**Airway fibrosis in rats induced by vanadium pentoxide**

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Although vanadium and vanadium-containing particles cause lung inflammation and airway hyperreactivity, it is not known whether vanadium exposure results in airway remodeling similar to that observed in individuals with chronic asthma. Asthma is a complex disorder characterized by airway hyperresponsiveness and progressive inflammation. The inflammatory response in the airways of asthmatic patients may lead to fibroproliferative changes, such as an increase in airway smooth muscle cell (SMC) mass (12, 15), mucous cell metaplasia within the lining of the airway epithelium (6), and the development of irreversible airway fibrosis (5, 6, 17, 27). An increase in airway wall smooth muscle results in an enhanced contractile response and an amplified narrowing of the airway lumen during an asthmatic attack (17). The deposition of extracellular matrix proteins by connective tissue cells accumulating and proliferating beneath the airway epithelium (i.e., airway fibrosis) contributes to chronic, irreversible narrowing of the airway lumen (5, 27).

The principal collagen-producing connective tissue cell type in the fibrotic response is likely a myofibroblast phenotype (i.e., contractile interstitial cell) (1, 34). Myofibroblasts possess characteristics of fibroblasts (e.g., positive for vimentin and procollagen) and SMC (e.g., positive for desmin and smooth muscle actin) (34). It has been suggested that myofibroblasts contribute to restrictive airway disease by depositing collagen and thereby promoting airway fibrosis (5, 27). Myofibroblasts also have the potential to differentiate into SMC (28), and ultrastructural studies of airways from asthmatic patients suggest that myofibroblast-to-SMC differentiation contributes to increased airway smooth muscle mass observed in asthma (14).

The purpose of this study was to investigate the progression of airway remodeling after a single intratracheal instillation of vanadium pentoxide ($\text{V}_2\text{O}_5$) to determine whether this metal causes constrictive airway pathology consistent with its asthma-like effects in humans and rodents. We report that $\text{V}_2\text{O}_5$ instillation causes airway smooth muscle thickening, mucous cell metaplasia in the airway epithelial lining, and a marked increase in the proliferation of peribronchiolar myofibroblasts. These proliferative events precede the development of airway fibrosis.

**METHODS**

Intratracheal instillation. $\text{V}_2\text{O}_5$ suspensions (pH 7.2) were vortexed thoroughly, then bath sonicated for 30 min at 25°C before instillation. Male Sprague-Dawley rats (Charles River)
weighing ~200 g were instilled intratracheally with 200 µl of sterile saline or 1 mg/kg (0.2 mg/rat) V_2O_5 (Aldrich Chemical, Milwaukee, WI) suspended in 200 µl of saline, as previously reported (3). Rats received a single injection of bromodeoxyuridine (BrdU; 50 µg/kg ip) 1 h before they were killed. At 3, 6, and 15 days after instillation, the animals (3 saline-instilled and 5 V_2O_5-instilled per time point) were overdosed with an intraperitoneal injection of pentobarbital sodium (Nembutal), and the lungs were removed en bloc. The lungs were instilled with neutral buffered Formalin in PBS, pH 7.2, the trachea was tied off, and the lungs were immersed in Formalin overnight. After fixation, the lung tissues were embedded in paraffin and cut into 6-µm-thick sections. Sections were mounted and stained for hematoxylin and eosin, Masson’s trichrome for collagen, and alcian blue-periodic acid-Schiff (PAS) for identification of mucin-containing goblet cells. Serial sections were used for desmin, vimentin, and BrdU immunohistochemistry.

Immunohistochemistry. Lung tissue was fixed overnight in 10% neutral buffered Formalin. Immunohistochemistry was performed using the avidin-biotin peroxidase method. Tissue sections (6 µm) were deparaffinized with xylene and dehydrated with a series of graded alcohol solutions to automation 10% neutral buffered Formalin. Immunohistochemistry was carried out on rats at 3, 6, and 15 days after instillation with V_2O_5. Sections were stained with antibodies against desmin, vimentin, and BrdU (Vector Laboratories) for identification of desmin-positive, vimentin-positive, and BrdU-positive bronchiolar SMC layer by BrdU immunohistochemistry. The majority of BrdU-positive cells were observed in the connective tissue layer surrounding the SMC band. Neighboring sections were stained with antibodies against desmin and vimentin (Vector Laboratories). V_2O_5 stimulates airway smooth muscle thickening. Desmin was demonstrated as a specific SMC marker and clearly stained SMC in normal bronchiolae from saline-instilled rats but did not stain the airway epithelium or lung cells residing within the lung parenchyma (Fig. 1A). V_2O_5 instillation caused a thickening of the desmin-positive bronchiolar SMC layer by day 6 (Fig. 1B). Desmin-positive peribronchiolar cells were also abundant 6 days after V_2O_5 instillation and were identified as myofibroblasts (see below). Proliferating airway SMC were detected by BrdU immunohistochemical staining at day 3, indicating that at least some of the SMC thickening observed at day 6 was due to replicating SMC (Fig. 1B). However, these BrdU-positive SMC were few in number and represented <15% of the total number of BrdU-positive cells surrounding the airway at day 3 after instillation. The majority of BrdU-positive cells were observed in the connective tissue layer surrounding the SMC band. Morphometric analysis. Morphometric evaluation was carried out on rats at 3, 6, and 15 days after instillation with saline or V_2O_5. Five airways were measured per rat, and at least three saline- and three V_2O_5-instilled animals were evaluated at each time point. Bronchioles that presented a closed circular or oval profile were selected. The thickness of the brown-staining, desmin-positive airway smooth muscle layer or the blue-staining, trichrome-positive peribronchiolar collagen layer was measured on digitized microscopic images (magnification ×400) of histological sections with the NIH Image Program (National Institutes of Health, Bethesda, MD), as described previously (8). The ratio of area to perimeter was used as an index of smooth muscle thickness or airway collagen thickness, where the area is defined as the entire ring of smooth muscle or collagen. The NIH Image Program allows for manual outlining of the desmin-stained smooth muscle layer or the trichrome-stained collagen layer and computes the area within the outlined ring of tissue. The perimeter is the airway basement membrane circumference. Thus we corrected for the variability in bronchiolar diameter (i.e., perimeter). Airway smooth muscle thickness was also verified by conventional morphometry, wherein the thickness of the smooth muscle layer from the base of the columnar epithelium to the proximal (inner) edge of the vimentin-positive adventitia was measured using an eyepiece reticle. The smooth muscle wall thickness was routinely evaluated at two points on opposite sides of the short axis of the elliptical profiles, and measurements were made at locations where cell borders appeared sharp to minimize tangential sectioning. Similar verification was performed to determine thickness of the trichrome-positive layer (i.e., airway fibrosis), where measurements with an eyepiece reticle were made from the base of the columnar epithelium to the distal (outer) edge of the blue-staining collagen surrounding the airway. For analysis of time-course data, one-way ANOVA was performed to determine an effect of exposure. If this analysis was significant, two-sample t-tests were performed on treatment effects at each time point. RESULTS

V_2O_5 stimulates airway smooth muscle thickening. Desmin was demonstrated as a specific SMC marker and clearly stained SMC in normal bronchiolae from saline-instilled rats but did not stain the airway epithelium or lung cells residing within the lung parenchyma (Fig. 1A). V_2O_5 instillation caused a thickening of the desmin-positive bronchiolar SMC layer by day 6 (Fig. 1B). Desmin-positive peribronchiolar cells were also abundant 6 days after V_2O_5 instillation and were identified as myofibroblasts (see below). Proliferating airway SMC were detected by BrdU immunohistochemical staining at day 3, indicating that at least some of the SMC thickening observed at day 6 was due to replicating SMC (Fig. 1B). However, these BrdU-positive SMC were few in number and represented <15% of the total number of BrdU-positive cells surrounding the airway at day 3 after instillation. The majority of BrdU-positive cells were observed in the connective tissue layer surrounding the SMC band. Quantitative morphometry of the bronchiolar SMC layer with use of the NIH Image Program showed a 2.2- to 2.5-fold increase in the area-to-perimeter ratio (i.e., thickness) of the smooth muscle layer that peaked 6 days after V_2O_5 instillation and remained thickened at day 15 (Fig. 2). Measurements made by eyepiece reticle were somewhat more variable and indicated 2- to 4.5-fold increases in smooth muscle thickness. To assess whether the increase in smooth muscle mass was due to hypertrophy or hyperplasia, we counted nuclear profiles on hematoxylin- and eosin-stained serial sections of the same airways that we used for smooth muscle area/perimeter measurements. The airway cross sections from saline-instilled rats contained 28 ± 5
nuclear profiles compared with 67 ± 17 nuclear profiles in airway cross sections from V$_2$O$_5$-instilled rats at day 6. Thus there was a 2.3-fold increase in airway SMC nuclear profiles. Because the morphometry data indicated a 2.2- to 2.5-fold increase in smooth muscle thickness, these data suggest that the increase in smooth muscle mass after V$_2$O$_5$ exposure is due primarily to SMC hyperplasia.

Identification of peribronchiolar myofibroblasts during V$_2$O$_5$-induced airway remodeling. Mesenchymal cells staining positively for desmin and vimentin were abundant around bronchioles possessing thickened airway smooth muscle at day 6 after instillation (Fig. 3).

Airway SMC stained only for desmin and not vimentin, whereas the airway epithelium was negative for both markers. Because vimentin is a well-known fibroblast marker and desmin is a marker of SMC, these results indicated that proliferating mesenchymal cells surrounding V$_2$O$_5$-injured airways were most likely myofibroblasts. To verify the identity of desmin-positive, vimentin-positive myofibroblasts, serial sections of the peribronchiolar region were viewed by high-magnification oil-immersion light microscopy. The majority of connective tissue cells within this region stained positively for cytoplasmic desmin and vimentin, which further indicated that these cells were mainly myofibroblasts (Fig. 4). Some inflammatory cells (e.g., macrophages) that infiltrated this region were positive for vimentin but not for desmin (data not shown). As mentioned above, few SMC were BrdU positive (Fig. 1, inset), and the majority of BrdU-positive cells were observed in the connective tissue layer surrounding the SMC band (Fig. 5). Quantitation of BrdU-positive peribronchiolar cells within the airway smooth muscle layer or underlying vimentin-positive layer of approximately equal thickness showed that the majority of proliferating cells were not SMC but connective tissue cells possessing a myofibroblast phenotype (Figs. 5 and 6).

Activation of the bronchiolar epithelium by V$_2$O$_5$. The bronchiolar epithelium could be important in driving the proliferation of the airway SMC or peribronchiolar connective tissue cells after airway injury (Figs. 1 and 5). V$_2$O$_5$ instillation caused activation of the airway epithelium, defined as mucous cell metaplasia (a serous cell-to-goblet cell phenotypic change), where mucin in the goblets was detected by alcan blue-PAS stain. BrdU-positive airway epithelial cells were rarely observed (Figs. 1 and 5), which indicated that activation...
of the airway epithelium did not involve hyperplasia. Saline-instilled control airways possessed a predominance of ciliated epithelial cells and no detectable goblet cells (Fig. 7). After V$_2$O$_5$ injury, the alcian blue-PAS stain showed that 30–40% of the airway epithelial cells had differentiated to goblet cells (Figs. 7 and 8). A quantitative assessment revealed that numbers of goblet cells increased maximally by day 6 after V$_2$O$_5$ instillation and declined to nearly saline control levels by day 15 (Fig. 8). Airway fibrosis during V$_2$O$_5$-induced airway injury. The deposition of mature collagen around bronchioles was detected by trichrome staining. In a previous study of V$_2$O$_5$-induced lung fibrosis, we showed that total lung hydroxyproline increased fourfold by day 15 after instillation (26), yet this quantitative measurement includes collagen deposited within lesions in the lung parenchyma as well as around airways. To measure changes in peribronchiolar collagen deposition, we used morphometry to quantitate the thickness of the trichrome-positive layer. The thickness of the subepithelial trichrome-positive layer increased by 3.1- to 3.9-fold at day 15 after V$_2$O$_5$ instillation, as determined by area/perimeter measurements with the NIH Image Program (Figs. 9 and 10). More variable measurements were obtained with eyepiece reticle measurements, and the magnitude-increase values for collagen thickness among saline- and V$_2$O$_5$-instilled groups ranged from 2.5- to 7-fold depending on the specific site within the airway wall that was measured (data not shown). This indicated that area/perimeter measurements obtained from the computer-assisted NIH Image Program were more reliable than eyepiece reticle measurements. Significant increases in the thickness of the trichrome-positive layer were not observed before day 15, and no increases were observed in the saline-instilled control animals (Fig. 10).

DISCUSSION
In this study, we have shown that the intratracheal instillation of V$_2$O$_5$, a transition metal associated with occupational asthma, induced remodeling of the rat bronchiolar architecture and caused fibroproliferative changes in the airway wall that are consistent with the pathology of asthma. These features included mucous cell metaplasia, airway smooth muscle thickening, proliferation of peribronchiolar myofibroblasts, and airway fibrosis. Our definition of airway fibrosis encompasses subepithelial fibrosis (i.e., basement membrane thickening) and peribronchiolar fibrosis surrounding the smooth muscle layer. Increasing attention has been focused on the significance of airway fibrosis in asthma, and it has been proposed that airway myofibroblasts participate in the increased production of collagen that leads to airway fibrosis (5, 13). To our knowledge, this is the first report of metal-induced airway remodeling that is consistent with the fibroproliferative pathology seen in asthma.
Myofibroblasts may contribute to vanadium-induced airway remodeling in at least two ways. First, the contractile nature of myofibroblasts might contribute to persistent narrowing of the airway lumen. Second, the deposition of collagen by peribronchiolar myofibroblasts could contribute to airway narrowing by forming scar tissue around the airway. We observed a transient appearance of peribronchiolar myofibroblasts that peaked at day 6 after V$_2$O$_5$ instillation (Fig. 3) and then declined by day 15, leaving a thickened collagen sheath around the airway (Fig. 9). In tissues other than lung, myofibroblasts appear transiently within days of injury and decrease in number as healing occurs (32). The loss of peribronchiolar myofibroblasts in our study between days 6 and 15 could be due to removal of cells by apoptosis or differentiation of myofibroblasts to airway SMC.

In addition to their role in promoting airway fibrosis, it has been suggested that myofibroblasts migrate and differentiate into SMC, and this is one possible explanation for the increase in smooth muscle wall mass seen in asthma (14). The more classic explanation for increased smooth muscle mass involves SMC hyperplasia and hypertrophy (12, 15). A hyperplastic growth response could arise when SMC are stimulated to proliferate in response to mitogens released by the activated airway epithelium, inflammatory cells such as macrop...
phages, or the SMC themselves. In the present study, the BrdU-labeling index in the airway smooth muscle layer at any given time was low compared with the numbers of BrdU-positive peribronchiolar cells in the surrounding connective tissue layer. However, it is possible that we missed the peak of proliferating SMC, since we did not investigate time points before day 3 after instillation. Our quantitation of nuclear profiles in the airway smooth muscle wall showed a 2.3-fold increase in cell number, which was nearly identical to the magnitude increase in area/perimeter measurements for SMC thickness caused by V2O5 instillation. This indicates that the SMC thickening that we observed is due mainly to cell hyperplasia. However, this does not rule out the possibility that some proliferating myofibroblasts adjacent to the smooth muscle wall migrated and differentiated into SMC, thereby increasing smooth muscle wall thickness. Our data suggest that SMC hypertrophy plays only a minor role in the thickening of the airway wall.

A variety of growth factors and cytokines have been reported to stimulate the proliferation or differentiation of myofibroblasts and SMC. For example, platelet-derived growth factor (PDGF) isoforms and transforming growth factor (TGF)-α are potent mitogens for mesenchymal cells (fibroblasts, myofibroblasts, and SMC) and are upregulated during fibroproliferative lung disease (16, 22). We recently reported that tyrrosine kinase inhibitors specific for PDGF or epidermal growth factor receptors reduced pulmonary fibrosis in rats instilled with V2O5 (26). Basic fibroblast growth factor (FGF-2) is normally sequestered within the basement membrane of airways (31) but, when released, is mitogenic for human airway SMC and also upregulates the PDGF receptor α-subtype to render these cells more responsive to the mitogenic effects of PDGF (2). Targeted expression of interleukin (IL)-11 to airways with the Clara cell 10-kDa promoter caused airway remodeling and subepithelial fibrosis that was characterized by increased collagen and increased desmin and smooth muscle α-actin-containing cells, including myofibroblasts and SMC (33). TGF-β1, a major inducer of collagen deposition by myofibroblasts and fibroblasts, is upregulated during the progression of lung fibrosis (18). Furthermore, TGF-β1 induces fibroblasts to differentiate to a smooth muscle α-actin-positive myofibroblast phenotype (10). Proinflammatory cytokines such as IL-1β and tumor necrosis factor-α (TNF-α) are also increased after lung injury, and neutralizing antibodies to TNF-α have been reported to

![Fig. 7. Alcian blue-periodic acid-Schiff (PAS) staining demonstrating mucous cell metaplasia in airway epithelium of V2O5-instilled rats. A: cross section of a bronchiole from a control (saline-instilled) rat at day 6 after instillation showing a predominance of ciliated epithelial cells lining airway lumen and a lack of mucus-producing goblet cells. B: bronchiole from a V2O5-instilled rat at day 6 after instillation shows numerous mucus-positive (purple-staining) goblet cells (arrowheads). Quantitation of alcian blue-PAS-positive goblet cells is shown in Fig. 8. Original magnification, ×200.](http://ajplung.physiology.org/)

![Fig. 8. Quantitation of alcian blue-PAS-positive goblet cells during course of airway fibrogenesis after V2O5-induced lung injury. Number of alcian blue-PAS-positive cells in each bronchiole cross section were counted in 5 bronchiole cross sections per lung from 3 animals per saline-instilled group or 5 animals per V2O5-instilled group at 3, 6, and 15 days after instillation. AB, automation buffer. Values are means ± SE. Statistical deviation represents variation among individual animals: *P < 0.05.](http://ajplung.physiology.org/)
block pulmonary fibrosis (25). Thus it appears that the fibrogenic response is orchestrated by a variety of cytokines and growth factors that mediate myofibroblast growth and collagen deposition.

It is likely that V$_2$O$_5$ stimulates several cell types in the airways to produce cytokines. For example, we found that V$_2$O$_5$ was a strong inducer of mucous cell metaplasia in vivo (Fig. 4), and activation of human airway epithelial cells in vitro by vanadium compounds has been reported to stimulate the secretion of IL-6, IL-8, and TNF-$\alpha$ (7). In addition, we previously reported that V$_2$O$_5$ stimulates the secretion of IL-1$\beta$ by rat alveolar macrophages (3). Therefore, epithelial cells and macrophages could function as effector cells in vanadium-induced airway fibrosis. Also, the mesenchymal target cell types (i.e., SMC and myofibroblasts) could themselves act as a source of cytokines and growth factors after V$_2$O$_5$ stimulation. The mechanisms through which vanadium compounds increase cytokine production have not been clarified, but several studies have reported that vanadium stimulates a variety of signaling events in epithelial cells and fibroblasts, including tyrosine phosphorylation (30), mitogen-activated protein kinase activation (29, 35), and activation of nuclear factor-$\kappa$B (20). One or more of these signaling pathways could be linked to induction of cytokine gene expression.

In summary, we have shown that V$_2$O$_5$ instillation causes airway remodeling similar to that observed in individuals with asthma and chronic bronchitis. These changes include airway SMC thickening, mucous cell metaplasia, and airway fibrosis. The transient appearance of peribronchiolar myofibroblasts, which were desmin and vimentin positive, coincided with an increase in airway smooth muscle mass and preceded the development of airway fibrosis. These data support the idea that myofibroblasts contribute to airway fibrosis. Because V$_2$O$_5$ is a cause of occupational asthma, this model should be useful for investigating the cellular and molecular mechanisms of airway SMC thickening and airway fibrosis.

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