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Mitochondrial Biogenesis in the Pulmonary Vasculature During Inhalational Lung Injury and Fibrosis

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ABSTRACT

Cell survival and injury repair is facilitated by mitochondrial biogenesis; however, the role of this process in lung repair is unknown. We evaluated mitochondrial biogenesis in the mouse lung in two injuries that cause acute inflammation and in two that cause chronic inflammation and pulmonary fibrosis. By using reporter mice that express green fluorescent protein (GFP) exclusively in mitochondria, we tracked mitochondrial biogenesis and correlated it with histologic lung injury, proliferation, and fibrosis. At 72 hours after acute LPS or continuous exposure to hyperoxia (Fio_2, 1.0), the lungs showed diffuse infiltration by inflammatory cells in the alveolar region. In reporter mice, patchy new mitochondrial fluorescence was found in the alveolar region but was most prominent and unexpected in perivascular regions. At 14 days after instillation of asbestos or bleomycin, diffuse chronic inflammation had developed, and green fluorescence appeared in inflammatory cells in the expanded interstitium and was most intense in smooth muscle cells of pulmonary vessels. In all four lung injuries, mitochondrial fluorescence colocalized with mitochondrial superoxide dismutase, but not with proliferating cell nuclear antigen. These data indicate that vascular mitochondrial biogenesis is activated in diverse inhalational lung injuries along with oxidative stress. This finding indicates a unique and unexpected mechanism of metabolic adaptation to pulmonary fibrotic injuries. *Antioxid. Redox Signal.* 10, 269–275.

INTRODUCTION

Acute lung injury (ALI) comprises a spectrum of damage ranging from airway inflammation to extensive fibrosis of the alveolar region. Although the exact molecular events underlying fibrogenesis in the lung are still unknown, deregulated inflammation and cellular damage are postulated to be important initiators of cell proliferation and fibrogenesis (27).

During inflammation, mitochondria are susceptible to oxidative damage, especially the circular mitochondrial genome (mtDNA), which lacks protective histones and is located near electron-transport complexes that produce reactive oxygen species (ROS) (16). Unopposed mtDNA oxidation interferes with mitochondrial transcription and OXPHOS protein synthesis, which not only impairs respiratory capacity but may also exacerbate ROS production (5). Endogenous ROS can directly damage mtDNA, leading to altered mitochondrial gene expression and function (28). These effects have been observed during injury to highly metabolic tissues such as the liver and heart (21, 22).

We have reported that mitochondrial biogenesis is an integral adaptive mechanism that is recruited during injuries associated with mitochondrial oxidative stress. Mitochondrial biogenesis is activated in the mouse brain after exposure to hyperbaric hyperoxia, and in the rodent liver and heart during sepsis (8, 21, 22). The transcriptional processes recruited to support biogenesis regulate the mitochondrial content of the cells, and biogenesis is a prosurvival response to support increased energy requirements or injury to the mitochondria (9). Mito-

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chondrial biogenesis is regulated by cross-talk between nuclear and mitochondrial genomes and is coordinated by nuclear coactivators such as PGC-1α and the nuclear respiratory factors (NRF-1 and NRF-2), which transactivate genes for oxidative phosphorylation, protein importation, and heme biosynthesis (11, 16, 18). These transcription factors also mediate mtDNA transcription and replication through two nuclear genes: mitochondrial transcription factor A (Tfam) and its cofactor, mitochondrial transcription factor B (11, 16). It appears that activation of these molecular regulators of biogenesis during adaptation to oxidative stress promote cell and organ survival (16, 21, 22).

The role of mitochondrial function in normal lung homeostasis and the importance of mitochondrial damage and biogenesis in lung injury and repair remain poorly understood, especially because the lung relies on glycolysis more than do other organs. Indeed, the lung is sparsely populated with mitochondria, although a few cell types, such as type II epithelial cells and bronchial and vascular smooth muscle cells, are rich in this organelle. Mitochondrial initiation of apoptosis has also been implicated as a factor in hyperoxic lung injury (14). Therefore, we hypothesized that mitochondrial biogenesis would be sparingly activated in the lung during injury, causing inflammation and fibrosis, and that it would be linked primarily to evidence of cell proliferation. To investigate this idea, we used two models of acute lung inflammation and two models of chronic pulmonary inflammation and fibrosis in mice to correlate lung injury with the regional localization of mitochondrial biogenesis. Mitochondrial biogenesis was tracked in the lung after injury by using a unique reporter mouse and compared with the location of the mitochondrial isoform of superoxide dismutase, cell proliferation, and fibrosis.

MATERIALS AND METHODS

Materials

Crocidolite asbestos fibers (>10 μm in length) were obtained from the National Institutes of Environmental Health Sciences (Research Triangle Park, NC). All other chemicals were from Sigma (St. Louis, MO), unless otherwise indicated.

Animal exposures

All animal experiments were reviewed and approved by the Duke University Institutional Animal Care and Use Committee. We used wild-type C57BL/6, 8 to 10 weeks old, or transgenic mice that express green fluorescent protein (GFP) exclusively in mitochondria (mtGFP-tg mice), obtained from Tokyo Metropolitan University and bred in our facility (20). The mice contain a genetic construct that incorporates GFP and the mitochondrial localization sequence of cytochrome c oxidase subunit VIII onto the β-actin promoter, which targets the GPF in mitochondria. Thus, GFP expression occurs in most mitochondria. Male mice were anesthetized briefly with halothane by using a nose cone and treated with LPS (0.5 mg), bleomycin (2 mg/kg), or asbestos (0.1 mg) by intratracheal instillation, as described (25). The animals then recovered, were returned to their cages, and were killed after 3 days (LPS) or 14 days (asbestos and bleomycin). For exposure to hyperoxia, the mice breathed Fio_2 1.0 in Plexiglas exposure chambers for 72 h and then were killed (26).

Histology and immunohistochemistry

Standard hematoxylin and eosin (H&E) staining was performed on 5-μm-thick lung sections, as previously described (4). Photos of random fields were taken from each slide at x40 magnification. Representative images are shown.

Fluorescence imaging

Fluorescence microscopy was performed with a Nikon Optiphot-2B microscope. Lung sections from mtGFP-tg mice were deparaffinized, rinsed in phosphate-buffered saline (pH 7.4), and coverslipped. Immunofluorescence was performed as previously reported (23). The anti-SOD2 (StressGen/ Assay Designs, Inc., Ann Arbor, MI) and anti-PCNA (Santa Cruz Biotechnology, Santa Cruz, CA) primary antisera were used at working dilutions of 1:500 and 1:50, respectively. Sections were then washed extensively in PBS and incubated with Alexa-Fluor 594 fluorescent secondary anti-rabbit IgG (Molecular Probes, Carlsbad, CA). Specific immunostaining was assessed by using one or both secondary antibodies without primary antibody. Multiple dilutions of each primary antibody were tested and optimized to minimize nonspecific adsorption, to ensure discrimination of fluorescent signals, and to set the fluorophore concentration. Conventional red and green fluorescence images were obtained by using a Nikon Microphot-FXA fluorescence microscope followed by electronic image merging.

RESULTS

We characterized the lung pathology and related it to the development of mitochondrial biogenesis in response to four unique pulmonary injuries that originate on the epithelial and/or endothelial surfaces of the alveolar region. Hyperoxia and intratracheal LPS instillation were studied as representative models of severe acute lung injury/inflammation, and intratracheal asbestos and bleomycin were used to study chronic inflammation and fibrosis.

Histologic changes were assessed with H&E and correlated with lung fibrosis, which was identified by using Masson's Trichrome stain for collagen. The two acute models of lung injury showed similar findings with abundant acute inflammatory changes, with relatively little collagen accumulation. At 72 h after exposure to hyperoxia, diffuse acute lung inflammation was characterized by the presence of numerous inflammatory cells and erythrocytes within the alveolar spaces and interstitium (Fig. 1A). Masson's Trichrome staining showed patchy collagen deposition within the alveolar spaces in some areas (Fig. 1B). A similar pattern was observed 72 h after intratracheal LPS instillation; we noted intra-alveolar and interstitial inflammatory cell accumulation, as well as patchy alveolar collagen deposition (Fig. 1C and D).

Fourteen days after intratracheal instillation of asbestos, the lungs showed an accumulation of mixed inflammatory cells in the alveolar region and confluent areas of marked interstitial

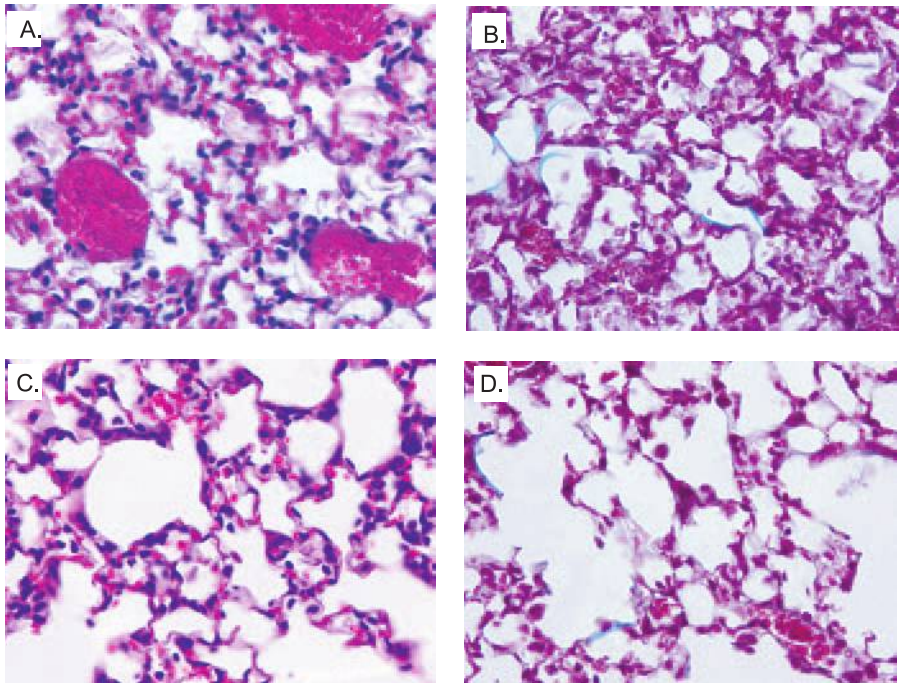

FIG. 1. Histology and Masson's Trichrome staining of the lung after exposure to hyperoxia or instillation of intratracheal LPS. Hematoxylin and eosin (H&E) staining or Masson's trichrome staining were performed on paraffin-fixed lung sections. (**A**) Mice were exposed to hyperoxia (FiO_2 1.0) for 72 h. Light microscopy showed acute lung inflammation with numerous inflammatory cells, mainly polymorphonuclear leukocytes, and erythocytes within the alveolar spaces and interstitium. (**B**) Masson's Trichrome staining showed patchy collagen deposition (blue staining) within the alveolar spaces in some areas. (**C**) H&E and (**D**) Masson's Trichome after LPS (1 mg/kg) was instilled *via* intratracheal instillation, and lungs were fixed 72 h later. Note the intra-alveolar and interstitial inflammatory cell accumulation, as well as the scarcity of alveolar collagen deposition. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

thickening (Fig. 2A). These changes had a pronounced peribronchial/alveolar distribution. Masson's Trichrome demonstrated substantial fibrosis, with collagen deposition in the alveolar region and within the expanded interstitium (Fig. 2B). Similar findings were present 14 days after intratracheal instillation of bleomycin, with peribronchial distribution of acute and chronic inflammatory cells in the alveolar region, and interstitial collagen deposition (Fig. 2C and D).

To determine whether mitochondrial biogenesis occurs in the lung during acute inflammation or during chronic inflammation and fibrosis, we performed fluorescence microscopy in mitochondrial reporter mice to assess the mitochondrial distribution and to evaluate the lungs for the appearance of new mitochondria (Fig. 3). During acute pulmonary inflammation from hyperoxia, we noted increased mitochondrial fluorescence in the smooth muscle of small blood vessels, along with occasional scattered fluorescence in the inflammatory cells of the alveolar region (Fig. 3A). A similar pattern of mitochondrial fluorescence was present after intratracheal LPS, with most intense staining in the perivascular regions and in inflammatory cells (Fig. 3B).

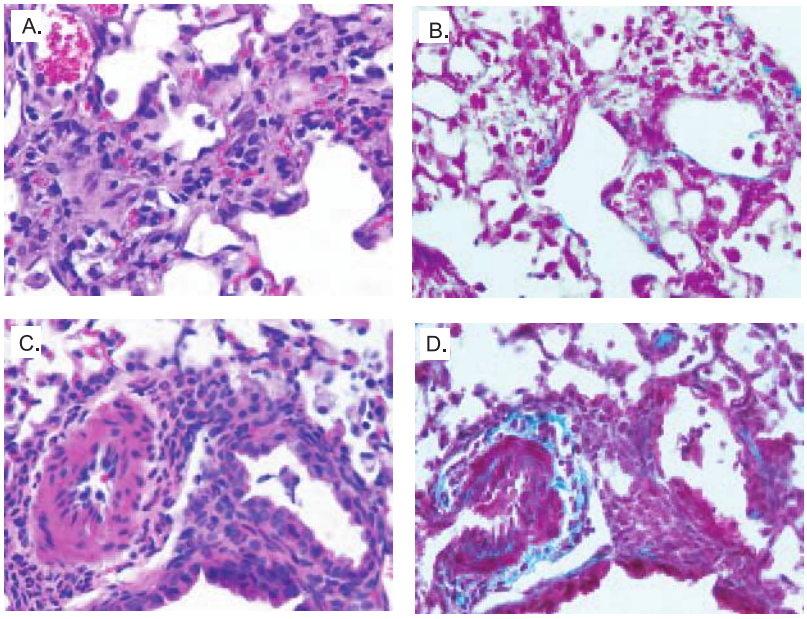

FIG. 2. Lung histology and fibrosis 14 days after intratracheal asbestos and bleomycin. Hematoxylin and Eosin (H&E) staining and Masson's trichrome staining were performed on lung sections 14 days after exposure. (**A**) Histological micrograph 14 days after intratracheal asbestos (0.1 mg) showed increased cellularity and lung inflammation characterizied by numerous inflammatory cells and erythrocytes within the alveolar spaces and interstitium, in a peribronchial distribution. (**B**) Masson's Trichrome staining after intratracheal asbestos showed interstitial collagen deposition (blue staining within the interstitium. (**C**) Micrograph of lung histology after intratracheal bleomycin (2 mg/kg) revealed lung inflammation. Note the numerous inflammatory cells and erythrocytes within the alveolar spaces and interstitium. Note presences of intraalveolar and interstitial inflammatory cells. (**D**) Masson's Trichrome staining after intratracheal asbestos showed diffuse collagen deposition (blue staining) in the interstitium. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

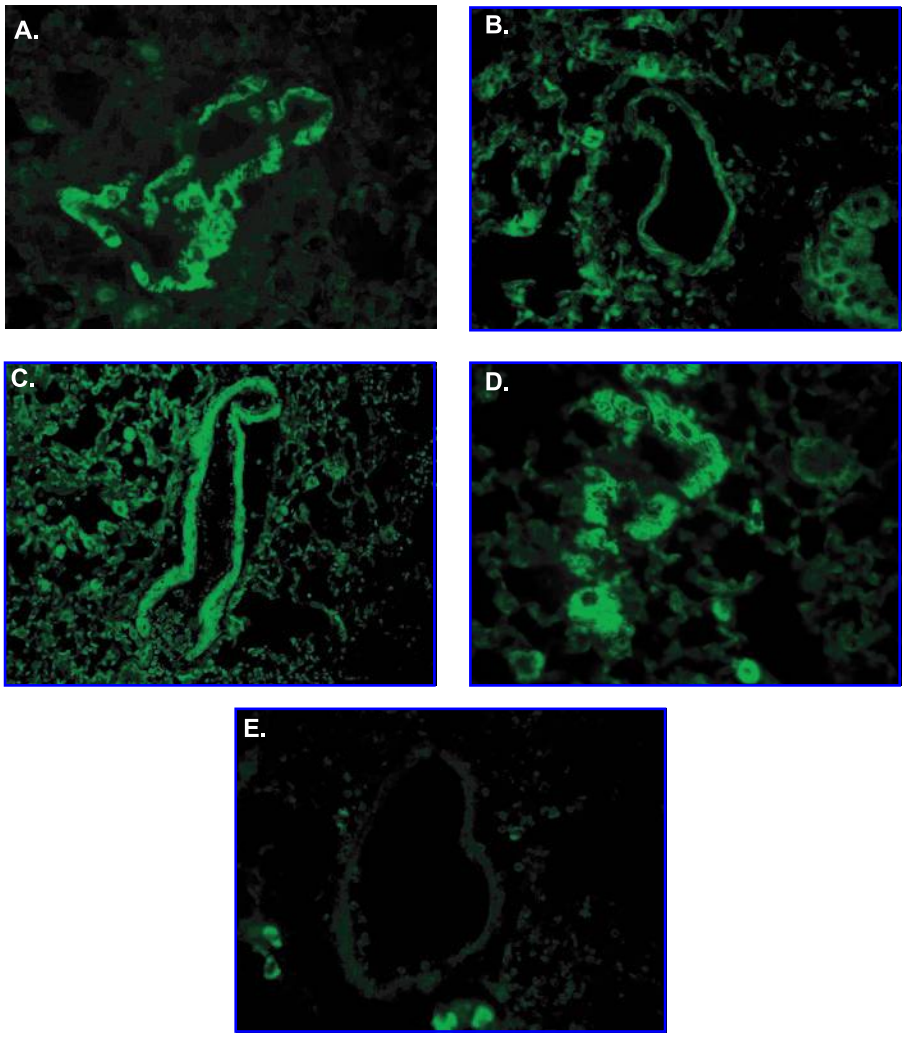

FIG. 3. Fluorescence microscopy of lungs of mtGFP-tg mice after one of four inhalational injuries. (**A**) Mice were exposed continuously to hyperoxia (FiO2 1.0) for 72 h. Microscopy showed intense mitochondrial fluorescence in the smooth muscle of small blood vessels, along with scattered fluorescence in the inflammatory cells in the alveolar region. (**B**) LPS (1 mg/kg) was instilled *via* intratracheal route, and lungs were fixed after 72 h. Intense mitochondrial fluorescence was present in the inflammatory cells. (**C**) Asbestos (0.1 mg) was instilled *via* intratracheal route, and lungs were fixed 14 days after intratracheal instillation of asbestos. There was a similar pattern of fluorescence in vascular smooth muscle and sparsely within the alveolar region. (**D**) Bleomycin (2 mg/kg) was instilled *via* intratracheal route, and lungs were fixed after 14 days. Small muscularized pulmonary vessels showed substantial increase in green fluorescence, primarily in the smooth muscle, inflammatory cells, and within the expanded interstitium. (**E**) Mitochondrial distribution in the lung of unexposed control mtGFP-tg mouse. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

Fourteen days after intratracheal instillation of asbestos, the small muscularized pulmonary vessels showed dramatic increases in green fluorescence, primarily in the smooth muscle layer (Fig. 3C). We also noted prominent mitochondria in the chronic inflammatory cells of the expanded interstitium.

Fourteen days after intratracheal instillation of bleomycin, we found a similar pattern of fluorescence in vascular smooth muscle cells and sparsely within the alveolar region (Fig. 3D). Figure 3E shows green fluorescence images from a control mouse, indicating that mitochondria in the normal lung are distributed in the vascular and bronchial smooth muscle and found in the bronchial epithelial and some alveolar epithelial cells, most likely in the type II cell.

To correlate mitochondrial biogenesis in the lung with mitochondrial oxidative stress during lung injury, we performed immunofluorescence microscopy by using red fluorescent staining for the mitochondrial isoform of superoxide dismutase (MnSOD, SOD2) (Fig. 4, Left panels) and in mtGFP-tg mice, by using green fluorescence to track mitochondrial distribution (see Fig. 3). The images were merged to identify the colocalization (Fig. 4, right panels). In acute lung injury caused by hyperoxia and LPS, intense staining for SOD2 was present in inflammatory cells and in vascular smooth muscle (Fig. 4A and B, left panel). During asbestos- and bleomycin-induced pulmonary fibrosis, intense SOD2 staining was also present predominantly in vascular smooth muscle and in inflammatory cells (Fig. 4C and D, left panels), and green fluorescence mitochondrial staining was most intense in vascular smooth muscle (Fig. 3). The merged images in both acute inflammatory and fibrotic lung injury show that mitochondrial biogenesis colocalizes with SOD2 (Fig. 4A–D, right panels), indicating an association of mitochondrial oxidative stress with formation of mitochondria in the lung. In Fig. 4E, a micrograph of lungs from a control mtGFP-tg mouse demonstrates that MnSOD is primarily distributed in alveolar (left panel) and bronchial epithelium (not shown), where it colocalizes with mitochondrial fluorescence (right panel).

Because mitochondrial biogenesis promotes cell survival and is necessary for normal mitosis, we wondered whether cell proliferation in the lung during inflammation and fibrosis is associated with biogenesis. As in Fig. 4, we performed immunofluorescence microscopy by using red fluorescence staining for proliferating cell nuclear antigen (PCNA) in mtGPF-tg mice. In the acute inflammatory injuries, only mini-

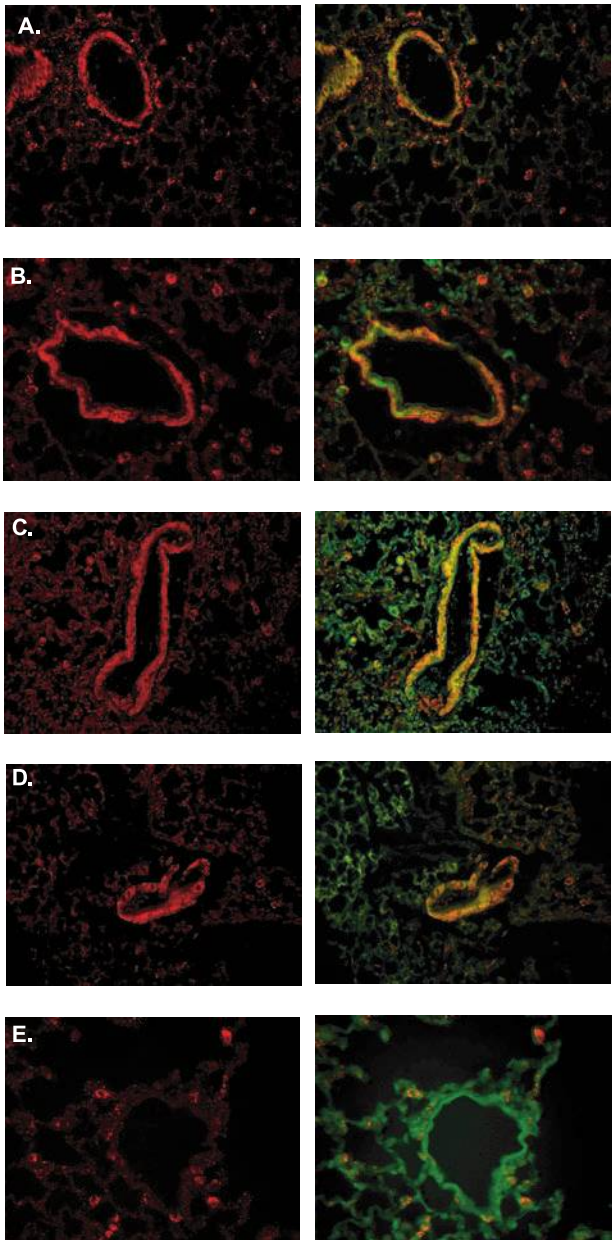

FIG. 4. Correlation of mitochondrial biogenesis with mitochondrial SOD during lung injury. Immunofluorescent microscopy was performed for mitochondrial superoxide dismutase (SOD2) using red fluorescent secodary antibody in the mt-GPF-tg mice, and images were merged to co-localize. In acute lung injury caused by (**A**) hyperoxia and (**B**) LPS (72 h), SOD2 was present in inflammatory cells and in vacular smooth muscle (*left panels*), and co-localized with mitochondia in the merged images (*right panels*). During (**C**) asbestos and (**D**) bleomycin-induced pulmonary fibrosis (14 days), intense staining for SOD2 was present in inflammatory cells and vascular smooth muscle (*left panels*), similar to distribution of green mitochondrial fluorescence. SOD2 co-localized with mitochondria in the merged images (*right panels*). (**E**) In control mtGFP-tg mice, MnSOD is distributed primarily in the alveolar epithelium (*left panels*), where it co-localizes with baseline mitochondrial fluorescence (*right panels*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

mal PCNA staining was detectable in the lung (images not shown). In pulmonary inflammation and fibrosis due to asbestos and bleomycin, intense PCNA staining was found in the interstitium and inflammatory cells (Fig. 5A and B, left panels), whereas the majority of green fluorescence indicating mitochondrial biogenesis occurred in vascular smooth muscle (see Fig. 3). The merged images show that localization of cell proliferation in the small blood vessels is distinct from that of mitochondrial biogenesis in this case (Fig. 5A and B, right panels). In both bleomycin- and asbestos-injured lungs, mitochondrial fluorescence colocalizes with PCNA in some of the parenchymal or inflammatory cells as well.

DISCUSSION

These novel data demonstrate for the first time that mitochondrial biogenesis occurs in the lung during acute and chronic inhalational injury. Interestingly, we found increased mitochondrial biogenesis primarily in the vasculature and in close association with mitochondrial SOD—presumably due to oxidative stress—not with cell proliferation. Notably, the increase in mitochondrial biogenesis associated with vascular smooth muscle did not localize with the PCNA cell-proliferation marker. This suggests that vascular smooth muscle cells increase mitochondrial biogenesis in response to both acute and chronic lung injuries and may be an important compensatory response. Biogenesis in vascular smooth muscle may be a hallmark of vascular remodeling and could contribute to the pulmonary hypertension frequently seen in patients with pulmonary fibrosis. In contrast to the vascular smooth muscle cells, some colocalization of mitochondrial biogenesis occurred with the PCNA proliferation marker in the alveolar parenchyma. This suggests that mitochondrial biogenesis may contribute to cellular regeneration in the injured alveolar parenchyma. In addition, increased mitochondrial fluorescence was present in the inflammatory cells. The pulmonary parenchymal findings suggest a unique mechanism of metabolic adaptation to inflammatory and fibrotic stress in the lung.

Although the lung is sparsely populated with mitochondria, they have an important role in lung homeostasis, particularly in the maintenance of type II alveolar epithelium (12). However, until now, the importance of mitochondrial damage and biogenesis during lung injury and repair has been unknown. The mt-GFP reporter mice show convincingly that several lung cell types express a high density of mitochondria, especially vascular smooth muscle cells. It is also known that endothelial and vascular damage is prominent in hyperoxic lung injury (7), and mitochondrial mechanisms have been implicated in apoptosis of epithelial cells in hyperoxic lung injury (14). Mitochondrial electron-transport chain activity also appears to be compromised in lung epithelial cells isolated from rats after exposure to hyperoxia (1). These data implicate mitochondrial damage as an important factor in lung injury and led us to investigate this possibility in several models of lung injury and fibrosis.

Our studies provide a novel demonstration that after several types of injury, mitochondrial biogenesis is activated largely in the pulmonary vascular smooth muscle, a location

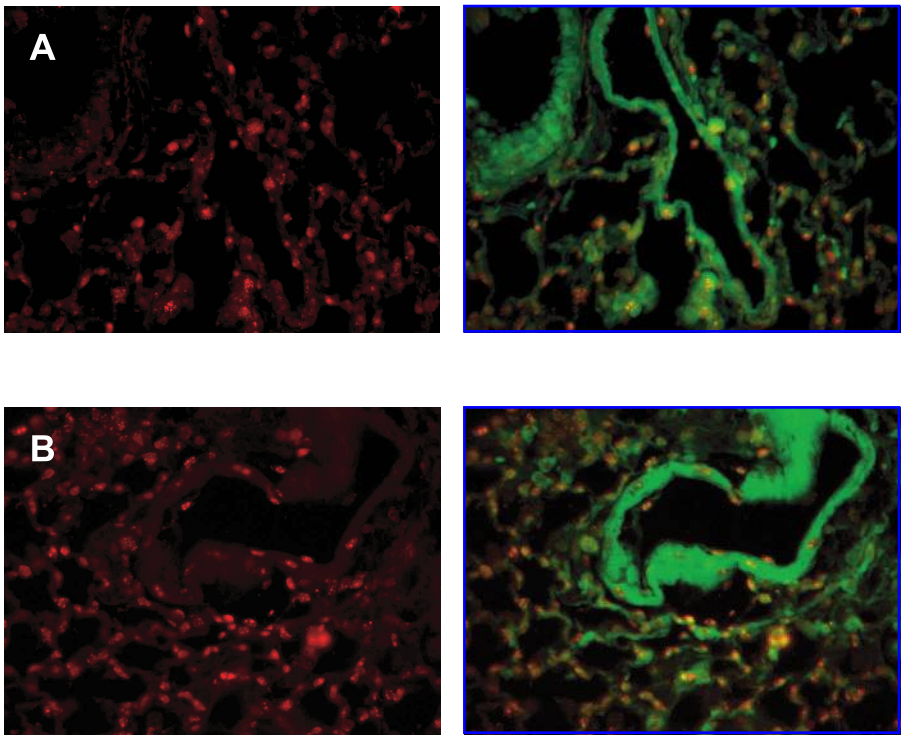

FIG. 5. Fluorescence microscopy for proliferating cell nuclear antigen (PCNA) in mtGPF-tg mice. Following intratracheal instillation of (**A**) bleomycin (2 mg/kg) or (**B**) asbestos (0.1 mg) in the mtGFP-tg mice, immunofluorescent microscopy was performed for PCNA on paraffin-fixed lung sections with red fluorescent secondary antibody labeling (*left panel*), and images were merged to co-localize. (**A**) *Right panel.* Merged images from lungs 14 days after bleomycin-exposure of the mouse. (**B**) *Right panel.* Merged images from lungs 14 days after asbestos-exposure of the mouse. Co-localization by the merged image shows that activation of cell proliferation is distinct from mitochondrial biogenesis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

that is clearly distinct from the epithelial site of injury initiation. This finding was common to all four models and implies that vascular mechanisms are recruited either to limit injury or to participate in lung repair. It is also possible that this early response to injury could contribute to vascular remodeling later in the progression of injury. The signals that govern these responses are unknown, but could include ROS, nitric oxide, or any of several inflammatory or mitogenic cytokine or chemokine pathways. The colocalization of new mitochondria with SOD2 indicates that mitochondrial oxidative stress accompanies biogenesis, although we cannot exclude that some of the staining for SOD2 is merely an indicator of new mitochondria. These observations in the lung are in keeping with our earlier findings in the heart and liver, where excessive ROS production and mitochondrial injury are opposed by the prosurvival pathways of mitochondrial biogenesis (21, 22, 24). We did not measure the transcriptional markers of biogenesis for these experiments, but the structural evidence of new mitochondria in the GFP mice is quite convincing when injured mice are compared with controls. Although mitochondrial biogenesis is clearly occurring in the vascular smooth muscle, we cannot determine whether the apparent increase in mitochondrial fluorescence in inflammatory cells is due to biogenesis or simply to the known influx of inflammatory cells during these injuries. As acute inflammatory cells are major factors in the initiation of these injuries and they do not proliferate, many of the cells in which mitochondria and PCNA colocalize are probably epithelial or endothelial cells or both. Further studies on the role of mitochondrial biogenesis in normal repair *versus* fibrosis are therefore warranted.

In experimental fibrosis caused by asbestos and bleomycin, a strong role is suggested for reactive oxygen species (ROS) (3, 10). ROS modify proteins, lipids, and nucleic acids, which alters the normal functions of these macromolecules (3, 10, 25). Oxidative injury caused by asbestos fibers is amplified by their iron content, and iron catalyzes the Haber–Weiss reaction, leading to hydroxyl radical formation (19). The importance of oxidative stress in the injury is strengthened by studies showing modulation of injury by the antioxidant activity of superoxide dismutase, catalase, and iron chelators such as desferoxamine (3, 6, 15). However, it remains an open question whether mitochondrial ROS also serve this important signaling function in lung cells. We have shown that mitochondria-derived ROS have signaling functions that activate biogenesis pathways in the heart (23), and it seems probable that this would also occur during oxidative stress in the lung.

The molecular events that initiate fibrogenesis in the lung are poorly understood, but the cornerstone of current thinking is that loss of alveolar epithelial cell integrity initiates fibroblast signaling (*e.g., via* TGF-β-linked cell migration and proliferation), which in severe cases, results in disordered repair (6, 13, 27). This paradigm has been established in asbestos-related fibrosis in animals, where injury evolution is a complex process involving both epithelial apoptosis and activation of prosurvival transcription factors such as NF-κB (10). Similar events occur in experimental lung injury and fibrosis caused by intratracheal instillation of bleomycin, which requires inflammation and oxidative injury, loss of alveolar epithelial cells, and exposure of alveolar basement membranes within a few days, followed by fibroblast proliferation (3).

We did not find that mitochondrial biogenesis colocalized with cell proliferation or fibrogenesis in the two fibrogenic models, again suggesting it has a protective prosurvival function in the pulmonary vasculature. It is interesting that over-

lapping prosurvival signaling pathways may regulate mitochondrial biogenesis and contribute to fibroblast proliferation, thereby raising the issue of how the relative balance of these diverse processes plays into fibrogenesis.

In closing, it must be mentioned that pathogenic correspondence between mouse models of pulmonary fibrosis (silica, asbestos, lung radiation, and bleomycin) and the human diseases is not established. Despite having differences from their clinical counterparts and the progression of idiopathic pulmonary fibrosis, these experimental exposures do produce lung scarring after intense inflammation (2), and in the case of asbestos, progressive severe fibrosis. Specifically, the role of acute and chronic inflammation in human idiopathic pulmonary fibrosis is not clear and is now considered less important than previously believed (13, 27). However, these well-characterized animal models do provide a basis to explore the biology of mitochondrial biogenesis in the lung and extend these observations to fibroproliferation in human lung injury.

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ABBREVIATIONS

H&E, hematoxylin and eosin; mt-GFP, mitochondrial green fluorescent protein; MnSOD, mitochondrial superoxide dismutase (SOD2); NF-κB, nuclear factor-kappa beta; PCNA, proliferating cell nuclear antigen; ROS, reactive oxygen species; PGC-1α, peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α.

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