Mitochondrial Rac1 GTPase Import and Electron Transfer from Cytochrome c Are Required for Pulmonary Fibrosis*

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Background: Rac1 activation is linked to H₂O₂ generation in macrophages.

Results: Two cysteine residues in Rac1 modulate mitochondrial H₂O₂ generation via import and electron transfer from cytochrome c.

Conclusion: Mitochondrial Rac1 activity in alveolar macrophages is associated with oxidative stress.

Significance: Rac1 directly mediates mitochondrial H₂O₂ production in alveolar macrophages, which is linked to pulmonary fibrosis.

The generation of reactive oxygen species, particularly H₂O₂, from alveolar macrophages is causally related to the development of pulmonary fibrosis. Rac1, a small GTPase, is known to increase mitochondrial H₂O₂ generation in macrophages; however, the mechanism by which this occurs is not known. This study shows that Rac1 is localized in the mitochondria of alveolar macrophages from asbestos patients, and mitochondrial import requires the C-terminal cysteine of Rac1 (Cys-189), which is post-translationally modified by geranylgeranylation. Furthermore, H₂O₂ generation mediated by mitochondrial Rac1 requires electron transfer from cytochrome c to a cysteine residue on Rac1 (Cys-178). Asbestos-exposed mice harboring a conditional deletion of Rac1 in macrophages demonstrated decreased oxidative stress and were significantly protected from developing pulmonary fibrosis. These observations demonstrate that mitochondrial import and direct electron transfer from cytochrome c to Rac1 modulates mitochondrial H₂O₂ production in alveolar macrophages pulmonary fibrosis.

An important and prototypical type of pulmonary fibrosis occurs after exposure to asbestos, which results in an interstitial pneumonitis and subsequent collagen deposition. Although strict regulatory controls are in place to limit exposure, more than 1.3 million workers continue to be exposed to hazardous levels of asbestos annually (1, 2). The development of pulmonary fibrosis is a complex process that results in aberrant remodeling of lung tissue. The modulation of lung remodeling during pulmonary fibrosis is poorly understood, and no effective therapeutic options have come about to prevent disease development. Thus, understanding the mechanism(s) by which aberrant remodeling is regulated may provide a potential target for therapy.

The generation of reactive oxygen species (ROS), including H₂O₂, plays a critical role in tissue injury and consequent fibrosis by modulating extracellular matrix deposition (3, 4). The production of ROS is accentuated by the inefficient phagocytosis of asbestos fibers by alveolar macrophages (5). We have shown that alveolar macrophages obtained from patients with pulmonary fibrosis produce high levels of H₂O₂ and that the primary source of H₂O₂ generated in alveolar macrophages in the setting of pulmonary fibrosis is the mitochondria (4). The generation of H₂O₂ is critical for the fibrotic response in lung injury because abrogating mitochondrial oxidant stress or administration of catalase attenuates the development of pulmonary fibrosis in mice (4, 6).

Rac1 is a member of the Rho family of guanosine 5’-triphosphate (GTP)-binding proteins. Rac1 regulates several cellular functions, such as actin polymerization and migration, cell adhesion, and phagocytosis in macrophages (7–9), which are all necessary processes to engulf asbestos fibers. The C-terminal cysteine residue in Rho GTP-binding proteins, such as Rac1, can be modified by geranylgeranylation. This post-translational modification is important for Rac1 activation and interaction with other proteins (10). The 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors, such as simvastatin, block the rate-limiting enzyme that converts HMG-CoA to mevalonate. Simvastatin and other statins are known to attenuate injury in different tissues (11–15). The therapeutic effects of HMG-CoA inhibitors have been attributed to a reduction in inflammation.

Rac1 activation increases the generation of H₂O₂ in multiple cell types (3, 16, 17), and Rac1 has been shown to indirectly

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2 The abbreviations used are: ROS, reactive oxygen species; micro-CT, micro-computed tomography; IMS, intermembrane space; t-BOOH, tert-butyl hydroperoxide.
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increase mitochondrial ROS generation via engagement of integrins or by increasing ceramide levels (18–20). We have previously shown that overexpression of constitutively active Rac1 in macrophages increased mitochondrial \( \mathrm{H}_2\mathrm{O}_2 \) (3, 6); however, the mechanism by which Rac1 mediates mitochondrial \( \mathrm{H}_2\mathrm{O}_2 \) generation in macrophages has not been described.

Complex III is a major site of ROS generation in mitochondria (21), and the heme group of cytochrome \( c \) accepts an electron from the cytochrome \( b-c1 \) complex and transfers the electron to the cytochrome oxidase (complex IV). Complex IV reduces \( \mathrm{O}_2 \) to water without production of ROS, but the partial reduction of \( \mathrm{O}_2 \), which results in the generation of ROS, can occur at multiple sites upstream of complex IV. Although cytochrome \( c \) is commonly linked to apoptosis, it is also known to play a role in mitochondrial \( \mathrm{H}_2\mathrm{O}_2 \) generation depending on its redox state (22, 23). We have previously shown that knockdown of the iron-sulfur protein, Rieske, in complex III attenuates \( \mathrm{H}_2\mathrm{O}_2 \) generation in macrophages (4). However, the role of cytochrome \( c \) in macrophage \( \mathrm{H}_2\mathrm{O}_2 \) generation in pulmonary fibrosis has not been investigated.

In this study, we discovered that Rac1 is localized and active in the mitochondria of alveolar macrophages obtained from asbestosis patients and that Rac1 mitochondrial import required the C-terminal cysteine residue (Cys-189) of Rac1. The presence of Rac1 in alveolar macrophage mitochondria was associated with the development of oxidative stress in the lung because the interaction of Rac1 with cytochrome \( c \) in mitochondria resulted in electron transfer between cytochrome \( c \) and Rac1, which increased \( \mathrm{H}_2\mathrm{O}_2 \) generation. Furthermore, mice harboring a conditional deletion of Rac1 in macrophages produced less \( \mathrm{H}_2\mathrm{O}_2 \) and demonstrated both less oxidative stress and fibrosis relative to wild type mice after asbestos exposure. These observations provide new insight into a novel mechanism whereby Rac1 is imported into the mitochondria and accepts electrons from cytochrome \( c \), which modulates mitochondrial \( \mathrm{H}_2\mathrm{O}_2 \) levels in alveolar macrophages contributing to the development pulmonary fibrosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chrysotile asbestos was provided by the North American Insulation Manufacturers Association Fiber Repository. \( p \)-Hydroxyphenyl acetic acid, horseradish peroxidase (HRP), and reduced \( \beta \)-NAD phosphate tetrasodium (NADPH) were purchased from Sigma.

**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. Seventy five percent of the normal subjects were male between ages 30–55 years-old. Alveolar macrophages were also obtained from patients with asbestosis. Patients with asbestosis had to meet the following criteria: 1) FEV1 and carbon monoxide diffusing capacity at least 50% predicted; 2) current non-smoker; 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. The asbestosis patients were all males, 50–65 years-old, and had moderate-to-severe restrictive physiology for at least 5–10 years. Fiberoptic 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 1st 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 Rac1 null C57BL/6 mice were used in these studies and all protocols were approved by the University of Iowa Institutional Animal Care and Use Committee. Rac1 null mice were generated by selectively disrupting the Rac1 gene in cells of the myeloid lineage, as described previously (24). Mice were administered 100 \( \mu \)g of chrysotile asbestos in 50 \( \mu \)l of normal saline intratracheally after being anesthetized with 3% isoflurane using a precision Fortec vaporizer (Cyprane, Keighley, UK). Twenty one days later, mice were euthanized with an overdose of isoflurane, and bronchoalveolar lavage (BAL) was performed. BAL cells were collected by centrifugation of the BAL for 10 min at 220 \( \times \) g, and cell differential was determined by Wright-Giemsa stain. Over 90% of the BAL cells were macrophages.

**Cells**—THP-1 and MH-S cells were obtained from American Type Culture Collection and maintained as described in the manufacturer’s instructions. All experiments were performed with 0.5% serum supplement.

**Plasmid and Transfections**—Full-length human Rac1 (NM_006908.3) was amplified by PCR using Rac1 cDNA in pUSEamp (Millipore) as a template plus forward and reverse primers containing 5’-BamHI or 3’-Sall sites. The resulting PCR product was cloned into pCR4-TOPO (Invitrogen), digested, and then ligated into the BamHI-Sall sites of pRK-Flag vector. The pRK-Flag Rac1 construct contains a Flag epitope on the N terminus. To generate the Rac1-V5-His\(_\alpha\) construct, Rac1 cDNA containing amino acid residues 1–189 was amplified by PCR and cloned into pcDNA3.1D/V5-His vector (Invitrogen). Single Cys \( \rightarrow \) Ser mutations in Rac1 were made by PCR using the QuikChange site-directed mutagenesis kit (Stratagene). The reading frame and sequence of vectors used in this study were verified by fluorescent automated DNA sequencing (University of Iowa DNA facility). The I MS-targeted HyPer was generously provided by Dr. Vadim Gladyshev (25).

**Adenoviral Vectors**—Replication-deficient recombinant adenovirus type 5 with the E1 region replaced with DNA containing the cytomegalovirus (CMV) promoter region alone (Ad.CMV) or constitutively active (Ad.V12Rac1) Rac1 (26) downstream of the CMV promoter were obtained from the Gene Transfer Vector Core at the University of Iowa (Iowa City).

**Determination of \( \mathrm{H}_2\mathrm{O}_2 \) Generation**—\( \mathrm{H}_2\mathrm{O}_2 \) concentration was determined as described previously (3, 4).

**Isolation of Membrane, Cytoplasm, Mitochondria, Mitochondrial Intermembrane Space, and Mitoplasts**—Cell fractions were obtained as described previously (4, 27).
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**Immunoblot Analysis**—Whole cell lysates or cell fractions were separated by SDS-PAGE. Immunoblot analyses were performed with the designated antibodies followed by the appropriate secondary antibody cross-linked to HRP.

**Determination of Apoptosis**—Macrophages were incubated with staurosporine (Sigma) at concentrations of 1 μM and 250 nm for 3 h, as a positive control. Whole cell lysates were prepared, and immunoblot analysis was performed using anti-caspase 3, active.

**Purification of Rac1-V5-His-tagged Protein**—Cells were transiently transfected with empty pcDNA3.1 vector or pcDNA3.1-Rac1-V5-His vectors. After 24 h cells were exposed to chrysotile asbestos 10 μg/cm² for 3 h. Cells were harvested in Buffer B (PBS, 0.5 M NaCl, 1% Triton X-100, protease, and phosphatase inhibitors); the lysates briefly sonicated on ice, and cellular debris was pelleted at 12,000 × g for 10 min at 4 °C. Talon metal (cobalt) affinity resin (Clontech) was added to each lysate, and samples were rotated overnight at 4 °C. The Rac1-V5-His proteins were eluted by adding protein sample buffer and heating at 95 °C for 5 min.

**Glutathione Assay**—Lung tissue that had been perfused to remove red blood cells was homogenized directly into 5-sulfosalicylic acid (5% w/v) and centrifuged, and the supernatant was saved at −80 °C overnight for the glutathione assay. Total glutathione content was determined as described (4). 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 (4). All glutathione determinations were normalized to the protein content of the lung homogenates.

**Hydroxyproline Assay**—Lung tissue was homogenized, dried to a stable weight, and then acidified with 6 N HCl, and hydrolyzed by heating at 120 °C for 24 h. Hydroxyproline measurements were determined as described and normalized to dry lung weight.

**In Vivo Micro-computed Tomography Imaging**—Following intraperitoneal injection of ketamine (87.5 mg/kg) and xylazine (12.5 mg/kg) for anesthesia and a nonresponsive pedal reflex test, a microsurgical tracheotomy was performed. Controlled respiration was achieved through the tracheotomy, via a computer controlled Flexivent (Scireq, Canada) ventilator. Respiratory paralysis was induced through the administration of 0.1 mg/kg pancuronium. To maintain sedation throughout the imaging process, 1.5% isoflurane was administered through the ventilator. A BioVet (Supertron Technologies, Newark, NJ) system was used to monitor the animal’s heart rate throughout the experiment.

A Micro-CT II (Siemens Pre-Clinical Solutions, Knoxville, TN) was used for in vivo scanning of the mouse. As imaging of the lungs during spontaneous respiration can affect the image quality produce, the Intermittent Iso-pressure Breath Hold, which is a custom-gated imaging process, was utilized (28, 29). This technique triggered image acquisition during a forced breath hold with periods of hyperventilation between acquisitions. The micro-CT scanner setting for in vivo imaging was 60 peak kV, 500 μA, and an exposure time of 600 ms. A total of 720 projections were acquired over 200°. Image reconstruction resulted in a volumetric dataset over the thorax with 28-μm isotropic voxels (1,536 × 1,536 pixels and 1,024 image slices).

**Statistical Analysis**—Statistical comparisons were performed using either an unpaired, one-tailed t test or one-way analysis of variance followed by Bonferroni’s multiple comparison test. Values in figures are expressed as means ± S.E., and p < 0.05 was considered to be significant.

**RESULTS**

**Alveolar Macrophages from Asbestosis Patients Have Increased Mitochondrial Rac1 Localization and Activity**—Because Rac1 has been indirectly linked to mitochondrial H₂O₂ generation (3, 18–20) and alveolar macrophages obtained from asbestosis patients produce high levels of H₂O₂ (4), we first asked if Rac1 was responsible, in part, for the H₂O₂ generation in alveolar macrophage mitochondria. Isolated mitochondria from alveolar macrophages obtained from patients with asbestosis demonstrated significantly greater immunoreactive Rac1 relative to macrophage mitochondria from normal subjects (Fig. 1A). In contrast, no significant differences in Rac1 expression were found in whole cell lysates of alveolar macrophages taken from normal subjects and asbestosis patients (Fig. 1B). When mitochondrial Rac1 localization was expressed as a ratio of whole cell Rac1, alveolar macrophages obtained from patients with asbestosis had nearly 2.5-fold greater mitochondrial Rac1 expression relative to normal subjects (Fig. 1C). Furthermore, the activity of mitochondrial Rac1 was found to be nearly 4-fold higher in alveolar macrophages obtained from patients with asbestosis compared with normal subjects (Fig. 1D).

To address the specificity of Rac1 mitochondrial localization in alveolar macrophage obtained from asbestosis patients, we determined if Rac2, a GTPase that is prevalent in macrophages, had a similar pattern of mitochondrial localization as Rac1. Isolated mitochondria from alveolar macrophages obtained from normal subjects demonstrated significantly greater immunoreactive Rac2 relative to macrophage mitochondria from asbestosis patients (Fig. 1E). This observation supports the fact that asbestos exposure induces Rac1 mitochondrial import and activation, as Rac2 is not activated in asbestos-exposed macrophages (data not shown).

To investigate whether the mitochondria were the primary site of Rac1 activation and whether asbestos exposure increased mitochondrial Rac1 activation, alveolar macrophages were obtained from asbestosis patients and exposed to chrysotile asbestos ex vivo. Mitochondrial and membrane fractions were isolated, and Rac1 activation was determined. Rac1 activation in the mitochondria increased significantly after 30 min and continued to increase further in a time-dependent fashion, whereas activation in the membrane fraction remained at basal levels (Fig. 1F). Based on these novel observations, we formulated the hypothesis that expression and activity of Rac1 in the mitochondria play a role in alveolar macrophage H₂O₂ generation, which is critically linked to the development in pulmonary fibrosis.

**Rac1 Regulates Oxidative Stress in Vivo**—To determine the biological significance of Rac1-mediated mitochondrial H₂O₂ generation in the pathogenesis of pulmonary fibrosis, we
exposed WT mice and mice with a conditional deletion of Rac1 in macrophages (Rac1 null) to chrysotile asbestos. After 21 days, the mice were euthanized; BAL was performed, and alveolar macrophages, which are the predominant (90%) cell type in BAL fluid (Fig. 2A), were isolated to measure H2O2 generation. Alveolar macrophages obtained from WT mice generated H2O2 at a significantly higher rate than cells obtained from Rac1 null mice (Fig. 2B). To determine the role of mitochondria in H2O2 generation in vivo, WT and Rac1 null mice were exposed to chrysotile asbestos in a similar manner; alveolar macrophages were obtained from BAL fluid after 21 days, and mitochondria were isolated. As expected, mitochondrial H2O2 was significantly greater in WT mice compared with Rac1 null mice (Fig. 2C).

Because of the significant differences in mitochondrial H2O2 levels in WT and Rac1 null alveolar macrophages, we questioned whether these observations had a role in modulating oxidative stress in the lungs. Mice were exposed to chrysotile asbestos. After 21 days, the mice were euthanized, and the lungs were excised and homogenized to determine the percentage of total GSH in the disulfide form (% GSSG). The lungs from WT mice had a significantly higher percent of total glutathione in the oxidized disulfide form (increased % GSSG) relative to the Rac1 null mice, indicating increased oxidative stress in the lungs of WT mice (Fig. 2D). In aggregate, these data demonstrated that alveolar macrophages obtained from asbestos-exposed WT mice had significantly greater mitochondrial H2O2 generation that mediated the increased oxidative stress in the lung parenchyma. Furthermore, the fact that the level of oxidative stress was reduced in the lungs of Rac1 null mice supports the hypothesis that mitochondrial Rac1 is a significant source of H2O2 in alveolar macrophages.

**Mitochondrial Rac1 Is Required for Developing Pulmonary Fibrosis**—To determine whether the relationship between mitochondrial Rac1, H2O2 generation, and oxidative stress was linked to the development of pulmonary fibrosis, WT and Rac1 null mice were subjected to live imaging of lungs by micro-CT scanning 21 days after exposure to chrysotile asbestos. Com-
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**FIGURE 2.** Rac1 modulates oxidant stress in vivo. WT and Rac1 null mice were exposed to chrysotile asbestos (100 μg) intratracheally. After 21 days, the mice were euthanized. A, BAL was performed, and cell differential was determined by Wright-Giemsa stain. WT (n = 3) and Rac1 null (n = 3); *, p < 0.001 macrophages versus polymorphonuclear neutrophil (PMN) and lymphs. B, measurement of alveolar macrophage H2O2 generation was performed in isolated BAL cells, which is expressed as a rate (nmol/min/106 cells). WT (n = 8) and Rac1 null (n = 8); *, p < 0.0055. C, alveolar macrophages were obtained by BAL after 21 days. Mitochondria were isolated, and H2O2 generation was measured. WT (n = 8) and Rac1 null (n = 12); *, p < 0.0023. D, lungs were removed and homogenized for glutathione assay. Total GSH in disulfide form was expressed as % GSSG. WT (n = 6) and Rac1 null (n = 7); *, p < 0.0110.

pared with Rac1 null mice, WT mice had increased interstitial markings in the lung parenchyma with septal thickening and traction bronchiectasis, both of which are indicative of pulmonary fibrosis (Fig. 3, A and B). Histological analysis with Masson's trichrome confirmed the micro-CT findings by showing dense collagen deposition and destruction of normal lung architecture in the lungs from WT mice, whereas the lungs from Rac1 null mice were essentially normal (Fig. 3, C and D). To verify the radiographic and histological observations, we determined the extent of fibrosis biochemically utilizing a hydroxyproline assay. Hydroxyproline in the lungs of WT mice was significantly higher compared with Rac1 null mice (Fig. 3E). Taken together, these data suggest that mitochondrial Rac1 contributes to increased oxidative stress, which modulates the development of pulmonary fibrosis.

Asbestos Induces Rac1 Import into the Mitochondria in Macrophages—Because Rac1 is highly expressed in the mitochondria of alveolar macrophages obtained from asbestos patients and is linked to pulmonary fibrosis, we determined if asbestos exposure affected whole cell Rac1 expression. Asbestos did not alter Rac1 protein levels in the whole cell lysates (Fig. 4A), so we investigated whether asbestos induced translocation of Rac1 into mitochondria by exposing macrophages to chrysotile asbestos for 3 h. Mitochondrial fractions were isolated, and an immunoblot for Rac1 revealed that Rac1 dramatically increased in the mitochondria of cells exposed to chrysotile asbestos (Fig. 4B).

To investigate if Rac1 was in the mitochondria and not attached to the outer mitochondrial membrane, we isolated the mitochondrial intermembrane space (IMS) and mitoplasts from asbestos-exposed macrophages. Rac1 was present in both the IMS and the mitoplasts; however, similar to the observation in whole mitochondria, cells exposed to chrysotile asbestos had a significant increase in Rac1 expression in the IMS (Fig. 4C). In contrast, Rac1 levels in the mitoplasts were not altered by asbestos. To correlate Rac1 expression with activity in mitochondria, a Rac1 activity assay was performed with isolated IMS and mitoplast fractions. The activity of Rac1 was more than 4-fold greater in the IMS (Fig. 4D). These data demonstrate that Rac1 is imported into the mitochondria and is active in the IMS in macrophages exposed to chrysotile asbestos.

C-terminal Cysteine Residue in Rac1 Is Required for Mitochondrial Import—Although Rac1 has been identified to be present in lymphocytes, betaTc3 cells, and the renal cortex (30–32), the mechanism by which Rac1 translocates into mitochondria is not known. The import of small proteins into the mitochondria requires conserved cysteine motifs (4, 33). The Rac1 protein contains seven cysteine residues, and to our knowledge, no cysteine motif in Rac1 has been described. We hypothesized that a specific cysteine residue(s) regulated Rac1 translocation into the mitochondria. Rac1 constructs were generated with single Cys → Ser mutations (Fig. 4E). Cells were transiently transfected with Flag-Rac1 constructs, and after 24 h the cells were exposed to chrysotile asbestos. Mitochondrial fractions were isolated, and an immunoblot analysis for the tagged protein revealed that all Flag-Rac1 mutants were imported into the mitochondria except for the C189S mutant (Fig. 4F). To verify that the C189S mutant was expressed, macrophages were transiently transfected with an empty vector, Flag-Rac1WT, or Flag-Rac1C189S and exposed to chrysotile for 3 h. Immunoblot analysis revealed that Flag was present in whole cell lysates obtained from cells expressing the WT and the C189S (Fig. 4G). Taken together, these data demonstrate that Rac1 translocates into the mitochondria and is active in the...
after exposure to chrysotile asbestos for 3 h. In cells expressing Flag-Rac1<sub>WT</sub>, asbestos increased localization of Flag-Rac1 into the mitochondria, and this was completely abrogated in cells incubated with simvastatin (Fig. 5A). In contrast, in cells expressing the C189S mutant, the vector remained in the cytoplasm, and incubation with simvastatin had no effect. To confirm that this observation was secondary to geranylgeranylation, an immunoblot analysis for Rap 1A, which recognizes only the nongeranylated protein, showed that simvastatin increased Rap 1A (Fig. 5B). These data indicate that Rac1 geranylgeranylation at the Cys-189 residue is necessary for mitochondrial import.

**Rac1 Increases H<sub>2</sub>O<sub>2</sub> Levels in the Mitochondrial IMS**—Because Rac1 activation was localized to the IMS, we determined if Rac1-mediated mitochondrial H<sub>2</sub>O<sub>2</sub> was generated in the IMS. The H<sub>2</sub>O<sub>2</sub> biosensor HyPer was targeted to the IMS by fusing it with a partial sequence of mouse glycerol phosphate dehydrogenase 2 (25). Macrophages were transiently transfected with the IMS-targeted HyPer construct and either Flag-Rac1<sub>WT</sub> or Flag-Rac1<sub>C189S</sub>. After 24 h, cells were exposed to chrysotile asbestos for 3 h, and fluorescence excitation spectra were determined by flow cytometry. Macrophages expressing the Rac1<sub>WT</sub> had significantly higher fluorescence in the IMS compared with cells expressing the Flag-Rac1<sub>C189S</sub> mutant (Fig. 5C). These data indicate that Rac1 translocation to the mitochondrial IMS is critical for asbestos-induced H<sub>2</sub>O<sub>2</sub> generation in macrophages.

To determine whether Rac1 import modulated H<sub>2</sub>O<sub>2</sub> levels in whole mitochondria, macrophages were transiently transfected with the Flag-Rac1<sub>WT</sub> or the Flag-Rac1<sub>C189S</sub> mutant. The cells were cultured for 3 h in the presence or absence of chrysotile asbestos, and mitochondria were isolated to measure H<sub>2</sub>O<sub>2</sub> generation. The mitochondria obtained from cells expressing the WT had a significant increase in mitochondrial H<sub>2</sub>O<sub>2</sub> generation that amplified in a time-dependent manner (Fig. 5D). In contrast, the mitochondria from cells transiently transfected with the C189S mutant had a marked reduction in H<sub>2</sub>O<sub>2</sub> levels indicating that Rac1 import is necessary for mitochondrial H<sub>2</sub>O<sub>2</sub> generation.

We next determined the effect of the other cysteine mutations on mitochondrial H<sub>2</sub>O<sub>2</sub> levels. Macrophages were transiently transfected with the Flag-Rac1 constructs. Cells were exposed to chrysotile asbestos for 3 h, and mitochondria were isolated to measure H<sub>2</sub>O<sub>2</sub> generation. The mitochondria obtained from cells expressing the WT, the C157S, and the C18S Rac1 constructs had a significant increase in mitochondrial H<sub>2</sub>O<sub>2</sub> generation, and the C81S and C105S mutants had a slight reduction in H<sub>2</sub>O<sub>2</sub> levels (Fig. 5E). The mitochondria from cells transiently transfected with the C189S mutant had a marked reduction in H<sub>2</sub>O<sub>2</sub> levels indicating that Rac1 import is necessary for mitochondrial H<sub>2</sub>O<sub>2</sub> generation.

IMS in macrophages. Furthermore, these data indicate that Cys-189 is required for mitochondrial import.

**Geranylgeranylation of Rac1 Is Necessary for Rac1 Mitochondrial Import**—The C-terminal cysteines of Rho GTPases are known to undergo geranylgeranylation, which is a post-translational modification that is important for activation and interaction with other proteins (10). Because the Cys-189 residue is the C-terminal cysteine, we investigated whether the mutation (C189S) altered Rac1 geranylgeranylation. Macrophages were transiently transfected with an empty vector, Flag-Rac1<sub>WT</sub>, or Flag-Rac1<sub>C189S</sub>. The cells were incubated in the presence or absence of simvastatin (10 μM) overnight. Simvastatin inhibits HMG-CoA reductase, the rate-limiting enzyme that converts HMG-CoA to mevalonate, which is a precursor to geranylgeranylpyrophosphate. Mitochondria and cytoplasm were isolated from cells transiently transfected with the C6S mutant had a marked reduction in H<sub>2</sub>O<sub>2</sub> levels (Fig. 5F). These data demonstrate that Rac1-mediated H<sub>2</sub>O<sub>2</sub> generation in macrophages is modulated by factors other than mitochondrial import and that Cys-178 has a critical role in regulating mitochondrial H<sub>2</sub>O<sub>2</sub> levels in macrophages.

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![Image of mitochondrial Rac1 localization in alveolar macrophages](https://example.com/image)

FIGURE 3. Mitochondrial Rac1 localization in alveolar macrophages is linked to the development of pulmonary fibrosis. WT and Rac1 null mice were exposed to chrysotile asbestos (100 μg) intratracheally. Micro-CT scan images were obtained on live mice (in vivo) 21 days after exposure. Images are representative of three WT (A) and three Rac1 null (B) mice. Mice were euthanized 21 days after exposure, and lungs were removed and processed for collagen deposition using Masson’s trichrome staining. Micrographs are representative of 10 WT (C) and 10 Rac1 null (D) mice. E, mice were euthanized 21 days after exposure, and lungs were removed and homogenized for hydroxyproline assay. WT (n = 4) and Rac1 null (n = 4); *, p < 0.0208.
Asbestos Induces Interaction of Rac1 with Cytochrome c in Mitochondria—Because Rac1 is active in the IMS, we asked whether Rac1 interacts with an IMS protein(s) linked to H2O2 generation. Cytochrome c is known to be associated with H2O2 generation (22, 23), so we exposed macrophages to chrysotile asbestos for 3 h and isolated mitochondria. Mitochondrial lysates were subjected to immunoprecipitation with the Rac1 monoclonal antibody, and the immunoprecipitated samples were separated on a polyacrylamide gel. An immunoblot analysis performed for cytochrome c revealed that cells exposed to asbestos had a significant increase in cytochrome c that immunoprecipitated with Rac1 in vivo (Fig. 6A). To substantiate that this binding occurred in the mitochondria, we isolated cytoplasmic and mitochondrial fractions from macrophages exposed to asbestos. An immunoblot analysis revealed that cytochrome c was retained in the mitochondria and not released into the cytoplasm (Fig. 6A).

Because of the fact that antibody-mediated immunoprecipitation of Rac1 may have other associated proteins, we determined if Rac1 interacted with cytochrome c in vitro by generating Rac1 constructs containing a C-terminal V5-His tag. Cells were transiently transfected with the Rac1-V5-HisWT and Rac1-V5-HisC6S and Rac1-V5-HisC178S vectors because they had a more significant effect on mitochondrial H2O2 generation. After 24 h, the cells were exposed to chrysotile asbestos for 3 h. Rac1 expression was significantly enhanced in cells transiently transfected with the Rac1-V5-His vectors (Fig. 6B). Cell lysates were subjected to His pulldown. Purified cytochrome c was incubated with beads alone or His pulldown beads for 30 min. The Rac1-V5-His constructs were eluted by heating. The WT, C6S, and C178S V5-His constructs interacted with purified cytochrome c in vitro (Fig. 6B). In contrast, no cytochrome c was present when incubated with the beads alone.

Although cytochrome c remained in the mitochondria, we investigated whether asbestos exposure or Rac1 activation induced macrophage apoptosis. To determine whether asbestos induced macrophage apoptosis, cells were exposed to chrysotile asbestos for 3 h. Immunoblot analysis showed that cells exposed to asbestos had an increase in active caspase 3, a key effector of apoptosis (Fig. 6C). The role of Rac1 was evaluated by infecting macrophages with either an empty (Ad5.CMV) or constitutive active Rac1 (Ad5.V12Rac1) adenoviral vector. After 48 h, an immunoblot analysis for activated caspase 3 was performed. Caspase 3 was not present in lysates obtained from cells expressing the empty vector, but cells overexpressing V12Rac1 had an increase in activated caspase 3. As a pos-
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Differential Oxidation and Reduction of Rac1 and Cytochrome c Secondary to Electron Transfer Is Required for Mitochondrial H$_2$O$_2$ Generation—Because the redox state of cytochrome c modulates mitochondrial H$_2$O$_2$, the relationship between the redox states of Rac1 and cytochrome c was explored. Macrophages were transiently transfected with the WT, C6S, and C178S Flag-Rac1 vectors and a cytochrome c vector. The cells were exposed to chrysotile asbestos for 3 h, and mitochondria were isolated in nonreducing conditions. Samples were separated on a polyacrylamide gel in the presence or absence of the reducing agent dithiothreitol (DTT, 20 mM). As a positive control, purified cytochrome c was oxidized in vitro with tert-butyl hydroperoxide (t-BOOH). DTT does not reduce all of the bands in the purified cytochrome c samples because the purification process results in dimerization or acid-modified structures of cytochrome c that do not contain a disulfide linkage. However, the corresponding upper band is reduced in the presence of DTT. Cytochrome c was completely reduced in cells expressing Flag-Rac1WT (Fig. 7A). In contrast, cells expressing C6S had some cytochrome c oxidation that did not change after exposure to chrysotile asbestos, and C178S had a significant increase in oxidized cytochrome c in both the presence and absence of asbestos exposure (Fig. 7A). No oxidized cytochrome c was present with the addition of DTT with any Rac1 construct. These data suggest that cytochrome c is predominantly reduced in cells expressing Rac1WT, whereas it becomes more oxidized if the Rac1 cysteines are mutated (i.e. C6S or C178S).
To evaluate the redox status of cytochrome c in a different manner, purified cytochrome c was incubated with t-BOOH (250 μM) or diamide (3 mM) to oxidize the protein in the presence or absence of DTT for 45 min prior to separating on a polyacrylamide gel. Both t-BOOH and diamide oxidized cytochrome c, and DTT attenuated t-BOOH-induced oxidation.
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and completely reversed the effect of diamide (Fig. 7B). These data suggest that purified cytochrome c can be oxidized effectively in vitro, and the effect of diamide can be completely reversed with DTT.

To investigate the oxidation of Rac1, macrophages were transiently transfected with an empty vector, Flag-Rac1WT, or Flag-Rac1C178S as a negative control. Cells were exposed to chrysotile for 3 h, and mitochondria were isolated in nonreducing conditions. Samples were separated on a polyacrylamide gel in the presence or absence of DTT, and an immunoblot analysis for the tagged protein was performed. Flag-Rac1WT was oxidized in nonreducing conditions, and this oxidation was enhanced by exposure to chrysotile (Fig. 7C). The oxidation state of Flag-Rac1WT was inhibited in the presence of DTT. As expected, the oxidation of the C178S mutant did not occur in the mitochondrial samples. Rac1 has seven cysteines, so several different intramolecular disulfides can be generated by oxidation that will result in oxidized Rac1 migrating at different levels. Rac1 may also form mixed disulfides with other proteins containing cysteine residues, such as cytochrome c, which will result in migration at different levels. Nonetheless, all of the oxidized forms of Rac1 are reduced by DTT. Taken together, the differential oxidation and reduction of Rac1 and cytochrome c suggest that electron transfer is responsible for their redox states.

To determine the site at which electron transfer occurred between cytochrome c and Rac1, macrophages were transiently transfected with an empty vector, Flag-Rac1WT, Flag-Rac1C6S, or Flag-Rac1C178S. Cells were exposed to asbestos for 3 h, and mitochondria were isolated in nonreducing conditions, and the oxidation status of Flag-Rac1 was evaluated. Flag-Rac1WT and Flag-Rac1C6S were oxidized in the nonreducing conditions, and this oxidation was enhanced with exposure to chrysotile in cells expressing the Flag-Rac1WT (Fig. 7D). In the presence of DTT, however, the WT and C6S were completely reduced, including the samples obtained from cells exposed to asbestos. In contrast, the C178S mutant was not oxidized in the nonreducing conditions in the presence or absence of chrysotile asbestos. The C178S mutant was present at the reduced level in the presence or absence of DTT (Fig. 7D). These data demonstrate that the C178S mutant is expressed in the mitochondria and suggest that electron transfer is responsible for the redox states.

To determine whether electron transfer between Rac1 and cytochrome c had an effect on mitochondrial H2O2 generation, macrophages were transiently transfected with Rac1-V5-His vectors containing WT, C6S, or C178S constructs. Samples were subjected to His pulldown. Purified cytochrome c was incubated with diamide in the presence or absence of β-mercaptoethanol to reversibly prevent disulfide bond formation by oxidizing or reducing the cysteine residues in cytochrome c, respectively. The purified cytochrome c preparations were then added to the His beads for 30 min, and H2O2 production was determined. Diamide inhibited H2O2 levels in vitro with the Rac1-V5-HisWT, and the addition of β-mercaptoethanol increased H2O2 more than 3-fold (Fig. 7E). H2O2 generated from the Rac1-V5-HisC6S was significantly inhibited by diamide, and β-mercaptoethanol enhanced H2O2 levels when compared with the incubation with diamide (Fig. 7E). In contrast, diamide had no effect on H2O2 production by the Rac1-V5-HisC178S, in vitro, and H2O2 levels were inhibited by the addition of β-mercaptoethanol. These data demonstrate that the import of Rac1 to the mitochondria, the interaction with cytochrome c, and the electron transfer to Cys-178 mediate the differential oxidation and reduction of Rac1 and cytochrome c that is necessary for mitochondrial H2O2 generation. These data also reveal that the redox state of critical cysteines in Rac1 modulates the development of pulmonary fibrosis.

DISCUSSION

Asbestos is a prototypical cause of pulmonary fibrosis in which ~200,000 cases of asbestos-related pulmonary disease are diagnosed each year, leading to 4,000 deaths annually (1). For unclear reasons, the incidence of pulmonary fibrosis has increased significantly over the past 10–15 years (34). Macrophages play a key role in the inflammatory response and oxidative stress that occurs after injury in multiple tissues that can result in aberrant repair. The macrophage-induced inflammation, oxidative stress, and repair processes that result in fibrosis are known to occur in cardiac, hepatic, renal, and pulmonary tissues. The development of pulmonary fibrosis from asbestos exposure and other causes is critically linked to the generation of ROS, including H2O2. In this study, we demonstrated that Rac1 is imported into the mitochondria in macrophages, and Rac1 is active in the IMS where it interacts with and accepts electrons from cytochrome c via Cys-178 to induce mitochondrial H2O2 generation. Furthermore, we demonstrated that mitochondrial Rac1 activity in alveolar macrophages is associated with increased oxidative stress, which modulates the development of pulmonary fibrosis. Taken together, our observations provide a unique target that has broad therapeutic implications.

Rac1 is a member of the family of Rho GTPases, and it regulates several cellular functions, such as the assembly of NADPH oxidase in nonphagocytic cells, actin polymerization, cell adhesion, and cell differentiation (35, 36). Rac1 is ubiquitously expressed, and its activation increases the generation of H2O2 (3, 6, 16, 17). Pulmonary fibrosis is characterized by aberrant tissue remodeling in response to injury, and this process can be influenced by H2O2. We have shown that macrophage-derived oxidative stress promotes the development of pulmonary fibrosis by increasing collagen deposition by human lung fibroblasts (3, 4). Previous studies provide evidence that Rac1 induces mitochondrial ROS production indirectly by engagement of integrins in fibroblasts and by increasing ceramide in endothelial cells (18–20). In this study, we demonstrate for the first time that Rac1 mediates mitochondrial H2O2 generation directly in macrophages by translocation and interaction with cytochrome c in mitochondria, which transfers an electron to Rac1 at Cys-178 leading to H2O2 formation. H2O2 levels are not only controlled by Rac1 because Rac1 null macrophages do not completely eliminate H2O2. Other sources of H2O2 production in BAL cells include the cell membrane and lysosome NADPH oxidase, mitochondrial respiratory complexes, and superoxide.
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dismutase (3, 4, 37). Thus, H$_2$O$_2$ production in BAL cells is controlled, at least in part, by mitochondrial Rac1.

Recent evidence has shown that Rac1 interacts with Bcl-2 in the mitochondria of lymphocytes, but Bcl-2, rather than Rac1, was coupled to mitochondrial ROS generation (32). In contrast, the ability of Rac1 to translocate into the mitochondria and accept electrons represents a new mechanistic insight into how Rac1 can facilitate the generation of H$_2$O$_2$ by partial reduction of O$_2$ in macrophages.

Similar to other small proteins, such as Cox17, TIM13, and Cu,Zn-SOD (4, 33), a conserved cysteine (Cys-189) in Rac1 is required for the import into the mitochondria. This cysteine residue is also important for geranylgeranylation, a lipidation process common to all Rho GTPases. This post-translational modification is important for activation and association with other proteins (10). The geranylgeranylation of the C-terminal cysteine residue in Rac1 has been shown to be necessary for interaction with the cell membrane and nuclear localization (38, 39). Our data verify that the C189S mutant was not imported into mitochondria due to its lack of geranylgeranylation because Flag-Rac1WT import was inhibited by simvastatin.

Limited data are known regarding the redox status of GTPases, especially as it relates to their ability to induce ROS generation. The oxidation of RhoA, rather than Rac1, after exposure to cisplatin was shown to dissociate it from GTP, thereby decreasing its activity (40). This oxidation was shown to be due to the presence of an additional cysteine in RhoA. It is not clear in this study what the effect oxidation had on ROS generation. Another study showed that in reducing conditions, Rac1 increased NADPH oxidase activity in neuronal cells, and when oxidized, its activity declined (37). Our *in vivo* and *in vitro* data, however, indicate that oxidized Rac1 in the mitochondria of alveolar macrophages is necessary for H$_2$O$_2$ generation.

The primary role of cytochrome c is the transfer of electrons from complex III to complex IV in the mitochondrial respiratory chain. Superoxide anion (O$_2^-$) and H$_2$O$_2$ generation are known to increase dramatically when cytochrome c is retained in the mitochondrial respiratory chain, which has been linked to apoptosis (41). In fact, cytochrome c is implicated as being responsible for activating programmed cell death upon release from the mitochondria (22, 41, 42). Cytochrome c also has antioxidant properties by eliminating O$_2^-$ and H$_2$O$_2$ from the mitochondria (41). Our results demonstrate that cytochrome c is retained in the mitochondria; however, macrophages undergo apoptosis after 3 h of asbestos exposure. This result corroborates other studies in that asbestos induces apoptosis (43–45). Rac1 has been shown to inhibit apoptosis in many cell types (32, 46–48), but we found that constitutively active Rac1 induces apoptosis. It is likely that macrophages undergo apoptosis in these conditions secondary to the high mitochondrial H$_2$O$_2$ levels generated.

The transfer of electrons from cytochrome c to Rac1 in mitochondria provides a novel mechanism by which Rac1 increases H$_2$O$_2$ levels. It also suggests that Rac1 removes electrons from the mitochondrial respiratory chain to partially reduce O$_2^-$ to p66$^{Shc}$, a pro-apoptotic protein, is known to generate mitochondrial ROS and was recently shown to oxidize cytochrome c to generate H$_2$O$_2$ and induce apoptosis in fibroblasts (22). The redox status of p66$^{Shc}$ after electron transfer is not clear, and the site that accepts electron transfer is not known. Rac1, however, was oxidized with the electron transfer to Cys-178. Because the interaction between Rac1 and cytochrome c appears to be critical for the differential oxidation and reduction reaction and electron transfer, studies are currently in progress in our laboratory to determine whether there is direct binding and, if so, to determine the domain in Rac1 that binds to cytochrome c.

Nonetheless, our data demonstrate that the differential oxidation and reduction of Rac1 and cytochrome c is critical for the generation of H$_2$O$_2$ in macrophages. This new mechanistic role of Rac1 is a prospective biomarker for pulmonary fibrosis, and it provides a potential target for intervention.

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