjects (30), and there is an even greater increase in cells from patients with COPD (45), which have greatly enhanced elastolytic activity (46). Neutrophils also secrete serine proteases, including neutrophil elastase, as well as MPP-9 (gelatinase B), which can contribute to tissue destruction (3). Cigarette smoke increased MPP-9 protein expression and net gelatinase activity in BALF of WT mice, correlating to the higher numbers of macrophages and neutrophils observed. It was no surprise that gpx-1−/− mice exposed to cigarette smoke had more BALF MPP-9 protein expression and net gelatinase activity given the greater numbers of macrophages and neutrophils. This is also in accordance with our previous study in which MPP-9 protein expression was increased in gpx-1−/− mice during cerebral ischemia-reperfusion injury (59).

Before using the gpx mimetic ebselen in vivo, we wanted to confirm that ebselen had antioxidant properties in vitro. An in vitro study of the human macrophage-like cell line Mono-Mac6 reported that CSE-mediated proinflammatory events are regulated by the redox status of the cells (62). Our study revealed that the mouse alveolar macrophage cell line (MH-S cells) had high levels of protein oxidation in response to CSE. Treatment of the cells with 10 and 100 μM ebselen markedly reduced CSE-induced protein oxidation. The oxidants O2− and NO in cigarette smoke can immediately react to form the highly reactive ONOO− molecule, which can directly oxidize proteins. One of the antioxidant properties of ebselen in mimicking gpx-1 is its ability to scavenge ONOO− (35). In addition, it has been reported that ebselen enhances pulmonary expression of both copper/zinc and manganese superoxide dismutases, which can contribute to a decrease in the formation of peroxynitrite by lowering the concentration of available O2− (23).
Ebselen has been shown to be protective in vivo in disease situations hallmarking by oxidative stress such as diabetes-associated atherosclerosis and cerebral ischemia-reperfusion injury (8, 59). In addition, ebselen has been used in clinical trials of acute ischemic stroke (38, 61). Specifically, Yamaguchi et al. (61) explored the effects of ebselen on the outcome of acute ischemic stroke in a multi-center, placebo-controlled, double-blind clinical trial. They demonstrated that early treatment (i.e., patients who started ebselen within 24 h of stroke onset) with ebselen (150 mg bid) improved the outcome of acute ischemic stroke (61). Similarly, Ogawa et al. (38) showed in a randomized, double-blind, placebo-controlled trial of ebselen conducted in patients with complete occlusion of the middle cerebral artery that ebselen protected the brain from ischemic damage in the acute stage. In this light, it is possible that ebselen may influence the key reactions involved in the inflammatory responses in COPD, but no studies have yet been reported on the protective role of ebselen in cigarette smoke-induced lung inflammation. We show for the first time that ebselen, when administered prophylactically, inhibits cigarette smoke-induced BALF neutrophilia and macrophage accumulation. Ebselen (10 mg/kg) produced comparable inhibition to that produced by the same dose of ebselen in ozone- (23), lipopolysaccharide- (19), and sephadex-induced (4) lung inflammation in rats.

In the present study, it is likely that ebselen reduced cigarette smoke-induced BALF inflammation by inhibiting the gene expression of a variety of neutrophil (IL-17A) and macrophage (MIP-1α, MCP-1) chemotactic factors pertinent to lung inflammation. Suppression of GM-CSF by ebselen would also have contributed to the reduced BALF cellularity given that GM-CSF is a survival factor for neutrophils and macrophages and that GM-CSF inhibits neutrophil apoptosis (20, 56). We have recently shown that neutralization of GM-CSF inhibits cigarette smoke-induced lung inflammation using this cigarette smoke exposure model (56). In addition, it could be possible that ebselen’s antioxidant properties of removing H2O2, scavenging ONOO−, and enhancing pulmonary expression of both copper/zinc and manganese SODs (which can contribute to a decrease in the formation of ONOO− by lowering the concentration of available O2•−) may have also contributed to reduced inflammation, although this was not measured in our study. It could also be possible that ebselen reduced cigarette smoke-induced BALF inflammation by inducing cell death as previously described by Guerin and Gauthier (17), although this was not measured in our study. Thus, targeting gpx-1 with mimetics such as ebselen might exert anti-inflammatory and antioxidant effects in vivo.

In our study, we found that prophylactic administration of ebselen reduced cigarette smoke-induced mRNA expression of the macrophage metalloelastase MMP-12. In patients with COPD, MMP-12 is produced in greater quantities in the lungs than in healthy patients, and emphysema induced by chronic cigarette smoke exposure is prevented in MMP-12−/− mice (21, 37). While the levels of MMP-12 protein were below detection using zymography in our study, we were able to show that cigarette smoke-induced BALF MMP-9 protein expression was reduced by ebselen. Lung proteolysis is actively counteracted by antiproteases. We did not measure antiproteases in this study. However, the zymographic and fluorogenic substrate approaches we used have the advantage of defining actual net matrix degrading activity over more sensitive but less informative methods such as immunocytochemistry and ELISA. These findings suggest that gpx-1 may have a role in the control of cigarette smoke-induced lung inflammation directed by the proteases MMP-9 and MMP-12 in mice.

It was interesting to note that similar to the prophylactic ebselen-treatment protocol, ebselen administered after the onset of cigarette smoke-induced lung inflammation was able to attenuate BALF inflammation, marked by reduced macrophages and neutrophils. We did not explore the mechanism behind this anti-inflammatory response but would assume there are reductions in the key neutrophil and macrophage chemotactic factors (MIP-1α, MCP-1, IL-17A), the neutrophil and macrophage survival factor GM-CSF, and the proteases MMP-9 and MMP-12. In addition, ebselen’s antioxidant properties could also be involved.

In summary, this study has shown that gpx-1 protects the lung from cigarette smoke-induced lung inflammation. In addition, we have shown that increasing the antioxidant capacity of the lungs by using a gpx-1 mimetic such as ebselen has proven to be beneficial in resolving inflammation induced by cigarette smoke. It is important to note that ebselen was effective when given prophylactically and perhaps more importantly when administered therapeutically (i.e., in established disease) as would be the case in clinical practice. Given that previous clinical trials with antioxidants such as NAC have had only modest effects on clinical parameters (reduction in exacerbations, hospitalization, and days of disability but no effect on decline in FEV1) and oxidative stress markers indicating lack of efficacy in COPD, it is clear that there is a need for more efficient and selective ways of targeting excessive oxidative stress in COPD (40). The striking effects of ebselen in our model suggest the possible therapeutic utility of targeting gpx-1 in human lung disease where high neutrophil and macrophage numbers, protease induction, and cytokine and chemokine overproduction are believed to be central agents in disease pathogenesis.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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