Increased ozone-induced airway neutrophilic inflammation in extracellular-superoxide dismutase null mice

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**Abstract** Extracellular-superoxide dismutase (EC-SOD) exists primarily in the tissue interstitium and the lung contains particularly large amounts of the enzyme. To determine the roles of EC-SOD and extracellularly formed superoxide radicals in the pulmonary response to the common air pollutant ozone, wild-type mice and mice lacking EC-SOD were exposed to 1 \(-\) 5 ppm ozone for 48 h. The exposure resulted in a marked neutrophilic inflammatory reaction observed both in the bronchoalveolar lavage fluid (BALF) and by histopathology of the lungs, which was much stronger in the mice lacking EC-SOD. Unlike the wild-type mice, the null mutants also showed increased levels of interleukin-6 in the BALF. The ozone exposure also resulted in increased airway mucosal permeability and cell damage as indicated by increased protein and lactate dehydrogenase in the BALF. There was, however, no difference between the two groups of mice. The results suggest that extracellular superoxide radicals are important inflammatory mediators in the pulmonary response to ozone, but in the present model, the radical and the infiltrating neutrophils contributed little to the pulmonary injury. The data, together with previous findings, support a role for EC-SOD as a modulator of inflammatory reactions.

**INTRODUCTION**

Extracellular-superoxide dismutase (EC-SOD) is a tetrameric, copper- and zinc-containing glycoprotein (1). An important property of EC-SOD is its affinity for heparin and heparan sulphate (2) and, in vivo, the bulk of the enzyme is anchored to heparan sulphate proteoglycans in the connective tissue interstitium and on cell surfaces (2, 3). A small fraction of the EC-SOD in the body is found in extracellular fluids such as plasma, lymph, synovial fluid and cerebrospinal fluid (4, 5). The two additional mammalian superoxide dismutase isoenzymes are located in other compartments: CuZn-SOD in the cytoplasm and Mn-SOD in the mitochondrial matrix. The physiological roles of the SOD isoenzymes should differ, since the substrate, the superoxide anion radical, crosses membranes poorly. Functional differences are also suggested by the large differences in the regulation of the expression of the enzymes (6). EC-SOD synthesis is primarily regulated by inflammatory cytokines (6). Mice lacking EC-SOD display no obvious spontaneous phenotype, but die prematurely from fulminant lung oedema when exposed to hyperoxic conditions (7).

In the mouse, the lung contains particularly large amounts of EC-SOD. Several studies have shown that the common air pollutant ozone, at low ambient levels, can adversely affect lung function, increase allergen sensitivity and cause neutrophilic airway inflammation in humans and animals (8). Ozone is strongly oxidizing and attacks compounds of the airway surfaces. A series of toxic compounds are thereby formed including reactive oxygen species, and antioxidants including EC-SOD (9) are induced in the lung following ozone exposure (10). The present study was undertaken to elucidate the roles of extracellularly formed superoxide radicals and EC-SOD in the pulmonary response to ozone. We examined bronchoalveolar lavage fluid (BALF) and morphological changes in the lungs after ozone exposure of mice lacking EC-SOD in comparison with normal wild-type mice.
METHODS

Mice and ozone exposure

The EC-SOD null mutant mice were generated by homologous recombination (7). Pilot experiments using subacute exposure (0–2 ppm) showed the mouse strains used in these studies (C57Bl/6 × 129/SV) to be relatively resistant to ozone (c.f. refs II, I2). In total, 16 EC-SOD null mutant and 16 wild-type mice were exposed to ozone. Two sets of experiments were conducted, each with eight EC-SOD null mutant mice and eight wild-type mice. The mice were 12 weeks old and all female. All 16 mice were placed together in a Perspex chamber with a 12-h light/dark cycle and were fed food and water ad libitum. The chamber was flushed with filtered air (relative humidity 50% and temperature 20°C) containing ozone at 2300 l h⁻¹ for 48 h. The ozone was generated by electric discharges in oxygen by a Fischer Ozone Generator (500 NM, Fischer Labor und Verfahrens-Technik-GmbH, Germany) and the chamber level was continuously measured photometrically and kept constant at 1.5 ppm. Eight EC-SOD null mutant mice and eight wild-type mice inhaling only air served as references for the ozone-exposed animals.

Bronchoalveolar lavage

The mice were sacrificed by cervical dislocation immediately following exposure, and the left lung lavaged with 2 × 0.3 ml 0.9% NaCl solution through a 1.1 mm catheter connected to a syringe. The fluid recovery was 73% in the null mutant group and 69% in the wild-type group. The total cell number was counted in a Burker chamber. The BALF was subsequently centrifuged at 400g for 15 min and cell slides were prepared using a Cytospin 3® (Shandon Southern Instruments Inc., Sewikly, PA, USA) at 96 g for 5 min. After staining according to May–Grönewald Giemsa for differential cell counts, 200 cells per slide were counted. Readings were done on blinded slides. The total protein concentrations were determined with Coomassie BB G-250 (Protein Assay, Biorad, Hercules, CA, USA), lactate dehydrogenase (LD) with a reagent kit (Boehringer Mannheim GmbH, Mannheim, Germany), and interleukin-6 (IL-6) by an ELISA (R&D systems, Minneapolis, MN, USA). The detection limit was <2 pg ml⁻¹. Interleukin-6 is a common marker of lung tissue inflammation after ozone exposure (13).

Morphology of the lungs

The right lung was perfusion-fixed through the trachea with a solution of 3.7% formaldehyde/4% glacial acetic acid at the pressure of a 20 cm water column. The specimens were fixed for 8 h, transferred to a graded series of ethanol baths for dehydration and subsequently processed for paraffin sectioning. Four-µm thick sections were stained with haematoxylin and eosin. Since the terminal bronchioli are known to exhibit the most pronounced morphological changes after oxidant exposures to air pollutants such as NO₂ and ozone, the microscopic scoring was focused on this area (14). Readings were done with the identity of the slides masked. At least three terminal bronchioli in close association with large venules in three different sections were read in each animal, and mean scores were used for comparison. The degree of inflammation in terms of neutrophil infiltration and oedema in the terminal bronchioli was given a score between 0 and 4, as follows: 0=no inflammation; 1=mild inflammation; 2=moderate inflammation; 3=severe inflammation; 4=very severe inflammation (15).

ASSESSMENT OF LUNG OEDEMA

A lower lobe of the right lung was dissected free and placed in a pre-weighed glass vial. After determination of the wet weight of the tissue, the vials were dried to constant weight at 60°C. The oedema was expressed as the ratio between the wet weight and the dry weight.

Statistical analysis

Data are generally presented as means ± SD. Analyses for statistical significance of difference were performed with the non-parametric Mann–Whitney U–Wilcoxon Rank Sum W test for independent groups, using the statistical package SPSS. A P value of < 0.05 was considered significant.

RESULTS

Cell response

In the BALF, there was a large increase in the number of neutrophil leukocytes in the mice exposed to 1.5 ppm ozone for 48 h, and mice lacking EC-SOD showed a significantly greater increase than did the wild-type mice [Fig. 1(a)] (1.31 ± 1.8 × 10⁴ and 0.48 ± 0.87 × 10⁴ cells ml⁻¹ of BALF for the EC-SOD null mutant mice and wild-type mice, respectively, P=0.017). The number of macrophages in the BALF did not differ significantly between the two groups of mice after the ozone exposure [Fig. 1(b)], nor did the number of lymphocytes [0.14 ± 0.39 × 10⁴ (n=13) and 0.045 ± 0.10 × 10⁴ (n=14) cells ml⁻¹ of BALF for the EC-SOD null mutant mice and wild-type mice, respectively, P=0.12)]. There was no statistically significant difference in the total number of lavageable cells from the airspace between mice from the two groups after exposure to ozone [4.07 ± 2.9 × 10⁴ (n=13) and 2.93 ± 1.4 × 10⁴ (n=14) cells ml⁻¹ of BALF, for the EC-SOD null mutant mice and wild-type mice, respectively, P=0.50]. In the air controls there were no
significant differences in the number of inflammatory leukocytes between the two groups of mice [Figs 1(a) and 1(b)].

Interleukin-6

The levels of IL-6 in the BALF of the air control mice were below the detection level in all individuals except one [Fig. 1(c)]. In contrast, there was a significant increase in IL-6 in the BALF of the ozone exposed mice lacking EC-SOD compared to the air controls \((P=0.02)\). The increase was also larger than that seen in the ozone-exposed wild-type mice \((P=0.04)\). The BALF IL-6 levels in these mice did not significantly differ from the air control mice \((P=0.96)\).

Tissue damage and permeability

There was a 3–4-fold increase of the intracellular enzyme LD in the BALF of mice exposed to ozone compared to the air controls. This indicates that the ozone inhalation caused damage to the cells in the lung, but there was no difference between the null and wild-type groups [Fig. 1(d)]. To assess the increase in airway mucosal permeability of the lungs following ozone exposure, the amount of protein present in the BALF was determined. As for the levels of LD, there was a difference between the ozone-exposed mice and the air controls, but not between the two groups of mice exposed to ozone [Fig. 1(e)].

Morphology of the lung tissue

There were no signs of inflammation in the lung tissue of EC-SOD null mutant mice and wild-type mice when inhaling air [Figs 1(f), 2(A) and 2(B)]. The interstitia were thin and normal, both in the alveoli and in the terminal bronchioli. In contrast, lung tissue from mice exposed to 1·5 ppm ozone for 48 h showed a marked inflammatory reaction [Figs 1(f), 2(C) and 2(D)], similar to what has been described previously (14). The inflammation was most pronounced in the terminal bronchiolar region, and showed oedema and infiltration of inflammatory cells from the vessels adjacent to the terminal bronchioli and into the interstitium and airway walls. In the separately evaluated BALF, this neutrophil traffic appeared as increased numbers of neutrophils in the air spaces [Fig. 1(a)]. In the tissue sections, more inflammatory cells were additionally seen in the alveolar capillaries, together with a mild alveolar oedema. In the airway epithelium and submucosa there were also increased numbers of inflammatory cells present.
The semiquantitative morphological analyses focused on the terminal bronchiolar changes. There was a relatively large variation in inflammation score between individual mice within the two groups. The overall inflammation score of the EC-SOD null mutant mice was significantly higher [Fig. 1(f)] ($1 \cdot 89 \pm 0.84$) than that for the wild-type mice ($0.64 \pm 0.97, P=0.0008$).

**Oedema**

There was no detectable oedema in the lungs of the mice exposed to ozone as determined by wet weight/dry weight ratios. The ratios did not differ significantly from the air controls ($4.8 \pm 0.5, n=16$ and $5.2 \pm 0.3, n=8$ for the EC-SOD null mutant mice exposed to ozone and air.

**Fig. 2** Tissue sections showing the terminal bronchioli of the mice. The photographs are representative for an individual with the mean inflammation score of each group. Arrows indicate venules in association with terminal bronchioles and asterisks terminal bronchioles. (A) air control EC-SOD null mutant mouse, (B) air control wild-type mouse, (C) EC-SOD null mutant mouse exposed to ozone, (D) wild-type mouse exposed to ozone.
respectively; $5.2 \pm 0.8$, $n=16$ and $5.2 \pm 0.2$, $n=8$
for the wild-type mice exposed to ozone and air, respectively.

**DISCUSSION**

Ozone exposure induced a marked inflammatory response, characterised by increased levels of neutrophils, IL-6, LD and protein in the BALF, as well as a marked neutrophilic inflammation of the lung tissue in full accordance with previous studies in animals and humans (10, 14). The mice lacking EC-SOD showed a greater neutrophilic inflammation and, unlike the wild-type mice, an increase in IL-6 in the BALF was observed. On the other hand, there were no significant differences in cell damage and airway mucosal permeability as indicated by leakage of protein and LD to the BALF.

The EC-SOD null mutant mice have previously been shown to be similar to wild-type mice in health status, in several antioxidant enzymes, and in a variety blood variables including leukocytes (7). Likewise, in the present study we found no significant differences in the BALF variables and histology between the wild-type and EC-SOD null mutant mice serving as air controls. Compensatory inductions of other antioxidant enzymes and differences in the other analysed variables should therefore not confound the interpretation of the present study.

The lungs (7) and arterial walls are the organs in mice that contain the highest activities of EC-SOD. The enzyme is expressed by a variety of cell types in the lung (16). Using immunohistochemistry, it has been shown both in the mouse (17) and the human lung (18) that EC-SOD is concentrated in the matrix under the epithelium, in the matrix surrounding vessels, around smooth muscle cells in larger airways and in alveolar septa. The enzyme thus exists in significant amounts in locations in which the inflammatory changes were found in the present study.

Ozone primarily reacts with a variety of components of the pulmonary epithelial lining fluid (19, 20). Several of the secondary products formed are very reactive and interact with cells of the airways, which respond by secreting pro-inflammatory factors (13, 20). In the resulting inflammatory reaction, a variety of sources of extracellular superoxide radicals can be envisaged, including activated NAD(P)H oxidases in neutrophils, macrophages, endothelial cells, fibroblasts, and smooth muscle cells (21) and as a by-product during prostaglandin and leukotriene biosynthesis (22). The significant difference in ozone-induced inflammatory response between the EC-SOD null mutant and the wild-type mice, suggests that superoxide radicals in the extracellular space contribute to the amplification of the inflammatory response, particularly the influx of leukocytes. The present findings may be related to the previous findings that injection of SOD reduces adhesion of leukocytes following tissue ischaemia/reperfusion (23), and infusion of oxidized low-density lipoprotein (24).

Despite the enhanced neutrophilic infiltration in the EC-SOD null mice, there was no evidence for enhanced cell damage and airway mucosal permeability, reflected by increased LD and protein levels in BALF compared to wild-type mice. The result is in accordance with a previous study on ozone-exposed mice in which prevention of neutrophil infiltration had no effect on the leakage of protein to the BALF (31). Possibly the neutrophils in the lung in the present model are only activated to a limited extent. Major activation of the neutrophils and secretion of superoxide radical should conceivably be more noxious in the EC-SOD null mice than in the controls.

EC-SOD null mice are more sensitive to hyperoxia and die prematurely with fulminant lung oedema (7). As with ozone, there is no major primary increased formation of superoxide radicals in the extracellular space. The enhanced response of the EC-SOD null mice to hyperoxia is apparently caused by superoxide radicals formed as a result of a secondary inflammatory reaction. The characteristic of the inflammatory reactions caused by ozone and hyperoxia, however, differ widely. The results from the two models, together with previous findings that the synthesis of EC-SOD is widely regulated by a variety of inflammatory cytokines (6), provide evidence that a physiological role of the enzyme may be to serve as a modulator of inflammatory reactions.

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