

Cardiovascular, Pulmonary and Renal Pathology

Loss of Extracellular Superoxide Dismutase Leads to Acute Lung Damage in the Presence of Ambient Air

A Potential Mechanism Underlying Adult Respiratory Distress Syndrome

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The extracellular superoxide dismutase 3 (SOD3) is highly expressed in both blood vessels and lungs. In different models of pulmonary injury, SOD3 is reduced; however, it is unclear whether this contributes to lung injury. To study the role of acute SOD3 reduction in lung injury, the SOD3 gene was deleted in adult mice by using the Cre-Lox technology. Acute reduction of SOD3 led to a fivefold increase in lung superoxide, marked inflammatory cell infiltration, a threefold increase in the arterial-alveolar gradient, respiratory acidosis, histological changes similar to those observed in adult respiratory distress syndrome, and 85% mortality. Treatment with the SOD mimetic MnTBAP and intranasal administration of SOD-containing polyketal microparticles reduced mortality, prevented the histological alterations, and reduced lung superoxide levels. To understand how mice with the SOD3 embryonic deletion survived without lung injury, gene array analysis was performed. These data demonstrated the up-regulation of 37 genes and down-regulation of nine genes, including those involved in cell signaling, inflammation, and gene transcription in SOD3^{-/-} mice compared with either mice with acute SOD3 reduction or wild-type controls. These studies show that SOD3 is essential for survival in the presence of ambient oxygen and that acute loss of this enzyme can lead to severe

lung damage. Strategies either to prevent SOD3 inactivation or to augment its levels might prove useful in the treatment of acute lung injury. (Am J Pathol 2008, 173:915–926; DOI: 10.2353/ajpath.2008.080119)

The superoxide dismutases (SODs) are major defenses against oxidative damage caused by the superoxide anion (O₂⁻).¹ There are three isoforms of SOD in mammalian cells. In most tissues, the copper/zinc superoxide dismutase (Cu/ZnSOD or SOD1), a cytoplasmic copper-containing enzyme, is predominant. Mice lacking SOD1 are predisposed to hepatic fat deposition, hepatic tumor development, motor neuron disease, abnormal vascular function, and vascular hypertrophy.^{2–6} Manganese superoxide dismutase (MnSOD or SOD2) is localized to the mitochondria, where it plays a major role in defending against O₂⁻ generated as a co-product of electron transport.⁷ Mice lacking MnSOD die of a cardiomyopathy within the first 10 days of life.⁸ The last discovered of the SOD isoforms is extracellular superoxide dismutase, or SOD3. SOD3 is similar to SOD1 in that it is a copper/zinc-containing enzyme; however, it contains a signal se-

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quence that targets it to the Golgi secretory apparatus and it is loaded with copper via two novel proteins, Atox-1 and Menkes.⁹ SOD3 is secreted via the trans-Golgi network and is bound to components of the extracellular matrix including heparin binding sites, fibullin-5,¹⁰ collagen-I, and hyaluronan.^{11–13} While SOD3 represents less than 5% of the total SOD in most cells, it is highly expressed in the lung.^{14,15}

The adult respiratory distress syndrome represents a major cause of morbidity and mortality in hospitalized patients and is observed in the setting of diverse clinical insults, including sepsis, hemorrhage, surgery, trauma, and extensive transfusion. The precise mechanisms whereby these various clinical entities predispose to lung injury remain poorly defined. Of note, several experimental models of lung injury, including hypoxia, asbestos exposure, bleomycin, and hyperoxia, are associated with reduced lung SOD3 protein and activity.^{16–19} These studies suggest that extracellular oxidative stress contributes to the pathogenesis of acute lung injury and that SOD3 protects against it.^{19–22}

In these cases of lung injury, it is unclear whether the loss of SOD3 contributes to or is simply a consequence of lung injury. Of note, lung injury caused by exposure to 100% oxygen is hastened in mice with embryonic deletion of SOD3 (SOD3^{-/-} mice). Embryonic SOD3^{-/-} mice tolerate exposure to ambient oxygen levels without difficulty; however, they have had a life-time to compensate for any deleterious effects of SOD3 deletion. These animals are therefore not informative in determining the role of acute SOD3 reduction in causing lung damage in the presence of normal oxygen concentrations.

The present study was performed to accomplish the following goals. First, we sought to determine whether acute reductions in SOD3, similar to that observed in experimental models of lung damage, could cause lung injury without exposure to hyperoxia. Second, we sought to develop a method of delivering exogenous SOD to prevent lung damage. To this end, we developed a novel polyketal particle that could be delivered intranasally and that could release SOD slowly over several days. Third, we examined potential compensatory mechanisms present in mice with embryonic deletion of SOD3 that could potentially prevent lung damage in these animals.

Materials and Methods

Creation and Study of $Tg^{cre/esr} \times ecSOD^{loxP/loxP}$ Mice

The targeting vector was created by modifying a 6.12-kb *Hind*III fragment of the mouse *SOD3* gene obtained from the BAC clone 229-C-20 on vector pBeloBAC (Genome Systems, St. Louis, MO). This was extended by cloning an additional 3 kb into the 5' end. A single loxP site was inserted into intron 1 at the *Aat*II site and a loxP-flanked neomycin resistance cassette was inserted at *Ap*I site 3' of exon 2. All three loxP sites were in the same orientation. The targeting vector used for homologous recombination was 10.7 kbp in length and the 5' and 3' homologous

sequences for targeting were 4.43 kb and 1.23 kb, respectively.

Electroporation of the linearized DNA fragment into embryonic stem cells yielded 306 clones resistant to G418. Southern blotting using a probe external to the targeting vector indicated that two clones had successful recombination. The neomycin cassette was removed by transfection of embryonic stem cells with Cre-recombinase containing plasmid, yielding cells that harbored a SOD3 targeted allele (see Supplemental Figure 1 available at <http://ajp.amjpathol.org>). A high percentage of chimeric mice were generated by blast injection and were bred to C57BL/6 mice to allow germ line transmission of the floxed SOD3 allele. Heterozygous $ecSOD^{loxP}$ offspring were backcrossed six generations to C57BL/6 wild-type mice before being crossed to the tamoxifen-Cre transgenic mice. These were then intercrossed a second time to generate $Tg^{cre/esr} \times ecSOD^{loxP/loxP}$ experimental animals. The control group for experiments was C57BL/6 mice that were purchased from the Jackson Laboratory (Bar Harbor, Maine). Mice were fed with regular chow diet (LabDiet, rodent diet no. 5001) *ad libitum* and studied between 10 and 12 weeks of age after CO₂ euthanization. The Emory University Animal Care and Use Committee approved the protocol for animal use. Experiments were performed when mice were 3 months of age.

Measurement of Blood Gases, Blood Pressure, and Cardiac Contractility

Mice were anesthetized with 1% to 2% isoflurane in 100% O₂, the abdomen opened, and ~150 μ l of blood was collected from the abdominal aorta into a heparinized syringe and analyzed on an ABL5 blood gas analyzer (Radiometer Copenhagen). Arterial blood samples were obtained as soon as the animals became ill. Total anesthesia time was held constant in mice from each treatment group. Partial pressures of oxygen, CO₂, and pH were measured to calculate arterial-alveolar gradient. Blood pressure was measured noninvasively via the tail cuff method and invasively using radiotelemetry as previously described.²³ Cardiac function was evaluated using the Siemens Sequoia Acuson 512 Sonograph (Siemens, Mountain View, CA) and 15L8 linear transducer (14 MHz). Two-dimensional images were acquired at a depth setting of 2 cm.

Tamoxifen Injection and Administration of MnTBAP and SOD Microparticles

Cre-recombinase was activated by the intraperitoneal injection of tamoxifen (3 mg/20 g of body weight) for 5 consecutive days. Control mice were studied in parallel. These animals received injections with an equivalent volume of the vehicle, corn oil, for 5 consecutive days. As another control, C57BL/6 mice were treated for a similar period of time with tamoxifen. In some experiments, the SOD mimetic MnTBAP (Calbiochem catalog no. 475870, purity >95%, 1 mg/20 g of body weight) was injected

intraperitoneally daily starting 2 days before tamoxifen injection, during tamoxifen treatment, and for 7 days after tamoxifen treatment. In other experiments, 80 μ l of polyketal particles containing SOD (5 mg/ml, described below) were administered intranasally while mice were briefly anesthetized with isoflurane (2%).

Histology and Immunostaining

After CO₂ euthanization, a cannula was placed in the left ventricle and the mice were initially perfused at 100 mmHg with saline to remove blood and then with 10% formaldehyde. Lungs and other organs were harvested after 10 minutes of perfusion and preserved in formaldehyde until embedded in paraffin, sectioned, and immunostained for SOD3 using a rabbit polyclonal antibody against SOD3 previously described.²⁴

Flow Cytometry Analysis of Lung Inflammatory Cells

Lungs were cleared of blood by perfusion with phosphate-buffered saline (PBS), excised, and digested using collagenase type IX (125 U/ml), collagenase type IS (450 U/ml), and hyaluronidase IS (60 U/ml) dissolved in 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-PBS buffer containing calcium. The digested tissue was then passed through a 70- μ m sterile cell strainer (Falcon, BD), yielding single cell suspensions. Cell labeling was performed using the following antibodies (all from BD PharMingen, apart from CD49b): fluorescein isothiocyanate anti-CD45 (30-F11); PerCP anti-CD45 (30-F11); PE anti-CD4 (GK1.5); APC anti-CD4 (GK1.5); PerCP anti-CD8 (53-6.7); APC anti-CD3 (145-2C11); fluorescein isothiocyanate anti-I-Ab (AF6-120.1); APC anti-CD11c (HL3); APC CD11b (M1/70); APC CD4 (RM4-5); PerCP CD4 (RM4-5); PE NK1.1 (PK136); PE CD49b (eBioscience). Cells were washed twice with 1% bovine serum albumin-PBS buffer and additionally incubated for 30 minutes in 37°C with complete media (RPMI 1640 with 10% fetal calf serum). Fluorescence-activated cell sorting analysis was performed using a Becton Dickson LSRII flow cytometer. An initial gate was applied to exclude cell debris from further analysis, and CD45 staining was used to identify leukocytes within the aortic cell suspension. Within the CD45⁺ gate individual subpopulations of leukocytes were identified with antibodies listed above. Data were analyzed using Flowjo software (Treestar) and expressed as an absolute number of cells per two lungs.

Western Blots and SOD Activity

Protein expression was examined using Western blot analysis as previously described. Antibodies used were the following: anti-SOD1 from Biodesign International; anti-SOD2 from Stressgen; and anti-SOD3 as previously described.²⁴ Equal gel loading was determined by blotting

for α -actin (Sigma). Activity of SOD isoforms was determined spectrophotometrically by monitoring the inhibition of the rate of xanthine oxidase-mediated reduction of cytochrome c. SOD3 was separated from intracellular SOD with concavalin A as described previously.²⁴

Detection of Extracellular Superoxide Using Electron Spin Resonance

To detect lung O₂⁻ production, we used electron spin resonance spectroscopy with the CAT1H spin probe as previously described.²⁵ The SOD-inhibited amplitude of the low-field component of the electron spin resonance spectra of oxidized CAT1H was used to quantify extracellular O₂⁻ production. Values were normalized to dry weight.

Formulation of SOD-Loaded Microparticles

SOD-loaded microparticles were formulated from the acid-sensitive polymer poly(cyclohexane-1,4-diyl acetone dimethylene ketal) using a modified water-in-oil-in-water (w/o/w) double-emulsion procedure. As reported previously, SOD-loaded poly(cyclohexane-1,4-diyl acetone dimethylene ketal) microparticles degrade in acidic environments and have been shown to decrease cellular superoxide *in vitro*.²⁶ Briefly, 15 mg of SOD (Sigma) was dissolved in 75 μ l of a 2.5% (w/v) polyvinyl alcohol (Sigma, 31–50 kd) solution prepared from a pH 8.0 sodium phosphate buffer (0.1 mol/L). This aqueous SOD/polyvinyl alcohol solution was dispersed via homogenization (21,000 rpm for 60 seconds.) into 1.0 ml of a 15% (w/v) poly(cyclohexane-1,4-diyl acetone dimethylene ketal)/CHCl₃ solution, generating the w/o primary emulsion. This SOD-containing w/o emulsion was then transferred by pipette to the bottom of a 24-ml vial containing 15 ml of an aqueous 5% (w/v) polyvinyl alcohol solution and homogenized (9500 rpm for 90 seconds.) to produce the w/o/w secondary emulsion. This w/o/w emulsion was then poured into a stirred 5% polyvinyl alcohol solution buffered at pH 8.0 by a 0.1 mol/L sodium phosphate buffer. This mixture was then continuously stirred for 3 hours to allow the CHCl₃ to evaporate, leaving the polymer hardened around the SOD to generate SOD microparticles. To remove the excess polyvinyl alcohol, the hardened particles were transferred to 50-ml centrifuged tubes and washed by successively isolating the particles via centrifugation, decanting the supernatant, and then resuspending the particles in distilled water. The particles were washed three times and then freeze-dried, producing approximately 30 mg of SOD microparticles. A scanning electron microscopic image of the SOD microparticles (see Supplemental Figure 2 available at <http://ajp.amjpathol.org>) shows that these particles have diameters from 1 to 25 μ m, with the majority of the particles having diameters in the 5- to 20- μ m range.

The amount of SOD loaded into the particles was determined by a commercially available BCA protein assay kit (BioRad) using the following procedure. The encapsulated SOD was recovered from the microparticles by

hydrolyzing 5 mg of SOD microparticles in a 1 N HCl solution containing 2% (w/v) sodium dodecyl sulfate to hydrolyze the acid-sensitive poly(cyclohexane-1,4-diyl acetone dimethylene ketal) polymer. This acidic solution was neutralized with 1 N NaOH and assayed for protein content using the BCA protein assay with various concentrations of SOD in 1% sodium dodecyl sulfate that had been acidified and subsequently neutralized as a standard. The protein loading assays showed that each milligram of SOD microparticles contained 32.1 μg of SOD. Further details regarding these microparticles have been described previously.²⁷

Statistical Analysis

Data are expressed as means \pm SEM. Comparisons between groups were performed using analysis of variance and a Bonferroni/Dunn post hoc test for comparison of selected groups.

Results

Conditional Reduction of SOD3

$Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice were phenotypically identical to C57BL/6 mice. Administration of tamoxifen had no effect on C57BL/6 mice or on $ecSOD^{loxp/loxp}$ mice not harboring Cre-recombinase. Likewise, administration of vehicle (corn oil) to either C57BL/6 or $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice had no effect on survival. In contrast, administration of tamoxifen to $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice led to striking mortality such that 85% of mice died within 7 days (Figure 1). The pattern of death in these animals was characterized by normal behavior without any evidence of illness until a few hours before death, when they be-

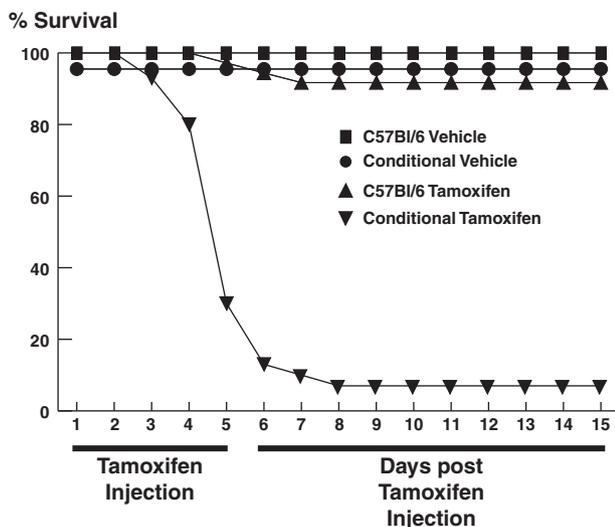


Figure 1. Effect of SOD3 deletion on survival. C57BL/6 and $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice were injected with tamoxifen (3 mg/20 g of body weight) or vehicle (equivalent volume of corn oil) intraperitoneally for 5 consecutive days. In additional experiments conditional SOD3 deletion, mice lacking the cre/esr transgene ($Cre^{-/-}$) were injected with tamoxifen.

came hunched, demonstrated limited motion, had tachypnea, and died shortly thereafter. After recognizing this, we began to sacrifice mice at the time that they became ill and harvest organs for study. The control animals (C57BL/6 receiving vehicle and tamoxifen and $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice receiving vehicle) were euthanized in parallel at this time. In general, this was between 3 and 7 days following onset of tamoxifen injections. Data in the figures therefore represent these time points.

Tamoxifen treatment reduced SOD3 protein without altering SOD1 levels in lungs of $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice while having no effect in C57BL/6 mice (Figure 2A). The decrease in SOD3 protein was paralleled by a reduction in its enzymatic activity, while there was no change in activity of intracellular SOD isoforms (Figure 2B). Polymerase chain reaction, using primers external to the loxP sites flanking exon 3, confirmed deletion of this exon following 5 days of tamoxifen injection (see Supplemental Figure 3 available at <http://ajp.amjpathol.org>). Immunostaining also showed a marked decrease in lung SOD3 following tamoxifen administration in $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice with no change in C57BL/6 mice (Figure 2C). This reduction of lung SOD3 was associated with a marked increase in lung O_2^- levels as measured using electron spin resonance (Figure 2D). Treatment with MnTBAP, while not inhibiting scission of exon 3, prevented this increase in O_2^- (Figure 2D and Supplemental Figure 3 available at <http://ajp.amjpathol.org>).

SOD3 Reduction Induces Lung Damage

Histological examination of the heart (Figure 3A), aorta (Figure 3B), kidney, and liver (data not shown) revealed no significant pathology in $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice following tamoxifen injection. Likewise, lung structure was not altered in C57BL/6 mice given either vehicle or tamoxifen or in $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice given vehicle (Figure 3C). In contrast, administration of tamoxifen to $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice caused a striking alteration of lung histology, with thickening of the alveolar septa, a marked inflammatory cell infiltrate, hemorrhage into the alveoli, and loss of patent alveoli (Figure 3, C and D).

Acute reduction of SOD3 was associated with a striking increase in lung levels of granulocytes, T cells, and natural killer cells. Among T cells, CD8 and to a lesser extent CD4⁺ cells were increased (Figure 4). Reduction of SOD3 did not change lung content of dendritic cells, B cells, or macrophages (data not shown). Injection of tamoxifen in C57BL/6 mice or vehicle in to $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice had no effect on lung inflammatory cells.

In additional studies, we measured arterial blood gases while the mice were anesthetized with isoflurane and breathing 100% O_2 . Deletion of the SOD3 gene resulted in a reduction in pO_2 and pH, an increase in pCO_2 , and a marked widening of the arterial-alveolar oxygen gradient (Table 1). Neither tamoxifen nor vehicle altered these parameters in C57BL/6 mice, and vehicle had no effect on blood gases in $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice.

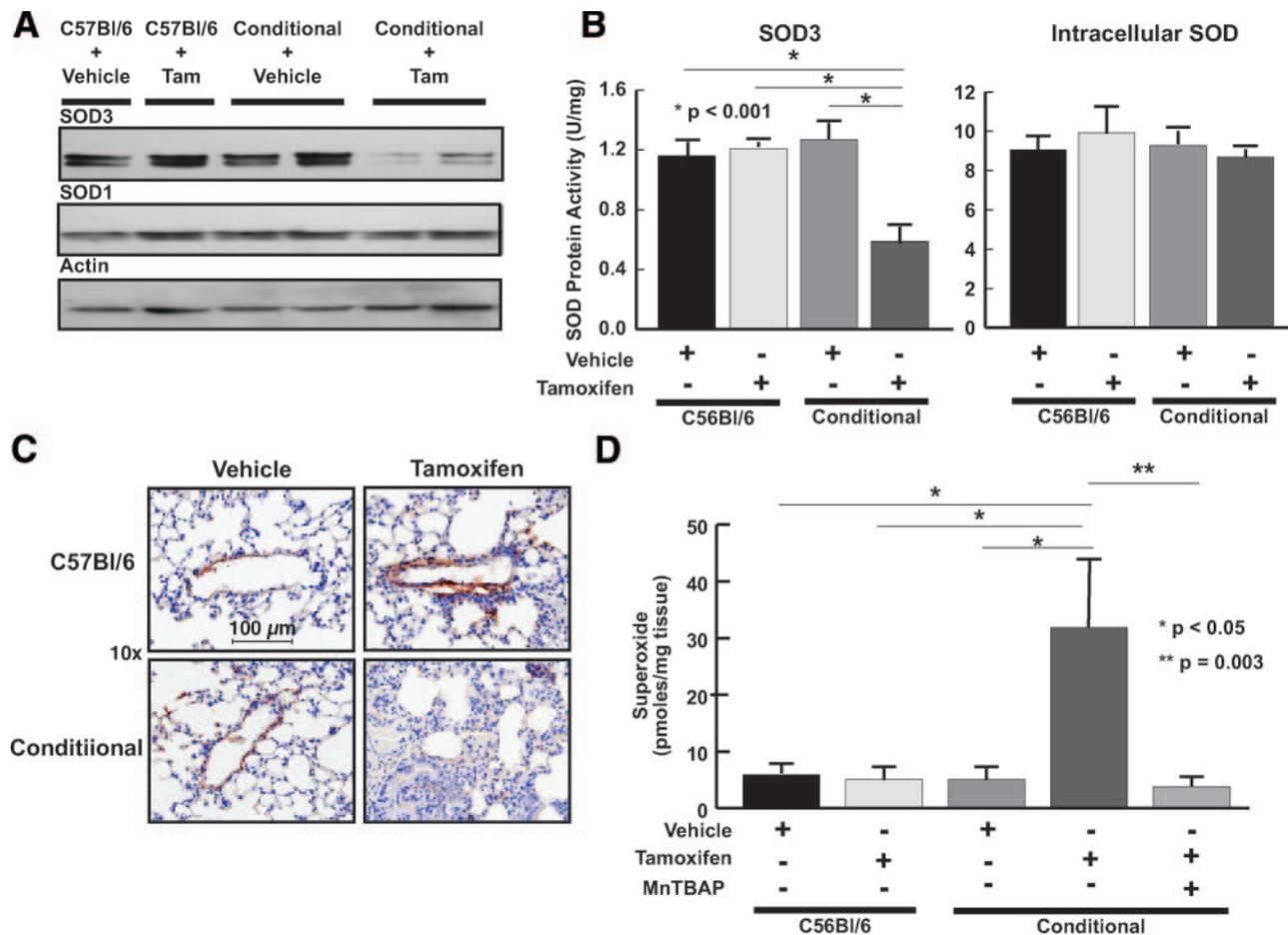


Figure 2. Effect of Cre-recombinase induction on SOD levels and activity and O_2^- levels in $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice. **A:** Western blot showing protein levels of SOD1 and SOD3 in lung tissue of C57BL/6 and $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice injected with either tamoxifen or vehicle. **B:** Effect of SOD3 depletion on intracellular SODs and SOD3 enzymatic activity in $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ and C57BL/6 mouse lung. Lung tissue was homogenized and SOD isoforms separated as described in the text. SOD activity was determined by measuring the ability to inhibit xanthine/xanthine oxidase cytochrome *c* reduction. Data are the means of three experiments. **C:** Immunohistochemistry and hematoxylin and eosin staining for SOD3 in lungs of C57BL/6 and $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice injected with tamoxifen (magnification, $\times 40$). **D:** Effect of SOD3 reduction on O_2^- production in C57BL/6 and $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice. Three or four 1-mm-thick slices were incubated in buffer containing the CAT1H probe for one hour at 37°C. Parallel slices were incubated with CAT1H and Cu/ZnSOD (100 U/ml). Aliquots of buffer were then examined using electron spin resonance ($n = 6$ or 7 per group).

Effect of Systemic and Locally Administered SOD on Survival and Lung Histology

In other experiments we determined if either systemic or local administration of antioxidants could prevent lung injury and mortality caused by deletion of ecSOD. Cotreatment of $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice with tamoxifen and intraperitoneal MnTBAP markedly reduced mortality and lung injury caused by SOD3 reduction (Figure 5, A and B). To directly demonstrate that pulmonary augmentation of SOD activity could prevent mortality and lung damage in these animals, we administered polyketal microparticles containing SOD1 via an intranasal route. This treatment also reduced mortality and alterations of lung morphology caused by acute SOD3 depletion (Figure 5B). Taken together, these data illustrate that a relatively moderate (50%) reduction of SOD3 in adult animals causes severe lung damage and death and that this can be prevented by either systemic augmentation of superoxide scavenging or by directed pulmonary delivery of SOD.

In additional experiments, we examined the effect of acute deletion of SOD3 on hemodynamics. Previous studies have shown that mice with embryonic knockout of SOD3 have normal blood pressure at baseline, but develop augmented hypertension during angiotensin II infusion.²⁵ Moreover, their aortas have higher levels of O_2^- and abnormal endothelium-dependent vasodilatation at baseline. We sought to determine whether acute deletion of SOD3 would cause an acute increase in blood pressure. Before the day on which the $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice became ill, blood pressure was not affected by tamoxifen injection as measured either noninvasively by the tail cuff method or invasively by radiotelemetry (Figure 6A). Likewise, cardiac contractility measured by echocardiography was preserved in $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice even when the animals became ill (Figure 6B).

To provide an understanding of compensatory mechanisms that might allow mice embryonically deleted SOD3 to survive, we compared mRNAs expressed in the lungs of mice with embryonic deletion of SOD3 to those in

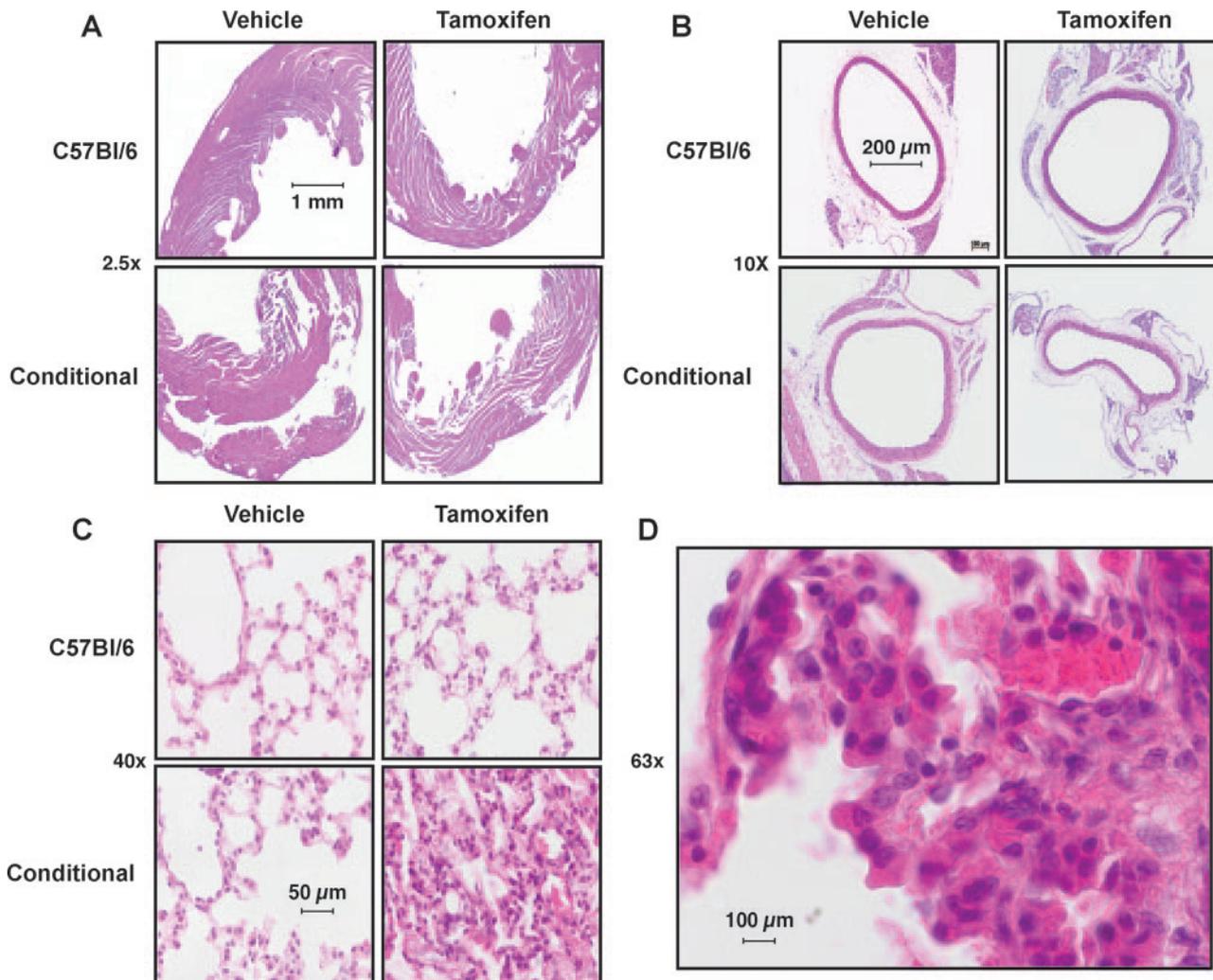


Figure 3. Effect of SOD3 deletion on histology of the heart, aorta, and lungs. Hematoxylin and eosin staining of 5- μ m paraffin-embedded slides of myocardium (A, $\times 10$); aorta (B, $\times 4$); and lung (C, $\times 40$) following either tamoxifen or vehicle injection ($\times 10$). D shows a higher magnification ($\times 63$) of eosin-stained lung with neutrophils, lymphocytes, and other inflammatory cells visible.

either normal wild-type mouse lungs or our $Tg^{cre/esr} \times ecSOD^{loxP/loxP}$ mice following tamoxifen injection using gene array studies. This analysis revealed 37 genes that were up-regulated in mice with embryonic deletion of SOD3 by more than twofold compared to either of the other groups (Table 2).

Discussion

There are several important new findings derived from the current study. First, in the presence of normal oxygen tension, an acute reduction of SOD3 causes severe lung disease. This adds to our current understanding of the role of SOD3, because prior studies of mice with embryonic deletion of SOD3 showed that lung damage only occurred on exposure to 100% oxygen or challenged with other insults. Our findings that acute loss of this protein in adult animals might have implications for other etiologies of lung disease, where SOD3 is also reduced. Second, we developed a novel method of locally delivering exogenous SOD using polyketal particles that was

effective in preventing lung disease. Given the similarity between the lung damage observed in our $Tg^{cre/esr} \times ecSOD^{loxP/loxP}$ mice and other models of acute lung injury, such a therapeutic approach might prove effective in treating additional causes of pulmonary injury. Third, our gene array analysis show a striking change in mRNA expression in the lungs of mice with embryonic SOD deletion, providing some insight into how mammals can compensate for a life-long loss of this gene.

The earth formed approximately 4.5 billion years ago, and it has been estimated that it was anoxic until about 2.3 billion years ago.²⁸ At that time, the first photosynthetic organisms, cyanobacteria, became capable of photosynthesis, leading to a sudden increase in oxygen in the oceans and atmosphere. This triggered the development of complex multicellular organisms that depended on oxygen;²⁹ however, a very complex biology arose as a result of this involving utilization of oxygen and protection against oxygen metabolites. Prokaryotic and eukaryotic cells adapted small molecules as antioxidants and developed enzymes that produce, scavenge, and

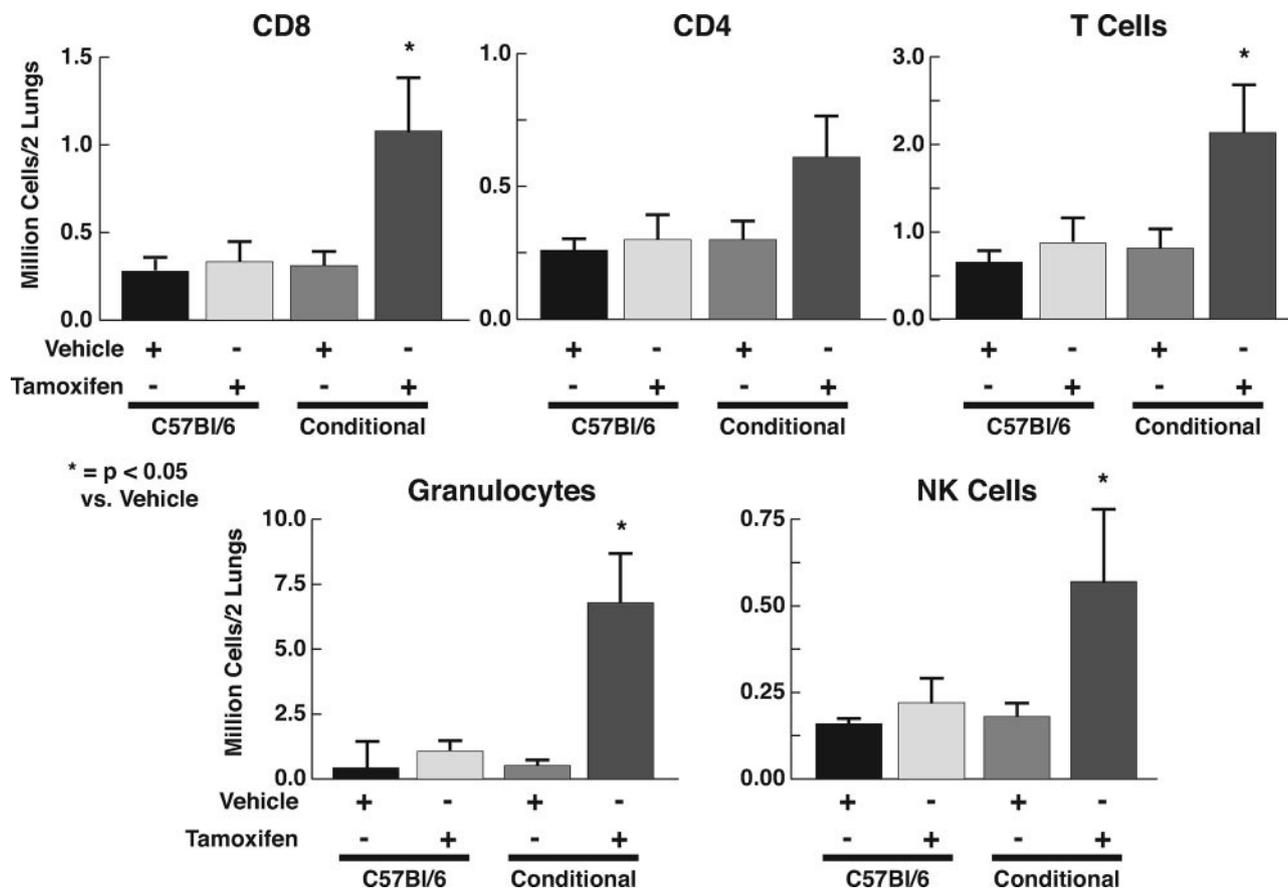


Figure 4. Lung infiltration of inflammatory cells following reduction of SOD3. Lungs were cleared of blood by perfusion with PBS, excised, and digested using collagenase and hyaluronidase as described in Materials and Methods. The homogenates were dissolved in 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-PBS and passed through a 70- μ m sterile cell strainer, yielding single cell suspensions. Cells were immunostained, resuspended in fluorescence-activated cell sorting buffer, and immediately analyzed via flow cytometry.

use reactive oxygen species and other enzymes that can prevent and repair oxidative damage. Interestingly, some of these enzymes are highly preserved from bacteria to humans, supporting the concept that the coexistence with oxygen metabolites has been essential for millions of years. One such adaptation that developed in mammals was expression of SOD3 in high amounts in specific organs such as blood vessels³⁰ and the lung.⁹ The present study shows that SOD3 is essential for survival in the presence of ambient oxygen and that acute deletion of this gene leads to severe acute lung disease. This is associated with a widening of the arterial-alveolar oxygen gradient, a reduction in blood oxygen partial pressure, an increase in plasma CO₂, and ultimately death. Systemic treatment with the SOD mimetic MnTBAP or local administration of microparticles containing SOD reduced mor-

tality in these animals and preserved lung architecture. We speculate that these novel SOD carrier microparticles could reduce lung damage and mortality in human lung injury. It has been proposed that SOD mimetics might have a protective role against smoking-induced lung injury and COPD in humans.³¹ Specific SOD3 polymorphisms have also been associated with varying courses of idiopathic pulmonary fibrosis in humans, further supporting the role of this protein in protecting against lung disease.³² We propose that loss of SOD3 might underlie the progression of lung disease observed following diverse stimuli that would impose an initial oxidant stress, such as infection, radiation, hemorrhage, or trauma.

The highest oxygen tension encountered *in vivo* is at the alveolar surface, and these high levels of oxygen could serve as a substrate for reactive oxygen species-

Table 1. Arterial Blood Gases in C57BL/6 and Tg^{cre/esr} × ecSOD^{loxP/loxP} Mice Injected with Tamoxifen or Vehicle

	pH	pO ₂ (mmHg)	pCO ₂ (mmHg)	Arterial-alveolar gradient (mmHg)
C57BL6 vehicle	7.37 ± 0.01	545 ± 40	38 ± 1	78 ± 20
C57BL6 tamoxifen	7.36 ± 0.02	596 ± 26	42 ± 4	55 ± 27
Conditional vehicle	7.37 ± 0.01	581 ± 19	42 ± 1	79 ± 19
Conditional tamoxifen	7.23 ± 0.05*†	409 ± 67†	53 ± 3†	237 ± 67*†

*P < 0.05 versus vehicle injection.

†P < 0.05 versus C57BL/6 mice treated with tamoxifen.

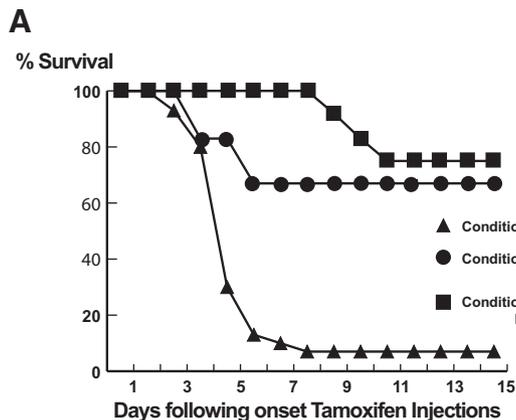
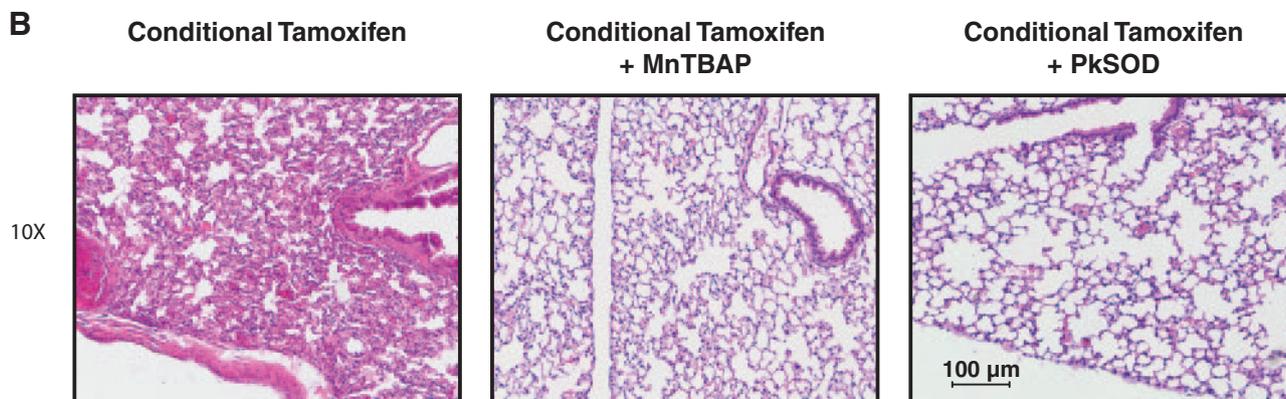


Figure 5. A: Effect of the SOD mimetic MnTBAP and SOD-containing microparticles on survival (**A**) and lung injury (**B**) in C57BL/6 and $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice. MnTBAP (1 mg/20 g of body weight) was dissolved in PBS and injected intraperitoneally for 3 consecutive days before starting the tamoxifen or vehicle injections and for 12 days thereafter. During isoflurane anesthesia, SOD-containing nanoparticles were administered intranasally (1 mg/20 g of body weight) 3 days before starting tamoxifen and vehicle injections, on the third day of treatment, and 3 days after finishing treatment. **B:** Lungs were fixed with formalin 10% and embedded in paraffin. Five- μ m slices were cut and stained with hematoxylin and eosin. Magnification, $\times 10$.



generating enzymes such as NADPH oxidases within macrophages or in alveolar epithelial cells,³³ mitochondria in epithelial cells,³⁴ or xanthine oxidase in epithelial cells.³⁵ The presence of O_2^- -scavenging enzymes would therefore seem to be critical at this site. Indeed, previous studies have shown that the alveolar surface is the major

site of lung SOD3.^{11,36} In keeping with this, we observed a striking increase in superoxide production as measured by electron spin resonance in mice following deletion of SOD3.

Mice with embryonic deletion of SOD3 are predisposed to lung damage when given 100% oxygen for

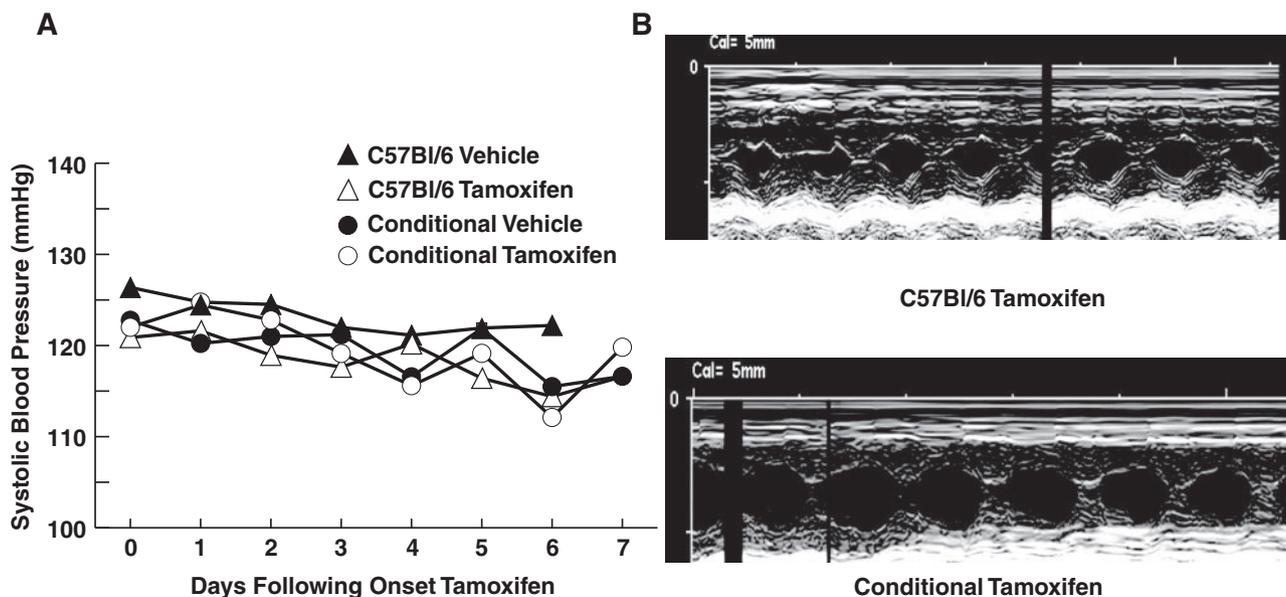


Figure 6. A: Effect of SOD3 deletion on blood pressure and cardiac function. Blood pressure (mmHg) measured by radiotelemetry in C57BL/6 and $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice before, during, and after treatment with either tamoxifen or vehicle is shown in **A** ($n = 6-10$ per group). **B:** Examples of echocardiography images obtained in C57BL/6 and $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice after tamoxifen treatment for 5 days.

Table 2. Gene Array in Lungs from Wild-Type $Tg^{cre/esr} \times ecSOD^{loxp/loxp/loxp}$ Mice Treated with Tamoxifen and $SOD3^{-/-}$

Gene name	Fold change $SOD3^{-/-}$ versus conditional knockout	Fold change $SOD3^{-/-}$ versus wild type
<i>Igh-VJ558</i>	11.06	30.39
<i>Fosb</i>	6.84	4.97
<i>Camk2β</i>	6.53	5.26
<i>Nr4a1</i>	4.21	3.46
<i>Egr3</i>	4.14	5.46
<i>Fgfr3</i>	3.70	2.43
<i>Ctse</i>	3.58	9.43
<i>Cd59a</i>	3.49	2.65
<i>Utrn</i>	3.23	3.02
<i>Egr1</i>	3.11	3.69
<i>Zfp236</i>	3.00	2.10
<i>Ndst1</i>	2.87	4.38
<i>Atf3</i>	2.70	2.92
<i>Cyr61</i>	2.65	2.81
<i>Apold1</i>	2.55	2.75
<i>Wdfy1</i>	2.52	3.35
<i>Nr1d2</i>	2.43	4.78
<i>Arf3</i>	2.42	3.26
<i>Cpd</i>	2.38	2.54
<i>Rassf3</i>	2.03	2.98
<i>Etv5</i>	2.00	2.39
<i>Ptprj</i>	-2.45	-2.40
<i>Peg3</i>	-2.54	-2.06
<i>Lrp1</i>	-2.74	-2.02
<i>Cd163</i>	-3.42	-2.25
<i>Il22ra2</i>	-4.13	-2.34
<i>Mid1</i>	-11.5	-8.84

prolonged periods²⁰ or when exposed to other oxidant injuries such as hypoxia or ozone.^{18,37} The histological appearance of the lungs in these animals following exposure to high oxygen has been reported to be similar to that observed in the current study following acute deletion of the gene in normoxic conditions.²⁰ Surprisingly, mice with embryonic SOD3 deletion tolerate exposure to ambient oxygen, although in preliminary studies, we have found that they have minor degrees of alveolar septal thickening and inflammatory infiltrates that denote a moderate degree of lung injury (data not shown). It is likely that embryonic deletion of SOD3 leads to compensatory mechanisms that allow survival despite structural changes we observed on acute deletion of this gene.

Previous studies have supported a role for reactive oxygen species such as superoxide in inducing chemotaxis of inflammatory cells³⁸ and that SOD3 prevents inflammation.^{39,40} Our fluorescence cell sorting analysis showed that acute deletion of SOD3 leads to a striking pulmonary infiltration of various inflammatory cells. Among T cell subtypes, the increase in CD8⁺ cells was greater than CD4⁺ cells. In keeping with these findings, Hashimoto et al have shown that bleomycin-induced lung injury is associated with a lymphocytic infiltrate in mice.⁴¹ These investigators have also shown high levels of mRNA for several products of CD8⁺ T cells, including granzymes A and B, perforin, Fas, and Fas ligand in the bronchoalveolar fluid of patients during the acute phase of lung injury.⁴² These molecules participate in apoptotic cytotoxicity induced by CD8 cells and could contribute to

lung injury. The large accumulation of granulocytes occurs in numerous types in acute lung injury.^{43,44}

As seen in Figure 4, tamoxifen injection caused a small increase in granulocytes in wild-type mice. We considered the possibility that tamoxifen could induce an inflammatory response in the absence of SOD3 and that the acute reduction in SOD3 caused by tamoxifen was not the cause of lung damage and death. To address this, we treated $SOD3^{-/-}$ mice for 5 days with tamoxifen ($n = 6$). This caused no death or change in lung histology (data not shown). We therefore do not believe that tamoxifen nonspecifically caused lung damage in the absence of SOD3 but that its effect was due to acute reduction of SOD3.

In $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice, induction of Cre-recombinase using tamoxifen did not completely eliminate SOD3 but reduced it by 50 to 60%, presumably due to an incomplete deletion of the gene in the target tissues. It is possible that SOD3 levels would have decreased further; however, mice became ill generally between 3 and 7 days following the initial tamoxifen injection, and we harvested organs at that time, such that it was impossible to determine the efficacy of Cre-recombinase induction on SOD3 levels in these animals at latter times. In $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice that survived tamoxifen injections, we found minimal (<30%) reduction of SOD3 in preliminary studies, suggesting that these animals might have survived simply because of persistent SOD3 expression. The reason why tamoxifen had minimal effect in these animals is unclear but might be due to technical problems due to intraperitoneal injection or variability of Cre-recombinase induction. Our data can also be interpreted as showing that as lung SOD3 levels fall, when they reach approximately 50% of normal levels, acute lung injury occurs. Despite this modest reduction of SOD3, there was a striking increase in lung O_2^- production as estimated by electron spin resonance, in keeping with the rapid kinetics of the reaction between SOD and O_2^- . It is unlikely that the lung damage occurred as a consequence of tamoxifen or the vehicle used, as tamoxifen had no effect on survival or lung histology in C57BL/6 mice or $ecSOD^{loxp/loxp}$ mice lacking Cre-recombinase. The fact that MnTBAP and SOD1 locally released from microparticles could prevent mortality in these mice strongly supports a role for O_2^- in the genesis of the lung damage. The role of MnTBAP as antioxidant has been already demonstrated in prior studies, in which MnTBAP has prolonged survival in SOD2-deficient mice and reduced injury in Paraquat-induced lung injury.^{45,46}

Several prior studies have shown that diverse injuries such as hyperoxia, asbestos injury, and hypoxia are associated with a decrease in lung activity and protein levels of SOD3 ranging from 30% to 50%.¹⁶⁻¹⁸ In these studies, it was not clear whether this decrease in SOD3 was the cause of lung injury or was simply a reflection of destruction of cells that produce this enzyme. Our present experiments indicate that a 50% decrease in SOD3 can cause lung injury even in the absence of an initiating insult. In addition to changes in protein levels, hydrogen peroxide can lead to inactivation of the Cu/ZnSODs via a reaction with the copper catalytic center of

these enzymes.^{47–49} It is therefore possible that hydrogen peroxide generated in response to stimuli such as infection or trauma could lead to inactivation of SOD3 without changing its protein levels.

To gain insight into mechanisms that allow survival of mice with embryonic deletion of SOD3 that are not present in the $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice, we performed gene array studies analyzing 47,000 mRNAs in these animals. Thirty-seven genes were up-regulated by more than twofold in the $SOD3^{-/-}$ lungs compared to either wild-type mice or the $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice after tamoxifen treatment. These encoded several factors involved in cell signaling, including the transcription factors FosB, Nr4a1, Erg1, and Etv5. FosB interacts with the DNA-binding complex AP1 and modulates nuclear gene transcription.⁵⁰ The transcription factor early growth response (Erg-1) has been shown to be involved in the tissue factor gene regulation,⁵¹ and Etv5 is a member of the Ets transcription factor family.⁴⁴ Other cell signaling genes that are highly expressed in the $SOD3^{-/-}$ lungs include the FGF receptor 3, which has been implicated in lymphatic vessel development⁵²; the Ras effector family member Rassf3, which can function as a tumor suppressor⁵³; and Arf3, a member of the small G-protein family that is involved in epithelial cell proliferation.⁵⁴ The extracellular matrix encoding mRNAs, utrophin, and Ndst were also increased in the $SOD3^{-/-}$ mice compared to the other animals. Of particular interest, CD59, a surface protein that conveys resistance to compliment attack, was up-regulated about threefold in mice with embryonic deletion of ecSOD. Another striking finding was an increase in immunoglobulin heavy chain VJ558 by 30-fold in the $SOD3^{-/-}$ mice and by threefold in the $Tg^{cre/esr} \times ecSOD^{loxp/loxp}$ mice compared to wild-type mice. This might implicate inappropriate expression of heavy chain in nonimmunological tissues in response to oxidant stress.

In contrast to the rather large number of genes up-regulated in the $ecSOD^{-/-}$ mouse lungs, a relatively small number were down-regulated. One notable gene that was reduced by two- to 2.5-fold in mice with both embryonic and acute deletion of SOD3 is Peg 3, which is involved in tumor necrosis factor- α signaling and promotes p53-mediated apoptosis.⁵⁵ In addition, the interleukin-22 receptor was reduced by fourfold in the mice with embryonic deletion. Interleukin-22 is a product of CD4⁺ T cells and is involved in autoimmune and proinflammatory diseases. Down-regulation of its receptor might convey protection. Another striking finding was a ninefold down-regulation of MID1, a microtubule-associated protein, in $SOD3^{-/-}$ mice. This protein is involved in formation of midline structures; however, its role in inflammation has not been defined.⁵⁶

The roles of these various genes in protection against oxygen toxicity remain undefined, and a precise definition of their roles is beyond the scope of this study. It is of interest, however, that virtually none of these are obviously involved in scavenging of reactive oxygen species or responses to oxidant stress, while many seem to be involved in protection against inflammatory reactions. It might have been expected that alternate reactive oxygen

species-scavenging enzymes would be up-regulated in $SOD3^{-/-}$ mouse lungs, such as one of the other superoxide dismutases or other molecules that could react with superoxide. This analysis emphasizes the unique aspects of the superoxide dismutases in that they seem to be the only enzymes that catalytically remove the superoxide radical and the unique extracellular location of SOD3, which prohibits compensation by intracellular superoxide dismutases. Thus the organism is unable to provide an alternate mode for removing extracellular superoxide dismutase and therefore compensates for the life-long loss of SOD3 by reducing inflammation. These studies therefore emphasize the link between oxidation and inflammation and provide insight into how mammals respond to a loss of a particular reactive oxygen species-scavenging enzyme.

In a prior study, we showed that mice with embryonic deletion of SOD3 develop augmented hypertension in response to angiotensin II infusion but had normal hemodynamics at baseline.²⁵ Given the large amount of SOD3 in vessels, we initially thought that the normal hemodynamics at baseline was due to compensatory mechanisms that could have developed in the $SOD3^{-/-}$ mice during development and that acute reduction of SOD3 might lead to hypertension. The limited data we obtained in mice before death failed to reveal any increase in blood pressure. It is difficult to exclude a role of SOD3 in modulation of basal blood pressure based on these studies because of the limited time before pulmonary pathology developed. A cross of our $SOD3^{loxp/loxp}$ mice with a vascular smooth muscle specific Cre-recombinase could be helpful in this regard. In preliminary studies, we found that blood pressure does not increase in mice given SOD-containing microparticles intranasally, suggesting that this modest decrease in SOD3 in organs other than the lung does not alter baseline blood pressure.

In summary, our current studies show that SOD3 is essential for survival in the presence of ambient oxygen, and even a modest reduction of this enzyme leads to profound lung injury and mortality. These findings have relevance to several causes of lung injury that are associated with decreased SOD3 protein levels and activity, and it is interesting to speculate that in these conditions, the loss of SOD3 is a perpetuating factor. Moreover, in the present study, we show that systemic treatment with MnTBAP or local administration of SOD using novel microparticles can reduce lung damage and reduce mortality. Therapies such as these might prove useful in treating acute lung injury in humans.

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