Mitochondrial Cu,Zn-Superoxide Dismutase Mediates Pulmonary Fibrosis by Augmenting H$_2$O$_2$ Generation*

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The release of H$_2$O$_2$ from alveolar macrophages has been linked to the development of pulmonary fibrosis, but little is known about its source or mechanism of production. We found that alveolar macrophages from asbestosis patients spontaneously produce high levels of H$_2$O$_2$ and have high expression of Cu,Zn-superoxide dismutase (SOD). Because Cu,Zn-SOD is found in the mitochondrial intermembrane space (IMS), we hypothesized that mitochondrial Cu,Zn-SOD-mediated H$_2$O$_2$ generation contributed to pulmonary fibrosis. Asbestos-induced translocation of Cu,Zn-SOD to the IMS was unique to macrophages and dependent on functional mitochondrial respiration and the presence of at least one of the conserved cysteines required for disulfide bond formation. These conserved cysteine residues were also necessary for enzyme activation and H$_2$O$_2$ generation. Cu,Zn-SOD-mediated H$_2$O$_2$ generation was inhibited by knockdown of the iron-sulfur protein, Rieske, in complex III. The role of Cu,Zn-SOD was biologically relevant in that Cu,Zn-SOD$^{-/-}$ mice generated significantly less H$_2$O$_2$ and had less oxidant stress in bronchoalveolar lavage fluid and lung parenchyma. Furthermore, Cu,Zn-SOD$^{-/-}$ mice did not develop pulmonary fibrosis, and knockdown of Cu,Zn-SOD in monocytes attenuated collagen I deposition by lung fibroblasts. Our findings demonstrate a novel mechanism for the pathogenesis of pulmonary fibrosis where the antioxidant enzyme Cu,Zn-SOD translocates to the mitochondrial IMS to increase H$_2$O$_2$ generation in alveolar macrophages.

Pulmonary fibrosis is a progressive disease characterized by aberrant repair that results in remodeling and destruction of the normal architecture of lung tissue. Asbestos exposure is a prototypical cause of pulmonary fibrosis. Approximately 200,000 cases of asbestos-related pulmonary disease are diagnosed each year, leading to 4,000 deaths annually, despite tight regulatory controls to limit exposure (1). Although asbestos remains an important cause of pulmonary fibrosis, the mechanism for asbestos-induced lung injury is poorly understood.

Reactive oxygen species (ROS), including H$_2$O$_2$, play an important role in the pathogenesis of asbestos-mediated pulmonary fibrosis. Generation of ROS can occur in a cell-free system by the reduction of oxygen on the surface of the asbestos fiber, but the production is amplified during phagocytosis of fibers by neutrophils, macrophages, and monocytes (2, 3). We have demonstrated that administration of catalase to wild-type (WT) mice attenuated the development of fibrosis after exposure to asbestos (4), signifying that H$_2$O$_2$ generation by alveolar macrophages is a critical factor in the pathogenesis of asbestosis; however, the source and molecular mechanism of asbestos-induced H$_2$O$_2$ generation in alveolar macrophages is unknown.

H$_2$O$_2$ generation primarily results from dismutation of superoxide anion (O$_2^-$), which occurs at a rapid rate ($10^5$–$10^6$ M$^{-1}$ s$^{-1}$) nonenzymatically, and superoxide dismutase (SOD) increases the dismutation reaction by 10$^4$-fold (5–7). There are three SOD enzymes: Cu,Zn-SOD (SOD1) is located in the cytosol and mitochondrial intermembrane space (IMS); Mn-SOD (SOD2) is located in the mitochondrial matrix; and EC-SOD (SOD3), which is an extracellular SOD (8, 9).

Alveolar macrophages obtained from patients with pulmonary fibrosis, including asbestosis, have been shown to resemble monocytes (10). These monocytes and young macrophages release ROS, including H$_2$O$_2$, which is associated with the persistent inflammatory response, cell injury, apoptosis, cell proliferation, and fibrogenesis (2, 11, 12). In addition, monocytes have a high level of Cu,Zn-SOD expression, which decreases with cell differentiation (13).

Coupled with our previous observations showing the role of mitochondria in collagen deposition (14), these data demonstrate a novel pathway by which mitochondrial H$_2$O$_2$ generation is augmented by translocation of Cu,Zn-SOD to the IMS in monocytic inflammatory cells. Increased mitochondrial Cu,Zn-SOD expression and activation in monocytic cells induces pulmonary fibrosis by increasing fibroblast collagen production. These results provide a potential target that could protect against the development of a prototypical form of pulmonary fibrosis.

EXPERIMENTAL PROCEDURES

Materials—Chrysotile asbestos was provided by the NAIMA Fiber Repository. $p$-Hydroxyphenyl acetic acid (pHPA), horse-radish peroxidase (HRP), $N,N'$-dimethyl-9,9'-biacridinium dinitrate (lucigenin reagent), and reduced $\beta$-NAD phosphate tetrasodium (NADPH) were purchased from Sigma.

acetic acid; TEMED, $N,N',N'$-tetramethylethylenediamine; GSNO, $N$-nitrosothioglutathione; ETC, electron transport chain; HPRT, hypoxanthine-guanine phosphoribosyltransferase; BAL, bronchoalveolar lavage; $\beta$-ME, $\beta$-mercaptoethanol; VDAC, voltage-dependent anion channel.
Cu,Zn-SOD−/− Mice Are Protected from Pulmonary Fibrosis

Human Subjects—The Human Subjects Review Board of the University of Iowa Carver College of Medicine approved the protocol of obtaining alveolar macrophages from normal volunteers. Normal volunteers had to meet the following criteria: 1) age between 18 and 55 years; 2) no history of cardiopulmonary disease or other chronic disease; 3) no prescription or non-prescription medication except oral contraceptives; 4) no recent or current evidence of infection; and 5) lifetime non-smoker. Alveolar macrophages were also obtained from patients with asbestosis. Patients with asbestosis had to meet the following criteria: 1) FEV1 and DLCO at least 60% predicted; 2) current nonsmoker; 3) no recent or current evidence of infection; and 4) evidence of restrictive physiology on pulmonary function tests and interstitial fibrosis on chest computed tomography. Fiber optic bronchoscopy with bronchoalveolar lavage was performed after subjects received intramuscular atropine, 0.6 mg, and local anesthesia. Each subsegment of the lung was lavaged with five 20-ml aliquots of normal saline, and the first aliquot in each was discarded. The percentage of alveolar macrophages was determined by Wright-Giemsa stain and varied from 90 to 98%.

Mice—WT and Cu,Zn-SOD−/− C57BL/6 mice (a generous gift from Dr. Steven Lentz, University of Iowa, Iowa City, IA) were used in these studies, and all protocols were approved by the University of Iowa Institutional Animal Care and Use Committee. Mice were intratracheally administered a dose of 100 μl of 0.9% saline solution containing 0.1 mg of lidoacetamide suspended in 50 μl of 0.6% intravascular atropine, 0.6 mg, and local anesthesia. Each subsegment of the lung was lavaged with five 20-ml aliquots of normal saline, and the first aliquot in each was discarded. The percentage of alveolar macrophages was determined by Wright-Giemsa stain and varied from 90 to 98%.

Cell Culture—Human monocyte (THP-1), mouse type II alveolar epithelial (MLE-12), and human lung fibroblast (HFL-1) cell lines were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 or DMEM with the following supplements: 2–10% fetal bovine serum supplement. 1640 or DMEM with the following supplements: 2–10% fetal bovine serum and 28 mM TEMED in the dark.

Isolation of Cytoplasts, Mitochondria, Mitochondrial Intermembrane Space, and Mitoplasts—Cellular compartment separation was performed as described previously (17, 18). The cytoplasm was isolated by suspending cells in 200 μl of lysis buffer (50 mM Tris, pH 8, 10 mM EDTA, protease inhibitor), sonicated for 10 s on ice, and centrifuged at 2,000 × g for 5 min. The supernatant was centrifuged at 100,000 × g for 10 min after which the supernatant containing the cytoplasmic fraction was collected. Mitochondria were isolated by lysing the cells in a mitochondria buffer containing 10 mM Tris, pH 7.8, 0.2 mM EDTA, 320 mM sucrose, and protease inhibitors. Lysates were homogenized using a Kontes Pellet Pestle Motor and centrifuged at 2,000 × g for 8 min at 4 °C. The supernatant was removed and kept at 4 °C, and the pellet was lysed, homogenized, and centrifuged again. The two supernatants were pooled and centrifuged at 12,000 × g for 15 min at 4 °C. The pellet was then resuspended in mitochondria buffer without sucrose. For mitochondrial IMS isolation, the mitochondria fractions were treated with digitonin (0.1 mg of digitonin/mg of mitochondria) for 1 h at room temperature, centrifuged at 10,000 × g for 10 min, and the supernatant was collected. 100 mM Iodoacetamide was added to prevent SOD1 activation while disrupting the outer membrane. For mitoplast isolation, mitochondria were incubated in a 5 × volume of cold hypotonic buffer (10 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM dithiothreitol) for 10 min on ice. 150 mM NaCl was added to the buffer for 10 min on ice. Samples were centrifuged at 18,000 × g for 20 min at 4 °C, and then the pellet was resuspended in sucrose-free mitochondria buffer.

Lucigenin Assay—The lucigenin assay was performed with cytosolic or mitochondrial protein (10 μg) as previously described (19).

Plasmids and Transfections—Human Cu,Zn-SOD cDNA (NM_000454) with no stop codon was amplified by PCR and inserted into pcDNA3.1D/V5-His-TOPO vector (Invitrogen). Mutations of cysteines in Cu,Zn-SOD-V5-His were generated using the QuikChange II Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). The following mutants were generated: Cu,Zn-SOD-V5-HisC257S, Cu,Zn-SOD-V5-HisC146S, and Cu,Zn-SOD-V5-HisC257S,C146S. To generate the Cu,Zn-SOD-GFP construct, SOD1 cDNA with no stop codon was amplified by PCR using a forward primer containing a NheI (underlined) site, 5′-GCT AGG ATG GGC AAG GCC GTG T-3′ and reverse primer having an EcoRV site, 5′-GAT ATC TTG GGC GAT CCC AAT TAC ACC-3′. The resulting PCR product was subcloned into pCR4-TOPO (Invitrogen). The pCR4-SOD construct was digested with Nhel and EcoRV and the product was ligated into Nhel-EcoRV sites of phMGFP vector (Promega) using T4 DNA ligase. The correct reading frame and sequence of plasmids used in this study were verified by fluorescent automated DNA sequencing performed by the University of Iowa DNA facility. Cells were transfected with vectors by using FuGENE transfection reagent (Roche Applied Science) according to the manufacturer’s instructions.

Adenoviral Vectors—THP-1 monocytes were infected with replication-deficient adenovirus type 5 with the E1 region replaced with DNA containing the cytomegalovirus (CMV) promoter region alone (Ad5.CMV) or Ad5.Cu,Zn-SOD vector.
(Gene Transfer Vector Core, University of Iowa Carver College of Medicine, Iowa City, IA) at a multiplicity of infection of 500 in serum-free RPMI medium. After 5 h, serum was added to the medium to a final concentration of 0.5%, and the cells were allowed to recover for 48 h.

**S-Nitrosoylation Analysis**—5 μg of purified Cu,Zn-SOD protein (Oxis Int., Portland, OR) was incubated with 1 mM S-nitrosoglutathione (GSNO) (Sigma) for 30 min at room temperature in the dark. Samples then were exposed to 312-nm UV lights for 5 min on ice. Samples were mixed with non-reducing sample buffer and separated by SDS-PAGE.

**Immunoblot Analysis**—Whole cells lysates were obtained as previously described (20) and separated by SDS-PAGE. Immunoblot analyses were performed with the designated antibodies followed by the appropriate secondary antibody cross-linked to HRP.

**Confocal Microscopy**—THP-1 cells were transfected with phMGFP-SOD vector in a coverglass chamber and allowed to recover for 24 h. After exposing to chrysotile asbestos for 3 h, medium was changed to Hanks’ balanced salt solution containing 500 nM MitoTracker Red (Molecular Probes, Eugene, OR) and incubated for 30 min at 37 °C. The 488- and 579-nm lines of a krypton/agron laser were used for measuring the fluorescence excitation of GFP and MitoTracker Red, respectively.

**Small Interfering RNA (siRNA)**—THP-1 cells were transfected with 100 nM scrambled (Santa Cruz Biotechnology, Santa Cruz, CA), human Rieske, or Cu,Zn-SOD siRNA duplex (IDT, Iowa City, IA) using DharmaFect 2 reagent (Dharmacon Research, Lafayette, CO) or together with Cu,Zn-SOD-V5-His or empty vector by using DharmaFect Duo reagent in antibiotics- and serum-free media, according to the manufacturer’s instructions. After 4 h, serum was added to a final concentration of 10%, and the cells were allowed to recover for 72 h.

**Quantitative Real Time PCR**—Total RNA from homogenized lungs or isolated BAL cells were obtained using TRIzol reagent (Sigma). After reverse transcription using the iScript reverse transcription kit (Bio-Rad), collagen Iα1, TGF-β, and HPRT mRNA expression were determined by quantitative real time PCR using SYBR Green kit (Bio-Rad) on an IQ5 Real-time PCR machine (Bio-Rad). The following primer sets were used: collagen Iα1, 5′-GAG TTT CCG TGC CTG GCC CC-3′ and 5′-ACC TCG GGG ACC CAT CTG GC-3′; TGF-β, 5′-CCG AGA GCC CTG GAT ACC A-3′ and 5′-TGC CGC ACA CAG CAG TTC-3′; and HPRT, 5′-CTT CAT AGA CTG ATG GAC-3′ and 5′-ATT CAG ATC AAA CTG GGC CTC ATC-3′. Data were calculated by the ΔΔCt method. Collagen I and TGF-β mRNA were normalized to HPRT and are expressed as arbitrary units.

**ELISA**—Active TGF-β in cell medium was measured by using a TGF-β ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Lipid Peroxidation**—Lipid peroxidation in BAL fluid was measured by using a thiobarbituric acid reactive substance kit (Cayman, Ann Arbor, MI) according to the manufacturer’s instructions.

**Glutathione Assay**—Lung tissue that had been perfused to remove red blood cells was homogenized directly into 5-sulfosalicylic acid (5% w/v), centrifuged, and the supernatant saved at −80 °C overnight for the glutathione assay. The protein pellet was dissolved in NaOH and the protein concentration was determined. Total glutathione content was determined as described (21). Reduced glutathione (GSH) and glutathione disulfide (GSSG) were distinguished by addition of 20 μl of a 1:1 mixture of 2-vinylpyridine and ethanol per 100 μl of sample, followed by incubation for 2 h and assayed as described previously (22). All glutathione determinations were normalized to the protein content of the lung homogenates.

**Statistical Analysis**—Statistical comparisons were performed using an unpaired, one-tailed t test. Values in the figures are expressed as mean ± S.E. and p < 0.05 was considered significant.

**RESULTS**

**Alveolar Macrophages Obtained from Patients with Asbestosis Generate High Levels of H2O2 and Have Increased Expression and Activity of Cu,Zn-SOD**—Alveolar macrophages obtained from the lungs of patients with pulmonary fibrosis are known to generate ROS, including H2O2 (23). To confirm that this phenomenon occurred in patients with asbestosis, we obtained alveolar macrophages from normal subjects and patients with asbestosis. We found that alveolar macrophages obtained from asbestosis patients had 10-fold greater rate of spontaneous H2O2 generation compared with normal subjects (Fig. 1A).

SOD increases H2O2 generation 104-fold from the dismutation of O2− compared with spontaneous dismutation, so we next determined if there was a difference in SOD expression and activity between patients and normal subjects. Whole cell lysates were separated by SDS-PAGE to determine SOD expression or by native gel to determine SOD activity. We found that alveolar macrophages obtained from asbestosis patients had similar Mn-SOD expression (Fig. 1B) and activity compared with the normal subjects (Fig. 1C). In contrast, the asbestosis patients had significantly more Cu,Zn-SOD expression and Cu,Zn-SOD activity than normal subjects (Fig. 1, B and D). Based on these results, we formulated the hypothesis that Cu,Zn-SOD is the primary determinant for increasing production of H2O2 in asbestosis patients.

**Cu,Zn-SOD in the Mitochondrial IMS Contributed to H2O2 Production**—To determine whether Cu,Zn-SOD was important in increasing H2O2, we overexpressed Cu,Zn-SOD and measured H2O2 generation. THP-1 monocytes were infected with a replicative-deficient adenovirus containing either an empty vector (Ad5.CMV) or Cu,Zn-SOD (Ad5.Cu,Zn-SOD). Extracellular H2O2 concentration was significantly increased in cells infected with Ad5.Cu,Zn-SOD compared with Ad5.CMV-infected cells (Fig. 2A). Due to the fact that Cu,Zn-SOD is known to be located in the cytoplasm and in the mitochondrial IMS, we isolated each cell compartment and performed an immunoblot analysis for Cu,Zn-SOD. Cu,Zn-SOD was distributed in both mitochondria and cytoplasm. In mitochondria, Cu,Zn-SOD was concentrated in the IMS (Fig. 2B).

Because we found Cu,Zn-SOD expression in different cellular fractions, we next determined the site and effect of Cu,Zn-SOD overexpression on O2− generation. Cells were transfected with either an empty vector or Cu,Zn-SOD-V5-His, and cytoplasmic and mitochondrial fractions were isolated. O2− genera-
tion progressively increased in the mitochondrial fraction, and overexpression of Cu,Zn-SOD-V5-His significantly inhibited the generation (Fig. 2C). In contrast, minimal \( \text{O}_2^- \) was generated in the cytoplasm, and overexpression of Cu,Zn-SOD-V5-His had no effect on cytoplasmic \( \text{O}_2^- \) levels. Overexpression of Cu,Zn-SOD also reduced the rate of mitochondrial \( \text{O}_2^- \) generation (Fig. 2D). In aggregate, these data demonstrate that Cu,Zn-SOD is highly expressed in the mitochondria of monocytes, and it enhances the dismutation of \( \text{H}_2\text{O}_2 \).

**Asbestos Induced the Translocation and Activation of Cu,Zn-SOD in the IMS of Mitochondria**

To further address the role of Cu,Zn-SOD in mitochondria after asbestos exposure, THP-1 monocytes were transfected with Cu,Zn-SOD-V5-His vector and exposed to chrysotile for 3 h. Mitochondrial fractions were isolated and an immunoblot assay was performed for the tagged protein. Cu,Zn-SOD-V5-His increased dramatically in the mitochondria obtained from cells exposed to asbestos (Fig. 3A). The observation of translocation was confirmed with confocal microscopy (Fig. 3B). Cells were transfected with pHMGFP-SOD vector. After 24 h, cells were exposed to chrysotile asbestos for 3 h, and MitoTracker Red was used to stain the mitochondria. In the absence of asbestos, the GFP expression had a diffuse distribution (upper left panel), whereas MitoTracker Red distinguished the mitochondria (upper middle panel). The merged image of GFP and MitoTracker Red (Fig. 3B, upper right panel) demonstrated that there was no aggregation of the Cu,Zn-SOD in the mitochondria. In contrast, in cells exposed to chrysotile for 3 h, GFP (bottom left panel) was concentrated in the mitochondria as seen with MitoTracker Red (bottom middle panel), and the merged panel demonstrated that Cu,Zn-SOD localized to the mitochondria (Fig. 3B, bottom right panel).

Alveolar epithelial cells are known to generate mitochondrial ROS after exposure to asbestos and other environmental toxins (24, 25), so we questioned whether asbestos increased translocation of Cu,Zn-SOD in epithelial cells. MLE-12 cells were transfected with the Cu,Zn-SOD-V5-His vector. After 24 h the cells were exposed to chrysotile for 3 h, and mitochondrial fractions were isolated. An immunoblot for the tagged protein demonstrated that Cu,Zn-SOD levels in mitochondria decreased after asbestos exposure (Fig. 3C), suggesting that the phenomenon of Cu,Zn-SOD translocation is unique to monocytes.

Only apo-Cu,Zn-SOD, an immature and inactive form, translocates to mitochondria (26). The import of proteins, such as Cox17 and TIM13 (translocase of the inner membrane), into the IMS requires the presence of conserved cysteine motifs (27). Cysteine 57 and cysteine 146 comprise the conserved cysteine motif in Cu,Zn-SOD that form an intramolecular disulfide bond (28). To determine whether the cysteines involved in forming the disulfide bond in Cu,Zn-SOD have a role in translocation, we generated Cu,Zn-SOD constructs with mutations in either cysteine 57 (Cu,Zn-SOD-V5-HisC57S) or cysteine 146 (Cu,Zn-SOD-V5-HisC146S) and a construct containing mutations in both cysteine residues (Cu,Zn-SOD-V5-HisC57S,C146S). THP-1 monocytes were transfected with Cu,Zn-SOD-V5-His mutants and exposed to chrysotile asbestos for 3 h. Mitochondrial fractions were isolated and an immunoblot assay was performed for the tagged protein. The single cysteine mutations in Cu,Zn-SOD did not alter translocation to the
Cu2Zn-SOD−/− Mice Are Protected from Pulmonary Fibrosis

IMS, but the construct containing mutations in both cysteines was not detected in the IMS in either the presence or absence of asbestos (Fig. 3D).

Because the Cu,Zn-SOD-V5-HisC57S,C146S was not imported to the IMS, we determined whether it was stably expressed in the cytoplasmic compartment. Cells were transfected with mutant Cu,Zn-SOD expression vectors. After 24 h, cytoplasmic fractions were isolated, and an immunoblot assay was performed for the tagged protein. The C57S and C146S mutants had much lower cytoplasmic expression compared with the C57S,C146S mutant (Fig. 3E). This observation confirms that Cu,Zn-SOD-V5-HisC57S,C146S is stably expressed and retained in the cytoplasm. In aggregate, these data demonstrate that asbestos exposure induces translocation of Cu,Zn-SOD to the IMS in monocytes, and translocation requires the presence of at least one conserved cysteine residue.

Translocation and Activation of Cu,Zn-SOD Was Redox Sensitive—The formation of the disulfide bond between cysteine 57 and cysteine 146 is necessary for activation of Cu,Zn-SOD (28). To determine whether the Cu,Zn-SOD was active in the mitochondria after asbestos exposure, THP-1 monocytes were transfected with the cysteine mutants (C57S, C146S, or C57S,C146S). After 24 h, cells were exposed to chrysotile asbestos. Mitochondrial fractions were isolated for linked Cu,Zn-SOD, whereas this activated form was significantly reduced with β-ME (Fig. 4A). To further confirm that Cu,Zn-SOD was active in the IMS, THP-1 monocytes were exposed to asbestos for 3 h, and mitochondrial fractions were isolated. The mitochondrial fractions isolated from cells exposed to asbestos had a significantly higher level of H2O2 production compared with mitochondria obtained from non-exposed cells (Fig. 4B).

In addition to oxidation, another post-translational modification of proteins containing cysteine residues is S-nitrosylation, which is known to influence multimer formation and signal transduction (29–31). To determine whether Cu,Zn-SOD was modified in this manner, purified Cu,Zn-SOD was incubated in the presence or absence of the NO donor GSNO. The Cu,Zn-SOD monomer was S-nitrosylated when incubated with GSNO, but it inhibited Cu,Zn-SOD multimer formation. Multimer formation was increased when the S-nitrosylated Cu,Zn-SOD was exposed to ultraviolet radiation (UV), which results in photolysis of the S-nitrosocysteine (Fig. 4C).

Because a conserved cysteine motif is necessary for translocation of Cu,Zn-SOD to the mitochondria, we determined the effect of cysteine mutations on the ability to form active multimers. THP-1 cells were transfected with the cysteine mutants (C57S, C146S, or C57S,C146S). After 24 h, cells were exposed to chrysotile asbestos. Mitochondrial fractions were isolated for...
Cu,Zn-SOD−/− Mice Are Protected from Pulmonary Fibrosis

the cells expressing C57S or C146S, and cytoplasmic fractions were isolated for cells expressing C57S,C146S. Samples were separated on a polyacrylamide gel in the presence or absence of the reducing agent DTT. We observed that none of the cysteine mutants formed multimers (Fig. 4D).

To determine whether Cu,Zn-SOD translocation is linked to mitochondrial H2O2 generation, we transfected THP-1 monocytes with an empty vector, Cu,Zn-SOD-V5-His (WT) or the cysteine mutants (C57S, C146S, or C57S,C146S). After 24 h, cells were exposed to chrysotile asbestos for 3 h. H2O2 generation in cells expressing the WT Cu,Zn-SOD was significantly higher than cells expressing the empty vector (Fig. 4E). Cells expressing either one of the single mutants (C57S, C146S), which are not able to form an active multimer, had a significant reduction in H2O2 generation near control levels. Cells expressing C57S,C146S had a similar reduction in H2O2 generation compared with cells expressing the WT Cu,Zn-SOD (Fig. 4E). Taken together, these data demonstrate that asbestos-induced translocation of Cu,Zn-SOD to the IMS requires at least one of the conserved cysteine residues; however, both cysteine residues are necessary for multimer formation, enzyme activation, and subsequent H2O2 generation after exposure to asbestos.

The mitochondrial electron transport chain (ETC) is a major site of O2− production in both quiescent and activated cells. Because Cu,Zn-SOD translocation required a conserved cysteine motif and cysteines are targets of oxidation, we questioned whether increased mitochondrial ROS generation after asbestos exposure regulated translocation. THP-1 monocytes were transfected with the empty or Cu,Zn-SOD-V5-His vector in combination with either scrambled or Rieske siRNA to determine the effect on H2O2 generation as a function of Cu,Zn-SOD activity. Rieske is the iron-sulfur protein component of the cytochrome bc1 complex (complex III). Overexpression of Cu,Zn-SOD increased H2O2 production in cells transfected with either Cu,Zn-SOD-V5-His alone or with the scrambled siRNA (Fig. 5A). In contrast, cells transfected with the Rieske siRNA had a significant inhibition of H2O2. Overexpression of Cu,Zn-SOD in cells expressing the Rieske siRNA resulted in marked inhibition of H2O2 generation compared with cells expressing the empty vector alone (Fig. 5A). Rieske knockdown also significantly abrogated the rate of H2O2 generation (Fig. 5B). These data demonstrate that knockdown of complex III inhibited the production of H2O2 after asbestos exposure. In addition, Cu,Zn-SOD-induced H2O2 generation after asbestos exposure required an active complex III.

To confirm that the mitochondrial-derived ROS was required for mitochondrial Cu,Zn-SOD translocation and activation, THP-1 monocytes were transfected with scrambled or Rieske siRNA. After 72 h, cells were exposed to chrysotile asbestos for 3 h. Mitochondrial fractions were isolated and separated on a polyacrylamide gel in the presence or absence of β-ME. In the presence of β-ME, the disulfide bond-linked Cu,Zn-SOD was not present. In the absence of β-ME, cells expressing the scrambled siRNA had the activated, disulfide bond linked in Cu,Zn-SOD. In contrast, cells expressing the Rieske siRNA had a significant reduction in the activated Cu,Zn-SOD in the mitochondria (Fig. 5C). In aggregate, these data demonstrate that Cu,Zn-SOD-mediated H2O2 generation is strikingly reduced with knockdown of a critical redox center of complex III. These data also show that translocation and
activation the Cu,Zn-SOD in the IMS are coupled to the activity of mitochondrial ETC.

**Cu,Zn-SOD Regulates Asbestos-induced Oxidative Stress in Vivo**—To better define the potential biological relevance of Cu,Zn-SOD in the pathogenesis of pulmonary fibrosis, we exposed WT and Cu,Zn-SOD\(^{-/-}\) C57BL/6 mice to chrysotile asbestos. The predominant cells seen in the BAL fluid at 21 days were alveolar macrophages (data not shown). To demonstrate that Cu,Zn-SOD had a role in the generation of \(\text{H}_2\text{O}_2\) in vivo after asbestos exposure, we isolated monocytes and macrophages from bone marrow from WT and Cu,Zn-SOD after asbestos exposure, we isolated monocytes and macrophages indicating that Cu,Zn-SOD is crucial for the \(\text{H}_2\text{O}_2\) generation after asbestos exposure in vivo (Fig. 6B).

Due to the significant differences in \(\text{H}_2\text{O}_2\) levels between WT and Cu,Zn-SOD\(^{-/-}\) cells, we questioned whether Cu,Zn-SOD had a role in mediating increases in parameters indicative of oxidative stress in the lungs after asbestos exposure. Unsaturated lipids are significant components of surfactant and have been suggested to be major targets for lipid peroxidation during inflammation and lung injury (32). WT and Cu,Zn-SOD\(^{-/-}\) mice were exposed to chrysotile asbestos, and lipid peroxidation was determined at 21 days utilizing the thiobarbituric acid reactive substances assay. WT mice had greater than 6-fold higher levels of lipid peroxidation in BAL fluid compared with Cu,Zn-SOD\(^{-/-}\) mice (Fig. 6C). In addition, when lung tissue from similarly treated animals was assayed for the percentage of total glutathione (GSH) in the disulfide form (% GSSG), which signifies an increase in oxidation of GSH, the lungs from WT mice again demonstrated significantly higher % GSSG relative to the Cu,Zn-SOD\(^{-/-}\) (Fig. 6D).

Taken together, these data are completely consistent with the hypothesis that monocyte/macrophages from the lungs of WT mice exposed to asbestos have significantly greater \(\text{H}_2\text{O}_2\) production, which resulted in increased oxidative stress in both the BAL fluid and lung parenchyma. Furthermore, given that asbestos induced high levels of \(\text{H}_2\text{O}_2\) and that the levels of oxidative stress detected in the lungs were reduced in the lungs of Cu,Zn-SOD\(^{-/-}\) mice strongly supports the conclusion that Cu,Zn-SOD was a significant source of \(\text{H}_2\text{O}_2\) following exposure to asbestos.

**Cu,Zn-SOD\(^{-/-}\) Mice Were Protected from Developing Pulmonary Fibrosis After Asbestos Exposure**—To determine whether the relationship between Cu,Zn-SOD and \(\text{H}_2\text{O}_2\) generation and oxidative stress in the lung had an effect on the
development of pulmonary fibrosis, WT and Cu,Zn-SOD$^{-/-}$ mice were exposed to chrysotile asbestos. After 21 days, the animals were euthanized and lungs were excised and processed for staining with Masson trichrome to visualize collagen deposition. The lungs of WT mice had widespread collagen deposition in both peribronchial and parenchymal portions of the lung (Fig. 7A). In contrast, the collagen deposition in Cu,Zn-SOD$^{-/-}$ mice was significantly attenuated (Fig. 7B).

To verify the histopathological observations, we determined the extent of pulmonary fibrosis biochemically. Total mRNA was isolated from homogenized lungs obtained from mice 21 days after asbestos exposure. Collagen Ia1 mRNA expression was greater than 5-fold higher in the lungs of WT mice compared with Cu,Zn-SOD$^{-/-}$ mice (Fig. 7C).

Because TGF-β is a pro-fibrotic cytokine produced by macrophages, we determined the role of Cu,Zn-SOD in regulating its expression in vitro and in vivo. THP-1 monocytes were infected with a replicative-deficient adenovirus containing either an empty vector or Cu,Zn-SOD. After 48 h, the supernatants were harvested, and active TGF-β was determined by ELISA. Cells expressing Cu,Zn-SOD produced significantly more active TGF-β than cells expressing the empty vector (Fig. 7D). In WT and Cu,Zn-SOD$^{-/-}$ mice exposed to asbestos, BAL cells were obtained and TGF-β mRNA expression was quantified. Cells collected from asbestos-exposed WT mice expressed greater than 4-fold more TGF-β mRNA compared with Cu,Zn-SOD$^{-/-}$ mice (Fig. 7E). These data suggest that mitochondrial Cu,Zn-SOD regulates TGF-β production in macrophages.

Because fibroblasts are the primary cell that produces collagen and to provide a direct link between Cu,Zn-SOD and pulmonary fibrosis, we transfected THP-1 macrophages with either a scrambled or Cu,Zn-SOD siRNA. After 72 h, the conditioned medium was collected. Human lung fibroblasts (HLF-1) were cultured for 24 h in the conditioned medium obtained from the transfected cells in the presence or absence of chrysotile asbestos. The conditioned medium from the fibroblast cultures was used to measure collagen I secretion. HLF-1 cells exposed to conditioned medium from THP-1 cells transfected with the scrambled siRNA had significantly more procollagen I and collagen I compared with cells exposed to the conditioned medium from the Cu,Zn-SOD siRNA-transfected cells (Fig. 7F). Taken together, these data demonstrate that the antioxidant enzyme Cu,Zn-SOD induces pulmonary fibrosis via translocation and activation in the mitochondrial IMS where it enhances the generation of H$_2$O$_2$.

**DISCUSSION**

Although H$_2$O$_2$ generation has been linked to pulmonary fibrosis, little is known about its source or mechanism of production. In this study, we demonstrate that Cu,Zn-SOD translocation and activation in the IMS is unique to monocytes and macrophages and is dependent on a conserved cysteine motif and mitochondrial ETC function. These data also demonstrate that H$_2$O$_2$ generation is regulated by the presence of Cu,Zn-SOD in the IMS, which, in part, modulates the development of pulmonary fibrosis after asbestos exposure. Evidence to sup-
port this pathway include (i) asbestos increased mitochondrial ROS generation and increases translocation of Cu,Zn-SOD to the IMS; (ii) a conserved cysteine motif was necessary for translocation and activation; (iii) knockdown of the iron-sulfur protein, Rieske, decreased Cu,Zn-SOD translocation and activation; (iv) mitochondrial H2O2 generation is dependent on complex III O2 \(^{-}\) generation as knockdown of Rieske inhibited H2O2 production by Cu,Zn-SOD; (v) Cu,Zn-SOD\(^{-/-}\) mice had reduced H2O2 generation, decreased oxidative stress in the BAL fluid and the lung parenchyma, and were protected from developing pulmonary fibrosis; and (vi) knockdown of Cu,Zn-SOD in monocytes inhibits collagen I deposition by lung fibroblasts. Taken together, these observations provide novel insight into the mechanism linking H2O2 generation to pulmonary fibrosis and delineate the role of Cu,Zn-SOD in regulating mitochondrial H2O2 production.

We have shown that mitochondrial H2O2 production promotes the development of asbestos-induced pulmonary fibrosis by increasing collagen production in fibroblasts, and intratracheal administration of catalase attenuates asbestos-induced pulmonary fibrosis (4, 14). The use of other antioxidant enzymes, such as PEG-SOD, have not been effective in preventing pulmonary fibrosis (33). It is unclear in this study, however, if PEG-SOD exacerbated the lung injury. Moreover, the effect of SOD on the development of pulmonary fibrosis is not known.

Alveolar macrophages obtained from the lung of patients with chronic lung disease, such as pulmonary fibrosis, are known to resemble monocytes (10, 34). This is likely secondary to persistent recruitment of monocytes to the site of injury. Cu,Zn-SOD is highly expressed in monocytes, and the expression decreases with differentiation to mature macrophages (13). Studies have shown Mn-SOD to be increased in animal models after exposure to asbestos (35, 36), whereas Cu,Zn-SOD expression was not altered with asbestos exposure. These studies, however, measured expression in whole lung homogenates, whereas we have focused on the role of inflammatory cells in the pathogenesis of pulmonary fibrosis. Our data suggest a new conceptual framework for understanding asbestosis, and potentially other forms of pulmonary fibrosis, in that the increased Cu,Zn-SOD in the alveolar macrophages from asbestosis patients is secondary to the presence of monocytes and young macrophages in the lung.

An observation in our study regarding the mechanism of Cu,Zn-SOD-mediated H2O2 generation is that asbestos triggers translocation of Cu,Zn-SOD into the IMS, and this translocation was regulated by the presence of at least one cysteine in the conserved cysteine motif and mitochondrial ETC ROS production. Only apo-Cu,Zn-SOD, an immature and inactive form, translocates to mitochondria (26). Activation of apo-Cu,Zn-SOD requires insertion of copper and zinc and the for...
formation of an intramolecular disulfide bond between cysteine 57 and cysteine 146. Although the mechanism of zinc insertion remains unknown, the copper chaperone for SOD1 (CCS) controls the insertion of copper, which is also necessary to activate Cu,Zn-SOD (9). However, in mammalian cells, a CCS-independent copper insertion and Cu,Zn-SOD activation pathway also exists (37). We found that there was no alteration of CCS in the mitochondria in cells exposed to asbestos (data not shown), suggesting activation of Cu,Zn-SOD was CCS-independent.

Intramolecular disulfide bond formation between the thiol groups of the Cu,Zn-SOD monomer is also necessary for the activation of Cu,Zn-SOD (38), and our data demonstrate that disulfide bond formation is regulated by the redox environment of the IMS. Complex III is the major ROS production site among all mitochondrial ETC complexes, and it is the only complex that generates O$_2^-$ in the IMS (39). The Rieske protein is one of the four redox centers in complex III that plays an important role of transferring electrons from ubiquinol in cytochrome b to the heme group in cytochrome c$_1$. Inhibition of the Rieske protein by myxothiazol has been shown to reduce the extracellular H$_2$O$_2$ level in intact heart mitochondria (40). Our data, however, demonstrate that knockdown of the Rieske protein inhibits Cu,Zn-SOD translocation and activation, which underscores a critical functional role of the mitochondrial ETC and redox environment for Cu,Zn-SOD uptake and subsequent activation in the IMS. In addition, although at least one of the conserved cysteine residues is required for translocation, both Cys-57 and Cys-146 are necessary for Cu,Zn-SOD activation.

Other disorders, such as Down syndrome and amyotrophic lateral sclerosis, which have either increased or altered Cu,Zn-SOD...
Cu,Zn-SOD−/− Mice Are Protected from Pulmonary Fibrosis

SOD activity, exhibit oxidative stress that is linked to disease development (41, 42). Our data demonstrate that WT mice have greater lipid peroxidation in BAL fluid and oxidized GSH in lung tissue than Cu,Zn-SOD−/− mice, suggesting H2O2 generated by Cu,Zn-SOD results in increased oxidative stress in the lung. Our data demonstrate that decreased mitochondrial H2O2 generation results in decreased collagen deposition and supports our notion that Cu,Zn-SOD-mediated H2O2 generation is, in part, responsible for the development of pulmonary fibrosis after exposure to asbestos. These observations provide a potential target that could protect against the development of a prototypical form of pulmonary fibrosis.

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