The American Journal of Pathology, Vol. 178, No. 6, June 2011

DOI: 10.1016/j.ajpath.2011.02.007

Immunopathology and Infectious Diseases

Extracellular Superoxide Dismutase in Macrophages Augments Bacterial Killing by Promoting Phagocytosis

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Extracellular superoxide dismutase (EC-SOD) is abundant in the lung and limits inflammation and injury in response to many pulmonary insults. To test the hypothesis that EC-SOD has an important role in bacterial infections, wild-type and EC-SOD knockout (KO) mice were infected with *Escherichia coli* to induce pneumonia. Although mice in the EC-SOD KO group demonstrated greater pulmonary inflammation than did wild-type mice, there was less clearance of bacteria from their lungs after infection. Macrophages and neutrophils express EC-SOD; however, its function and subcellular localization in these inflammatory cells is unclear. In the present study, immunogold electron microscopy revealed EC-SOD in membrane-bound vesicles of phagocytes. These findings suggest that inflammatory cell EC-SOD may have a role in antibacterial defense. To test this hypothesis, phagocytes from wild-type and EC-SOD KO mice were evaluated. Although macrophages lacking EC-SOD produced more reactive oxygen species than did cells expressing EC-SOD after stimulation, they demonstrated significantly impaired phagocytosis and killing of bacteria. Overall, this suggests that EC-SOD facilitates clearance of bacteria and limits inflammation in response to infection by promoting bacterial phagocytosis. ***(Am J Pathol 2011, 178:2752–2759; DOI: 10.1016/j.ajpath.2011.02.007)***

Acute infection of the lower respiratory tract is the leading infectious cause of premature death, with a greater disease burden than cancer.[1–3] In the lung, alveolar macrophages and neutrophils are phagocytic inflammatory cells that have a central role in the elimination of bacterial infections. As the first line of defense, macrophages and neutrophils produce potent molecules such as reactive oxygen species (ROS) and proteases in an attempt to eliminate microbes. While this antimicrobial arsenal is beneficial for eradicating the infectious organisms, these toxic agents can directly cause pulmonary damage and contribute to complications such as acute respiratory distress syndrome.[4–6]

Endogenous antioxidant enzymes are normal cellular defenses that can protect against ROS-induced tissue injury. Extracellular superoxide dismutase (EC-SOD) is a 135-kDa antioxidant enzyme that scavenges the free radical superoxide.[7] This isozyme of the superoxide dismutase family is highly expressed in the lung and arteries and is bound to the extracellular matrix via its positively charged heparin/matrix-binding domain.[7–10]

EC-SOD acts as both an anti-inflammatory and antifibrotic agent in a number of pulmonary diseases including bleomycin- and asbestos-induced pulmonary fibrosis,[11–14] hyperoxia,[15–17] lipopolysaccharide-induced inflammation,[18] and pulmonary infection.[19,20] One mechanism by which EC-SOD inhibits inflammation is directly binding to and inhibiting oxidative fragmentation of several components in the extracellular matrix including collagen, heparan sulfate, and hyaluronan after interstitial lung injury.[11,21–24] The importance of endogenous pulmonary EC-SOD was recently highlighted in a study that demonstrated that acute loss of EC-SOD resulted in 85% mortality secondary to the spontaneous development of acute respiratory distress syndrome.[25] This indicates that EC-SOD is essential for protecting the lungs against inflammation and injury even in ambient air. In addition to its localization on matrices and in extracellular fluids, EC-SOD is also present intracellularly in neutrophils and macrophages.[13,19,26] However, the local-

Supported by grants #09PRE2230108 from the American Hospital Association (M.L.M.) and R01 HL63700 from the National Institutes of Health (T.D.O.).

Accepted for publication February 1, 2011.

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ization and role of EC-SOD in these inflammatory cells have not been investigated.

Few studies have investigated the role of this potent antioxidant in bacterial infections; therefore, the present study investigated the response of wild-type and EC-SOD knockout (KO) mice to live *Escherichia coli* inoculation. To better understand the role of EC-SOD in infection, the subcellular localization and functional role of EC-SOD in inflammatory cells was also investigated. These findings are important for innate lung defense against *E. coli* in a murine model of pneumonia and possibly in other gram-negative bacterial infections.

Materials and Methods

Pneumonia Studies

Nine-week-old sex-matched C57BL/6 mice (Taconic Farms, Inc., Hudson, NY) and EC-SOD KO mice (congenic in the C57BL/6 background[13]) were used for all animal studies. The animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Mice were intratracheally instilled with approximately 1×10^6 colony-forming units (CFUs) live *E. coli* (ATCC 25922; American Type Culture Collection, Manassas, VA) in 50 μL PBS[27] and sacrificed immediately (0 hours) or at 6 or 24 hours after inoculation. The entire left lung was removed and placed in 1 mL sterile water for homogenization as previously described.[19] Bronchoalveolar lavage fluid (BALF) was collected via instillation and recovery of 0.6 mL 0.9% saline solution from the right lung, and the right lung was then inflation-fixed with 10% formalin for histologic analyses.

Sample Processing

Cell counts with differential values in the BALF and cell differential counts were performed as described previously.[12] BALF supernatants were stored at −70°C until analyses. The left lung was homogenized as previously described,[19] and the homogenates were separated into aliquots for quantitative cultures of bacteria, Western blot analysis, and myeloperoxidase assays. In brief, for Western blot analyses, buffer was added to an aliquot of lung homogenate to a final concentration of buffer containing 0.5% Triton X-100, 150 nmol/L NaCl, 15 mmol/L Tris, 1 mmol/L Ca Cl_2, and 1 mmol/L $MgCl_2$ (pH 7.4), incubated on ice for 30 minutes, and centrifuged at 10,000 × *g* for 20 minutes. The supernatants were stored at −80°C for later use. For myeloperoxidase activity measurements, the lung homogenate was added to a buffer to a final concentration of solution containing 50 mmol/L potassium phosphate (pH 6.0), 5 mmol/L EDTA, and 5% w/v hexadecyltrimethyl ammonium bromide. This mixture was then sonicated and centrifuged at 12,000 × *g* for 30 minutes. The supernatant was then stored at −80°C until use.

Recovery of Bacteria

Serial 10-fold dilutions of lung homogenates were cultured in Luria-Bertani agar using the pour plate method to determine the number of CFUs.

Myeloperoxidase Activity Assay

Lung homogenates in 50 mmol/L potassium phosphate (pH 6.0), 5 mmol/L EDTA, and 5% w/v hexadecyltrimethyl ammonium bromide were assayed in triplicate at a ratio of 1:30 v/v in assay buffer [50 mmol/L potassium phosphate buffer (pH 6.0), 0.167 mg/mL *o*-dianisidine dihydrochloride, 0.0005% hydrogen peroxide, and 0.01% hexadecyltrimethylammonium bromide] in a 96-well plate. Immediately after addition of assay buffer, myeloperoxidase activity was monitored by measuring the absorbance at 460 nm over 2 minutes. Relative myeloperoxidase activity was calculated as the change in absorbance over time per left lung (dA/min/left lung).

Isolation of Macrophages

Macrophages derived from bone marrow were isolated and cultured from wild-type and EC-SOD KO mice as previously described.[28,29] Twenty-four hours before use, macrophages were washed and cultured with media lacking both antibiotics and L929 supplement. Peritoneal macrophages were isolated from the peritoneum via instillation and recovery of sterile PBS without calcium or magnesium. Alveolar macrophages were harvested via instillation and recovery of 0.8 mL cold sterile PBS from the lungs. Both cell types were allowed to adhere at 37°C in a humidified 5% CO_2 atmosphere for 2 hours. Nonadherent cells were removed by means of gentle washing, and remaining cells were cultured overnight in media lacking both antibiotics and L929 supplement for experiments. The purity of the cell populations was verified at flow cytometry using macrophage marker phycoerythrin-conjugated anti-mouse F4/80 (Molecular Probes, Eugene, OR). For detection of EC-SOD, cell lysates were collected in ice-cold lysis buffer with protease inhibitors [1% Triton, 25 mmol/L HEPES, 150 mmol/L NaCl, 5 mmol/L EDTA (pH 7.5) with 100 μmol/L 3,4-dichloroisocoumarin, 10 μmol/L E-64, and 1 mmol/L 1,10-phenanthroline].

Immunolabeling and Western Blot Analysis

EC-SOD in human phagocytes in normal lung was localized using electron microscopy with immunogold labeling as previously described.[30] Western blot analysis of EC-SOD in BALF, cell lysate, or lung homogenates was performed as previously described,[12,23] and standardized to β-actin as a loading control when appropriate. The EC-SOD antibodies used recognize both full-length and proteolyzed forms of this antioxidant enzyme.

Oxidant Production by Phagocytes

Electron paramagnetic resonance (EPR) spectroscopy was performed to determine the location and amount of

oxidant production in wild-type and EC-SOD KO macrophages 10 minutes after stimulation with 15 μg/mL phorbol 12–myristate 13–acetate (PMA). Peritoneal macrophages isolated from wild-type and EC-SOD KO mice were resuspended (1×10^6 cells/mL) in Krebs HEPES buffer (pH 7.4). Identification of ROS production (superoxide, peroxynitrite, and other downstream single-electron reactive-oxygen intermediates) was accomplished by exposing the stimulated cells to either 50 μmol/L cell-permeable CMH (1-hydroxy-3-methoxy-carbonyl-2,2,5,5-tetramethylpyrrolidine) or 50 μmol/L cell-impermeable PPH (1-hydroxy-4-phosphono-oxy-2,2,6,6-tetramethylpiperidine) spin probes (Noxygen Science Transfer & Diagnostics GmbH, Elzach, Germany) and analyzed using a table-top EPR spectrometer (eScan; Bruker BioSpin, Billerica, MA).[31–33] Various amounts (in micromoles per liter) of CM radical were measured to generate a standard curve to quantify the amount of oxidants produced by wild-type and EC-SOD KO cells. EPR settings were as follows: field sweep, 50 G; microwave frequency, 9.78 GHz; microwave power 20 mW, modulation amplitude, 2 G; conversion time, 327 ms; time constant, 655 ms; and receiver gain, 1×10^5. All buffers were treated with Chelex resin, and contained 25 μmol/L deferoxamine. Absence of transition metals was confirmed by the inability to detect the ascorbyl radical on exposure of buffer to 100 μmol/L ascorbic acid. Extracellular superoxide generation produced by PMA-stimulated peritoneal macrophages isolated from wild-type and EC-SOD KO mice was also measured via reduction of partially acetylated cytochrome *c*, which was calculated as the rate of change in absorbance at 550 nm over 2 minutes.

Culturing of Bacteria

Enhanced green fluorescent protein (EGFP)–expressing *E. coli* (BL21 strain)[34,35] were grown overnight in Luria-Bertani broth with 50 μg/mL ampicillin at 37°C with continuous shaking. Cultures were refreshed and grown for 2 hours to reach log phase, washed with Dulbecco's PBS without calcium and magnesium, and used for phagocytosis and bacteria killing studies.

Bacteria Killing Assay

Macrophages derived from bone marrow from wild-type and EC-SOD KO mice (500,000 cells per tube) were incubated in suspension with EGFP-expressing *E. coli* at 37°C (cell/bacteria, 1:100) in media lacking both antibiotics and L929 supplement.[28,29] Wild-type and EC-SOD KO cells and bacteria were incubated independently as controls. After 1 hour of incubation in a static tube, the bacteria remaining were cultured in Luria-Bertani agar using the pour plate method to determine the number of CFUs. The percentage of bacteria killed was calculated as the mean number of CFUs of the bacteria alone minus the CFUs remaining after 1 hour of incubation with cells, divided by the mean number of CFUs of the bacteria alone, multiplied by 100.

Live-Cell Microscopy of Phagocytosis

Macrophages derived from bone marrow (1×10^5 cells per dish) were plated onto 35-mm collagen-coated glass-bottomed dishes (MatTek Corp, Ashland, MA). Multimode imaging (dimensions: X, Y, Z, time, and color) was used to image cellular interactions between phagocytes and bacteria in a temperature-controlled chamber (Tokai HIT Co., Ltd., Tokyo, Japan) at 37°C. Movies were obtained using a Nikon Ti inverted microscope (×40 magnification) running NIS-Elements AR 3.1 software (Nikon Instruments Inc., Melville, NY). Images were collected sequentially using a CoolSNAP HQ2 camera (Photometrics Ltd., Tucson, AZ) using shuttered illumination in both fluorescence and differential interference contrast. Movies were analyzed using NIS-Elements software. Phagocytosis was quantified over 1 hour by an observer (T.D.O.) blinded to the experimental groups.

Endocytosis Analyses

Macrophages derived from bone marrow (250,000 cells per tube) were incubated with 5 μg/mL Alexa Fluor 555 dextran (Molecular Probes) for 1 hour at either 4°C or 37°C. Cells were then fixed, and flow cytometric analysis was performed using a FACS Vantage along with DiVa and CellQuest analytical software (Becton Dickinson & Co., Franklin Lakes, NJ).

Cell Viability

To determine whether there were differences in cell viability between macrophages from wild-type and EC-SOD KO mice, bone marrow–derived macrophages were treated with various concentrations of PMA (Sigma-Aldridge Corp., St. Louis, MO), lipopolysaccharide (*E. coli* O26:B6; Sigma-Aldrich Corp.), and EGFP-expressing *E. coli.*[34,35] Cell viability was measured using the CellTiter 96 AQueous Non-Radioactive Assay (Promega Corp., Madison, WI) according to the manufacturer's instructions or by means of visual assessment using trypan blue.

Statistical Analyses

Data were analyzed using commercially available software (PRISM version 5; GraphPad Software Inc., San Diego, CA). Animal experiments were analyzed using two-way analysis of variance with a Bonferroni posttest. Comparisons with one variable were analyzed using an unpaired Student's *t*-test. The number of cells that phagocytosed bacteria were compared using Fisher's exact test. Unless otherwise noted, all values are given as mean ± SEM. $P < 0.05$ was considered statistically significant.

Results

EC-SOD Limits Inflammation after E. coli *Inoculation*

EC-SOD attenuates inflammation and oxidative injury in numerous pulmonary diseases; however, few studies

have investigated its role in limiting injury in response to bacterial infections. To determine the role of EC-SOD in *E. coli* pneumonia, wild-type C57BL/6 and EC-SOD KO mice (congenic with the C57BL/6 strain) received an intratracheal instillation of *E. coli* and were euthanized immediately (0 hours) or at 6 or 24 hours after inoculation. The expression of EC-SOD in the airspace and lung parenchyma was evaluated in wild-type mice via Western blot analyses of the BALF and lung homogenates. Consistent with previous work,[19] bacterial pneumonia leads to a significant increase in EC-SOD in the alveolar lining fluid (data not shown).

To evaluate the importance of EC-SOD in response to bacterial infection, pulmonary inflammation was assessed in the BALF and lung tissue of infected wild-type and EC-SOD KO mice. There was no significant difference in inflammation between wild-type and EC-SOD KO animals at 6 hours after *E. coli* inoculation based on the cellular content of the BALF and the level of myeloperoxidase in the lung (data not shown). However, histologic examination of the lungs at 24 hours after inoculation with *E. coli* revealed an increased number of inflammatory cells in the EC-SOD KO mice compared with the wild-type mice (Figure 1A). EC-SOD KO mice also had more inflammatory cells in the BALF than did wild-type mice at 24 hours after infection (Figure 1B). Most cells in the lung and BALF were neutrophils, although macrophages were also present. At 24 hours after *E. coli* inoculation there were significantly greater numbers of neutrophils in the BALF from EC-SOD KO mice compared with wild-type mice ($9.604 \times 10^5 \pm 1.625$ versus $5.270 \times 10^5 \pm 0.635$; Figure 1C) and higher myeloperoxidase activity in the lungs of EC-SOD KO mice compared with wild-type mice (Figure 1D). EC-SOD KO mice also exhibited significantly more protein in the BALF than did wild-type mice at 24 hours after infection (Figure 1E). These results are consistent with those of previous studies that demonstrated that EC-SOD expression in the lung inhibits inflammation in response to a wide variety of pulmonary injuries.[11–13,19]

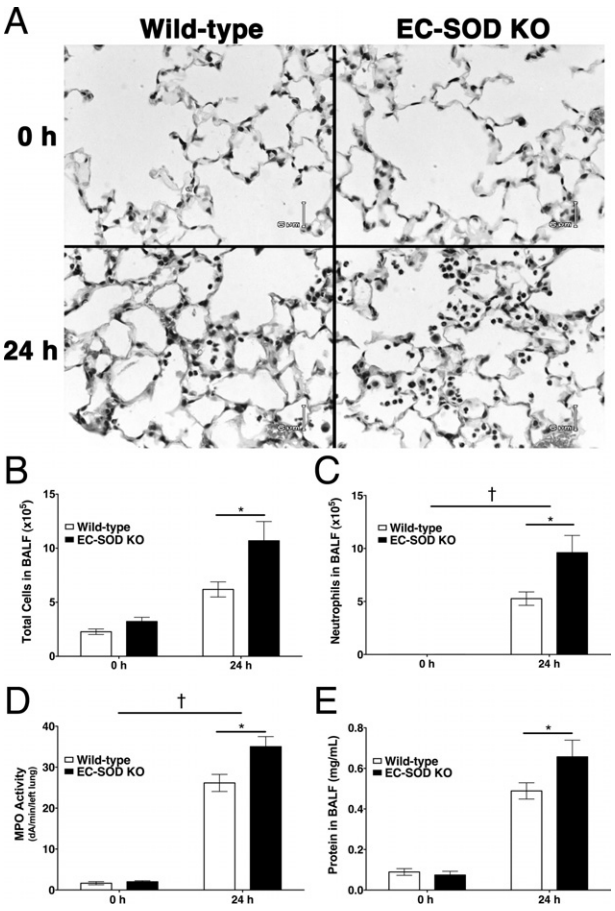

Figure 1. Compared with wild-type mice, EC-SOD KO mice exhibit increased inflammation. **A:** H&E staining of lung sections from wild-type and EC-SOD KO mice sacrificed at 0 and 24 hours after inoculation with *E. coli* demonstrates increased acute inflammation in the EC-SOD KO mice compared with wild-type mice. **B:** There was more inflammation (total cells) in the BALF from EC-SOD KO mice than from wild-type mice at 24 hours after infection. **C:** Differential counting demonstrated that this inflammatory response was primarily due to an increase in neutrophils in the BALF. **D:** Myeloperoxidase activity in lung homogenates also showed that EC-SOD KO mice exhibited significantly greater neutrophilic inflammation when compared with wild-type mice after inoculation with *E. coli*. **E:** Protein in the BALF was measured to assess injury, and compared with wild-type mice, the lungs of EC-SOD KO mice demonstrated significantly more protein in the alveolar lining fluid after infection. Data are expressed as mean ± SEM, analyzed using two-way analysis of variance with a Bonferroni post test. $^{*}P < 0.05$ indicates a significant difference between groups. $^{\dagger}P < 0.05$ indicates interaction, and hence a significant difference between wild-type and EC-SOD KO mice (n = six per strain for each time point).

Mice Lacking EC-SOD Have a Greater Bacterial Burden in the Lung after Pneumonia

Left lung homogenates from wild-type or EC-SOD KO mice at 0, 6, and 24 hours after *E. coli* inoculation were plated onto Luria-Bertani agar to determine the bacterial burden in the lungs. This burden is reported as the percentage of bacteria cleared from the lungs over time relative to the mean initial burden at 0 hours. Wild-type mice cleared more bacteria than did EC-SOD KO mice at both 6 hours (71.55 ± 4.694 versus 51.31 ± 6.637; n = 11 per strain) and 24 hours (99.90 ± 0.025 versus 99.80 ± 0.019; n = 6 per strain) after inoculation with bacteria (Figure 2). Therefore, even though EC-SOD KO mice demonstrated greater inflammation than did wild-type mice (Figure 1), the EC-SOD KO mice had significantly more viable bacteria in the lungs than did wild-type mice (Figure 2). Although the differences between EC-SOD KO and wild-type mice were statistically significant at both 6 and 24 hours after inoculation with bacteria ($P = 0.0217$ and $P = 0.0095$, respectively), the biological significance of this difference at 24 hours is unclear because the bacterial clearance of both strains at 24 hours is nearly 100%.

EC-SOD Is Present in Human and Murine Phagocytic Inflammatory Cells

EC-SOD is expressed in inflammatory cells[13,19,26]; however, the subcellular location and function are unknown. In the present study, electron microscopic immunolocalization of EC-SOD in human neutrophils and macrophages in human lung sections revealed that EC-SOD is located in

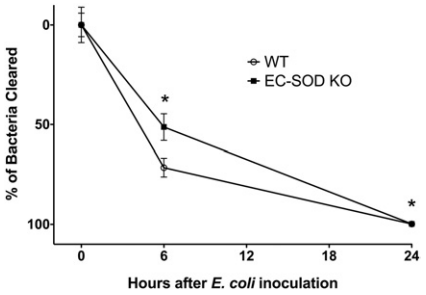

Figure 2. EC-SOD KO mice demonstrate greater bacterial burden than do wild-type (WT) mice after inoculation with *E. coli*. Left lung homogenates from wild-type and EC-SOD KO mice after exposure to *E. coli* were plated on Luria-Bertani agar to determine the bacterial burden in the lungs. Percent clearance of bacteria for each strain was calculated as the mean number of CFUs at 0 hours minus the CFUs remaining in the lung at 6 or 24 hours, divided by the mean number of CFUs at 0 hours, multiplied by 100. $^*P < 0.05$, unpaired Student's *t*-test ($n = 11$ per strain at 6 hours, $P = 0.0217$; and $n = 6$ per strain at 24 hours, $P = 0.0095$).

membrane-bound vesicles of these cells (Figure 3, A–C). Similarly, Western blot analysis of extracts from alveolar macrophages isolated from untreated wild-type and EC-SOD KO mice revealed expression of both proteolyzed and full-length EC-SOD in wild-type cells (Figure 3D). In addition to protein expression in these cells, EC-SOD activity was detected in the cell lysates from alveolar, peritoneal, and bone marrow–derived macrophages using a nitro blue tetrazolium SOD active stain (data not shown) as previously described.[9]

Macrophages Lacking EC-SOD Produce More Intracellular Oxidants

Inasmuch as phagocytic cell granules contain a potent arsenal of compounds involved in antimicrobial defense, the novel finding that EC-SOD is stored in membrane-bound vesicles in these cells suggests that EC-SOD may also have an important role in host defense against invading microbes. Peritoneal macrophages were isolated from wild-type and EC-SOD KO mice, and the purity of the macrophage populations was determined at flow cytometry using phycoerythrin-conjugated F4/80. The mean percentage of cells expressing F4/80 was 87.9 ± 1.3 and 91.133 ± 2.318 for wild-type and EC-SOD KO mice, respectively (mean ± standard deviation; $n = 3$ per strain).

EPR spectroscopy was performed to determine the intracellular and extracellular oxidant production of wild-type and EC-SOD KO macrophages at 10 minutes after stimulation with PMA (Figure 4). In the presence of the cell-permeable spin probe CMH, EC-SOD KO macrophages stimulated with PMA produced approximately 2.5-fold more oxidants than PMA-stimulated wild-type cells (2.691 μmol/L ± 0.5989 versus 1.049 μmol/L ± 0.2240; $n = 2$ per strain). Using the cell-impermeable spin probe PPH, there was no detectable extracellular oxidant production in PMA-stimulated wild-type or EC-SOD KO cells (Figure 4), which suggests that the oxidants produced by PMA stimulation were localized predominantly intracellularly. No oxidant production was detected with nonstimulated wild-type and EC-SOD KO macrophages exposed to either CMH or PPH spin probes (Figure 4). In addition, PMA stimulation of wild-type and EC-SOD KO macrophages did not lead to reduction of acetylated cytochrome *c* added to the culture medium (data not shown). The differences observed in oxidant production are not due to cell viability because

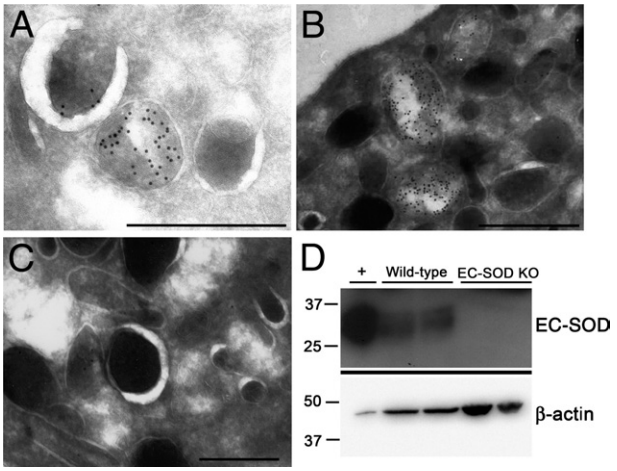

Figure 3. EC-SOD is present in human and mouse phagocytic cells. Immunogold labeling demonstrates the presence of EC-SOD in membrane-bound vesicles of a polymorphonuclear leukocyte (**A**) and an alveolar macrophage (**B**). **Black dots** represent protein A–gold (10-nm particles) bound to rabbit anti-human EC-SOD immunoglobulin. **C:** Nonimmune rabbit IgG was used as control and resulted in the absence of labeling. **D:** Western blot analysis of cell lysates from wild-type and EC-SOD KO alveolar macrophages revealed EC-SOD expression in wild-type but not EC-SOD KO alveolar macrophages. +, Mouse EC-SOD positive control. Scale bar = 0.5 μm.

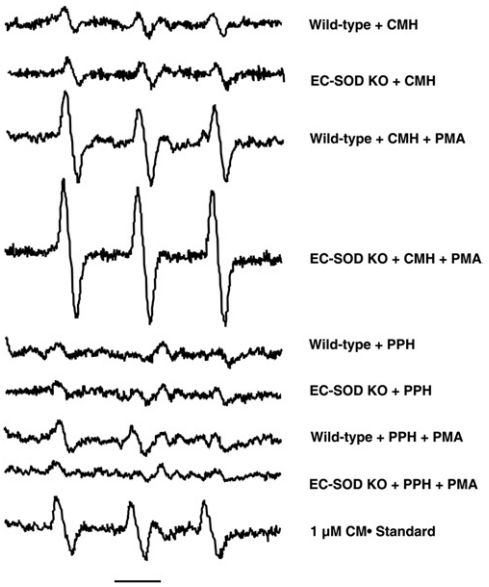

Figure 4. Macrophages lacking EC-SOD demonstrate more intracellular ROS production. Peritoneal macrophages isolated from wild-type and EC-SOD KO mice in Krebs HEPES buffer (pH 7.4) were exposed to 50 μmol/L cell-permeable spin probe CMH or 50 μmol/L non–cell-permeable spin probe PPH and analyzed for the CM radical using a spectrometer. Oxidant generation was measured in these cells after stimulation with 15 μg/mL PMA. The 1-μmol/L CM radical standard is also shown as a point of reference. Spectra represent five additive scans over 2 minutes at 37°C and are representative of three independent experiments. Bar = 10 G.

cells that express and lack EC-SOD have similar viability when exposed to PMA (data not shown). These data provide strong evidence that EC-SOD in macrophages reduces oxidant levels produced during the respiratory burst in response to stimuli.

Lack of EC-SOD Impairs Macrophage Function

Whereas the beneficial effects of releasing EC-SOD from activated neutrophils and macrophages for the surrounding host tissue are apparent, it is less clear what the role of EC-SOD will be on the actual antimicrobial function of these cells; however, *in vivo* data suggest that EC-SOD improved clearance of bacteria (Figure 2). *In vitro* studies were performed to assess clearance of bacteria in wild-type and EC-SOD KO cells. Macrophages derived from bone marrow were isolated from wild-type and EC-SOD KO mice, and the purity of the populations was determined to be 95.688 ± 2.247 and 93.513 ± 4.224, respectively, by flow cytometry using phycoerythrin-conjugated F4/80 (mean percentage of F4/80 expressing cells ± standard deviation, $n = 4$ per strain).

Oxidant production by stimulated phagocytes is important in clearance of bacteria because patients with chronic granulomatous disease demonstrate an increased incidence of bacterial infection, which is due to impaired NADPH oxidase function in phagocytes. However, *in vitro* assays of killing of bacteria reveal that macrophages lacking EC-SOD do not kill bacteria as efficiently as do wild-type macrophages (Figure 5). Specifically, macrophages containing EC-SOD killed a significantly greater percentage of bacteria than did cells lacking EC-SOD (51.48 ± 8.756 versus 6.471 ± 6.587; $P = 0.0143$). These *in vitro* findings are consistent with those of *in vivo* studies that demonstrated decreased clearance of bacteria in EC-SOD KO mice in a model of bacterial pneumonia (Figure 2). Together these findings, in conjunction with the EPR studies, suggest that EC-SOD has an important role in macrophage-mediated clearance of bacteria by regulating oxidants produced during the oxidative burst.

To further delineate the role of EC-SOD in macrophage-mediated clearance of bacteria, live-cell imaging

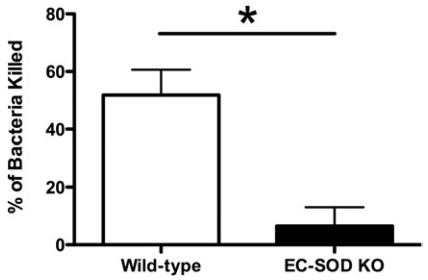

Figure 5. Macrophages lacking EC-SOD have decreased ability to kill bacteria. Bone marrow–derived macrophages from wild-type and EC-SOD KO mice were isolated and incubated for 1 hour with EGFP-expressing *E. coli* to assess the bacteria-killing ability of these cells. After incubation, the bacteria remaining were determined by colony counting, and the percentage of bacteria killed was calculated as the mean number of CFUs of the bacteria alone minus the CFUs remaining after 1 hour of incubation with cells, divided by the mean number of CFUs of the bacteria alone, multiplied by 100 (n = three per strain; $^*P = 0.0143$, unpaired Student's t-test).

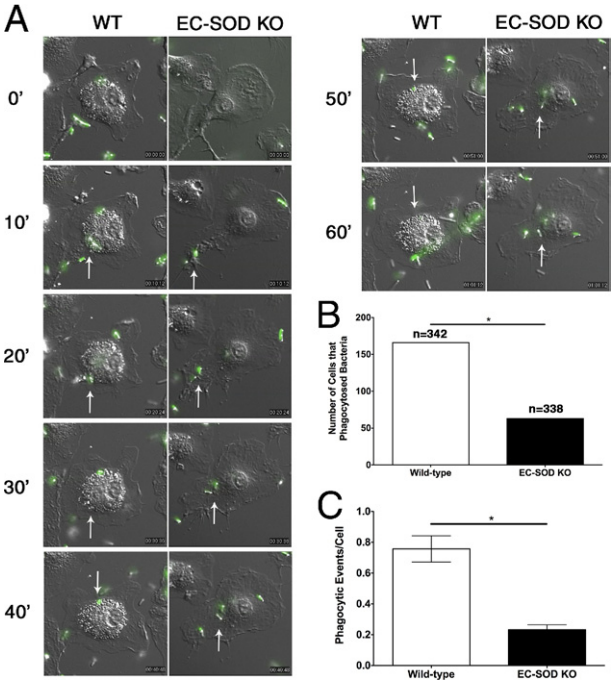

Figure 6. Lack of cellular EC-SOD impairs bacterial phagocytosis. **A:** Live-cell imaging of bacterial phagocytosis was performed by incubating bone marrow–derived macrophages isolated from wild-type (WT) and EC-SOD KO mice with EGFP-expressing *E. coli* (green). Images (obtained at 10-minute intervals; 40× magnification) show bacteria associating with and being phagocytosed by wild-type macrophages. **Arrows** represent bacteria being internalized (phagocytosed) by macrophages. However, although bacteria associate with EC-SOD KO macrophages, they are not phagocytosed, as is evident because the bacteria remain on the cell surface of these macrophages over 1 hour after addition of bacteria. Phagocytosis was quantified as the number of macrophages that phagocytosed bacteria (**B,** Fisher's exact test) and the number of bacteria that were phagocytosed (phagocytic events) per cell (**C,** unpaired Student's t-test) (n = total cells analyzed). $^*P < 0.05$.

was performed to visualize the dynamic interactions between EGFP-expressing *E. coli* and phagocytes expressing or lacking EC-SOD over 1 hour. Phagocytosis was assessed by enumerating the number of cells that phagocytosed at least one bacterium and the number of bacteria phagocytosed per cell in 10 to 15 random fields. These studies revealed that macrophages expressing EC-SOD (wild-type cells) are able to effectively phagocytose EGFP-expressing *E. coli*, whereas macrophages lacking this antioxidant enzyme (EC-SOD KO cells) exhibit impaired phagocytosis (Figure 6). This difference in macrophage function is not due to reduced cell viability because no difference in viability was observed between cells that expressed or lacked EC-SOD after treatment with lipopolysaccharide or EGFP-expressing *E. coli* (data not shown). This effect was also found to be specific for phagocytosis because endocytosis of fluorescent-conjugated dextran over 1 hour was not affected by the absence of EC-SOD (data not shown). These results suggest that macrophage expression of EC-SOD has an important role in promoting efficient phagocytosis of bacteria.

Discussion

Production of ROS is an important antimicrobial defense mechanism; however, this response must be carefully

regulated to minimize damage to the surrounding tissue. The present study demonstrated that lack of EC-SOD increases inflammation while decreasing clearance of bacteria in a murine model of bacterial pneumonia. Of note, EC-SOD KO mice are completely devoid of EC-SOD. Thus, it is unclear from these studies alone whether the effects on inflammation and clearance of bacteria are a result of inflammatory cell EC-SOD, pulmonary EC-SOD, or a combination of both sources. However, *in vitro* studies indicate that one mechanism in which EC-SOD contributes to clearance of bacteria is by promoting phagocytosis and killing of bacteria by macrophages. These novel findings demonstrate that production of oxidants is not sufficient to eliminate an infection; however, regulation of oxidant production by EC-SOD may be necessary for effective phagocytosis and killing of bacteria.

Recent studies have highlighted the importance of oxidant production in the induction and regulation of innate immunity, in particular in the context of chronic granulomatous disease. Patients with chronic granulomatous disease demonstrate defective phagocyte NADPH oxidase activity, resulting in decreased superoxide generation, which strongly correlates with increased incidence of life-threatening infections and excessive inflammation. Macrophages from these patients exhibit abnormal function because these cells release higher levels of anti-inflammatory cytokines and lower levels of proinflammatory cytokines in response to bacterial stimuli.[36] Similarly, a study using a murine model of chronic granulomatous disease further implicated oxidants as a critical regulator of the innate response because NAPDH oxidase activated the anti-inflammatory transcription factor Nrf-2 and inhibited the proinflammatory transcription factor NF-κB, thereby limiting inflammation.[37] Together, these findings demonstrate that oxidants produced via NADPH oxidase have a crucial role in regulating inflammation and host defense.

Numerous studies have also directly demonstrated that antioxidant activity is necessary for regulation of macrophage function. Specifically, EC-SOD attenuates lipopolysaccharide-induced inflammation in the lung by decreasing proinflammatory cytokine release from phagocytes.[18] Similarly, the antioxidant *N*-acetylcysteine modulates the macrophage phagocytic response to endotoxin by decreasing the production of ROS and the release of the proinflammatory cytokine tumor necrosis factor-α.[38,39] This effect was found to be potentially due to its ability to undergo cellular uptake and, therefore, to influence the oxidant-induced intracellular signal transduction activity in the phagocyte.[39] A more recent study found that hyperoxia reduces macrophage phagocytosis and that exogenous SOD treatment preserved actin cytoskeleton organization and phagocytosis of *Pseudomonas aeruginosa*.[40] These findings suggest that decreased superoxide scavenging may directly contribute to the defective phagocytosis observed in these cells. The present study further supports these previous studies in observing that regulation of oxidant production is important in promoting macrophage function. Whereas previous studies observed that administration of antioxidants or oxidative stress altered macrophage phagocytosis, the present study demonstrated that endogenous expression of EC-SOD in membrane-bound vesicles in these cells directly regulates oxidant production during the respiratory burst and is necessary to promote effective phagocytosis and killing of bacteria. However, it is still unclear whether unregulated or elevated oxidant production directly affects the ability of phagocytes to recognize and/or ingest the microbes; however, live-cell imaging studies suggest that bacteria are associating with macrophages but they are not being phagocytosed (Figure 6). Further research will need to be conducted to fully understand how EC-SOD located in phagocytic inflammatory cells modulates phagocytosis by controlling the oxidative environment in these cells.

A recent study demonstrated that an antioxidant mimetic, MnTE-2-PyP, was able to significantly decrease the number of *Mycobacterium abscessus* organisms growing in infected macrophage-like cells by promoting increased fusion of *M. abscessus*–containing phagosomes with lysosomes.[41] Thus, promoting phagolysosome formation may be one mechanism in which EC-SOD stimulates clearance of bacteria by macrophages.

The present data describe a novel and important function for EC-SOD in membrane-bound vesicles of macrophages in the innate inflammatory defense against bacterial infection. Western blot analysis of alveolar macrophage cell lysates from wild-type mice demonstrated the presence of both full-length and proteolyzed EC-SOD in these phagocytes. While protease inhibitors were present during the isolation of proteins from these cells, macrophages contain high amounts of proteases. Therefore, it is unknown whether the EC-SOD in macrophages is all full-length EC-SOD that underwent adventitious proteolysis during cell lysis or whether Western blot analysis truly reflects that macrophages contain a mixture of both proteolyzed and full-length EC-SOD. Regardless, the present findings suggest that EC-SOD in phagocytic inflammatory cells is enzymatically active and has a central role in promoting killing of bacteria by modulating phagocytosis. Although EC-SOD in phagocytic cells contributes to innate host defense against gram-negative bacteria, future investigations into the role of EC-SOD in other models of infection will need to be conducted to determine whether its role is conserved for other bacterial infections as well.

All models of lung injury examined to date demonstrated marked accumulation of EC-SOD in the alveolar lining fluid. On the basis of findings of the present study, accumulation of EC-SOD in response to pulmonary injury is likely an adaptive response that is important in innate immune defense against bacterial infection. Thus, supplementation of EC-SOD may also be useful as an adjuvant to standard antibiotic therapy in the treatment of microbial infections.

Acknowledgments

We thank Bruce Freeman, Ph.D., for guidance, and Beth Ganis, B.S., for technical assistance with maintaining the EC-SOD KO murine colony.

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